ASSESSMENT OF BACTERIOPHAGE-INSENSITIVE CULTURE BACTERIA FOR CHEDDAR CHEESE MAKING AND SUBSEQUENT DISCRIMINANT ANALYSIS FOR OBJECTIVE FLAVOUR EVALUATION

by

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ABSTRACT

The first part of this two-part study deals with six defined strains of bacteriophage-insensitive Streptococcus cremoris used over a period of 10 months to produce more than 2 million kg of Cheddar cheese on continuous cheesemaking equipment. Flavour development in this cheese was less than that in cheese made with conventional bulk starter. Proteolysis and rheological properties were examined. Pattern recognition techniques were used to analyze the multivariate data. Texture was affected by the mechanical process, moisture content and yield point. Casein proteolysis, age, culture type and firmness were the most discriminating variables affecting maturity.

In part two, it is well recognized that a minimum maturation period is required before Cheddar cheese is acceptable to the consumer. This is a lengthy and costly procedure in which quality is based on subjective evaluation. Classically, trained graders and sensory panels have performed this duty, but because of the variable, subjective and time consuming nature or organoleptic methods, a simple, more objective and reliable method for accurately assessing cheese flavour is proposed. Sensory evaluations by trained graders were done on more than 60 commercially produced cheese samples, and water soluble fractions prepared from all samples. A ternary gradient system was used to elute the non-volatile flavour components from an Adsorbosphere C_8 reverse phase column. A new mapping simplex optimization technique was applied to the HPLC profiles to optimize separation of the multi-component mixture. More than 45 peaks were obtained using an initial solvent volume ratio of 44.6:0:55.4 of trifluoroacetic acid (0.1%), acetonitrile and water. Over 56.6 min the ratio was changed to 0:36.6:63.4 at a flow-rate of 0.97 mL/min. The

optimized gradient system was superior to previous isocratic separations and the elution patterns differed for the various categories of cheese. Statistical pattern recognition techniques - principal component analysis and stepwise linear discriminant analysis - were used to interpret the HPLC profiles. Cheese samples were correctly classified according to their discriminant functions into groups. The technique differentiated between first grade and downgraded samples and was capable of assessing cheese flavour quality at an early age.

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I wish to thank my wife Barbara for her continued support during this difficult time, without which this work could not have been completed.

Finally, I would like to dedicate this thesis in loving memory of my father, Jack.

INTRODUCTION

Improved starter cultures using bacteriophage-insensitive mutants for Cheddar making have received much attention during the past ten years and major advantages have been claimed. Moreover, it is generally recognized that good quality Cheddar cheese is largely dependent on the starter strains selected (McDowall and Whelan, 1933). It is for this reason and because of the economic significance of Cheddar cheese as well as consumer acceptance that the dairy industry has placed great emphasis on cheese flavour quality control.

The aim of the present study was to evaluate the performance of six defined strains of bacteriophage-insensitive Streptococcus cremoris grown in whey-based starter media and subsequently used for commercial cheese production on automated continuous equipment. The performance of these strains were compared to conventional S. lactis culture propagated and used in the same manner. Unlike other studies that assess culture performance by evaluating the cheese using traditional methods with classically trained graders or sensory panels with little or no importance given to analytical methodology or statistical techniques, the present study proposes a more objective and reliable approach. Moreover, this approach is necessary in that it has been shown that the sensory evaluation of cheese may not be reliable and varies from grader to grader (Lelievre and Gilles, 1982).

The primary objective, therefore was to assess the effect of starter culture on texture and degree of cheese ripening while at the same time evaluating flavour quality. The texture of a cheese is one of the important characteristics that determines the identity and quality. However, most studies relating to cheese texture involve cheese maturity,

test temperature, crosshead speed, sample height and surface area (Culioli and Sherman, 1976). Few studies have dealt with the interrelationships among manufacturing parameters, compositional data and rheological properties. Lawrence and Gilles (1982) restricted their study to only Cheddar cheese which had been manufactured to various compositions. They found that the force compression curves were different for different cheese samples and were affected by the moisture content, pH and extent of casein proteolysis that had taken place. However, most studies are carried out on laboratory scale making it sometimes difficult to relate these results to commercial production.

The present study was restricted to only Cheddar cheese. Unlike other studies, the cheese was produced on continuous commercial scale equipment and varied in composition. Lawrence and Gilles (1980) showed that the quality of cheese was influenced by moisture in the non-fat-substance (MNFS), salt-in-moisture (S/M) levels and pH and proposed optimum specification limits for production of quality cheese. Therefore, the present studies placed emphasis on quantitative measurement of the cheese composition.

Because of the subjective nature of evaluating cheese flavour by sensory analysis a procedure outlined by McGugan et al. (1979) was adopted. They proposed that the non-volatile water extractable fraction was responsible for the intensity of cheese flavour. Therefore, analysis of these extracts from cheeses of approximately the same composition, age and manufacturing procedures but made by using the different cultures would provide valuable information about the flavour of the respective cheeses. Reversed phase high performance liquid chromatography (RP/HPLC) shown to be

a valuable analytical tool in assessing cheese flavour development (Pham and Nakai, 1984) was selected to analyze the extracts. The objective was to quantitatively relate proteolytic culture activity in the respective cheeses to their HPLC profile.

An additional aim of the study was to interpret the multivariate data by statistical pattern recognition techniques. Although the technique is well recognized and widely used in other disciplines it has only recently been applied to Food Science. Thus, the objective was to select from all the data the most discriminating factors or conditions that significantly affected cheese texture and/or taste.

LITERATURE REVIEW

The basic process for making Cheddar cheese has changed little: coagulation, cooking, dry-stirring, matting or cheddaring, milling, salting and pressing, while the time required for making has remained about the same. Mechanization of the process for making Cheddar cheese to reduce labour costs and physical labour has received much attention. Unfortunately, little data exists for critically evaluating these systems with respect to quality of cheese and to uniformity of composition (Emmons, 1978).

Starter cultures remain a vital necessity for manufacturing Cheddar cheese. Simple fool-proof methods of eliminating bacteriophage, developing bacteriophage-insensitive strains or avoiding the consequences of phage infection have been elusive goals of the cheese industry. Research efforts to improve starter culture technology for Cheddar cheese making has received much attention during the past several decades.

The flavour of a good quality Cheddar cheese is largely dependent on the starter strains selected (McDowall and Whelan, 1933). Although it is recognized that the primary function of a starter culture is to produce lactic acid, the selection of the culture to be used is very essential to good cheese manufacturing procedures. The culture must meet and satisfy a number of important requirements including essential acid production and flavour development (Lawrence et al., 1976), phage insensitivity (Heap and Lawrence, 1976; Lawrence et al., 1978; Thunell et al., 1981), absence of bacteriocins (Babel, 1977) and sensitivity to Cheddar cheese cook temperatures (Lowrie et al., 1974; McDowall and Whelan, 1933).

Since its development more than 40 years ago, cheese starter systems based on daily rotation of phage-unrelated single strains of lactic organisms and aimed at prevention of bacteriophage attack during cheese making has been a principal concern of cheese manufacturers. Heap and Lawrence (1976) provided a better understanding of bacteriophage relationships. They suggested that the major source of phages was the starter culture itself and phage levels in a commercial plant could be greatly reduced by avoiding the use of strains which could act as hosts for the phages present.

Limsowtin and Terzaghi (1976) isolated three S. cremoris mutants based on their specific phage resistance and suitable acid production. They were used in rotations, each paired with another single strain and successfully used in cheese production for more than nine months. A new multiple starter approach using six carefully selected strains was developed (Limsowtin et al., 1977) and continuously used for eight months. Czulak and co-workers (1979) isolated a single phage resistant strain and reported its use in cheese manufacture for three years without failures caused by phage attack. Thunell and co-workers (1981) developed phage insensitive, multiple-strain starters by plaquing cheese whey against potential starter strains. The six defined strains of Streptococcus cremoris have been used to produce more than 68 million kilograms of Cheddar cheese with no plants experiencing starter failure from phage attack.

Phage-insensitive mutant strains have been used successfully singly (Czulak et al., 1979) in paired rotations (Limsowtin et al., 1977) and multiple strains (Lawrence et al., 1978; Limsowtin and Terzaghi, 1976; Thunell et al., 1981).

The major advantage of the multiple strain culture is that it can be used continuously, at a lower innoculum with consistent activity despite strain variation and seasonal changes in milk composition (Limsowtin et al., 1977). Moreover, cheese flavour uniformity is obtained while the cheesemaker is able to select and mix compatible strains together to improve cheese quality by controlling starter activity (Lawrence et al., 1978; Thunell et al., 1981).

PROPAGATION, SELECTION AND COMMERCIAL APPLICATION

The principal function of lactic acid bacteria during cheese making is fermentation of lactose into lactic acid. However, this product of metabolism can reduce activity of lactic bacteria especially when the bacterial cells are held at a pH below 5.0 for extended periods of time. Beneficial effects of neutralizing acids in lactic culture media have been recognized for some time, however, commercial application has occurred only within the past five years (Richardson et al., 1981).

A number of pH control systems have evolved: continuous neutralization during culture growth maintaining the pH between 5.9 to 6.1 by injection of liquid or gaseous ammonia or ammonium hydroxide into the medium (Richardson et al., 1980); use of buffered starter media, several which are available and proven successful commercially (Mermelstein, 1982). A variation of pH control (Limsowtin et al., 1980), in which cultures are grown in reconstituted nonfat-dry-milk (10 percent solids) until the pH reaches between 4.5 to 5.0, requires approximately 18 h of incubation. The starter is then neutralized to pH 7.0 with sodium hydroxide and incubated for an additional two hours. The pH falls to about 5.0 during this period and remains static for at least two hours.

Generally, starter cultures produced with pH control and nutrient exhaustion can be stored for intervals that are considerably longer then have previously been possible (Hong et al., 1977). These cultures can be used at lower inoculum levels, 30-60% of that of conventional cultures, and because of the high population of uninjured cells with a shorter lag time the cheese develops acid faster (Mermelstein, 1982).

Procedures to determine the suitability of a strain for use in multiple starters has been outlined (Lawrence et al., 1978). Cultural characteristics of phage-insensitive mutants were compared to those of their respective parent strains and found to possess similar desirable characteristics and stabilities (Thunell et al., 1981).

Richardson and co-workers (1983) selectively grew proteinase-negative (Prt^-) variants of Streptococcus cremoris in pH controlled, buffered media and showed a reduced potential for the development of bitter flavour when used exclusively to manufacture Cheddar cheese. The Prt^- variants were more resistant to bacteriophage and antibiotics than proteinase positive (Prt^+) strains as well as being less sensitive to higher temperatures which retard normal culture growth.

Normal Cheddar flavour developed when S. cremoris numbers were controlled (lower) without markedly retarding acid production (Lowrie et al., 1974). These authors suggested acidity development by the starter partly determined the environment in which the maturation process occurred as well as controlling the growth of non-starter bacteria and hence any off-flavours that they might produce. Adda et al. (1982) showed that the pH of the cheese controlled the ripening process and it together with the moisture content affected texture.

In spite of the many advantages claimed for bacteriophage-insensitive mutants and pH controlled culture systems, bacteriophage insensitivity is not a permanent property of these strains (Czulak et al., 1979; Lawrence et al., 1978). The sensitivities of the strains may change when subcultured over a long period of time (Limsowtin et al., 1980). A critical aspect therefore of this starter system involves daily monitoring of the whey for bacteriophage against each of the strains and those showing phage sensitivity are replaced (Keogh, 1972; Hull, 1977).

Application and commercialization of bacteriophage-insensitive mutant strains grown in pH controlled starter systems have been used successfully in cheesemaking (Czulak et al., 1979; Limsowtin and Terzaghi, 1976; Lawrence et al., 1978; Thunell et al., 1981; Daniell and Sandine, 1981) and advantages to the industry has been reported (Richardson et al., 1983). Consistent starter performance and economic savings due to improved quality have been reported (Thunell et al., 1981). Moreover, it is possible to put together compatible mixtures of strains that will result in even better quality cheese (Lawrence et al., 1978) with reduced starter variability and more reliable cheesemaking (Daniell and Sandine, 1981).

COMPOSITION

It is generally recognized that traditional procedures for assessing cheese quality at an early age are not a reliable guide to its acceptability by consumers when the cheese is mature (McBride and Hall, 1979). Lawrence and Gilles (1980), in an excellent review, showed that the quality of cheese was influenced most by three compositional factors: MNFS, S/M levels and pH. The pH is influenced by the S/M and depends on the starter cultures used, the manufacturing conditions and the buffering

capacity of the curd (Pearce and Gilles, 1979; Lawrence and Gilles, 1982). Salt content is an important determinant of Cheddar cheese quality. High concentrations have a direct effect on culture growth, lactose fermentation and subsequent acid production as well as proteolysis (Thomas and Pearce, 1981).

Cheese composition has been shown to influence yield, grade and financial return when deviations from optimum specification limits (MNFS, 52-56%; fat-in-dry-matter (FDM), 52-55%; S/M, 4.0-6.0%; and pH 4.95-5.10) result during manufacture (Lelievre, 1983; Lelievre and Gilles, 1982).

TEXTURE

Most studies involving cheese starter cultures have dealt with selection, isolation, rate of acid production of the strains and flavour development in the cheese, while the quality of the cheese has been assessed by classical subjective evaluation. It is important to note, however, that the majority of cheeses are identified according to texture. The latter has been recognized as a multidimensional quality attribute important for consumer preferences. In spite of the importance of cheese texture it has not on the whole been very extensively studied.

Since the development of analytical equipment allowing texture profile analysis (TPA), (Szczesniak, 1975) a better and more complete understanding of the textural properties of the food is possible (Bourne, 1978).

Important subjective textural evaluations of cheese including firmness, springiness, smoothness and softness have been considered and measured objectively. Moreover, these results have been used to determine the identity and quality of the cheese (Baron and Scott Blair, 1953).

Preliminary observations using analytical instrumentation indicated that normal curds for several types of cheese recovered elastically a very high proportion of their original compression deformation upon unloading. The elastic deformation was related to the shear modulus and inelastic deformation was reported to be related to the viscosity of the cheese curd. A value inversely proportional to the shear modulus was suggested as the best single criterion of firmness (Baron and Scott Blair, 1953).

The texture of a large range of cheese samples and varieties has been tested objectively using a variety of instruments. These analytical measurements provide objective values of elastic deformation, plastic deformation, elasticity, forces required to compress or penetrate the cheese and energy required to rupture the material. Attempts to correlate these objective parameters with subjective evaluations have had various degrees of success. Shama and Sherman (1973) reported that the Instron Universal Testing Machine could be meaningfully employed to evaluate textural properties of foods. However, it is necessary to select the correct instrumental test conditions in order to correlate with sensory responses.

Many variables have been shown to influence the force compression behaviour of cheese (Culioli and Sherman, 1976). These include sample shape, dimensions, the location on the larger block of cheese, from which the sample was taken, rate that the compression force is applied, the cohesiveness of the sample, age, the nature of the interface between the samples edge and the Instron plates as well as test temperature. Lee et al. (1978) determined the important sensory textural characteristics of cheese and related them to objective measurements obtained with an Instron Universal Testing Machine and found good correlation with sensory panel

evaluations. Sensory preference indicated that firmness was the important characteristic of the cheese. Carter and Sherman (1978) evaluated the firmness of Leicester cheese and concluded that the force compression curves are influenced by the same factors previously found by Culioli and Sherman (1976).

Chen et al. (1979) measured six textural characteristics with an Instron Universal Testing Machine for eleven varieties of cheese and the objective measurements correlated with sensory panel results. Using stepwise multiple linear regression analysis, cheese texture was found to be influenced by the following compositional sequence: protein > NaCl > water > pH > fat.

The effect of compression ratio on the mechanical properties of cheeses of various textural characteristics was studied (Imoto et al., 1979; Lee et al., 1978) and correlations between sensory evaluations of cheese texture and mechanical properties determined. The pattern of changes in mechanical properties in relation to compression ratio was unique for each type of cheese tested.

Dickinson and Goulding (1980) studied the yield behaviour of three cheeses (Cheddar, Cheshire and Leicester) selected for their "crumbly" texture as a function of deformation rate, temperature and pre-yield compression history and showed that simple non-linear viscoelastic models were not satisfactory to predict yield behaviour.

Examination of the interrelationships between composition and microstructure in full-fat and reduced-fat cheeses have been studied (Emmons et al., 1980) and related to texture. Electron microscopy and compositional analysis revealed about 30% more protein matrix in the reduced-fat cheese which was responsible for the firmer and more elastic texture.

Green et al. (1981) studied the structure and texture of Cheddar cheese made with milk concentrated to different extents and found a direct relationship between concentration factor and the rheological behaviour and structure. They found that protein hydrolysis decreased with an increase in concentration. Scanning electron micrographs confirmed that the protein constituted a network in which the fat was entangled. Any modification of the nature or the amount of the protein present in the cheese would modify its texture. These results were in agreement with those of Emmons et al. (1980).

MATERIALS AND METHODS

1. CHEESE MANUFACTURE

Cheddar cheese was manufactured (1339 kg/h) with commercial scale equipment using 8500 litre OST vats and Alpha Matic continuous cheddaring equipment (Alpha-Laval Cheddar Systems Limited, 10 Oxford Road, Yeovil, Somerset, Great Britain BA21 5HR) or purchased from retail outlets.

Fresh, unstandardized raw milk was processed using high temperature short time (HTST) pasteurization (72°C; 16 sec) and filled directly into the vats at 30°C or heat treated (63°C; 16 sec) and then filled into the vats. Microbial rennet from Mucor miehei (Novo Industries, Weston, Ont., Can.) was used for all cheese manufactured and diluted 1:20 with water before adding to the milk under slow but constant agitation.

The label declaration on the purchased cheese was used as a parameter for age, assuming mild to be at least 100 days old and correspondingly medium 180 days and aged greater than 250 days.

2. COMPOSITIONAL ANALYSIS

All cheese samples were analyzed in duplicate for fat, moisture, total solids and salt according to official AOAC methods (AOAC, 1980). Samples for analysis were taken from the inside area of the piece of cheese and shredded finely using a 'home-style' grater. Butter fat was determined by the Roese-Gottlieb method.

Moisture was determined by weighing 2-3 g of the shredded cheese sample in a weighed flat bottom metal dish. The sample was dried in a vacuum oven held constant at 100°C (ca 4 h). After cooling the dishes in a desiccator, samples were quickly weighed. Loss in weight was expressed as moisture and % residue as total solids.

Salt was determined by a modification of the AOAC method for routine analysis during cheese manufacture. About 2 g of the shredded prepared cheese sample was accurately weighed into a 300 mL erlenmeyer and add 100 mL boiling H_2O. The flask was allowed to stand, swirling occasionally for 5-10 min while cooling to 50-55°C. After adding 2 mL K_2CrO_4 indicator the mixture was titrated with 0.1N $AgNO_3$ until an orange-brown colour persisted 30 seconds.

$$\% \text{ NaCl} = \text{mL } 0.1\text{N } AgNO_3 \text{ X } 0.585/\text{g sample}$$

For accurate determination of NaCl in the cheese sample a spectrophotometric method was used (Kupke and Sauer, 1970). To 0.2 mL of the water portion containing the salt, 4.0 mL ferric perchlorate working solution was added and absorption at 366 nm compared to a standard curve made with NaCl. [0.8g $Fe(ClO_4)_3$ was dissolved in 1.0 mL deionized H_2O and made up to volume (100 mL) with 70% $HClO_4$. Then 20 mL of 10% $HClO_4$ was added to 75 mL of this solution to prepare the working solution.]

3. SENSORY EVALUATION

Approximately 21d after production, samples were officially graded for flavour and physical characteristics by Federal Graders as outlined by Agricultural Canada Guidelines for the examination of Dairy Products. Retail purchased cheese was officially graded as soon as possible after purchase. Sensory attributes for all cheese included:flavour, texture, colour, moisture and salt.

4. CULTURE PROPAGATION

Six selected strains of bacteriophage-insensitive Streptococcus cremoris were purchased from Northwest Culture Tech. Inc. (P.O. Box 1991,

Corvalis, Oregon, 97339) and propagated in a pH controlled whey-based starter system (Biolac Inc., 750 West 200 North, P.O. Box 3490, Logan, Utah 84321) as shown in Figure 1.1.

The starter media was made by mixing freshly separated whey with water to adjust the lactose to 4.0%. Stimulant powder (Northwest Culture Tech. Inc., P.O. Box 1991, Corvallis, Oregon, 97339) was added at a rate of 4.5 kg per 450 litre of diluted whey (titratable acidity 0.55). The mixture was then pasteurized at 85°C for 45 min, cooled to 27°C and aseptically inoculated. The inoculated whey-base was continuously stirred during incubation and automatically controlled at 27°C by circulating hot or cold water as required through the jacket. The pH was controlled between 5.9 and 6.1 by the addition of 29.4% aqueous ammonia. After lactose depletion (approximately 18 h) the cultured media was cooled to 4°C and used at an inoculation level of 0.8%. Starter activity was measured before inoculation.

5. STARTER ACTIVITY

Eleven grams of antibiotic free non-fat-dry milk powder (NDM) was reconstituted in 100 mL of water. Ten mL was added to a series of 16 x 150 mm test tubes and sterilized (121°C; 15 min). To one 10 mL sterile NDM tube was added 0.1 mL of the starter to be tested and 0.3 mL of the starter to another 10 mL sterile NDM tube. The procedure was repeated for each starter to be tested and one 10 mL sterile NDM tube was retained as a blank. After mixing, the tubes were incubated at 30°C for 1.5 h after which pH readings recorded. The difference in pH between the blank and the inoculated tubes served as a quantitative reference and was an indication of the potential activity of the starter (Table 1.1). The procedure was

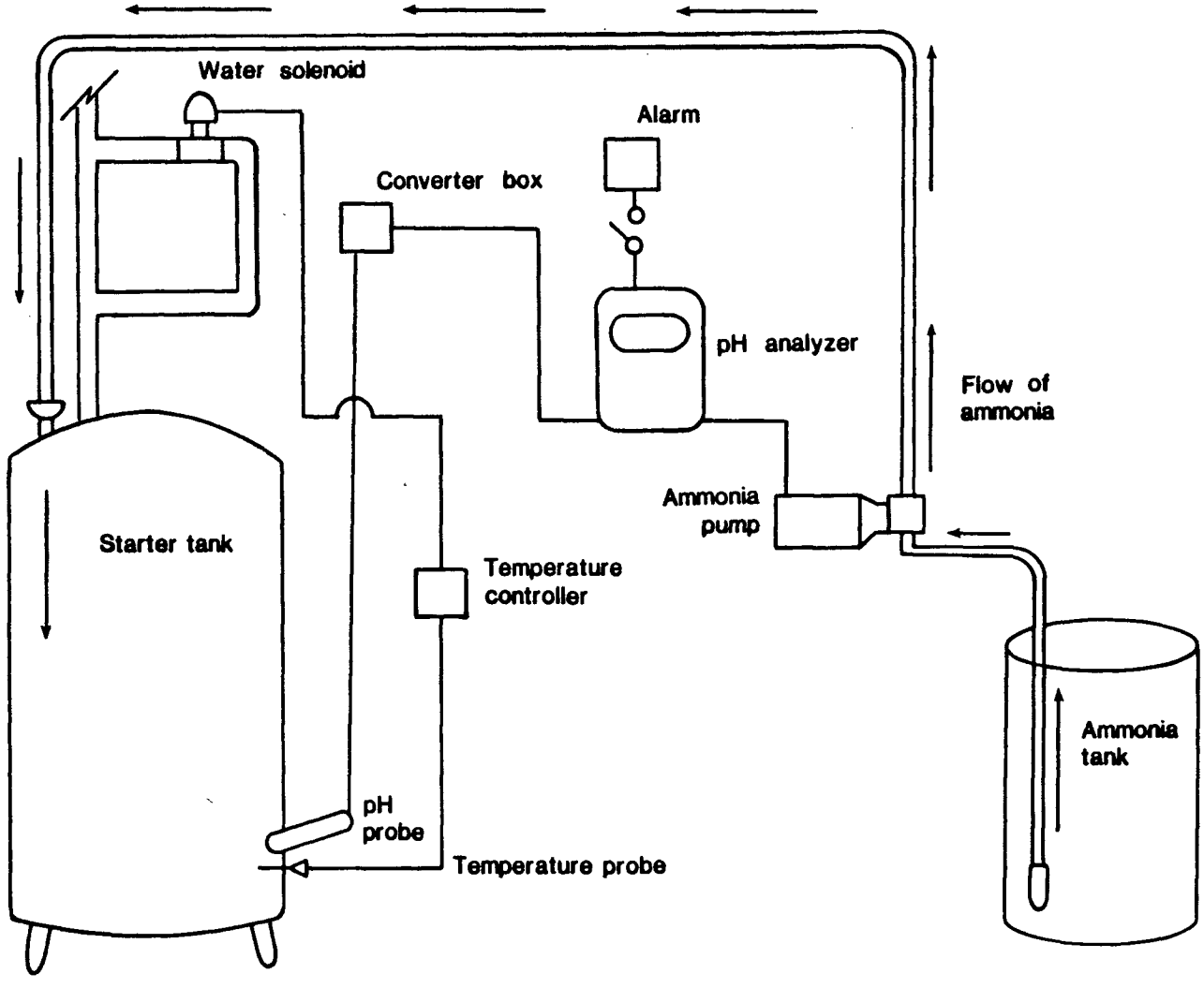


Figure 1.1 Schematic outlining the whey-based starter system.

Table 1.1. 1 and 3% active culture inoculated into reconstituted non-fat-dry-milk powder containing bromocresol purple and showing effect on pH after 1.5 and 3.0 h incubation at 30°C respectively.

	TIME (h)	INOCULUM	
		1%	3%
pH of blank	0.0	6.43	6.43
pH of 1% tube	1.5	6.17	
pH of 3% tube	3.0		5.80
difference		0.26	0.63

repeated after 3 h for the 3% inoculation. Only cultures with the same activity were used for cheese making.

6. BACTERIOPHAGE TESTING

To 500 mL of H_2O, 55 g of NDM (10% solids) and 0.75 g bromocresol purple (Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, NJ) were added. Ten mL of this indicator medium was then added to a series of test tubes 16 x 150 mm and sterilized (121°C; 15 min). A sample of cheese whey was filtered through a 0.45 μm Millipore filter to remove bacterial cells. Serial dilutions of the filtered whey were added to the sterile tubes containing 0.2 mL of active starter culture. The tubes were incubated for 5 h at 30°C before reading results. No colour change, a purplish-blue hue, indicated no acid development whereas a greenish-yellow hue, was a result of uninhibited growth of the starter culture. Phage titre was indicated by the degree of colour intensity (Figure 2.1) and served as a qualitative measurement.

7. ASSAYING FOR FREE AMINO GROUPS

Proteolysis in the trichloroacetic acid (TCA) soluble cheese fraction was measured by a modified micromethod with fluorescamine (Kwan et al., 1983). Four grams of cheese was centrifuged (15,000 X g; 30 min; 25°C)to remove fat. Precipitate, 0.3 g was added to 10 mL of TCA buffer containing 0.075M TCA, 0.15M sodium acetate and 0.225M acetic acid (Nakai et al., 1964) After mixing thoroughly the mixture was centrifuged (10,000 X g; 5 min; 25°C). To 0.4 mL of the supernatant, 10 mL of TCA buffer was added. To 0.1 mL of the diluted supernatant 0.3 mL of 3M K_2HPO_4 was added followed

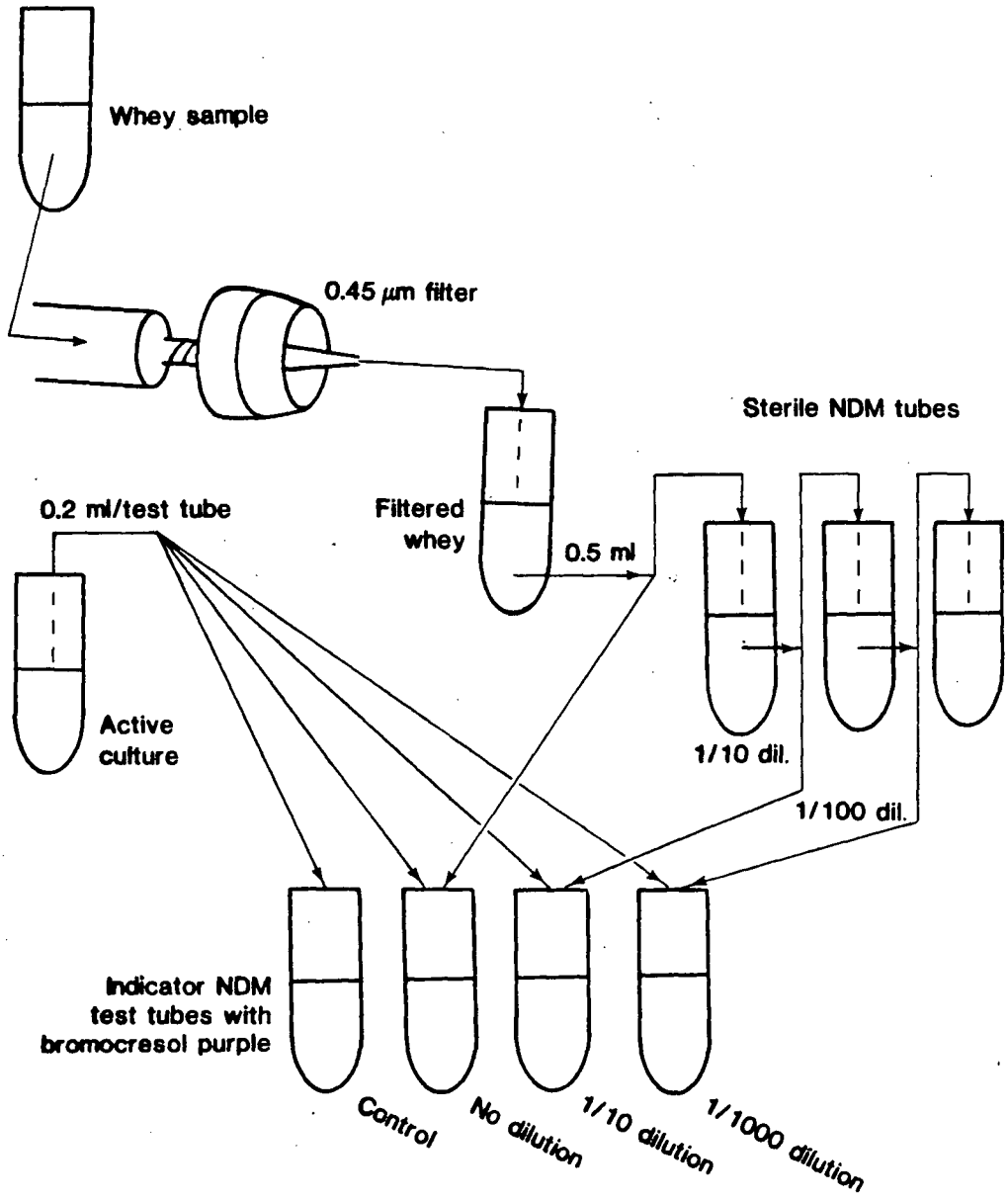


Figure 2.1 Method used to detect the presence of bacteriophage in whey with bromocresol purple as an indicator.

by 0.15 mL of 0.03% (wT/vol) fluorescamine (Chemical Dynamics Corp., South Plainfield, NJ) in acetone. The mixture was immediately mixed. To adjust the volume to the cuvette size, 3.0 mL of distilled water was added. A standard curve was made with tryptophan. Fluorescence (excitation = 395 nm; emission = 480 nm) was measured with an Aminco Bowman 4-8202 spectrophotoflurometer (American Instrument Co., Inc., Silver Spring, MD) and results reported in μg NH_2/gm cheese.

8. PREPARATION OF CHEESE SAMPLES

Cheddar cheese samples were prepared from the central regions of whole blocks (19 kg) to minimize the effect of moisture gradients which have been shown to exhibit non-symmetrical deformation on compression (Carter and Sherman, 1978; Culioli and Sherman, 1976). Samples (4°C) were cut into 2 cm cubes (Creamer and Olson, 1982) with a wire cheese cutter. All samples were weighed to confirm uniformity of size and placed in plastic sealed cups to prevent moisture losses. The cubes were allowed to equilibrate to room temperature (ca 22°C) for ca 6 h prior to compression testing.

9. TEXTURAL EVALUATION

Ten replicates of each sample of Cheddar cheese were compressed at ambient temperature (22°C) between flat parallel metal plates of a larger cross-sectional area on an Instron Model 1122 Universal Testing Machine. Force-compression data were recorded with the standard Instron recorder at a chart speed of 200 mm/min. All data reported were obtained using a constant crosshead speed of 50 mm/min.

10. SAMPLE PREPARATION FOR HPLC ANALYSIS

Commercially produced samples of Cheddar cheese made with either S. cremoris or S. lactis strains and of the correct composition (Lawrence and Gilles, 1982) and approximately the same age were selected for analysis. Extraction of the non-volatile water-soluble fraction was carried out according to the method of McGugan et al. (1979) except a smaller amount of sample was used (Pham and Nakai, 1984). The complete extraction procedure is described in Section 1 of Materials and Methods in Part II of this thesis.

11. STATISTICAL ANALYSIS

Computer analyses were performed on an Amdahl 470 V/8. Programs used were: BMD: 4M - Principal Component Analysis; BMD: 7M - Stepwise Linear Discriminant Analysis, BMDP Statistical Software Inc., 1981 Westwood Blvd., Suite 202, University of California. Description for analysis is outlined in Section 7 of Materials and Methods in Part II of this thesis.

12. HPLC ANALYSIS

A Spectra-Physics 8100 HPLC and 8400 variable wavelength detector (Spectra-Physics, Santa Clara, California) operated at a wavelength of 220 nm were used for the analysis with modification discussed in Part II - 4.

A reversed-phase column (250 x 4.6 mm I.D.) packed with Adsorbosphere C_8 (5 μm) purchased from Applied Sciences Laboratories (State College, Pennsylvania) was used for chromatographic runs. The volume of the sample loop was 50 μL.

All chromatographic runs were performed at ambient temperature at a flow rate of 0.97 mL/min. An optimized gradient elution system was used as

described in Section 5 in Part II of this thesis. The initial solvent composition was 44.6:0.0:55.4 for trifluroacetic acid (0.1%), acetonitrile and water. Over 56.6 min the ratio was changed to 0.0:36.6:63.4. Solvents were prepared as described in Section 4 in Part II of this thesis.

RESULTS AND DISCUSSION

RHEOLOGICAL PROPERTIES

The force-compression curves obtained for mild, medium and aged Cheddar samples subjectively evaluated first grade and with essentially the same moisture, fat, salt and pH are shown in Figure 3.1. In all cases the cheese deformed on compression and the force on the cheese increased until a yield point was reached. The initial portion of the curves were slightly convex then became concave. This is in agreement with Creamer and Olson (1982) who showed that low pH cheese has a steep convex force-compression curve relative to the Y-axis and crumbles at the yield point while a high pH cheese has a less steep, concave, force-compression curve and at the higher yield point it splits into large fragments.

The rheological properties of cheese have been shown to be related to the composition. Moreover, the degree of proteolysis determines the structure which in turn is responsible for the texture. Any alterations in the composition therefore can affect the final rheological properties. Cheese is essentially made up of a network of fat surrounded by protein (casein). Emmons et al. (1980) showed that reduced-fat cheese was considerably firmer and more elastic than full-fat cheese even though the moisture levels in the nonfat matter were the same. The textural differences found were related to moisture content, degree of proteolysis and pH of the cheese.

The pH, brought about through the production of lactic acid by starter culture, affects the physico-chemical properties of the cheese. A plausible explanation of the distinctly different textures of high and low pH cheeses was proposed by Creamer and Olson (1982). They concluded that fat stability in the protein/fat matrix of the cheese is not be affected by pH, however,

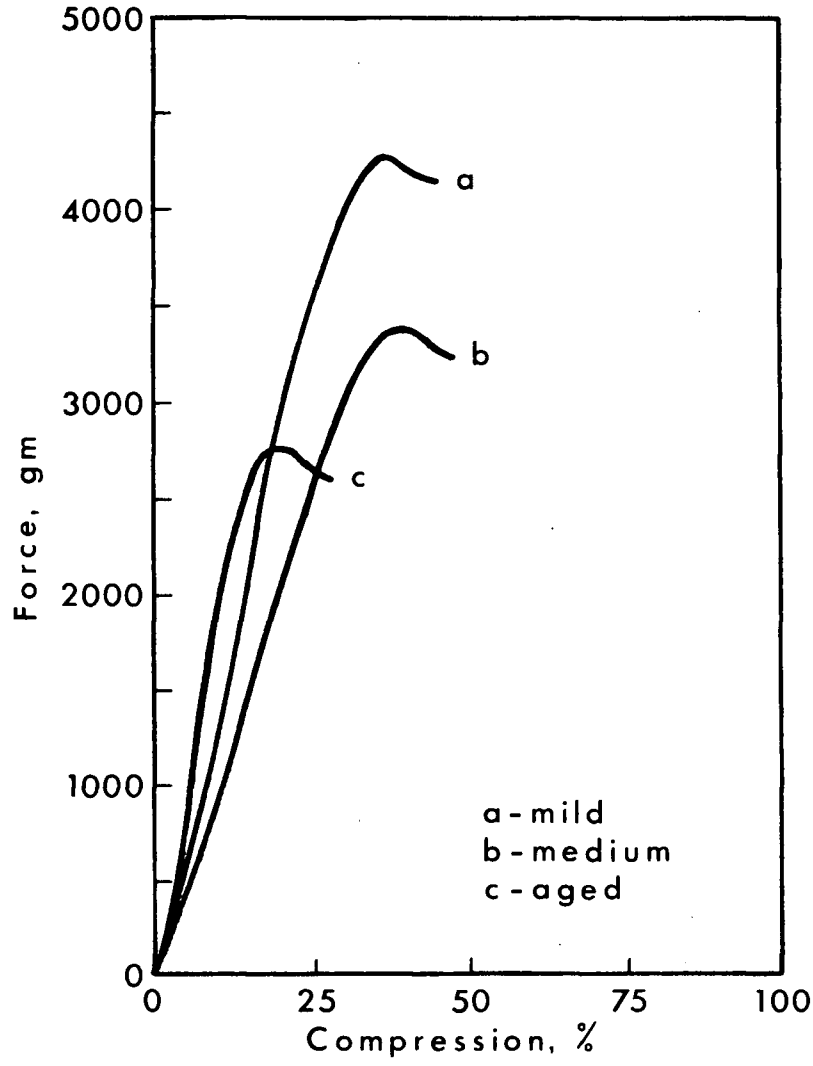


Figure 3.1 Force-compression curves for a mild, medium and aged cheese (samples number 18, 19 and 17 respectively from Table 4.1).

the protein fraction would be influenced. The major caseins have isoelectric points near 4.5. At this pH the casein components form compact aggregates which are held together by hydrophobic intra-aggregate forces while the inter-aggregate forces are weaker. Most of the water in such a system is ~~inert~~ bound and interstitial. At the higher pH, the casein molecules acquire a net negative charge resulting in repulsion and the tight protein aggregates absorb water. The dramatic differences obtained in the force-compression curves (Creamer and Olson, 1982) between experimentally produced high and low pH cheese were not found in this study. As shown in Table 2.1, differences in pH in commercially produced Cheddar was not as extreme. This was assumed to be due to the fact that all cheeses evaluated were within an intermediate pH range of 4.95-5.10 considered to be a low pH cheese (Lelievre and Gilles, 1982) with the exception of samples 13 and 36. Sample 36 was an extra-aged sample approximately 400 days old which did show a convex force-compression curve typical of low pH cheese and agreed with others.

Voisey (1977) explained that the initial concave then convex nature of the force-compression curve was due to adhesion between the sample and the cell surface in the case of the former. After compression the slope changes becoming convex and continues at a steady rate until the yield point. His study made no reference to age, composition or pH of the sample cheese. It is reasonable from the results of the present study and in agreement with others (Lelievre and Gilles, 1982) that Voisey's work was done on a low pH cheese and the particular force-compression curve is by no means representative of all Cheddar cheese.

The yield point for cheese samples occurred at greater force and greater compression for mild and medium cheeses (Figure 3.1). Rheological

CHEESE (sample no.)	FAT (%)	MOISTURE (%)	pH	SALT (%)	MNFS[1]	FDM[2]	S/M[3]
1	33.3	36.8	4.98	1.85	55.17	52.69	5.02
2	33.5	36.7	5.00	1.88	55.19	52.92	5.12
3	33.7	36.1	5.04	1.73	54.45	52.74	4.70
4	31.0	38.6	4.99	1.90	55.94	50.49	4.92
5	33.8	36.2	5.00	1.70	54.68	52.98	4.70
6	32.6	37.3	4.98	1.75	55.34	51.99	4.69
7	31.2	38.5	5.10	1.80	55.96	50.73	4.68
8	32.3	37.8	5.02	1.75	55.83	51.93	4.63
9	31.6	38.7	4.97	1.88	56.58	51.55	4.86
10	31.5	38.0	5.00	1.95	55.47	50.81	5.13
11	32.1	37.6	4.97	1.89	55.38	51.49	5.02
12	34.9	36.0	5.00	1.73	55.30	54.53	4.80
13	32.7	37.8	4.90	1.90	56.17	52.57	5.03
14	33.6	36.5	5.10	1.60	54.97	52.91	4.38
15	34.9	36.0	5.15	1.70	55.30	54.53	4.72
16	35.0	36.0	5.08	1.72	55.38	54.69	4.78
17	35.1	35.3	4.95	1.70	54.39	54.25	4.82
18	34.8	36.0	5.08	1.80	55.21	54.38	5.00
19	33.2	37.0	5.05	1.85	55.39	52.70	5.00
20	34.8	36.1	5.01	1.70	55.06	54.46	4.71
21	34.5	35.9	5.05	1.65	54.80	53.82	4.59
22	33.6	36.5	5.05	1.75	54.97	52.91	4.79
23	34.5	36.0	5.04	1.70	54.96	53.90	4.72
24	33.9	36.9	5.07	1.81	55.82	53.72	4.90
25	34.5	36.0	5.07	1.72	54.96	53.90	4.78
26	32.2	36.4	5.05	1.77	53.68	50.63	4.86
27	32.5	36.2	5.10	1.75	53.63	50.94	4.83
28	33.5	37.0	5.06	1.80	55.64	53.17	4.86
29	34.6	36.0	5.07	1.73	55.04	54.06	4.80
30	34.5	36.1	5.08	1.78	55.11	53.99	4.93
31	34.5	35.5	4.98	1.77	54.19	53.49	4.89
32	33.5	36.0	5.00	1.79	54.14	52.34	4.97
33	34.0	35.9	4.98	1.70	54.39	53.04	4.74
34	32.5	36.2	4.96	1.75	53.63	50.94	4.83
35	33.0	36.3	5.06	1.75	54.18	51.80	4.82
36	34.3	35.5	4.90	1.80	54.03	53.18	5.07

[1]MNFS - moisture in the non-fat substance; [2]FDM - fat-in-dry-matter; [3]S/M - salt-in-moisture.

Table 2.1. Compositional analysis of the commercially produced cheeses.

CHEESE (sample no.)	AGE (days)	YIELD POINT (g)	DISTANCE (mm)	% COMPRESSION AT YIELD POINT	DEFORMATION (mm)	FIRMNESS (g/mm)
1	199	3139.17	24.46	30.55	6.11	514.15
2	199	3427.00	24.65	30.80	6.16	556.21
3	116	4763.30	33.44	41.80	8.36	570.03
4	174	2997.50	30.92	38.65	7.73	389.42
5	160	3874.00	25.90	32.40	6.48	598.63
6	139	3546.00	29.40	36.75	7.35	483.47
7	185	2863.00	30.30	37.90	7.58	378.24
8	276	2234.00	20.75	25.95	5.19	430.97
9	227	2906.00	38.64	48.30	9.66	302.50
10	212	2507.00	29.60	37.00	7.40	339.93
11	221	2797.00	27.60	34.50	6.90	405.89
12	136	3944.00	29.70	37.15	4.73	531.89
13	300	3212.00	27.65	34.60	6.92	465.54
14	115	4361.00	30.85	38.55	7.71	567.44
15	36	4680.00	29.80	37.25	7.45	631.22
16	100	3425.00	25.70	32.15	6.43	535.32
17	424	2752.00	17.30	21.65	4.33	639.00
18	96	4292.00	30.50	38.15	7.63	563.18
19	150	3409.00	32.05	40.05	8.01	426.55
20	180	3650.00	32.00	40.00	8.00	456.25
21	180	3650.00	32.00	40.00	8.00	456.25
22	180	3550.00	33.00	41.25	8.25	430.30
23	180	3450.00	32.00	40.00	8.00	431.25
24	100	4400.00	31.00	38.75	7.75	567.74
25	100	4500.00	31.50	39.40	7.88	571.06
26	100	4480.00	81.00	37.50	7.50	597.33
27	100	4580.00	31.00	38.75	7.75	590.00
28	100	4550.00	29.00	36.25	7.25	627.59
29	100	5300.00	28.00	35.00	7.00	757.14
30	300	3200.00	28.00	35.00	7.00	457.14
31	300	3053.00	28.00	35.00	7.00	435.72
32	300	3390.00	27.50	34.40	6.88	492.73
33	300	3250.00	26.50	33.15	6.63	490.19
34	300	3290.00	28.00	35.00	7.00	470.00
35	300	3350.00	28.50	35.65	7.13	469.85
36	424	2752.00	17.30	21.65	4.33	639.00

Table 3.1. Rheological data for the commercially produced cheeses of different ages, composition and culture type.

data (Table 3.1) are consistent with the fact that younger cheese is more resistent to deformation. This is discussed later in 'Effects of Proteolysis'.

Deformation curves of the cheese samples were concave during compression due to the nature of the contact surface between the cheese and the plates of the Instron. This was assumed to be due to the oil (butterfat) present in the cheese which lubricated the metal plates of the Instron thus preventing a typical barrel shape during compression found by others (Culioli and Sherman, 1976).

EFFECT OF MOISTURE CONTENT

Table 2.1 shows the compositional analysis of the 36 commercially produced cheeses which were all subjectively evaluated by the Federal Graders as first grade. It can be seen that moisture in the non-fat-substance, fat-in-dry-matter, salt-in-moisture and pH were within optimum specifications for a quality cheese (Lelievre, 1983; Lelievre and Gilles, 1982).

Cheeses varying in moisture but of approximately the same composition, age and degree of proteolysis (Figure 4.1) show force-compression curves that are affected by moisture content alone. As the moisture content increases, the firmness of the cheese as indicated by the slope decreases. Figure 5.1 shows the yield force vs moisture content for cheese samples 1, 2, 4-14, 16 that were subjectively evaluated (Table 4.1) as being weak or slightly weak texture. The coefficient of determination for the regression line was 0.788. With the exception of samples number 12, 13 and 16 all others were evaluated as mild cheese regardless of age. It seems reasonable to assume, therefore, that moisture content is an important

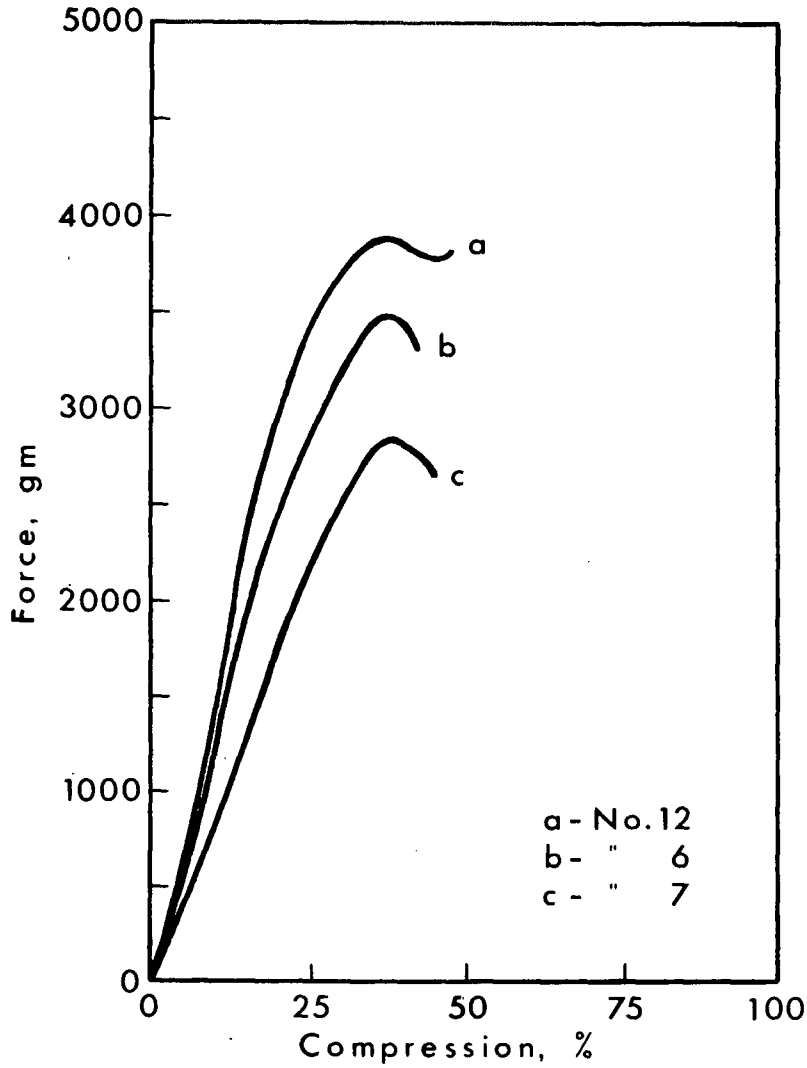


Figure 4.1 Force-compression curves for cheese of approximately the same age, composition and degree of proteolysis but varying in moisture content. Samples number 12, 6 and 7 with moisture of 36.0, 37.3 and 38.5% respectively. See Tables 2.1, 3.1, 4.1.

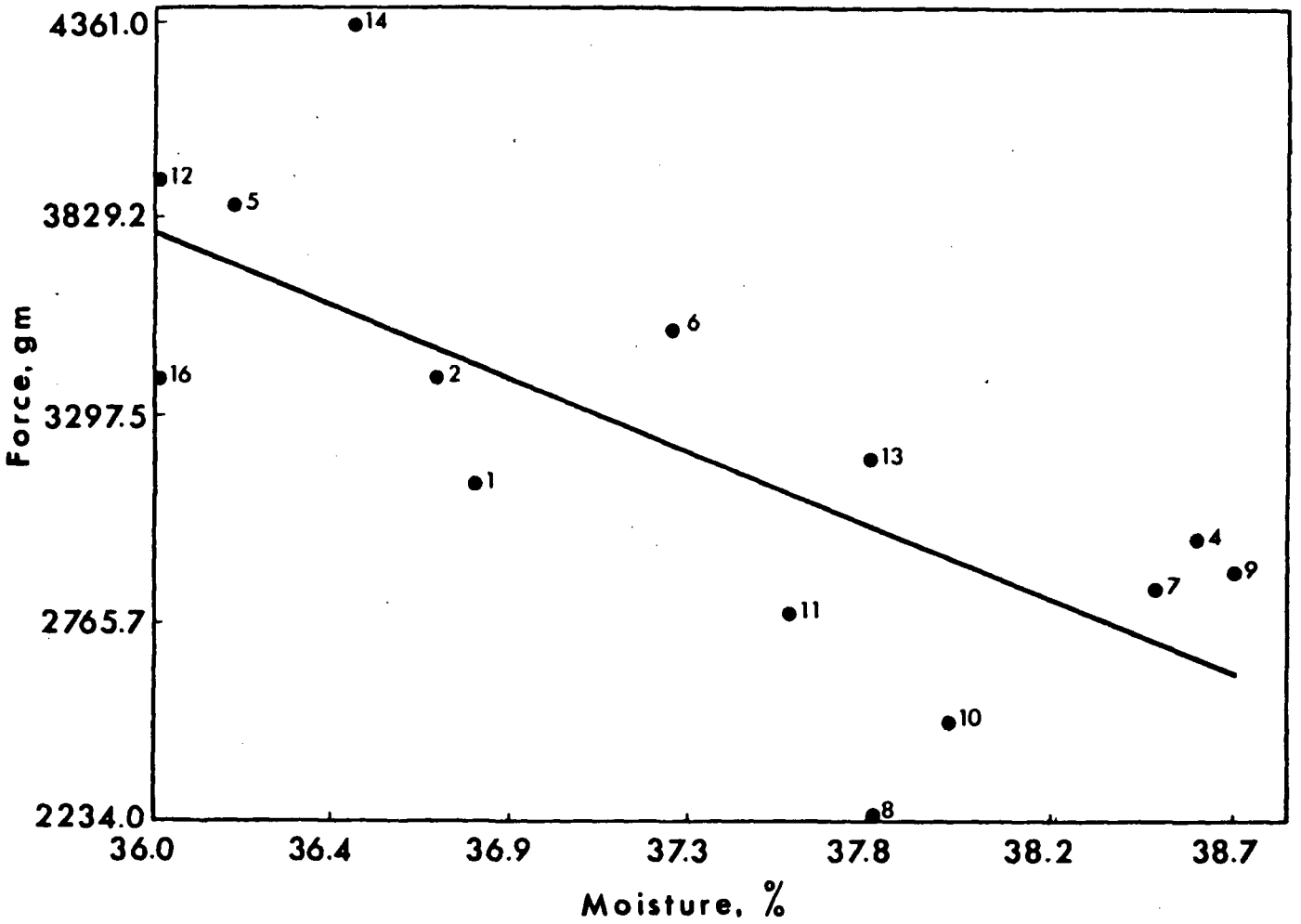


Figure 5.1 Variation in the force at the yield point for cheese samples number 1, 2, 4-14, 16 subjectively evaluated weak of slightly weak texture. See Table 2.1 for composition (r^2 = 0.788).

Table 4.1. Rheological, compositional and sensory evaluation of the commercially produced cheese samples of various ages using S. cremoris or S. lactis culture strains on both conventional and automated cheese processing equipment.

	A	B	C	D	E	F	G	H	I	J	K_1	K_2
NO.	YIELD POINT (g)	DISTANCE (mm)	% COMPRESSION AT YIELD POINT	DEFORMATION (mm)	FIRMNESS (g/mm)	MOISTURE (%)	$-NH_2$	CULTURE[a]	AGE (days)	PROCESS[b]	CATEGORY[c]	TEXTURE[d]
1	3139.17	24.46	30.55	6.11	514.15	36.8	46.0	1	199	1	1	2
2	3427.00	24.65	30.84	6.16	556.21	36.7	48.1	1	199	1	1	2
3	4763.30	33.44	41.80	8.36	570.03	36.1	38.0	2	116	1	1	1
4	2997.5	30.92	38.65	7.73	389.42	38.6	39.1	1	174	1	1	3
5	3874.00	25.90	32.40	6.48	598.63	36.2	41.0	2	160	1	1	2
6	3546.00	29.40	36.75	7.35	483.47	37.3	35.0	2	139	1	1	2
7	2863.00	30.30	37.90	7.58	378.24	38.5	40.0	1	185	1	1	3
8	2234.00	20.75	25.95	5.19	430.97	37.8	54.1	1	276	1	1	3
9	2906.00	38.64	48.30	9.66	302.50	38.7	47.0	1	227	1	1	3
10	2507.00	29.60	37.00	7.40	339.93	38.0	48.5	1	212	1	1	3
11	2797.00	27.60	34.50	6.90	405.89	37.6	50.3	1	221	1	1	3
12	3944.00	29.70	37.15	7.43	531.89	36.0	59.7	2	136	1	2	2
13	3212.00	27.65	34.60	6.92	465.54	37.8	61.0	1	300	1	2	3
14	4361.00	30.85	38.55	7.71	567.44	36.5	42.6	2	115	1	1	2
15	4680.00	29.80	37.25	7.45	631.22	36.0	35.6	2	36	1	1	1
16	3425.00	25.70	32.15	6.43	535.32	36.0	51.6	2	100	1	2	2
17	2752.00	17.30	21.65	4.33	639.00	35.3	94.2	2	424	2	4	1
18	4292.00	30.50	38.15	7.63	563.18	36.0	38.5	2	96	2	1	1
19	3409.00	32.05	40.05	8.01	426.55	37.0	60.1	2	150	2	2	1
20	3650.00	32.00	40.00	8.00	456.25	36.1	59.0	2	180	2	2	1
21	3650.00	32.00	40.00	8.00	456.25	35.9	57.8	2	180	2	2	1
22	3550.00	33.00	41.25	8.25	430.30	36.5	61.3	2	180	2	2	1
23	3450.00	32.00	40.00	8.00	431.25	36.0	58.6	2	180	2	2	1
24	4400.00	31.00	38.75	7.75	567.74	36.9	34.0	2	100	2	1	1
25	4500.00	31.50	39.40	7.88	571.06	36.0	35.0	2	100	2	1	1
26	4480.00	31.00	37.50	7.50	597.33	36.4	36.1	2	100	2	1	1
27	4580.00	31.00	38.75	7.75	590.00	36.2	35.8	2	100	2	1	1
28	4550.00	29.00	36.25	7.25	627.59	37.0	37.2	2	100	2	1	1
29	5300.00	28.00	35.00	7.00	757.14	36.0	38.0	2	100	2	1	1
30	3200.00	28.00	35.00	7.00	457.14	36.1	57.8	2	300	2	3	1
31	3053.00	28.00	35.00	7.00	435.72	35.5	59.0	2	300	2	3	1
32	3390.00	27.50	34.40	6.88	492.73	36.0	60.9	2	300	2	3	1
33	3250.00	26.50	33.15	6.63	490.19	35.9	59.7	2	300	2	3	1
34	3290.00	28.00	35.00	7.00	470.00	36.2	61.0	2	300	2	3	1
35	3350.00	28.50	35.65	7.13	469.85	36.3	58.5	2	300	2	3	1
36	2752.00	17.30	21.65	4.33	639.00	35.5	94.2	2	424	2	4	1

a 1 - S. cremoris; 2 - S. lactis.
b 1 - automated; 2 - conventional.
c 1 - mild; 2 - medium; 3 - aged; 4 - X-aged.
d 1 - firm; 2 - slightly weak; 3 - weak.

factor affecting texture and is not related to culture type. Indeed, relationships between various cheese-making parameters and grade established clearly that MNFS and S/M were the single most important parameters affecting the grade score of the cheese (Pearce and Gilles, 1979; Lawrence and Gilles, 1980). This information is in agreement with the present study.

The effect of moisture was found to be more closely related to the amount of moisture per unit of casein than with the absolute percentage of moisture in the cheese (Lawrence and Gilles, 1980) and it is in the mixture of moisture and casein that enzymatic reactions responsible for ripening largely take place. Small increases in the MNFS leads to the availability of free moisture as seen in Table 2.1. This free moisture increases the activity of both micro-organisms and enzymes and yet has a detrimental effect on cheese texture.

Cheeses with the lower S/M tended to mature quicker because of the reduced inhibitory effect of the salt on both starter culture bacteria and normal milk flora. During aging, however, the low S/M cheese deteriorated in quality and was downgraded due to the production of undesirable flavour components.

While small proportions of NaCl have stimulatory effects on lactic cultures, larger proportions exhibit toxic properties (Meister and Ledford, 1979). Walter *et al.* (1958) reported that strains of *S. lactis* were not significantly inhibited in Cheddar cheese curd by 1.6 to 2.0% NaCl, whereas most *S. cremoris* strains were inhibited slightly at 1.4%, definitely at 1.6% and almost completely at 2.0%.

The trained cheese graders had no difficulty in distinguishing cheeses of different moisture contents. This is reasonable since much of the water in Cheddar cheese is "bound" specifically to the caseins, their degradation

products and to the calcium lactate and sodium chloride present. Thus, a small increase in MNFS leads to a relatively large increase in freely available moisture which has been shown to be easily detected by the trained graders. Increases in salt content were also easily detected because of the coarseness and other detrimental effects it has on texture.

Although microstructure examination was not the scope of this study, Emmons et al. (1980) showed that increased moisture content decreased firmness and elasticity and that a relationship between microstructure, composition and texture existed. Increased moisture, specifically MNFS was responsible for a reduced firmness in the cheese texture confirming the results of the present study.

Lawrence and Gilles (1980) found with some modern cheesemaking systems, that both the curd particle size and the curd matrix are not uniform. This variable structure results in curd of differing moisture content since a highly structured curd will retain significantly more moisture than a non-cheddared curd. Although increased moisture was directly related to a decrease in yield force (Figure 4.1), the results were not dramatic, while a definite tendency was shown. This was due to the fact that the variability in composition did not exceed the ranges for a quality cheese (Lelievre and Gilles, 1982).

EFFECT OF PROTEOLYSIS

It has been considered that proteolysis occurring during ripening could be due to enzymes from two major sources: namely rennet and the starter culture. Although most of the rennet is removed with the whey, a small amount does remain with the curd (Holmes et al., 1977). The action of this residual clotting enzyme combined with that of enzymes from starter

bacteria and normal milk flora enzymes largely determine the overall proteolytic pattern in ripening cheese. The principal pathway of proteolytic breakdown during the ripening process suggested by Lowrie and Lawrence (1972) is one in which rennet causes a primary, limited degradation of caseins to high molecular weight non-bitter peptides that are further hydrolyzed by enzymes from the bacteria in the starter culture to smaller peptide fragments and free amino acids.

The importance of rennet for cheese flavour development and subsequent texture has been extensively reviewed (Fox, 1981). Throughout this study, in order to minimize variation in cheese flavour and/or texture, extracellular proteinases from Mucor mehei were used as the rennet source. The success of using this enzyme in commercially produced cheeses has been well documented. (Visser, 1981).

As shown in Table 4.1, the degree of proteolysis, expressed as free amino groups, and moisture content have an effect on the yield point of Cheddar cheese. As the cheese ages, proteolysis increases (Figure 6.1) and yield point decreases.

Electrophoretic studies (Creamer and Olson, 1982) have related the content of intact α_{s1}-casein to yield force, while de Jong (1975) developed a quantitative electrophoretic method to study proteolysis. They found that as the cheese ages, α_{s1}-casein is degraded to α_{s1}-I-casein while β-casein remains essentially intact. Although electrophoresis is a valuable analytical technique, fluorescamine which reacts specifically with free amino groups at the appropriate pH serves as a more sensitive assay method for proteolysis of protein in cheese by proteinases from the starter culture. The procedure which is highly sensitive and related to the number of peptide bonds hydrolyzed clearly suggests that all other parameters

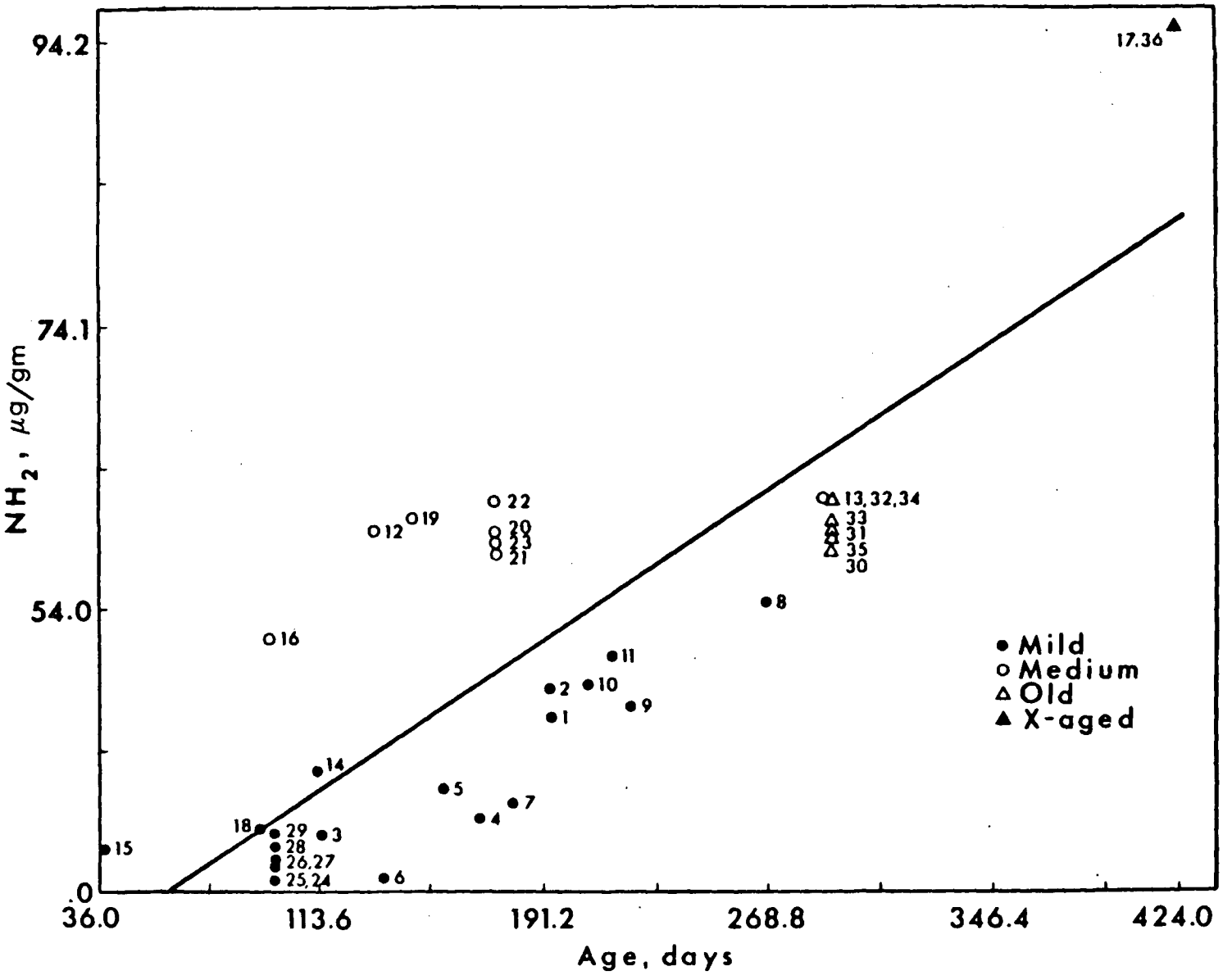


Figure 6.1 Variation in the free amino groups for cheeses of different ages commercially produced using either S. cremoris or S. lactis culture strains. See Table 4.1.(r^2 = 0.862).

being equal, cheeses manufactured using S. cremoris strains have a lesser degree of proteolysis and subsequent flavour development for approximately the same age than those produced with S. lactis cultures although initial acid production was the same (Figure 6.1). A possible explanation is that the buffered pH controlled starter media with added nitrogenous stimulants allowed selective growth of Prt^- cells (Richardson et al., 1983) to at least 93% of their Prt^+ counterparts. Prt^- variants are deficient in cell wall-bound proteinase and have been found to reach only 10-25% of the maximum cell density attained by Prt^+ organisms (Thomas and Mills, 1981). Cell wall-bound proteinases allow the starter cells to hydrolyze the milk proteins to peptides which are then small enough to diffuse into the cell wall and further degraded by the intracellular peptidases to free amino acids. These free amino acids are essential for the growth of the starter bacteria. Therefore, the greater the cell density the higher is the concentration of proteinase which increases exponentially with the growth of the culture. Indeed it is feasible that the S. cremoris strains used in this study did not reach maximum cell densities in the milk (in spite of acid production) compared to their S. lactis counterparts thus providing a possible explanation for the limited proteolytic activity due to lower amounts of cell wall-bound proteinases. Cheese made with S. cremoris and of approximately the same age as that made with S. lactis starter culture was subjectively graded mild while the latter was graded medium. Generally cheeses made with S. lactis ripened to medium in approximately 6 months, whereas those using S. cremoris strains required an additional 3 months to reach the same degree of maturity after which flavour differences were minimal. In case no 8 (Tables 3.1 and 4.1) the cheese was 276 days old

yet still classified as mild and case no 13, a 300 day old cheese was classified as medium.

The pH of cheese is influenced by the bacterial culture used. Moreover, Adda et al. (1982) showed that the pH of the cheese controlled the ripening process and together with the water content affected texture. As the pH drops during lactose fermentation, the acidity of the curd leads to solubilization of the phosphates and calcium linked to the protein micelles while at the same time lowering the activity of lipolytic and proteolytic enzymes. The lactic acid produced during ripening serves as a substrate for natural milk flora, not destroyed by cheese cook temperatures (37.8°C), to raise the pH to optimal levels for enzyme activity. Assuming the interrelationship between pH and proteolytic activity their effect on cheese texture clearly showed that cheese made with S. cremoris had force yield values typical of mild cheese regardless of age (Table 4.1) and required additional ripening time compared to cheese made with S. lactis before rheograms were typical of medium or aged cheese. (Figure 4.1, sample no. 7).

Rate of initial acid development in spite of the lower inoculum of 0.8% was the same for both S. lactis and S. cremoris cultures due to the large number of organisms inoculated, which is in agreement with others (Thunell et al., 1981; Lawrence et al., 1978).

EFFECT OF HEAT TREATMENT

Force-compression curves for cheese of approximately the same composition, pH and age were affected by the initial heat treatment of the milk. Heat-treated milk (63°C/16 sec) had a higher yield force (Figure 7.1) compared to pasteurized milk (72°C/16 sec). In cheese made from

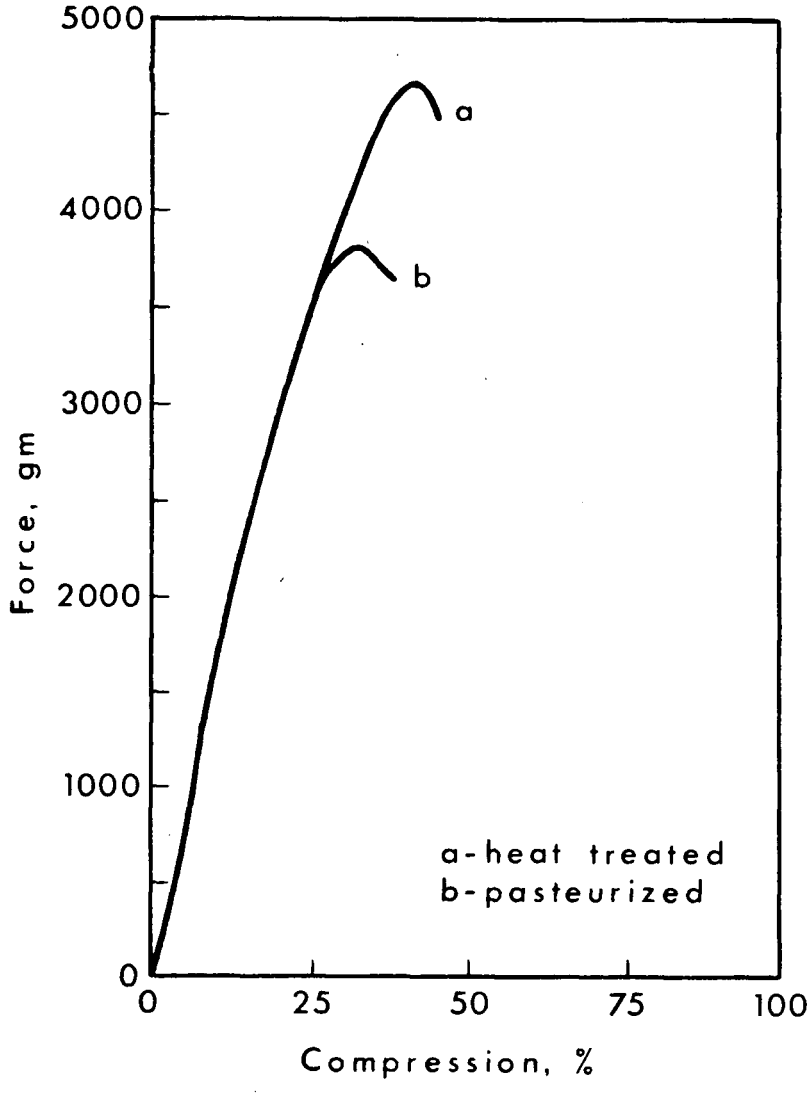


Figure 7.1 Effect of heat treatment for cheese milk on force-compression curves for commercially produced cheese of approximately the same age, composition and degree of proteolysis. a, 63°C/16s; b, 72°C/16s; (Samples no. 3 and 5 respectively Table 4.1).

pasteurized milk the serum proteins, particularly β-lactoglobulin, react through SH bonding with the casein thus destroying the capacity of the casein to form a firm curd resulting in a weaker texture cheese (Figure 7.1). In addition to the irreversible protein denaturation, the elevated temperatures have been implicated in altering heat-labile enzyme substrates (Visser, 1981). Thus the balance between active enzymes, substrates and resulting proteolytic breakdown products are altered or inhibited. This may explain why heat treated milk has been traditionally considered to produce more flavourful cheese.

Differences in the force-compression curves between heat-treated (63°C/16 sec) and pasteurized milk (72°C/16 sec) cheeses were obtained (Figure 7.1). It was assumed that cheese texture and enzyme activity were due to the composition of the cheese and the culture type used respectively and not affected by the heat treatment. Indeed both S. cremoris and S. lactis were used for making pasteurized milk cheese yet their proteolytic activities were different (Figure 6.1).

STEPWISE LINEAR DISCRIMINANT ANALYSIS (AGE)

Data from individual cheese analysis appear in Table 4.1, rows A through J. However, classification of the cheese samples into distinct groups of mild, medium, old and X-aged as well as distinct categories for texture (weak, slightly weak and firm) was assessed by Grader sensory evaluation.

The data from the 36 commercially produced cheese samples (Table 4.1) were analyzed by stepwise linear discriminant analysis (SLDA). The technique is based on the concept of classification of objects on the basis of the patterns formed by a set of observations or results. In the

analysis each cheese sample was considered as a point in a p-dimensional space (p being the number of variables appearing in Table 4.1, A through J). Each cheese sample analyzed, therefore, has a set of results which constitutes a pattern. Since groups of similar samples will have similar patterns, this information can be used to identify groups of similar samples or it can be used to characterize a group of similar samples in order to enable one to classify the group to which a new sample belongs. In the regression analysis, K_1 assessed by Grader evaluation was used for classification of the cheeses into age categories. All other data were considered independent variables. The program selects the most significant independent variable first followed by the second most significant etc., in a stepwise fashion. The F-value of each variable is computed and the variable with the highest F-value is the one entered at the first step. This variable is then paired with each of the other available variables, one at a time and the one giving the highest increase in discrimination is the next variable chosen. This is then entered in the next step along with the first variable and the same procedure is continued until the F-probability of deletion becomes too high (Coomans et al., 1979).

The significant difference among the four age groups was observed as shown in Table 5.1. By reduction of the original number of independent variables from 11 to 4, 100% of the sample cases were correctly identified (Table 6.1).

Because of the difficulty in visualizing the multivariate data obtained in a p-dimensional space, the intent of SLDA is to reduce p to 2 so that data obtained can be represented in a two dimensional plot or space (X- and Y- axis). For this purpose two main canonical variables were calculated. The first canonical variable which is a linear combination of

Table 5.1. F-matrix among the four age groups (mild, medium, old, X-aged) for the cheese samples, after discriminant analysis.

Age	Age		
	Mild	Medium	Old
Medium	76.14**		
Old	67.54**	0.10	
X-Aged	220.33**	86.62**	77.39**

**P < 0.01, F (4, 29; 0.01) = 4.04

Table 6.1. Classification-matrix for age generated by stepwise discriminant analysis. 100% of the cases were correctly classified using F-to-enter of 4.0.

		NUMBER OF CASES CLASSIFIED INTO GROUP			
GROUP	PERCENT CORRECT	MILD	MEDIUM	OLD	X-AGED
MILD	100.0	20	0	0	0
MEDIUM	100.0	0	8	0	0
OLD	100.0	0	0	6	0
X-AGED	100.0	0	0	0	2
TOTAL	100.0	20	8	6	2

observations that best discriminate among the samples is plotted on the X-axis. The second canonical variable which is the next best linear combination, orthogonal to the first one is plotted on the Y-axis. Therefore, each cheese sample is represented by a pair of canonical variables. A geometric interpretation for age classification (Figure 8.1) shows the coordinates for all samples which are clearly separated into mild, medium, old and X-aged categories. In addition to the graphic representation, SLDA enables classification of unknown samples by using discriminant functions characteristic of each group of cheese.

Perfect agreement for age classification between subjective sensory evaluation and SDLA was obtained. The degree of proteolysis ($-NH_2$), age of cheese (days), culture type and firmness were selected as the most important variables in this order for discrimination among sample cases according to age. The degree of proteolysis, age of cheese and firmness have already been shown to affect age classification, yet the statistical techique used suggests the important contribution of culture type to degree of age (i.e. mild, medium, aged and X-aged).

STEPWISE LINEAR DISCRIMINANT ANALYSIS (TEXTURE)

Data from individual cheese analysis appear in Table 4.1. Texture (K_2) in Table 4.1 was assessed by sensory Grader evaluation. The cheeses were divided into three classes: weak, slightly weak and firm and used for classification in SLDA. The significant difference among the three texture classifications is observed as shown in Table 7.1. By reduction of the original 11 variables to 3, 94.4% of the cases are correctly classified (Table 8.1).

A geometric interpretation for texture classification (Figure 9.1)

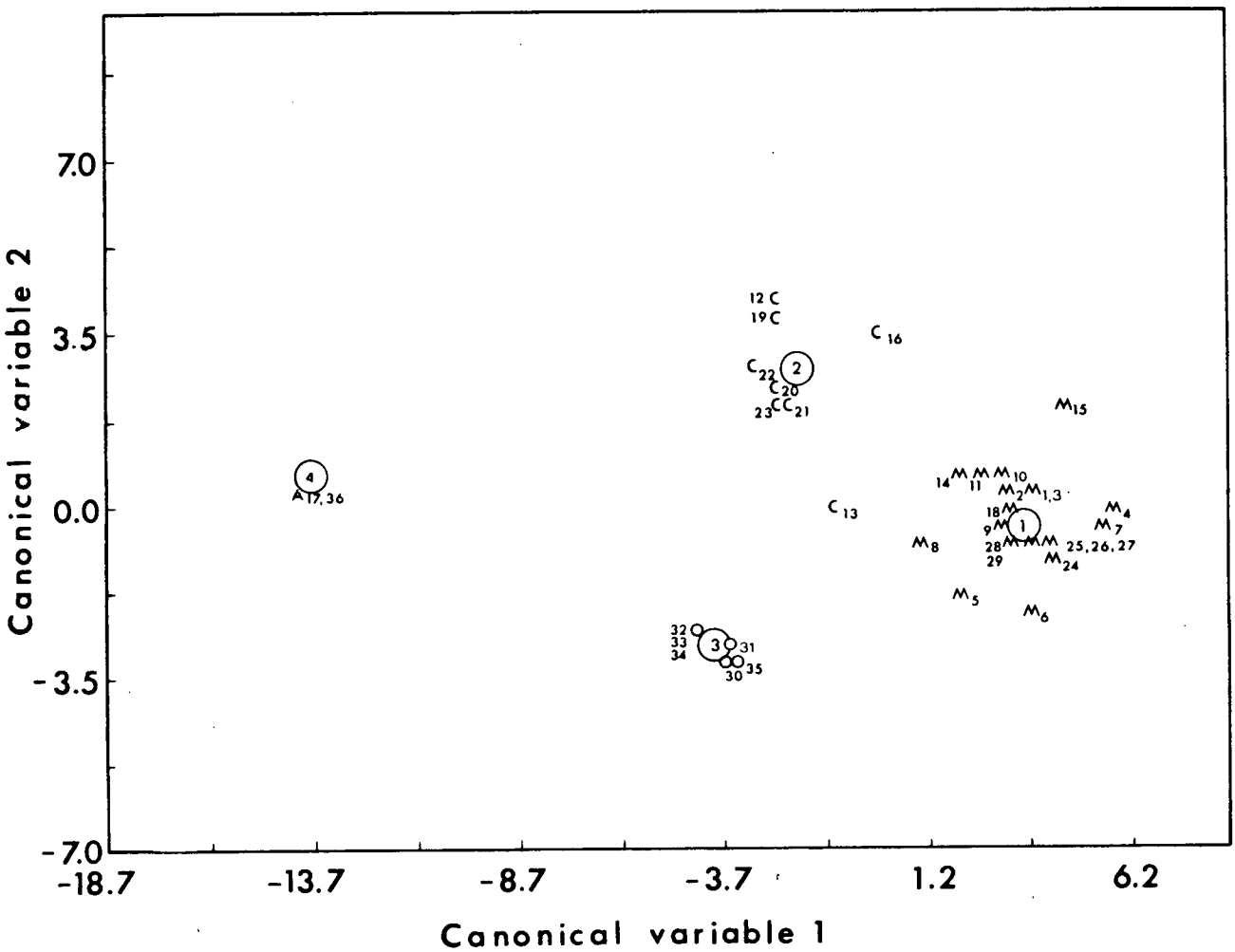


Figure 8.1 Canonical plot for age for the 36 commercially produced cheeses using the multivariate data in Table 4.1. A, X-aged; O, old; C, medium; M, mild. Group means indicated by circles.

Table 7.1. F-matrix among the three texture groups (firm, slightly weak, weak) for the cheese samples after discriminant analysis.

TEXTURE	Texture	
	Firm	Sl. Weak
Sl. weak	41.22**	
Weak	95.08**	22.93**

**P < 0.01, F (3, 31; 0.01) = 4.51

Table 8.1 Classification-matrix for texture generated by stepwise discriminant analysis. 94.4% of the cases were correctly classified using F-to-enter of 4.0.

GROUP	PERCENT CORRECT	NUMBER OF CASES CLASSIFIED INTO GROUP		
		FIRM	SL. WEAK	WEAK
FIRM	90.0	20	2	0
SL. WEAK	100.0	0	7	0
WEAK	100.0	0	0	7
TOTAL	94.4	20	9	7

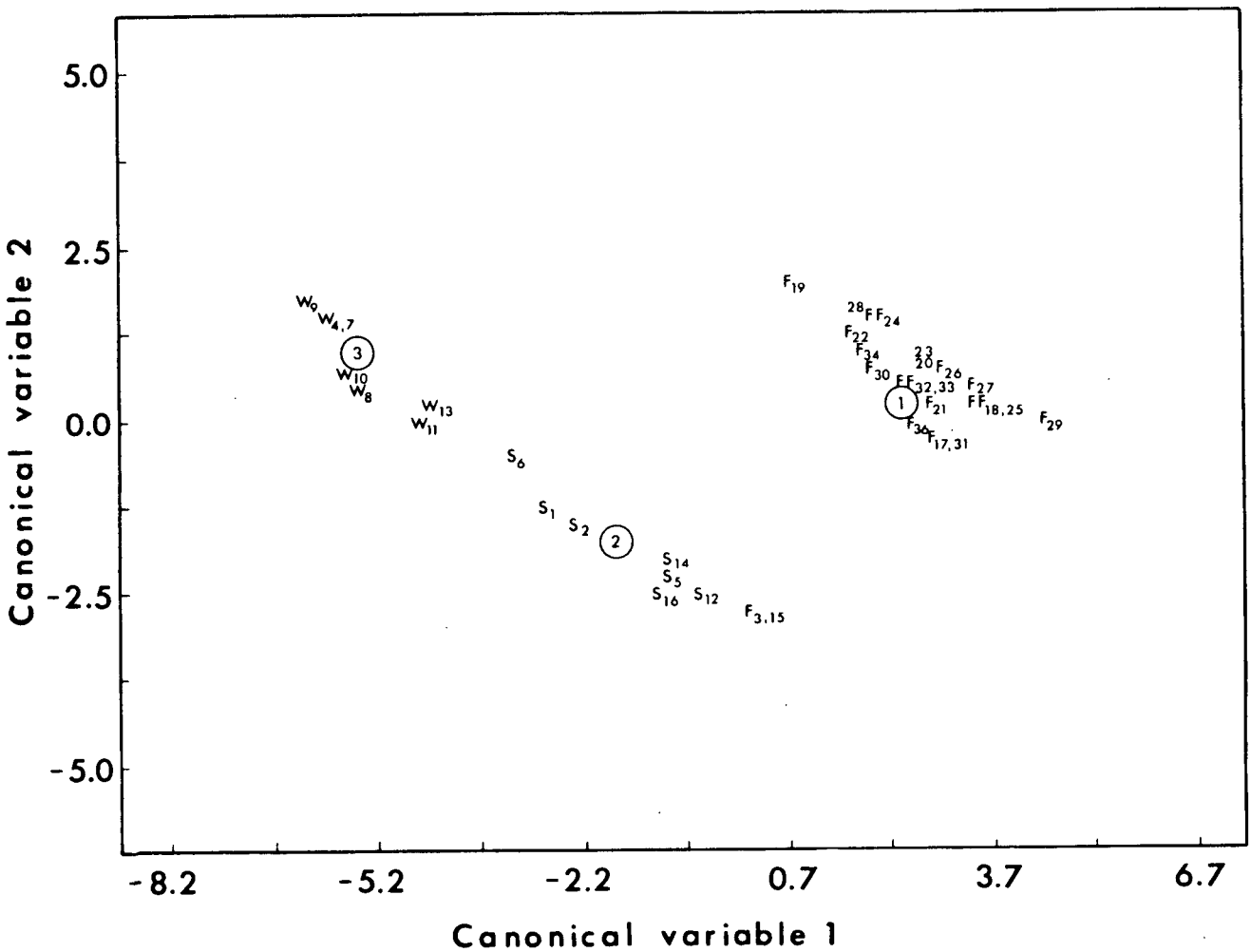


Figure 9.1 Canonical plot for texture for the 36 commercially produced cheeses using the multivariate data in Table 4.1. F, firm; S, slightly weak; W, weak. Group means indicated by circles.

clearly separates all samples into three distinct groups. Two misclassifications, samples 3 and 15, occurred based on the data provided and would be better classified as slightly weak. It is reasonable to assume that the sensory evaluation was incorrect and SLDA provides a more objective assessment in that it is based entirely on the chemical, physical and processing data. In fact, Lelievre and Gilles (1982) in their study of the relationship between grade and composition of young commercial Cheddar cheese showed that sensory evaluation varied from grader to grader.

Results of the discriminant analysis indicated that moisture, process (conventional or automated) and yield point were the most important variables for discrimination among sample cases according to texture. The cheese making conditions particularly with automated equipment tend to result in cheese whose composition may change significantly throughout the course of a single day's production (Lawrence and Gilles, 1980). This does not suggest that the cheese is of poorer quality; however, it may mature at a different rate. Although only 16 of the 36 cheese samples were manufactured on automated equipment, the statistical technique selected the process as the second most important variable affecting texture. Figure 10.1 shows the yield force vs. moisture for samples 1 - 11, 14, 15, 18, 24 - 29 grouped as mild by discriminant analysis. It would appear that cheeses of similar composition and degree of proteolysis have yield forces that are influenced by moisture alone. Samples number 4, 7 - 11, 13 were assessed as being weak texture cheese by sensory evaluation and in all cases were analyzed as high moisture. Yield force vs. moisture content for cheese identified as weak or slightly weak by SLDA (samples number 1 - 16) is shown in Figure 11.1. The only difference between the statistical analysis of cheese texture (r^2 = 0.817) and that of subjective evaluation

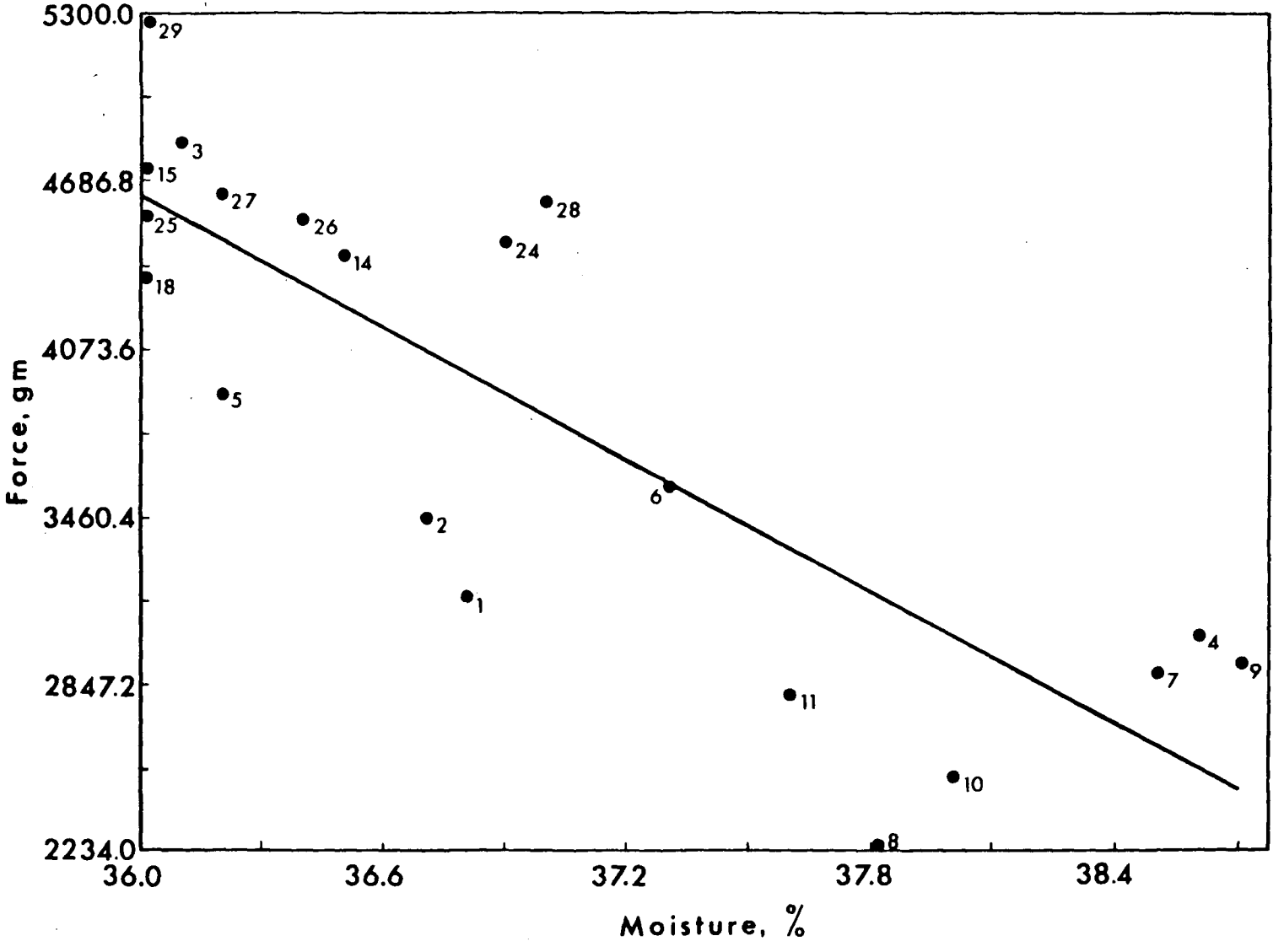


Figure 10.1 Variation in the force at the yield-point for cheese samples number 1-11, 14, 15, 18, 24-29 grouped as mild by SLDA and approximately same composition but varying moisture. See Table 3.1 for composition (r^2 = 0.853).

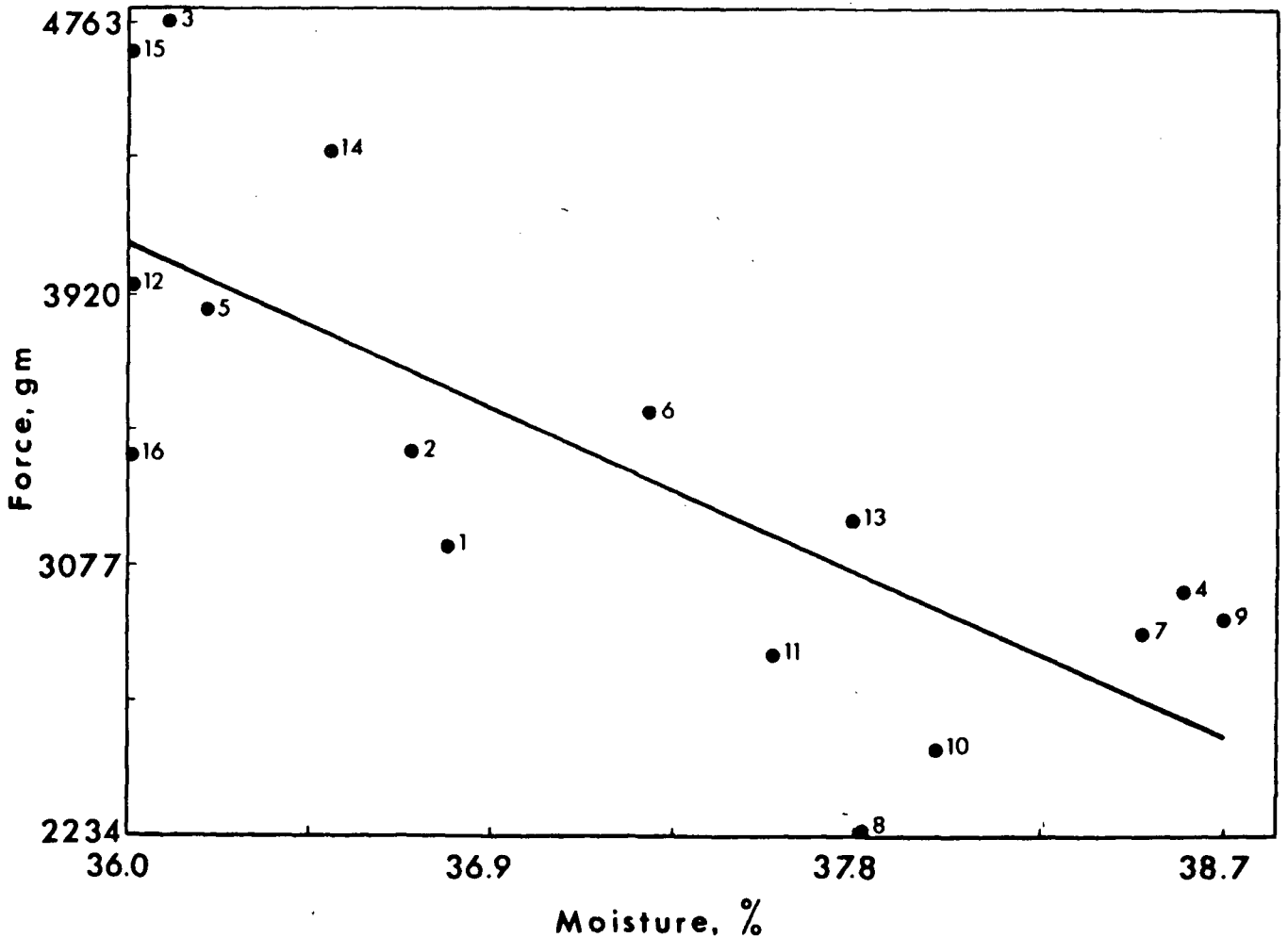


Figure 11.1 Variation in the force at the yield-point for cheese samples number 1-16 (r^2 = 0.817). Numbers 4, 7-11, 13 of approximately the same composition but varying moisture classified by SLDA as weak or slightly weak texture. See Table 3.1 for composition.

(r^2 = 0.788) was that samples no. 3 and 15 were classified as slightly weak by the former technique.

HPLC ANALYSIS

The non-volatile flavour components containing the salts, amino acids and peptides and shown to contribute to Cheddar flavour (McGugan et al., 1979) were extracted from samples number 2 and 12 (Table 4.1). These samples were selected firstly because of their similar composition and same manufacturing process but more importantly to demonstrate the effect of starter culture type on cheese taste. Sample 2 made with S. cremoris was 199 days old and assessed by sensory evaluation as mild whereas sample 12 made with S. lactis was 136 days old and graded as medium. Quantitative determination of proteolysis by assaying for free amino groups clearly showed less proteolysis in cheese made with S. cremoris compared to that made with S. lactis (Table 4.1) although the former was 63 days older than the latter. The HPLC profile using an Adsorbosphere C_8 column and optimized gradient elution as discussed in Part II indicated less protein degradation products in cheese made with S. cremoris (Figure 12.1) compared to that made with S. lactis (Figure 13.1). Indeed, based on the respective HPLC pattern obtained and using stepwise linear discriminant analysis (Part II), the cheeses were classified into mild and medium categories respectively regardless of age.

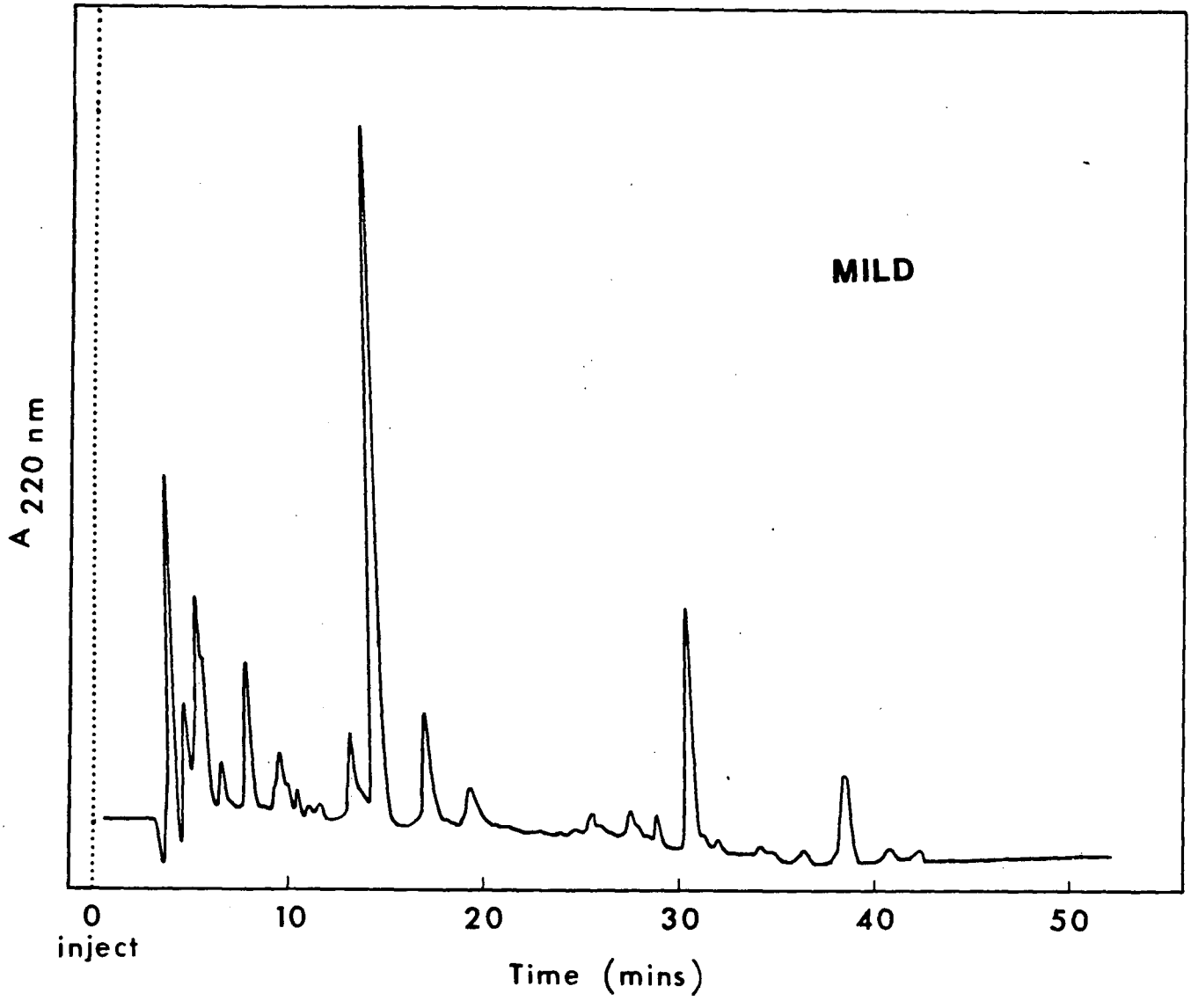


Figure 12.1 HPLC profile of the water extract from a 199 day old cheese, made with S. cremoris and subjectively graded mild. See Table 4.1 (sample no 2).

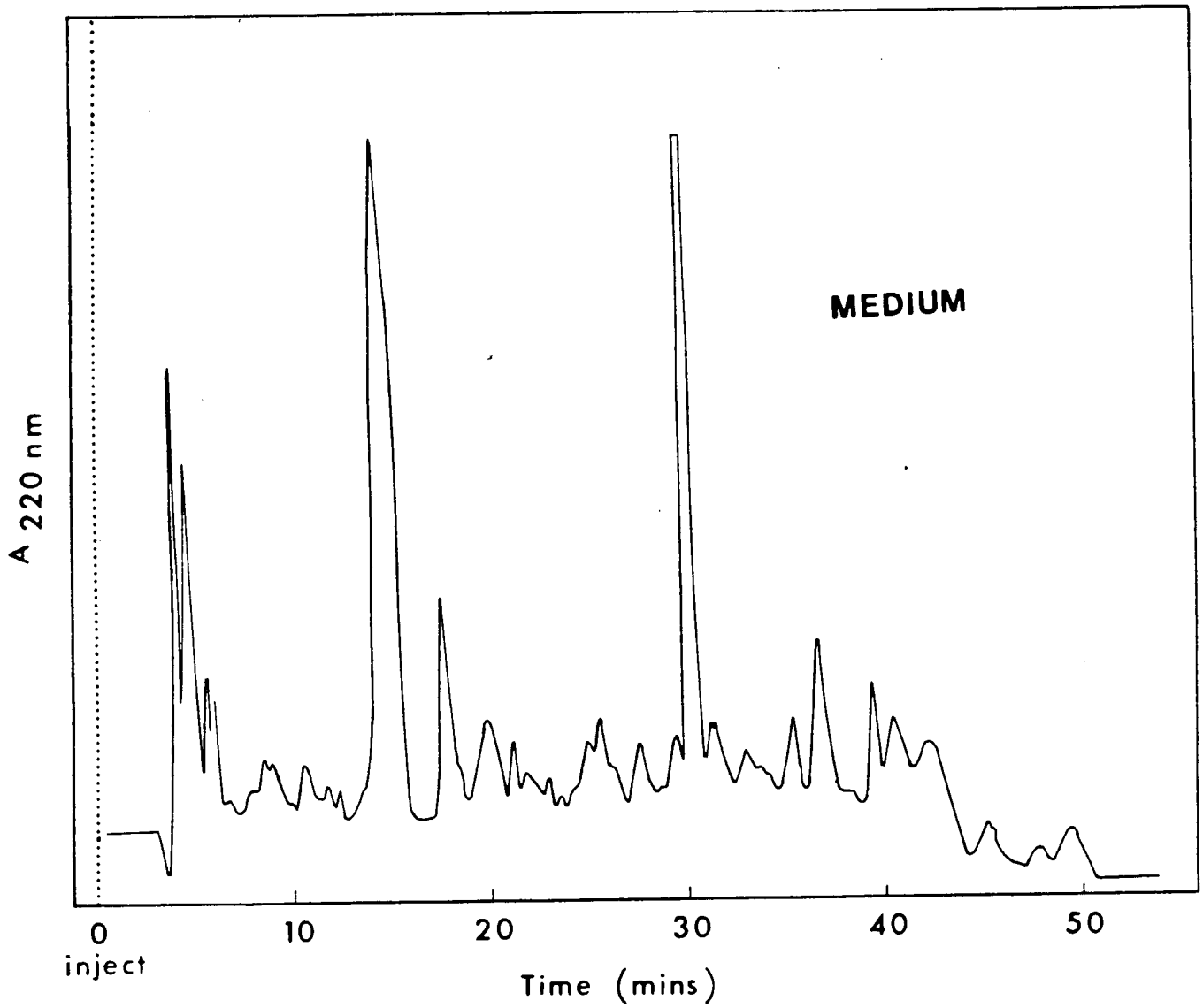


Figure 13.1 HPLC profile of the water extract from a 136 day old cheese, made with S. lactis and subjectively graded medium. See Table 4.1 (sample no 12).

CONCLUSIONS

Six defined culture strains of bacteriophage-insensitive Streptococcus cremoris propagated in whey-based starter media were successfully used over a period of 10 months to produce more than two million kg of Cheddar cheese on commercial continuous cheesemaking equipment.

1) The cultures grown in the pH controlled whey-based media retained their activity for up to six days.

2) Texture differences in the cheese were significant and were related to variation in composition and degree of proteolysis. Heat treating compared to pasteurization of the milk resulted in differences between the force-compression rheograms of the resultant cheeses.

3) Cheese made with S. cremoris strains required a longer ripening time to develop the same flavour intensity compared to cheese made with S. lactis cultures. This was related to proteolytic activity and quantified by measurement of free amino groups. Moreover, HPLC profiles of the non-volatile water extracts from cheeses made with S. cremoris were shown to have patterns characteristic of mild cheese.

4) Pattern recognition techniques (SLDA) used to analyze the multivariate data suggested texture was affected by the automated process, moisture content and yield point. Casein proteolysis, age, culture type and firmness were the most discriminating variables affecting maturity.

5) Application of multivariate analysis demonstrates a technique for possible use in evaluations of experiments or processes to detect systematically the significance of each of the experimental variables. This approach also provides a method for evaluation of multidimensional data. In both age and texture assessment the number of significant independent variables required to obtain correct classification was reduced from 11 to 4 and 11 to 3

respectively without loss of information. This technique indeed provides a powerful tool in extracting significant variables and in deriving useful information from a multitude of data.

PART II

INTRODUCTION

Flavour is the sensation produced by a material taken in the mouth, perceived principally by the senses of taste and smell and also by the general pain, tactile and temperature receptors in the mouth. Flavour also denotes the sum of the characteristics of the material which produce that sensation (Hall, 1968). Flavour therefore is a sensory phenomenon and is by far the most important factor which governs our appreciation of the foods we eat. The dairy industry is aware of the importance of flavour as an essential factor in food selection and acceptance and therefore have attached much value to flavour quality control (Badings and Neeter, 1980).

The texture and flavour properties of cheese are not obtained until after a ripening period, the length of which varies with the type of cheese and cannot be maintained at their best for an indefinite period of time. This means that what we have to observe is not constant with time. As a consequence of the heterogeneous nature of the product and the complexity of its constituents, the chemical basis of cheese flavour and aroma has not been eluciated despite a large number of studies. Most studies have placed emphasis on the volatile aroma, permitting, in some cases, one to obtain an insight into the broad mechanism of flavor and aroma development, but regrettably still leaving many questions unanswered (Adda et al., 1982).

Cheese ripening is essentially the controlled slow decomposition of the constituents of milk in a physically complex matrix. The precise nature of the reactions which produce flavour compounds and the way in which their relative rates are controlled is poorly understood. This has been due firstly to the lack of knowledge of the compounds which impart typical flavour to Cheddar cheese and secondly to the complexity of the cheese microflora as the potential producers of flavour compounds.

It becomes evident therefore that the most elusive and variable parameter in cheese is the concept of flavour itself. Not only have different research groups expressed differences of opinions on which compounds are important to cheese flavour but considerable differences can occur among sensory evaluation panelists, which is how cheese is usually assessed. The need for sensory panel training and strict statistical control is therefore imperative. Sensory evaluation is also applied in research, but has obvious limitations when comparing samples over an extended time period or between research laboratories.

A major problem, therefore, encountered in cheese maturation is the lack of a reliable method for estimating the extent of flavour development. Classically, trained graders and sensory panels have performed this duty, but because of the variable, subjective and time consuming nature of organoleptic methods, a simple, more objective and reliable method for accurately assessing cheese flavour is required. A chemical index or indices of maturation would allow better comparisons of ripening effects and more objective universal assessment of cheese flavour intensity and quality. The analysis of cheese for flavour enhancing compounds which might serve as indices has been extensively conducted. Hence, this study assesses the contribution of the water-soluble cheese fraction containing the salts, amino acids and peptides which have been shown to be responsible for the intensity of cheese flavour (McGugan et al., 1979). The non-volatile water fraction was analysed by reversed-phase high performance liquid chromatography (RP/HPLC).

The use of reversed-phase high performance liquid chromatography (RP/HPLC) as an analytical technique for protein separation, isolation and characterization is rapidly gaining interest (Hearn et al., 1982; Tweeten

and Euston, 1980) because of the potential to permit rapid and highly selective separations of peptides, polypeptides, proteins and amino acids. However, a formidable task exists to select the correct mobile phase as well as the operating conditions. Many liquid chromatographic separations have been developed by random selection of various mobile phase solvent mixtures with little theoretical considerations. Systematic optimization of the mobile phase for selectivity showed distinct advantages in achieving good resolution of all components of a mixture (Glajch et al., 1980). However, the systematic optimization of the mobile phase for selectivity in liquid chromatography is a relatively new development and is not yet widely practiced. Therefore, a further aim of the present research was to use Snyder's (1974, 1978) empirical classification of solvent properties for selectivity and a new mapping simplex optimization technique (Nakai et al., 1984) to improve resolution of the HPLC profile. These more efficient and effective procedures have the potential for both saving considerable development time and increasing the information content of the final HPLC profile.

Moreover, because of the complexity of the HPLC profiles it is somewhat difficult to determine or predict significant peaks responsible for taste and/or classification of cheese samples. Therefore, statistical pattern recognition techniques: principal component analysis and stepwise linear discriminant analysis were applied to the HPLC peaks in order to classify unknown cheeses into categories, or to separate cheeses into categories.

Significant peaks established by pattern recognition techniques were collected by using RP/HPLC. The amino acid contents of the fractions were evaluated to provide further information on the properties of peptides,

thus providing a better understanding of the peptides and/or amino acids implicated in the development of the complexity of cheese flavour and bitter taste.

The present study recognizes taste in a purely physiological sense of the term (i.e. sweet, sour, salt, acid and bitter) and that flavour is a complex combination of taste, smell, appearance, texture, etc. and that the analytical technique proposed may be used for the assessment of Cheddar cheese taste.

LITERATURE REVIEW

CHEDDAR CHEESE FLAVOUR

Early research on Cheddar cheese flavour sought a single compound or class of compounds responsible for characteristic flavour. When no such compound or class was found, Mulder (1952) proposed what was known as the Component Balance Theory. This theory suggested that Cheddar flavour was made up of a balance of flavours contributed by a number of different compounds. When the balance was upset by an excess or lack of one or more of the component compounds, atypical flavour was produced.

Cheese flavour results from the action of microorganisms and enzymes on the carbohydrates, fat and proteins of the milk and curd. The spectrum of compounds produced is often so wide and complex that those involved in the flavour remain unknown even after the application of sophisticated chemical analysis.

Because of the economic significance of Cheddar cheese and consumer acceptance, the dairy industry has placed great emphasis on flavour quality control. Despite the intensive research into Cheddar cheese flavour the agents responsible and their modes of action are still largely unknown. Many aspects of cheese chemistry and flavour have been rigorously investigated and thoroughly reviewed (Aston and Dulley, 1982; Adda et al., 1982; Law, 1981; Badings and Neeter, 1980; Forss, 1979; McGugan et al., 1979; Evans and Mabbit, 1974, Kristoffersen, 1973). However, attempts to relate the characteristic flavour and flavour quality of a given variety to a single or a group of closely related compounds have not been successful.

AMINO ACIDS

Amino acids, peptides and polypeptides produced during cheese ripening

are the result of the action of proteolytic enzymes originating from cheese milk, rennet and added bacterial cultures.

The principal pathway of proteolytic breakdown during the ripening process is thought to be the one by which rennet causes a primary, limited, degradation of the caseins after which the starter bacteria produce smaller peptide fragments and free amino acids (Visser, 1981; Lowrie and Lawrence, 1972). The mechanism of protein breakdown during ripening is well documented (Desmazeaud and Gripon, 1977). It begins with the action of rennet which cleaves the Phe_{105} - Met_{106} bond of κ-casein, thus inducing clotting. In addition, rennet cleaves the Phe_{23} - Phe_{24} and Phe_{24} - Val_{25} bonds of α_{s1}-casein during the early stage of ripening, with subsequent appearance of an α_{s1}I fraction. The action of the rennet goes on during the whole ripening period, inducing mainly the release of large molecular weight peptides, but no free amino acids are produced by the enzyme.

Law (1981) summarized the sequence of enzymatic protein breakdown by rennet, milk proteinase and starter proteinase (Figure 14.2). Free amino acids were the first compounds investigated for their contribution to cheese flavour. Glutamic acid has been shown to be responsible for the "brothy" flavour in cheese (Mulder, 1952; Harper, 1959) and Kosikowski (1951) found that as the total free amino acids increased, cheese flavour increased. Jarrett et al. (1982) like others (Ney, 1971; Nieuwoudt, 1977; Pettersson and Sjoström, 1975) recognized the importance of free amino acids to flavour and proposed a method for measuring phosphotungstic acid-soluble amino nitrogen (PTA-soluble amino N). The amino N level in the PTA filtrate was shown to be a good estimate of the total free amino acid levels. They showed that although the technique did not measure dibasic amino acids and proline there was a linear relationship between cheese ripening and free amino acids.

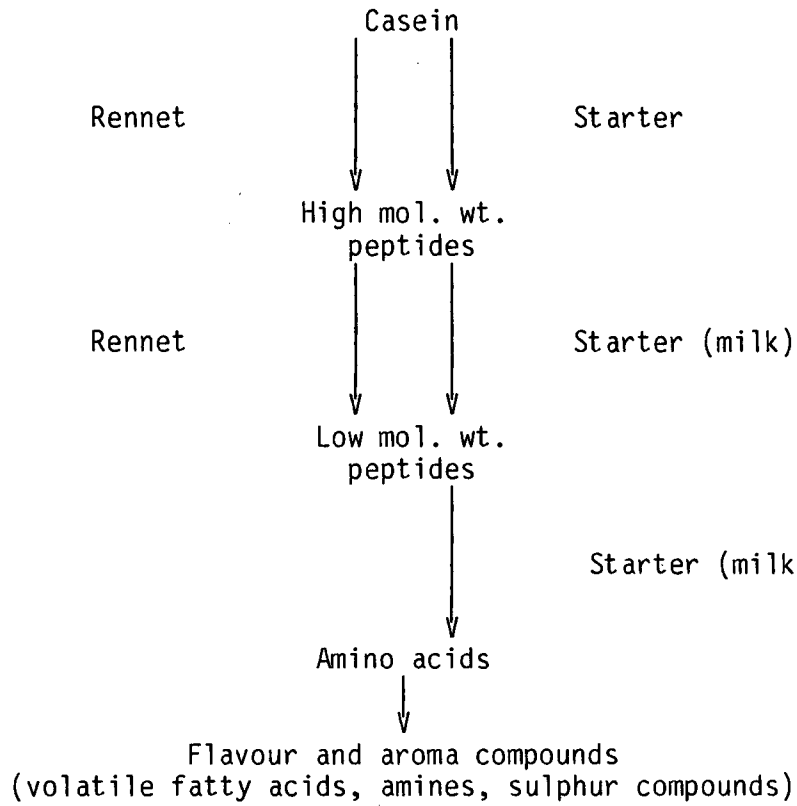


Figure 14.2 Schematic showing enzymatic hydrolysis of casein by rennet, milk proteinase and starter proteinase.

Prior to 1979 it was generally concurred that free amino acids made little or no direct contribution to Cheddar cheese flavour. However, McGugan et al. (1979) have recognized the importance of the non-volatile water extractable fractions. This group fractionated a mild and aged cheese and recombined the fractions in various combinations to assess the flavour of each fraction. The water-soluble fraction containing the salts, amino acids and peptides contributed most to the intensity. No significant difference was found between the deodorised and undeodorised fat fractions, indicating that the loss of volatiles was not important, at least not to flavour intensity. They concluded that the volatiles may contribute to the quality of flavour while the water-soluble fraction provides the intensity of flavour.

FREE FATTY ACIDS

Free fatty acids are hydrolyzed from milk triglycerides by two major sources: (1) breakdown of the fat by lipolysis either by milk, starter bacteria and possibly rennet lipases and (2) metabolism of carbohydrates and amino acids by bacteria Adda et al. (1982). However, evidence indicates that lipolysis is the major contributor of free fatty acids. Forss (1979) showed that butyric acid in cheese was present at twice the amount of that in milk and suggested that it should make a major contribution to cheese flavour while some of the minor free fatty acids enhanced flavour by synergism. Mabbit (1961), Patton (1963), and Forss and Patton (1966) also recognized the importance of free fatty acids as constituents to Cheddar cheese flavour. Yet, like others, Bills and Day (1964) and Law and Sharpe (1977) found no correlation between Cheddar cheese flavour and

free fatty acids. The literature on the subject is therefore vast and conflicting.

The importance of free fatty acids to the flavour of cheeses such as Romano and Parmesan is well accepted yet their role in Cheddar flavour is much less apparent. Aston and Dulley (1982) summarized techniques used to relate Cheddar flavour to free fatty acids:

(1) determination of their levels individually, collectively or as ratios in order to correlate these with flavour development;

(2) addition of fatty acids, sometimes with other compounds to bland bases to determine whether Cheddar flavour can be produced or improved;

(3) selective removal of the free fatty acids from extracts possessing Cheddar flavour to detect any alteration in flavour and

(4) manufacture of Cheddar cheese whose fat content has been decreased or replaced by a fat of vegetable origin.

The latter technique has been used by a number of workers (Lück and Downes, 1972; Foda et al., 1974; Harper, et al., 1979) and the cheese was found to have reduced levels of free fatty acids and subsequent lack of typical Cheddar flavour. Anders and Jago (1970) manufactured cheese from polyunsaturated fat fortified milk and found the lack of Cheddar cheese flavour due to the inhibitory effects of linoleic acid which inhibits the pyruvate dehydrogenase system in starter Streptococci.

Increasing the fat content above a certain limit does not improve flavour and may result in lipolysis and oxidation.

VOLATILE SULFUR COMPOUNDS

Volatile sulfur compounds (VSC's) have been considered important in cheese flavour for some time but with the introduction of more sensitive flame photometric detectors for gas chromatographic analysis, the very low levels in cheese can now be detected.

VSC's are unstable to conditions commonly encountered in flavour chemical studies and this may account for the incomplete and contradictory data on the roles of sulfur compounds in cheese flavour (Manning, 1979; Lamparsky and Klimes, 1981).

Kristoffersen (1973) postulated that cheese flavour quality resulted from a definite relationship between the relative concentrations of free fatty acids and hydrogen sulphide. Cheese manufactured from milk with the sulphur groups in the -S-S- state developed fuller and superior flavour in comparison with cheese made with the sulphur groups in the -SH state. The balance of -SH and -SS- groups modifies enzyme activity in that hydrogen resulting from oxidation-reduction reactions during the ripening process cannot be deposited by reducing the -SS- groups. However, Law and Sharpe (1977) found no relation between free fatty acids and H_2S to cheese flavour.

Methanethiol is present in Cheddar in amounts sufficient to contribute to flavour (Day et al., 1960). The absence of methanethiol from cheese and its removal from headspace volatiles results in the absence of typical cheese flavour and aroma, respectively (Manning, 1974; Manning and Price, 1977). Hydrogen sulphide contributes to aroma (Manning and Price, 1977) yet high concentrations result in 'sulphide' off-flavour.

Although sulphur compounds may be involved in cheese flavour (Kristofferson, 1973) they cannot give balanced Cheddar flavour and aroma

and may be responsible for undefined contributions to the overall flavour profile (Law, 1981).

NON-SULFUR VOLATILE COMPOUNDS

In mold cured cheese the production of methylketones may provide characteristic flavour. The mechanism of formation, involvement of spores and environmental conditions have been reviewed by Adda et al. (1982). The contribution of methylketones to Cheddar flavour was investigated by Walker and Keen (1974).

Gamma and delta lactones have been quantitatively determined in Cheddar cheese by gas chromatography-mass spectrometry of acetonitrile extracts and used to provide an overall index for maturation and flavour development (Wong et al., 1975). They suggested that the lactones not only contributed a flavour note of their own but also when blended with other compounds produced an additive and smoother flavour. Concentration of lactones was higher in rancid cheeses, suggesting that different pathways for lactone formation were possible when rancidity occurred.

As pointed out in the preceding review appreciable work has already been done to better understand Cheddar cheese flavour. However, it is generally agreed that more basic research into chemical and biochemical aspects of cheese ripening is required. In addition to the complexity of the analytical data already available and the differences of opinions on which compounds are important to Cheddar cheese flavour considerable differences occur among sensory panel results. Thus, with the data and analytical procedures presently available a more objective method of assessing Cheddar cheese flavour quality is imperative.

ANALYTICAL TECHNIQUES

Proteolysis and in some cases lipolysis appear to be key processes determining the rate of texture and flavour development in most varieties of cheese (Law, 1978) and the extent of proteolysis has been used as an indication of the degree of maturity of ripening cheese (Ney, 1971; Nieuwoudt, 1977).

Various electrophoretic methods presently available, employing different separation media (paper, starch gel, polyacrylamide gel) and different procedures (SDS, high voltage, isoelectrofocusing, bidimensional) have been used to study protein degradation during cheese ripening, but the most popular is the polyacrylamide gel electrophoresis (PAGE) method (Park et al., 1978; Thomas and Pearce, 1981).

A quantitative method for estimation of non-soluble N substances was developed using PAGE (de Jong, 1975). The method is relatively simple and from the densitograms the measurement of the degree of unhydrolyzed, native α_s- and β-caseins and to a certain extent the identification of major breakdown products including α_{s1}-I-casein can be made. As pointed out by Olson and co-workers (1983) variations of the method have been published and standardization of the procedure is required before it can be used as a quantitative assessment of cheese ripening.

Trieu-Cuot and Gripon (1982) used isoelectric focusing and 2-dimensional electrophoresis to study the pH 4.6 insoluble fraction during Camembert cheese ripening. Gel-filtration (Salji and Kroger, 1981) has been used as a separation technique for free amino acids and is routinely used to fractionate bitter peptides from Cheddar cheese (Champion and Stanley, 1982; Edwards and Kosikowski, 1983). However, in spite of a variety of the conventional chromatographic procedures available they

generally lack speed, resolving power and recovery. Therefore, high performance liquid chromatography (HPLC) is becoming increasingly important in quantitative analysis. The versatility of this technique is well recognized and technical publications are numerous. Bican and Blanc (1982) showed a great similarity between the electrophoretic profile of whey protein and casein to their respective HPLC analysis and suggested it as an alternative method to study casein degradation in a ripening cheese.

REVERSED-PHASE LIQUID CHROMATOGRAPHY

Reversed-phase high performance liquid chromatography utilizing a nonpolar stationary phase, usually a fully porous microparticulate silica in the range of 5-10 µm chemically bonded with alkyl chains and an aqueous polar mobile phase used to elute the strongly retained compounds, permits the rapid and highly selective separations of amino acids, peptides, polypepides and proteins (Tweeten and Euston, 1980; Hearn et al., 1979).

Although the number of studies using RP/HPLC are numerous (Heukeshoven and Dernick, 1982; Hancock and Sparrow, 1981; Hancock et al., 1981; Hearn, et al., 1982; Gazdag and Szepesi, 1981; Wilson et al., 1981; Bishop et al., 1980) and excellent reviews are available (Regnier and Gooding, 1980), the analysis of proteins is relatively new. This has been due to the relatively recent development of HPLC columns having support particles with 300 or 500 Å pores allowing interaction of the proteins with the stationary phase. Diosady et al. (1980) described the separation of whey protein and a quantitative estimation using RP/HPLC was obtained. Recently, Bican and Blanc (1982) studied the separation of whey proteins and suggested that the technique could provide HPLC profiles which are prerequisites when studying

protein degradation products, during cheese ripening. Pearce (1983) using a short alkyl chain C_8 reverse phase column completely resolved the major whey proteins in 30 min using an acid saline/acetonitrile gradient.

Champion and Stanley (1982) extracted water soluble Cheddar cheese flavour compounds using the separation method of Harwalkar and Elliot (1970). A water/91% methanol linear gradient at a rate of change of 1.14% methanol/min was used. The extract contained at least seventy-one compounds and from the HPLC elution pattern they suggested that hydrophobicity influences separation. High molecular weight compounds eluted earlier. There was however, no relationship between molecular weight of the compound in the bitter extract and their retention on the column.

Pham and Nakai (1984) studied the importance of the non-volatile flavour components in Cheddar cheese thought to be responsible for the intensity of Cheddar cheese flavour (McGugan et al., 1979). Using a reversed-phase column packed with Adsorbosphere C_8 Alkylsilica and an isocratic elution (0.1 M phosphate buffer pH 6.0), 13 clearly resolved peaks were obtained. They suggested that RP/HPLC analysis of the water-extract could be used as an objective evaluation of ripening of Cheddar cheese.

MOBILE PHASE

A considerable body of experimental work has been addressed to the investigation of mobile phase effects in the RP/HPLC of amino acids, peptides and proteins. Taken in isolation, these studies provide a vast array of different elution systems that makes the selection of the mobile phase difficult. Due to the number of studies available, it is possible to

make a rational interpretation in selecting eluting mobile phase conditions that provide good separation. However, in spite of the theoretical background, optimized analytical methods are seldom used (Glajch et al., 1980).

It is well recognized that in RP/HPLC the more hydrophobic the compound the greater is its interaction with the hydrophobic bonded stationary phase at a given eluant composition. Snyder (1974, 1978) proposed a general scheme for classification of common solvents according to their "polarity" or chromatographic strength and to their selectivity or relative ability to engage in hydrogen bonding. Various solvents could then be grouped into a selectivity-triangle concept and the strategy is to investigate solvents from the apices of the solvent triangle.

The systematic optimization of the mobile phase for selectivity to improve resolution in liquid chromatography (Snyder and Kirkland, 1979) is a relatively new development and not widely practised. A number of methods have been used for optimization of chromatographic systems (Morgan and Deming, 1975). The chromatographic response function (CRF) is used to measure the performance of both gas and liquid chromatography. The CRF has the general form:

$$CRF = \sum_{i=1}^{k} \ln (P_i)$$

Where P_i is a measure of separation between adjacent peaks in a chromatogram for k peak pairs, where k is one less than the total number of peaks. The largest CRF value generally indicates the best separation of the mixture.

A major problem encountered during optimization is a way of measuring

the quality of the analysis. Four parameters are generally used to evaluate the quality of the liquid chromatographic system. These parameters are k^1, the capacity factor; α, the separation factor; N, the number of theoretical plates; and R, resolution. Mathematically these are:

$$k^1 = \frac{V_i - V_o}{V_o}$$

$$\alpha = \frac{V_2 - V_o}{V_i - V_o} = \frac{k'_2}{k'_1}$$

$$N = 16 \left(\frac{V}{W}\right)^2$$

$$R = \frac{2(V_2 - V_1)}{W_1 + W_2}$$

The k^1 is a measure of a compound's retention in terms of the column volume. The separation factor α, is the ratio of the net retention time for any two components. N, is the number of theoretical plates in a column and can be increased by increasing column length (Pomeranz and Meloan, 1980).

Glajch et al. (1980) reviewed optimization techniques for chromatographic systems. They proposed a new method of data analysis called overlapping resolution mapping (ORM) which combines the Snyder solvent selectivity-triangle concept with a statistical simplex design technique for optimizing the mobile phase composition in RP/HPLC. Advantages claimed were that all pertinent information regarding a peak or pair of peaks is used in the optimization. In addition, crossovers and fused peaks can be handled thus providing good resolution.

MULTIVARIATE ANALYSIS

A major problem encountered in cheese ripening is the lack of a reliable method for estimating the extent of flavour development. Classically, trained graders and sensory panels have performed this duty, but because of the variable and subjective nature of organoleptic methods, a simple more objective method for accurately assessing cheese flavour is required. Almost all analytical techniques proposed to assess cheese flavour development are compared to Graders' results and/or a trained sensory panel (Aston et al., 1983; Grieve and Dulley, 1983; McGugan et al., 1979; Harwalkar and Elliot, 1970). Data obtained from sensory evaluation are usually subjected to merely routine statistical analysis. Seldom are factors being used as descriptors, examined first to determine whether they are truly critical to the evaluation (Derde and Massart, 1982).

The difficulty in interpreting the results of analytical techniques which produce simultaneously a large number of parameters has led to increasing interest in pattern recognition. Excellent reviews have been presented (Kowalski, 1980; Frank and Kowalski, 1982) on the topic. These authors have introduced an interdisciplinary science, Chemometrics, which has been defined as the application of mathematical and statistical methods to chemical measurements.

Pattern recognition techniques are a method for analyzing multivariate data in order to classify unknown objects into categories, or to separate objects into categories (Varmuza, 1980) while giving data reduction with minimal loss of information.

Multivariate data analysis offers various methods for efficient simplification and interpretation of many different variables

simultaneously. Pattern recognition for multivariate analysis has been used in chemistry, microbiology and medicine for identification of chemical compounds, classification of bacteria species and diagnosis of diseases respectively. The multivariate data analysis technique is finding increased application in food science and technology. Moreover, because of the interest in computer aided analysis of multivariate food research data an interdisciplinary IUFoST-symposium was held in Oslo, Norway (1982). The proceedings to that symposium were prepared in an excellent review on the subject (Martens and Russwurm, 1983).

Powers and Keith (1968) recognized the importance of gas chromatographic differences in the volatiles of foods and how these differences are related to quality. They observed that attempts to correlate gas chromatographic data with organoleptic flavour did not use the entire chromatogram but rather one or relatively few peaks and suggested that visual inspection or even simple mathematical evaluation resulted in poor correlations between the flavour and chemical compound. Using stepwise discriminant analysis (SLDA, a pattern recognition technique) four lots of roasted coffee with different organoleptic qualities were examined by gas chromatography (GC). By calculating all possible ratios among peak heights and subjecting these ratios to SLDA the coffee was correctly classified into the four flavour categories. Smeyers-Verbeke et al. (1977) applied discriminant analysis to the classification of milk samples according to their origin. The analysis was based on GC data for fatty acids in milk fat samples from cows, sheep and goats. Between the pure samples, SLDA allowed complete discrimination. Extension of the investigation to mixtures of these samples showed a high degree of correct classifications between the mixtures and the pure milks.

They concluded that the method should allow a higher proportion of correct classifications than is possible by visual inspection of the chromatograms.

The data for sixteen different chemical components of wine were analyzed by pattern recognition techniques and the samples classified according to vintage year and wine region (Kwan and Kowalski, 1978).

Aishima (1979) applied SLDA and principal component analysis (PCA) to GC profiles of soy sauce volatiles in order to compare soy sauce quality assessed by sensory evaluation and GC data. The soy sauce brands were roughly separated into four groups by PCA whereas SLDA correctly classified each sample into the proper group of the eight brands. A highly significant relationship between the principal components and sensory score were obtained (r^2 = 0.915). Aishima (1979, 1982, 1983), using multivariate analysis correctly classified soy sauce samples based on their GC profiles into the correct brands and found a close relationship with sensory evaluation. Eight principal components were extracted from the 39 GC peaks as significant factors responsible for the soy sauce aroma.

Multielemental data of wine determined by atomic emission spectrometry was analyzed by pattern recognition techniques to extract key elements and combination of elements which were charactistic of geographic origins and to investigate subtle differences in elemental concentrations due to intraregional and vintage variations (Kwan et al., 1979).

The quantitative determination of volatile compounds of grape brandies were analyzed by GC and using SLDA the cognacs from the respective brandies of different regions were definitely distinguished (Schreier and Reiner, 1979). Kwan and Kowalski, (1980b) using gas chromatography-mass spectrometry data classified Pinot Noir wines according to their geographic origins. These same workers in a subsequent study (Kwan and Kowalski,

1980a) correlated organic and elemental compositions with sensory evaluations and by using PCA were able to reduce the large number of chemical components to only a few key components related to overall quality of the wine. PCA applied to scores obtained from the sensory evaluation of wine provided information on the consistency of the panelist and areas requiring improvement (Kwan and Kowalski, 1980a).

Recently Woo and Lindsay (1983) developed a method for detecting and predicting hydrolytic rancidity off flavours in butter by correlating sensory data with free fatty acid concentrations by using stepwise regression and pattern recognition techniques. They suggested that the procedure developed should allow the dairy industry to practice more valid quality control programs for butter flavours.

Pham and Nakai (1984) analyzed water extracts from Cheddar cheese samples by RP/HPLC. SLDA applied to the HPLC profiles classified the cheeses into mild, medium, old and extra-old based solely on the water-soluble compounds. Discriminant functions were calculated and used to classify unknown samples of cheese into their correct age categories.

MATERIALS AND METHODS

1. SAMPLE PREPARATION

Cheddar cheese samples from different manufacturers and representing different ages and quality were purchased from retail outlets. The extraction method as outlined by McGugan et al. (1979) was followed (Figure 15.2) with some minor modifications as described by Pham and Nakai (1984).

Four grams of the commercially produced cheese sample was centrifuged at 30,000 X g for 25 min at 25°C. After decanting the fat, the residue was extracted to remove residual fat by mixing first with 2 mL methyl alcohol, then adding 0.2 mL water and mixing. After centrifuging the mixture (1000 X g; 30 min) the solvents were decanted and the residue was extracted twice more, as above, with 1.0 mL methyl alcohol, 1.0 mL methylene chloride and 0.6 mL water for each extraction.

The water-soluble fraction was prepared by adding 2.0 mL water to the combined solvent extracts. Two solvent layers and a precipitate were separated by centrifuging (1000 X g; 30 min) and the resulting precipitate added to the previous residue. The methyl alcohol fraction was extracted with two 0.25 mL portions of methylene chloride to ensure removal of fat-soluble material. The methyl alcohol-water fraction was evaporated by placing the tubes in a Silli Therm™ heating module (Pearce Chemical Company, Box 117, Rockford, IL) set at a temperature of 45°C under a steady stream of nitrogen. Water (2.0 mL) was added and evaporation continued. The procedure was repeated a further six times to remove volatiles from the water-soluble fraction. This fraction was combined with the water-soluble extract obtained by extracting the residue. The combined water-soluble extracts were further evaporated to approximately 1.0 mL and then made up

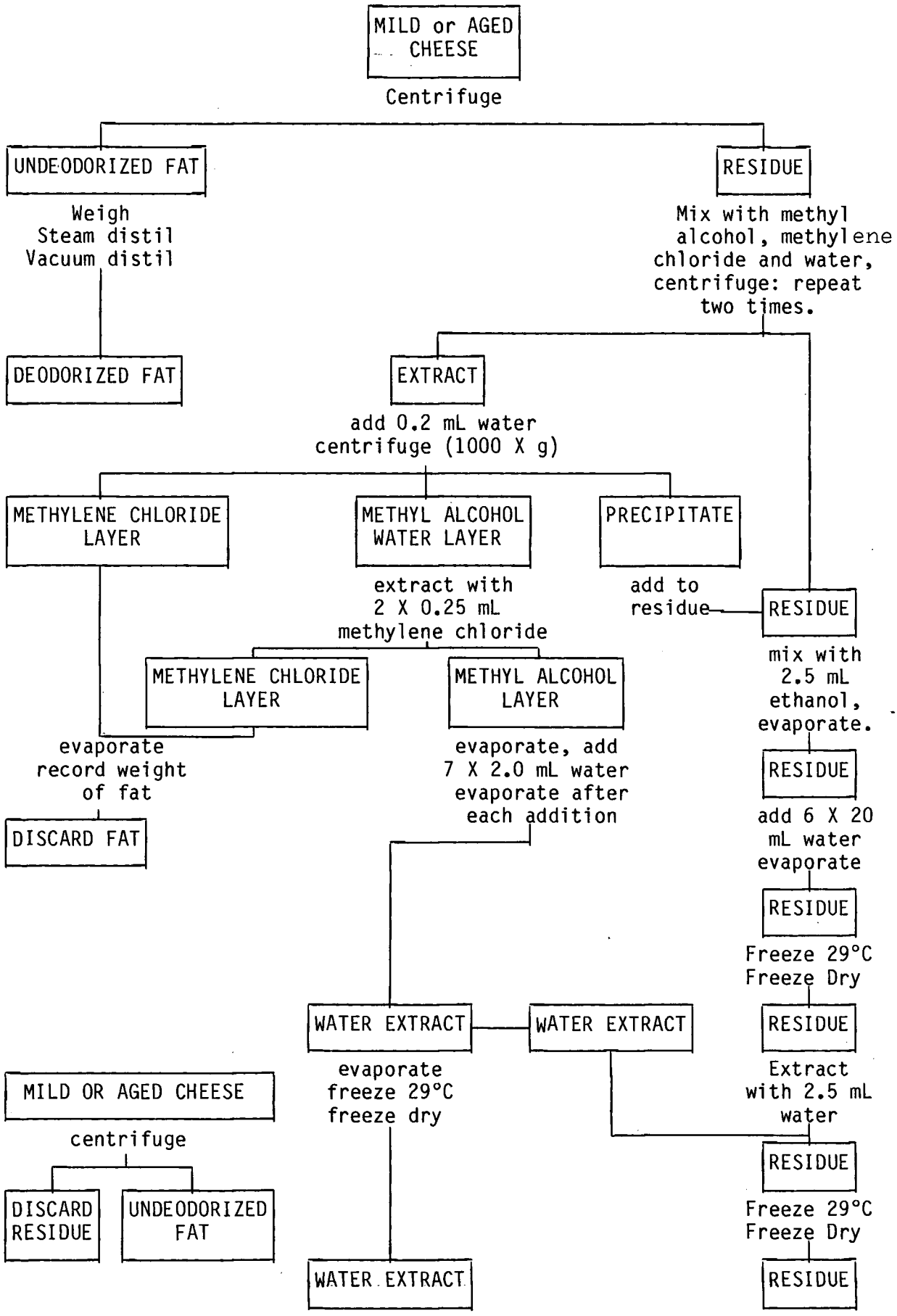


Figure 15.2. Flow diagram of preparation of cheese fractions.

to a final volume of 2.0 mL. The samples were filtered through 0.45 μm Millipore filter and stored frozen.

2. NITROGEN DETERMINATIONS

A Technicon Auto Analyser (Technicon Instrument Corporation, Tarrytown, New York) was used to analyse nitrogen (N) in the cheese, washed residue, methylene chloride layer and water extract obtained in the extraction procedure outlined in section 1. Non-protein nitrogen (NPN) was determined by centrifuging ca 4g cheese (30,000 X g; 25 min; 25°C) to remove fat. To 0.1 g of the defatted cheese, trichloroacetic acid (TCA) was added to a final concentration of 12%, centrifuged (10,000 X g; 10 min) and the supernatant analyzed for NPN as above.

3. COMPOSITIONAL ANALYSIS

All cheese samples were analyzed in duplicate for fat, moisture, total solids and salt according to official AOAC methods (AOAC, 1980). Samples for analysis were taken from the inside area of the piece of cheese and shredded finely using a 'home style' grater.

(a) Fat

Butter fat was determined by the Roese-Gottlieb method.

(b) Moisture

Described in Part I-2 of Materials and Methods.

(c) Salt

Described in Part I-2 of Materials and Methods.

(d) pH

pH was measured at ambient temperature (20-22°C) using a hydrogen specific ion sensitive electrode purchased from Fisher Scientific (Fair Lawn, NJ).

Compositional parameters including moisture in the non-fatty substance (MNFS), salt-in-moisture (S/M), and fat in dry matter (FDM) were calculated (Lawrence and Gilles, 1982).

4. SENSORY EVALUATION

All cheese samples were officially graded by Federal Graders for flavour and physical characteristics as soon as possible after purchase according to the guidelines established for the examination of dairy products (Agriculture Canada, Ottawa, Ontario).

Fifteen members from the Department of Food Science, University of British Columbia, who liked Cheddar cheese were screened for their ability to discriminate between mild, medium and aged Cheddar cheese samples. Ten panelists were chosen from the fifteen for their sensitivity to Cheddar cheese taste according to the following criteria: typical aged Cheddar taste, bitterness, rancidity, acid tendency and stale. Triangle tests were used in order to identify the odd sample classified according to age within each group (i.e. group 1: mild, medium, medium; group 2: medium, medium, aged). Replication of the evaluation on the same sample was conducted to provide an estimation of experimental error and the order of presentation was randomized to avoid any positional effects. Concurrent with the sensory analysis, two Federal Graders evaluated duplicate samples according to their standard methods. The graders were asked to classify the samples according to age and also to report overall grade and any taste or texture

defect. Correlations between the trained sensory panel and Federal Graders were calculated.

5. HPLC ANALYSIS

A Spectra-Physics 8100 HPLC and 8400 variable wavelength detector (Spectra-Physics, Santa Clara, CA) operated at a wavelength of 220 nm were used for the analysis. One modification to the equipment was a gas de-bubbler (Terochem Laboratories, Edmonton, Alberta) and installed between the ternary pump and the column.

As described by Hearn et al. (1982) and established by Pham and Nakai (1984) a reversed-phase column (250 X 4.6 mm I.D.) packed with Adsorbosphere C_8 (5 μm) (Applied Sciences Laboratories, State College, PA) was used for all chromatographic runs. The volume of the sample loop was 50 μL.

A ternary gradient system was used to elute the non-volatile taste components from the column. The initial solvent volume ratio was 44.6:0.0:55.4 for trifluroacetic acid (0.1%), acetonitrile and water. Over 56.6 min the ratio was changed to 0.0:36.6:63.4. All chromatographic runs were performed at ambient temperature (20-22°C) at a flow rate of 0.97 mL/min. Doubly distilled deionized water filtered through a Milli-Q system (Millipore, Bedford, MA) was used to prepare the trifluroacetic acid. Acetonitrile and all other sample solutions were filtered through a 0.45 μm Millipore filter. Prior to use, all solutions and water were evacuated for about 15 min and further degassed for an additional 10 min with helium. During gradient elution a fine steady stream of helium was allowed to continually degas the solutions.

A Spectra-Physics SP 4100 computing integrator was used to calculate

peak areas. Under BASIC control, the SP 4100 integrator controls the pumping system and the variable wavelength detector. In addition to computing integration functions, SP 4100 is capable of graphical presentation in X-Y axes. A full alphanumeric keyboard and a LED display facilitates entry, review and editing of all file and run data as well as operating status and system diagnostics.

6. OPTIMIZATION TECHNIQUES

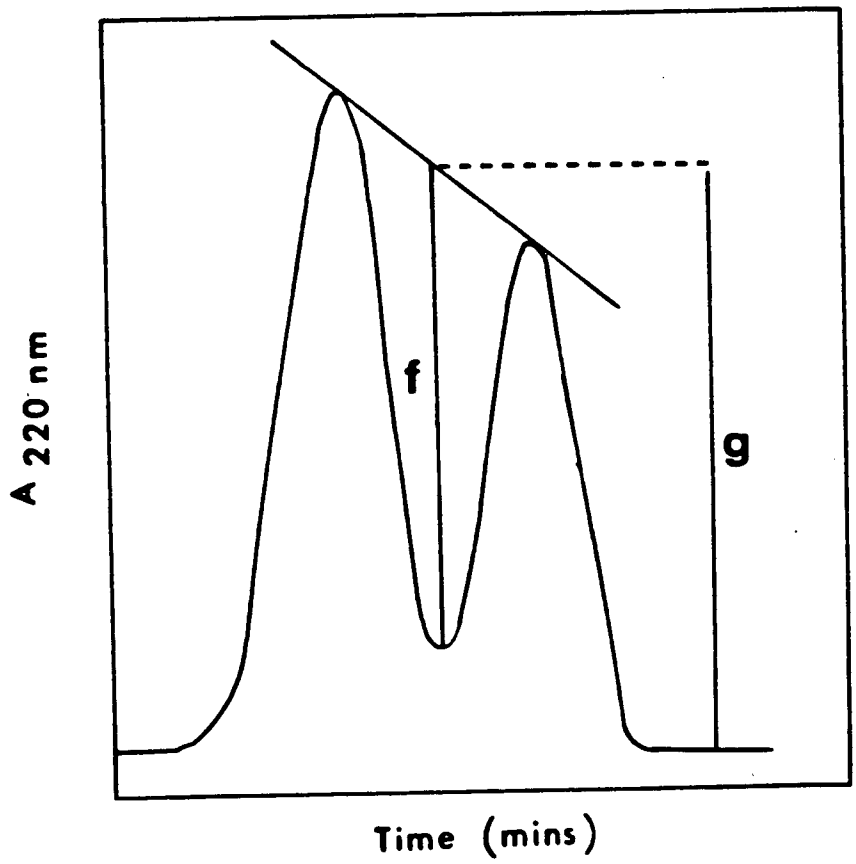
(a) Super Modified Simplex

A Super Modified Simplex (SMS) proposed by Routh et al. (1977) and written for a Monroe 1880 calculator (Monroe, The Calculator Company, Orange, NJ) was used to find optimum conditions for HPLC resolution. The method is a modification of a simplex optimization (Nelder and Mead, 1965) incorporating a quadratic curve fitting to determine the vertex immediately following the reflection vertex to replace the worst vertex in the preceding simplex.

To measure the quality of the HPLC peak separation as well as criteria to compare one mobile phase with another the method of Glajch et al. (1980) was used where P_i is a measure of the peak separation of the ith pair of peaks in a system with k total pairs of interest. Peak separation, P_i is simply defined:

$$P_i = \frac{f}{g}$$

where f is the depth of the valley below a straight line connecting two adjacent peak maxima and g is the height of the straight line above the baseline at the valley, as illustrated in Figure 16.2. The ΣP_i was used as the response for the Super Modified Simplex.



Resolution $P_i = \frac{f}{g}$

Figure 16.2 Schematic outlining method used to calculate peak resolution, adapted from Glajch et al. (1980).

(b) Mapping Super Simplex and Simultaneous Factor Shift.

In order to speed up the iterative optimization procedure and graphically illustrate the experimental response surface the Mapping Super-Simplex introduced by Nakai et al. (1984) and written for an Amdahl 470 V/8 computer was used. For each factor, the level values used in the optimization are divided into four groups based on their locations on a scale within large, medium and small limits. The large and small limits were determined from individual plots of response value (P_i) vs. each factor level (initial and final concentration of trifluoracetic acid and acetonitrile, and time). The medium limit is an average of both large and small limits. These limits were used for grouping the data. Data points for one factor which belongs to the same groups of other factors were joined together thus giving an estimate of the response surface. The maps for all factors provided target level values where the optimum mobile phase conditions were located.

A Simultaneous Factor Shift Program (Nakai et al., 1984) written for a Monroe 1880 programmable calculator was used. Target values (estimated best mobile phase conditions) were determined from the graphs. The program is designed to shift all factor levels obtained from the mapped graphs simultaneously one fifth the distance between the present best value and the target value. The new experimental conditions (vertices) resulting from the Simultaneous Factor Shift Program were investigated and their response values calculated as described previously.

7. STATISTICAL ANALYSIS

Statistical pattern recognition techniques (principal component analysis (PCA), stepwise linear discriminant analysis (SLDA) and regression

on principal components) were used to interpret the HPLC profiles. Programs employed were BMD:4M, BMD:7M and BMD:4R respectively, (BMDP Statistical Software Inc., University of California). Computer analyses were performed on an Amdahl 470 V/8 computer.

Pattern recognition is a method for analyzing multivariate data. For each sample analyzed, a set of results constituting a pattern is obtained. Similar samples will therefore have similar patterns. These patterns can be used to identify groups of similar objects or they can be used to characterize a group of similar objects in order to enable one to classify a new object in the group it belongs to based on regularities in the data.

The characteristic feature of multivariate analysis is the consideration of a set of n objects, on which are observed the values of p variables. Each cheese sample was considered as a point in a p-dimensional space (p being the number of peaks from the HPLC profile).

a) Principal Component Analysis

Principal component analysis, a direct pattern recognition technique, was applied to the HPLC data obtained for each cheese sample. All peak areas were entered into the analysis to select the most important variables (peak) for discrimination among the cheese samples. Each variable (peak) has a variance (a measure of dispersion of values around the mean) and usually the variables are associated with each other i.e. covariance between pairs of variables. The data set as a whole has a total variance which is the sum of the individual variances. PCA transforms the data to describe the same amount of variability (total variance) with the same number of axes, where each variable measured is an axis of variability. The first axis accounts for as much of the total variance as possible. The second axis accounts for as much of the remaining variance as possible,

while being uncorrelated with the first axis. This continues until all variables have been accounted for (Daultry, 1976). This results in a few large axes accounting for most of the total variance, and a larger number of small axes accounting for very small amounts of the total variance. These small axes are normally discounted from further consideration. The original set of correlated variables (HPLC peak areas) were transformed into a smaller set of uncorrelated principal components.

The interdependence of the variables was investigated by the correlation coefficient matrix (positive one, perfect positive correlation; zero, no correlation; negative one, perfect negative correlation). Eigenvalues greater than one were considered significant in describing total variance and used for the basis for the selection of the components.

Factor loadings from the significant principal components were plotted to give a two dimensional representation of the data.

b) Stepwise Linear Discriminant Analysis

The objective of the analysis is to weigh and combine linearly the discriminating variables so that the groups are forced to be as distinct as possible and the number of variables are reduced with minimum loss of differentiation between the groups.

Some linear combinations provide better separations than others and therefore the weight attached to each variable must be optimized so that orientation will be used, which will provide maximum differentiation between the groups. The optimal weight coefficients are obtained by maximization of the ratio of between-groups variance of the samples to their within-group variances and the correlation between the variables must be as small as possible (Coomans et al., 1979).

The cheese samples were classified into correct taste categories by the

Federal Graders and trained sensory panel and used for the development of the discriminant and classification functions (learning set).

Because of the difficulty in visualizing the multivariate data (p, peaks) obtained from the HPLC pattern for each cheese sample, SLDA was used to reduce the p-variables in a p-dimensional space to two.

The classification of the groups is represented graphically in a two-dimensional space (X and Y-axis) by a plot of the first canonical variable against the second canonical variable. The first canonical variable plotted on the X-axis is a linear combination of peaks that best discriminates among the groups. The second canonical variable, which is the next best linear combination orthogonal to the first one, is plotted on the Y-axis. Therefore, each cheese sample is characterized by the co-ordinates for the canonical variables.

In addition to canonical variables used for graphic representation of the data, SLDA yields a new set of variables which are linear combinations of the original parameters. These discriminant functions enable classification of unknown samples. The discriminant functions can be represented by the equation:

$$Z = a_1x_1 + a_2x_2 \ldots a_ix_i,$$

where a_1 .... a_i are coefficients of the discriminant function for each group i.e. young, mild, medium, old and X-aged. $x_1 \ldots x_i$ are the peak areas 1 to i for the unknown sample. Z is calculated in order to maximize the ratio of between-group variation to within-group variation. Z can be calculated by substituting $x_1 \ldots x_i$ by their respective peak areas for the unknown cheese sample obtained from the HPLC pattern.

The discriminant functions provides a maximum differentiation between groups of cheese. The unknown sample is then assigned to the group with the largest discriminant score.

8. AMINO ACID ANALYSIS

The water-soluble extracts (section 1) from a young, mild, medium, old and X-aged cheese were freeze dried in a Labconco Model 75018 freeze dryer (Labconco, Kansas City, MO). 2.5 mg dry protein sample (calculated by N determination, section 2) was added to 1.0 mL digestion mixture of 3-(2-aminoethyl) indole and p-toluene sulfonic acid (Simpson et al., 1976) in Pierce vacuum hydrolysis tubes (Pierce, Rockford, IL). Following evacuation and nitrogen flushing (three times) the contents of the hydrolysis tube were hydrolyzed at 110°C for 24 h. After hydrolysis, 2 mL 1N NaOH was added to the digested mixture then transferred to a 4 mL volumetric flask, pH adjusted to 2.1 and made to volume with H_2O. The sample was filtered through a 0.22 μm Millipore filter (Millipore Corp., Bedford, MA).

A Beckman System 6300 high performance amino acid analyzer (Beckman Instruments Inc., Palo Alto., CA) was used for analysis. The volume of the sample loop was 100 μL delivering 50 μL to the column. A Hewlett Packard 3390A Reporting Integrator (Hewlett Packard, Avondale, PA) was used to calculate peak areas of the particular amino acid and compared to that of a standard.

9. ANALYSIS OF HPLC FRACTIONS

Peaks selected to be most effective for discrimination by SLDA were fractionated by injecting an aged sample into the HPLC using the same gradient elution. After repeated injections of the sample the individual fractions were concentrated and injected to determine homogeneity. Amino acid analyses, as outlined in Section 8, were carried out on the specific fractions collected.

RESULTS AND DISCUSSION

More than 60 samples of cheese were used in the study and purchased from retail outlets representing different manufacturers across Canada. A group of 'young' cheeses, one week old, made on continuous cheddaring equipment were obtained from a local dairy.

SAMPLE PREPARATION

The method of McGugan et al. (1979) used to extract the non-volatile water extractable flavour components containing the salts, amino acids and peptides. Their method was selected because of the contribution of the non-volatile components to Cheddar cheese flavour. Sensory evaluation of the non-volatile water extracts from a mild and aged cheese were correctly identified.

Harwalkar and Elliot (1970) also suggested that the nonvolatile components contributed to Cheddar flavour. Although they were interested in the bitter and astringent fractions, their method of fractionation was essentially the same as that of McGugan et al. (1979). The methylene chloride: methyl alcohol: water extraction method of the latter workers was basically the only difference compared to chloroform: methyl alcohol: water extraction procedure of the former.

NITROGEN DETERMINATIONS

Pham and Nakai (1984) showed these non-volatile water fractions to have a positive reaction with the ninhydrin reagent and suggested they were protein degradation products from cheese ripening. Total nitrogen was determined on freeze dried samples of a mild and aged cheese. As well,

total nitrogen was determined for fractions collected and outlined in section 2. The results (Table 9.2) indicated that N was not detected in the methylene chloride fat fraction suggesting that the repetitive methyl alcohol water extractions removed any residual water soluble N components from the fat. McGugan et al. (1979) showed that the weight recovery of the water-soluble extract from an aged cheese was two times that of a mild. The findings of the present study confirmed these results that as the cheese ages, protein degradation products accumulate in the water-soluble fraction. Table 9.2 shows that aged cheese has a higher percentage of soluble N compared to mild cheese (21.0 and 13.6% respectively).

Soluble N has been used as a method to study proteolysis during cheese ripening and was found to increase with age (Park et al., 1978). Various extraction procedures have been compared (Kuchroo and Fox, 1982; Kuchroo and Fox, 1983) however despite the extensive use of proteolysis products as ripening indices little work has been done to relate these to flavour development.

COMPOSITIONAL ANALYSIS

All cheeses were analyzed for moisture, fat, salt and pH. Lawrence and Gilles (1982) showed that the quality of cheese was influenced most by moisture in the non-fat-substance (MNFS), fat-in-dry matter (FDM), salt-in-moisture (S/M) levels and pH. Therefore, only cheeses with the optimum specifications: MNFS, 52-56%; FDM, 52-55%; S/M, 4.0-6.0%; and pH 4.95-5.10 were selected for the initial study. Cheeses that did not fall into these specification limits were generally downgraded for flavour and/or texture by the Federal graders.

Table 9.2. Nitrogen (N) distribution of the various fractions from mild and aged cheese.

Sample	% Nitrogen		% Protein[1] (%N X 6.25)	
	Mild	Aged	Mild	Aged
Cheese	3.87	3.75	24.19	23.44
Washed residue	3.31	2.92	20.68	18.25
Water soluble fraction	0.53	0.79	3.31	4.94
Methylene chloride fraction	0.00	0.00	0.00	0.00

1 wet basis

SENSORY EVALUATION

Lawrence and Gilles (1980) demonstrated how small increases in MNFS leads to free moisture in the cheese. Without exception the trained cheese graders had no difficutly in distinguishing textural differences in cheeses of different moisture contents. No significant difference ($P > 0.05$) was found for cheese flavour assessment for the more than 60 samples of cheese between the subjective sensory evaluation by the trained panel to that of the Federal graders. Sensory analysis for the 15 panelists to determine the odd mild sample from two medium was significant ($P < 0.05$). Randomizing the same samples resulted in no significant difference ($P > 0.05$). Clearly the dynamic nature of cheese flavour is difficult to assess particularly in the mild to medium categories. In fact Lelievre and Gilles (1982) showed that sensory evaluation of particularly young cheese varied from grader to grader. McBride and Hall (1979) recognized that the traditional procedures for assessing cheese flavour quality at an early age are not a reliable guide to its acceptability at maturity.

Significant differences ($P < 0.01$) between medium and aged cheeses were found in this study suggesting that the flavour components responsible are more pronounced and possibly easier to distinguish. This is substantiated by the fact that cheese flavour and texture are obtained only after a ripening period (Adda et al., 1982) and the length of time is characteristic for the particular cheese. Notwithstanding, the spectrum of compounds is often so wide and complex that those involved in the flavour remain unknown (Law, 1981).

HPLC ANALYSIS

An Adsorbosphere C_8 reversed-phase column was chosen since it provided

the best resolution and greatest number of peaks using isocratic separation of water extractable flavour components (Pham and Nakai, 1984). Moreover, it is generally recognized that RP/HPLC permits the highly selective separation of amino acids, peptides, polypeptides and proteins (Hearn et al., 1982).

Figures 17.2-21.2 represent HPLC profiles for young, mild, medium, old and X-aged cheese respectively. From visual examination, the profiles are complex with more than 48 peaks. Peaks with similar retention times were considered to be the same. Repeated injection (10 times) of the water extract from a X-aged sample, chosen because of the greatest number of peaks, and integration of the peak areas at the respective retention times confirmed the reproducibility of the technique. The calculation of average and standard deviation for HPLC peak resolution resulted in reproducible separation (27.5 ± 0.23). The variation in column performance was minimized by repeated washing with first, organic solvent followed by water. This procedure ensured complete elution of any strongly bound hydrophobic compounds not eluted during the initial run. Regeneration of the column to the same starting working pressure (ca 600 psi) using the starting mobile phase suggested complete regeneration. Indeed, it is well recognized that in RP/HPLC, as the polarity of the substance increases, the weaker is its interaction with the hydrophobic bounded stationary phase. Peaks o, q, ff, mm and nn increased with age while peak f remained relatively constant in comparison from visual examination. Significant variation in other peaks was difficult to interpret visually because of the complexity of the chromatograms. Pham and Nakai (1984) using isocratic elution fractionated 13 peaks from the water soluble fractions of mild, medium, old and X-aged cheese and suggested changes in compositon with

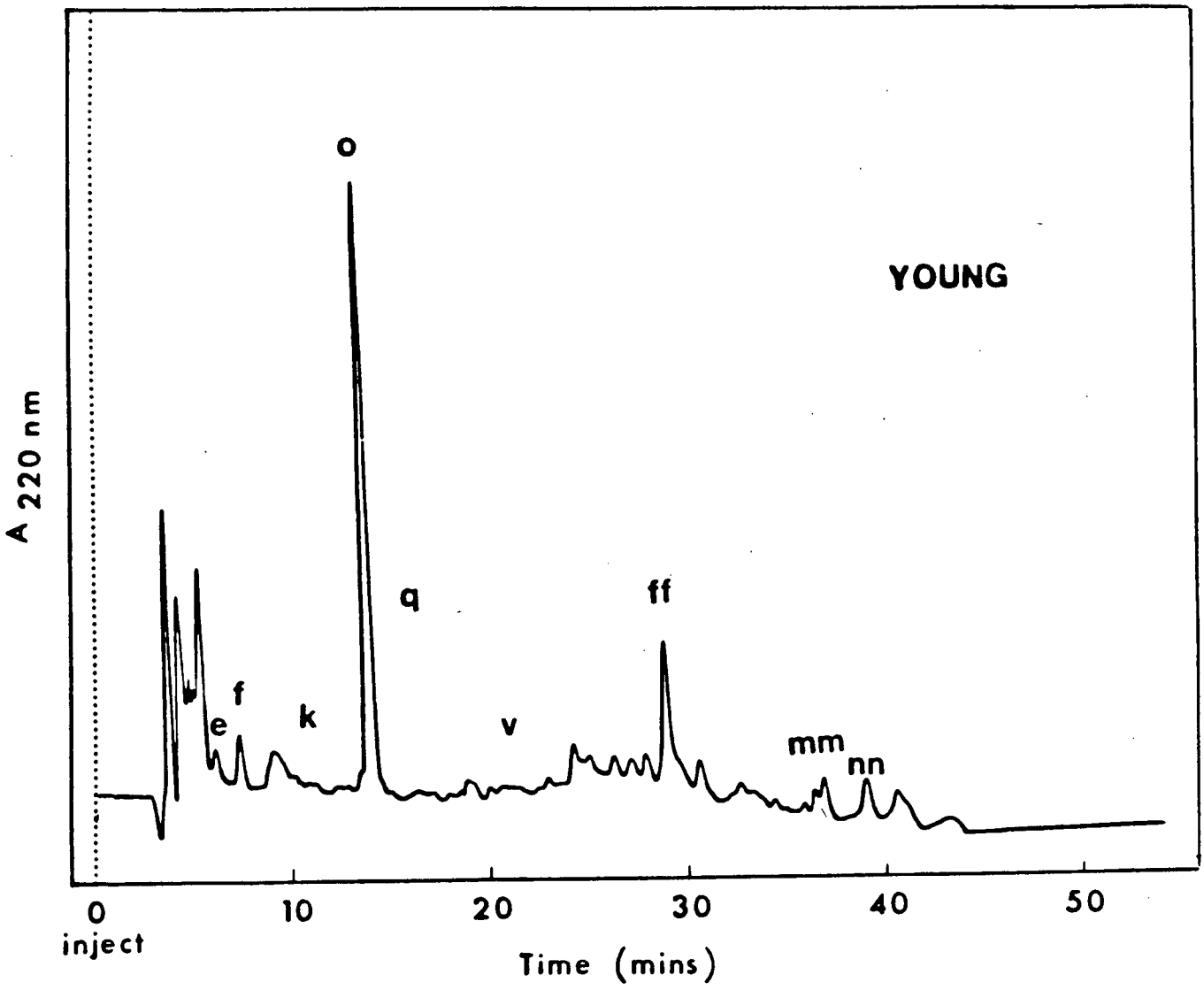


Figure 17.2 RP/HPLC separation of the water-soluble fraction from a young cheese.

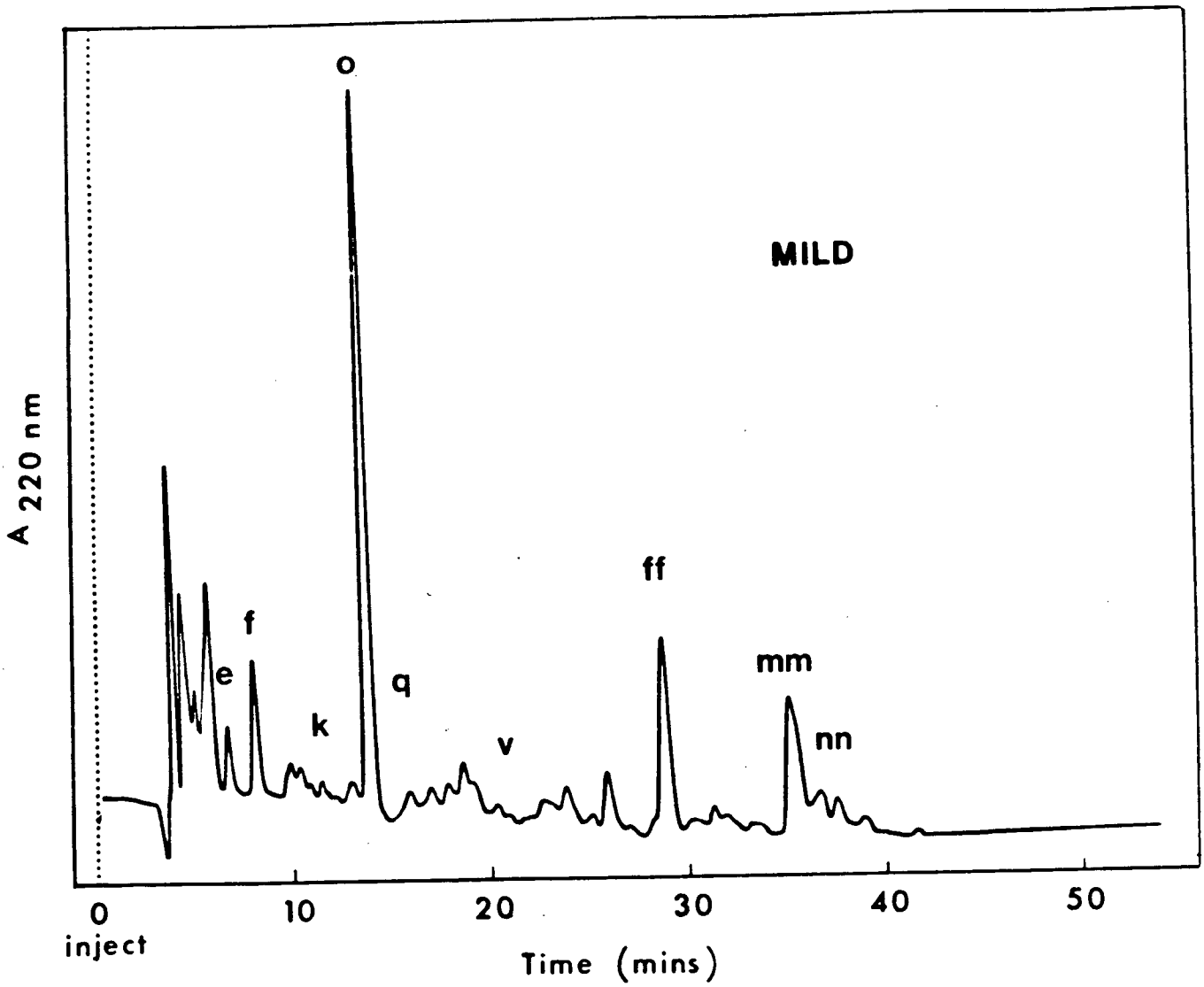


Figure 18.2 RP/HPLC separation of the water-soluble fraction from a mild cheese.

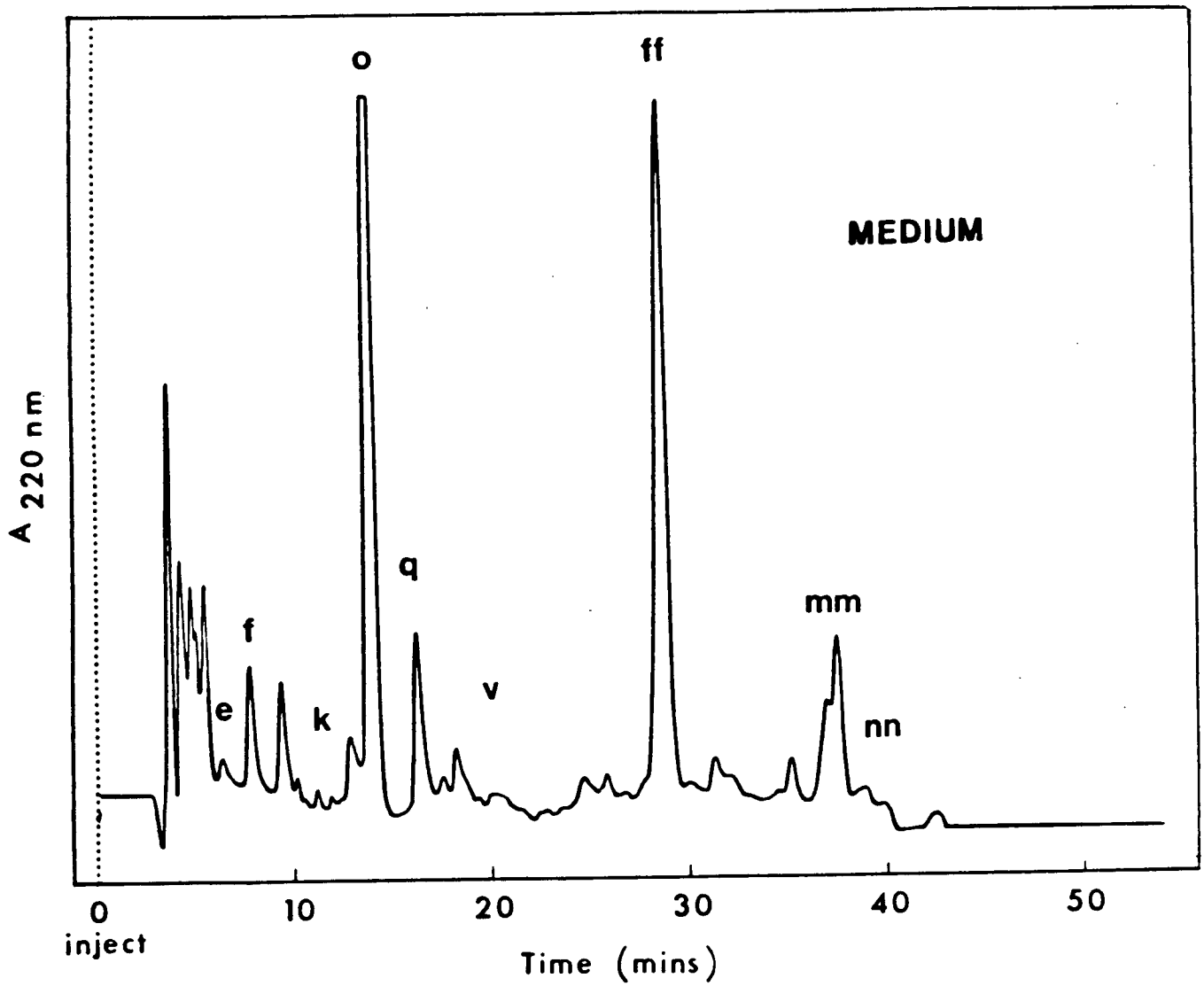


Figure 19.2 RP/HPLC separation of the water-soluble fraction from a medium cheese.

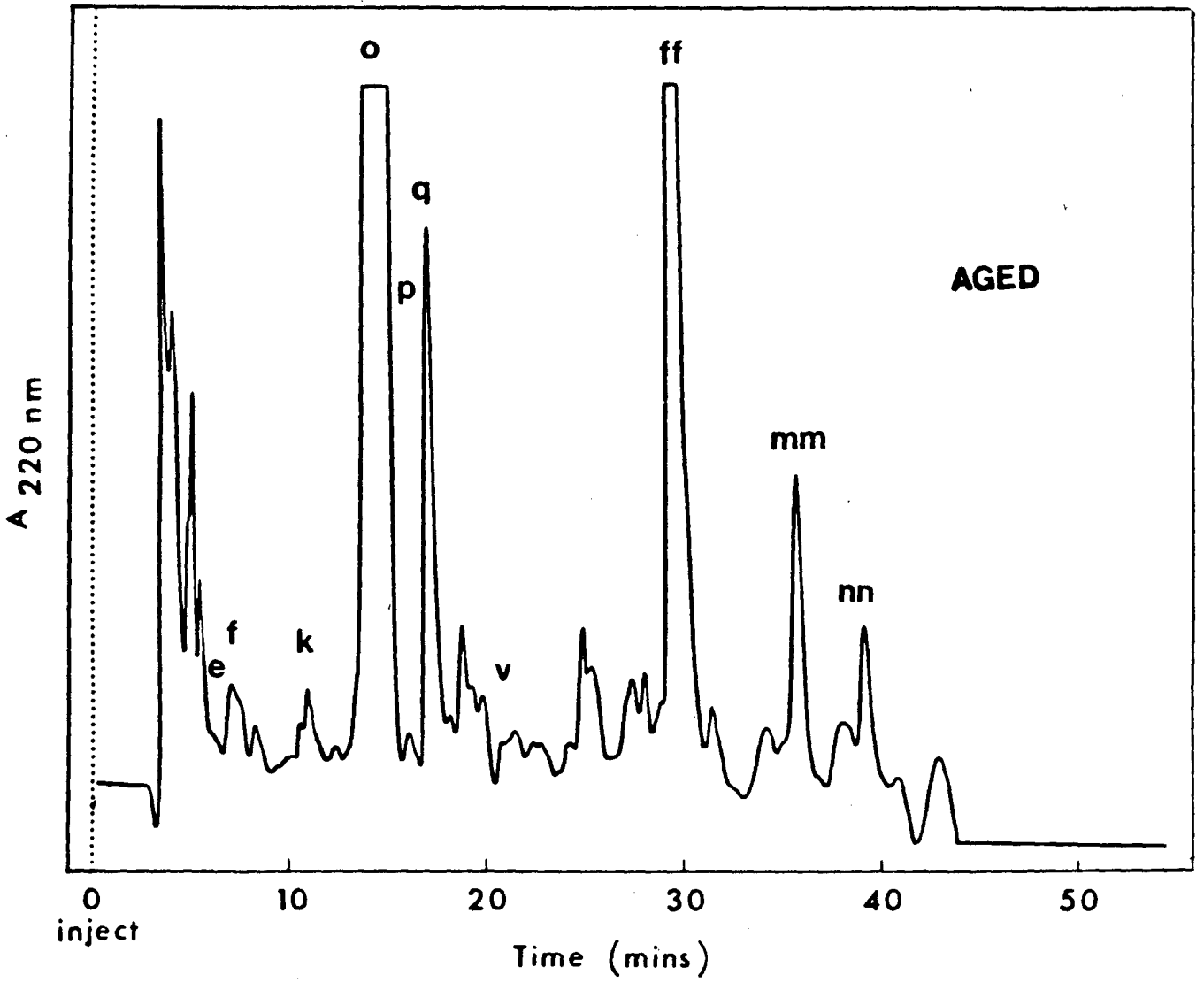


Figure 20.2 RP/HPLC separation of the water-soluble fraction from an old cheese.

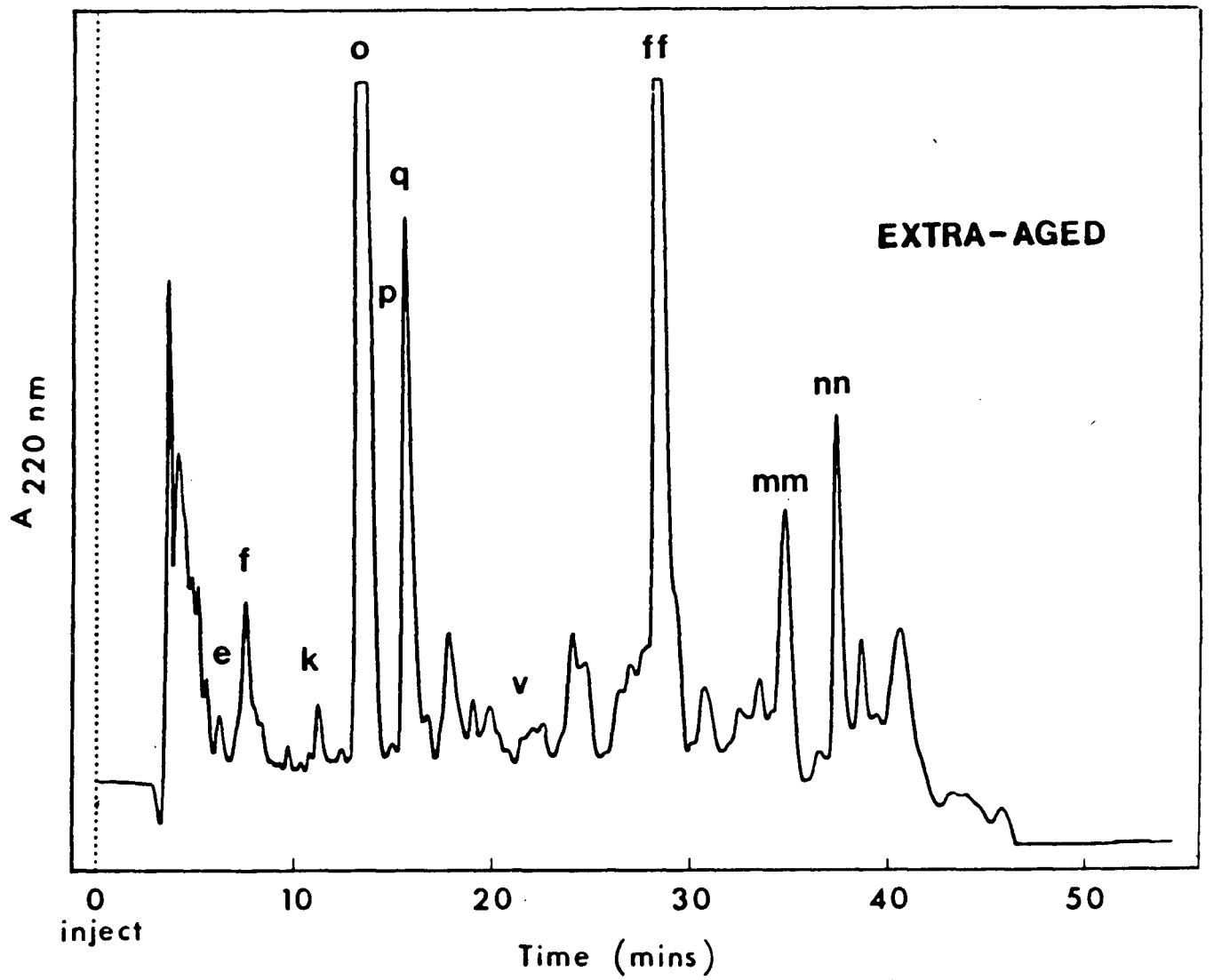


Figure 21.2 RP/HPLC separation of the water-soluble fraction from a X-aged cheese.

ripening. Generally, the older the cheese the greater the concentration of more hydrophobic compounds (Figures 20.2 and 21.2, peaks mm and nn). Champion and Stanley (1982) fractionated bitter peptides from Cheddar cheese and found that fractions with higher hydrophobicity were bitter. Although it can be assumed that these strongly retained fractions represent bitter peptides in the aged cheeses, their concentration was not enough to elicit bitterness and subsequent detection by sensory evaluation. The HPLC profile for a typical bitter X-aged cheese subjectively evaluated is shown in Figure 22.2. The pattern is essentially identical to the non-bitter X-aged sample (Figure 21.2). However, at the higher retention time, there was an increased number of peaks with a significant peak (rr) appearing at about 45 min. These strongly bound hydrophobic compounds, eluted only at the high concentrations of acetonitrile, are assumed to be responsible for the bitterness detected in the cheese. Furthermore, the most characteristic difference between the HPLC profiles of bitter cheese and non-bitter were these strongly retained peaks suggesting that RP/HPLC with the present mobile phase conditions permit separation of bitter peptides from the water soluble extract.

OPTIMIZATION

a) Super Modified Simplex

Differences in elution characteristics were obtained with different solvent systems. Mobile phases containing isopropanol, or propanol resulted in components eluting quickly with poor separation. This was due to the higher solvent strength and solubilization of the peptides. The use of acetonitrile as an organic modifier in spite of its low viscosity and UV-absorption combined with 0.1% trifluoroacetic acid (TFA, pH 2.1) significantly increased the efficiency of separation. When TFA was used,

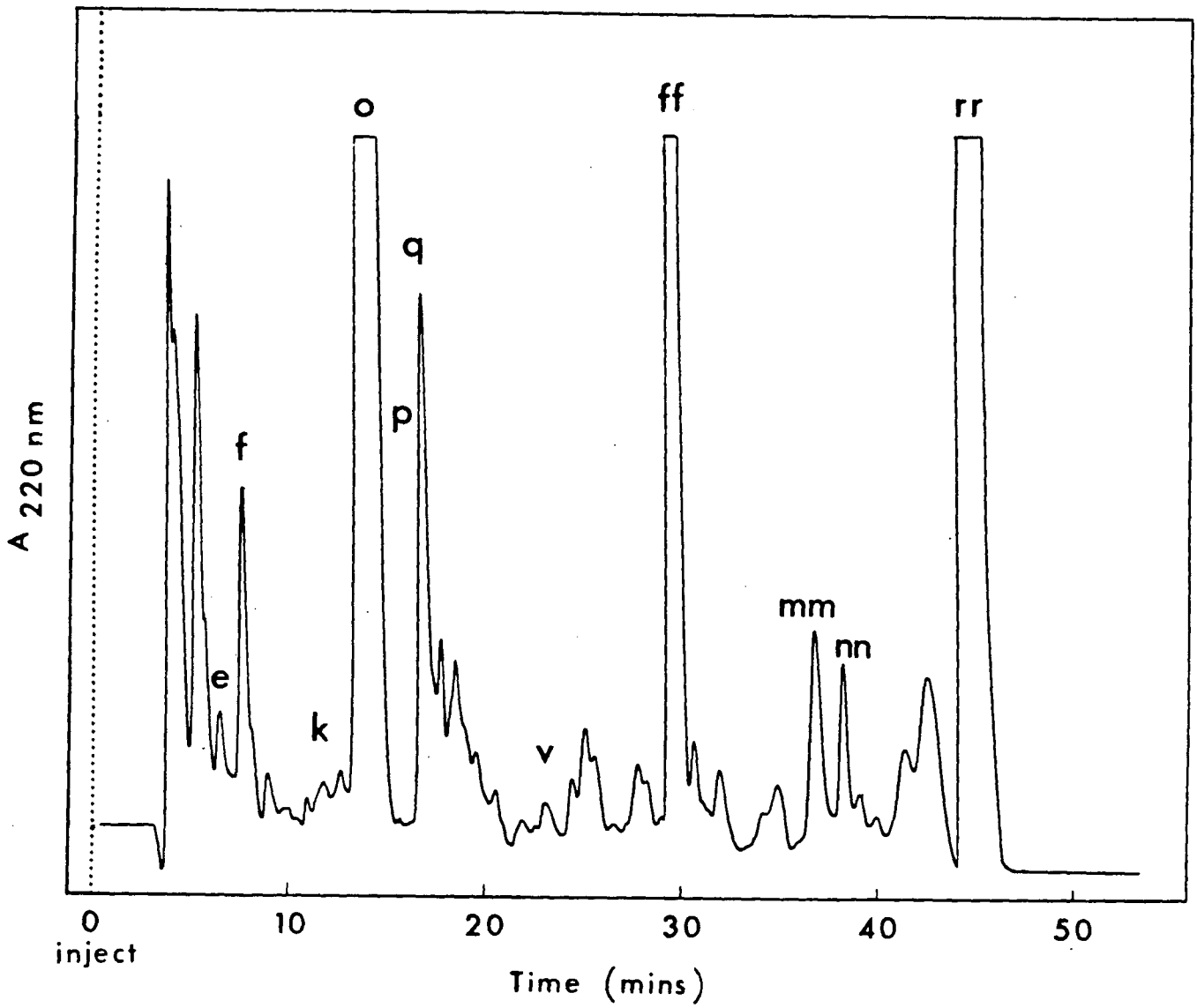


Figure 22.2 RP/HPLC separation of the water-soluble fraction from a bitter cheese.

the carryover of hydrophobic peptides was greatly reduced. Acharya et al. (1983) in their study on the tryptic peptides of hemoglobin suggested that TFA serves as an ion-pairing reagent and influences the retention times of histidine-containing peptides. Therefore, the basic groups in the presence of TFA makes the peptides relatively more hydrophobic.

Hearn et al. (1979) investigated the effect of pH and ion-pair formation on the retention of peptides. At the low pH, hydrophobic anionic reagents result in increased retention of the peptide samples whereas hydrophobic cationic reagents caused decreased retention. Thus retention was explained on the basis of either ion-pairing or ion-exchange interactions of the reagent with the protonated peptide.

The capacity ratios, k, of polypeptides are generally very sensitive to the concentration of the organic modifier. However, only a small difference in selectivity can be achieved by changing the organic solvents (Gazdag and Szepesi, 1981). Therefore, methanol and acetonitrile are generally accepted solvents in the HPLC analysis of polypeptides.

The pH stability of the Adsorbosphere C_8 column (pH 2-8) and the precipitation of the water-soluble extracts at the higher concentration of acetonitrile were used in developing constraints for the optimization. Levels chosen were: acetonitrile, 0-40%; TFA, 0-100%; flow rate, 0.5-2.0 mL/min; time, 30-60 min. Generally peptides were eluted within one hour at flow rates of approximately 1.0 mL/min. Temperature was not a factor in that Wilson et al. (1981) studied the influence of various experimental parameters on the behaviour of peptides on RP/HPLC and showed peptide retention to be temperature-independent between 20 and 55°C. All runs for the present study were carried out at room temperature (21-23°C).

An ultimate goal of most scientific research and investigation is to find the optimum conditions for achieving the objectives (Nakai, 1981). Therefore, the super modified simplex optimization introduced by Routh et al. (1977) with the experimental constraints previously discussed was employed.

The basic principle of the simplex optimization is to move away from the experimental conditions (Vertex) which has yielded the worst result in each simplex consisting of (n + 1) experiments, where n is the number of factors. Algorithms to find the next experimental conditions which move the experiment in the opposite direction from the worst result in the previous simplex can be programmed into a computer (Nakai et al., 1984).

The advantages of computer assisted optimization techniques are many: improved productivity by fewer experimental runs; multifactors including mutual interactions can be investigated without missing the true optimum, and possibly most important the optimum experimental factors can be found without relying on human intervention. The experimental conditions (Vertex) were generated by computer and after each experiment the peak resolution of the HPLC profile was calculated as described to provide the response. Each response was subsequently used in the algorithm to automatically direct the conditions to the optimum. Figure 23.2 shows the response values of the pattern search. Clearly the initial mobile phases, experiments 1 to 5 (Starting Simplex) resulted in poor peak resolution. As the optimization continued, resolution improved with the best condition obtained for experiment no. 9. It is a general trend in iterative optimization procedures that the speed of approaching the optimum is quickest at the beginning and progressively slows down thereafter (Nakai et al., 1984). This situation is true for mathematical models, however, in

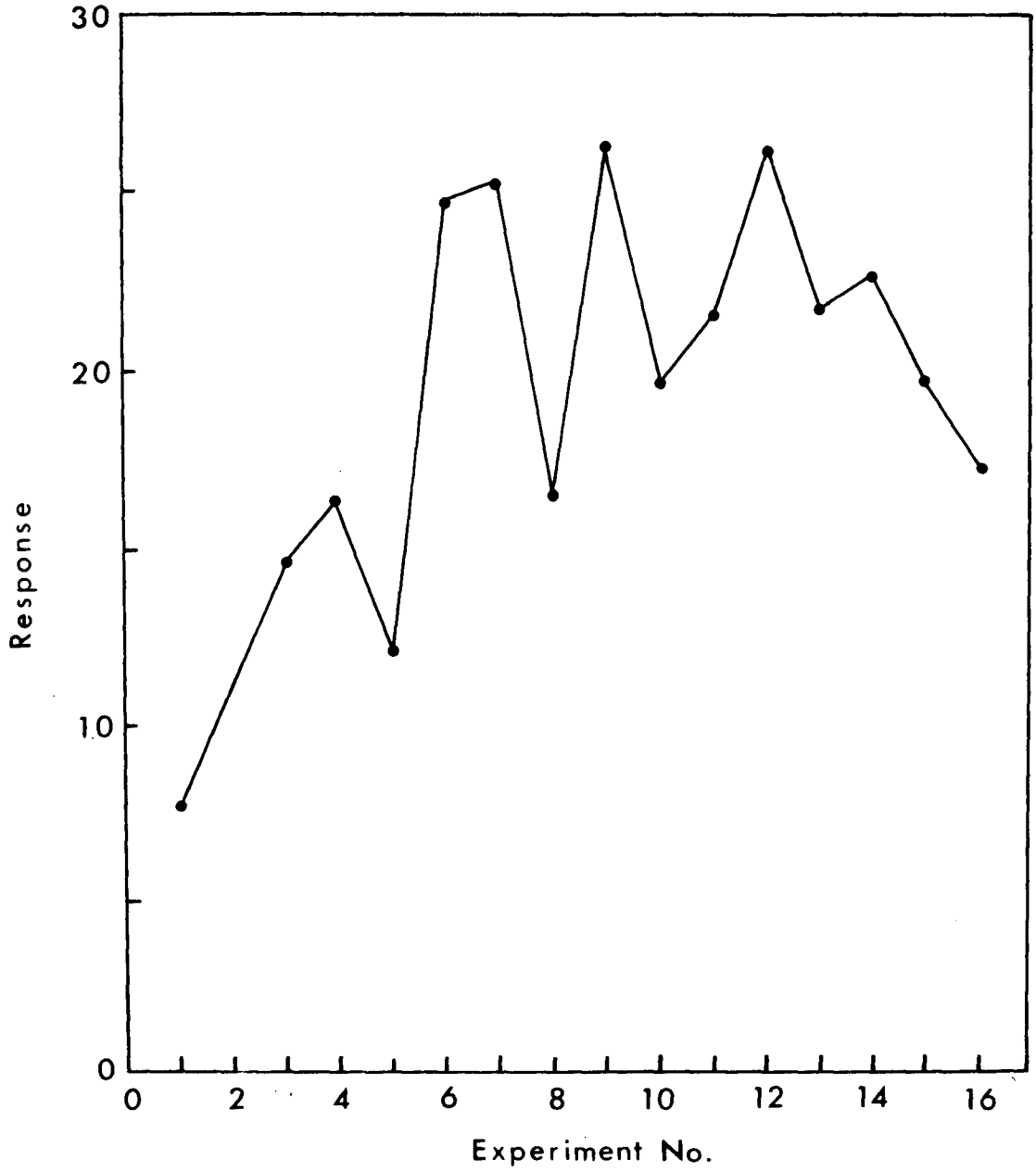


Figure 23.2 Optimization of the mobile phase to increase peak resolution of the water-soluble extract from a X-aged cheese sample. An Adsorbosphere C_8 column was used for all chromatographic runs.

real life situations converging at the optimum may not happen as shown in Figure 23.2. It is postulated that poor response values obtained after experiment 9 were due to the fact that some important factor may have been overlooked in the optimization. To verify that the mobile phase conditions of experiment no. 9 did in fact elicit the true optimum, repeated injections (5X) using the optimized conditions were carried out. Results obtained confirmed this as the true optimum with an average value of 106 and standard deviation of 1.58. It is interesting to note that experiment no. 12 resulted in a response similar to that of no. 9. Comparison of the mobile phase conditions for experiments no. 9 and 12 showed that the only significant difference was 100% trifluroacetic acid (TFA) for the latter and 44.6% for the former. It is reasonable to speculate that the increased concentration of TFA exceeded the amount required to make the peptides hydrophobic through ion-pairing and concentrations greater than 44.6% had no effect on improving resolution.

b) Mapping Super Simplex (MSS)

This technique allows the graphic representation in two dimensions of the response surface of the simplex optimization. The mobile phase conditions or factors were: flow rate, acetonitrile concentration, time and TFA concentration and their constraints selected as previously discussed. The response value for each experiment in the MSS is plotted against the individual factor as shown in Figures 24.2-27.2. The data points for one factor but also belonging to the same group of other factors are referred to as matched data points. Therefore joining these points together with lines establishes the response surface and the direction or location of the optimum level is indicated by where the lines converge on the graph. Sometimes the lines converge decisively as shown in Figure 26.2

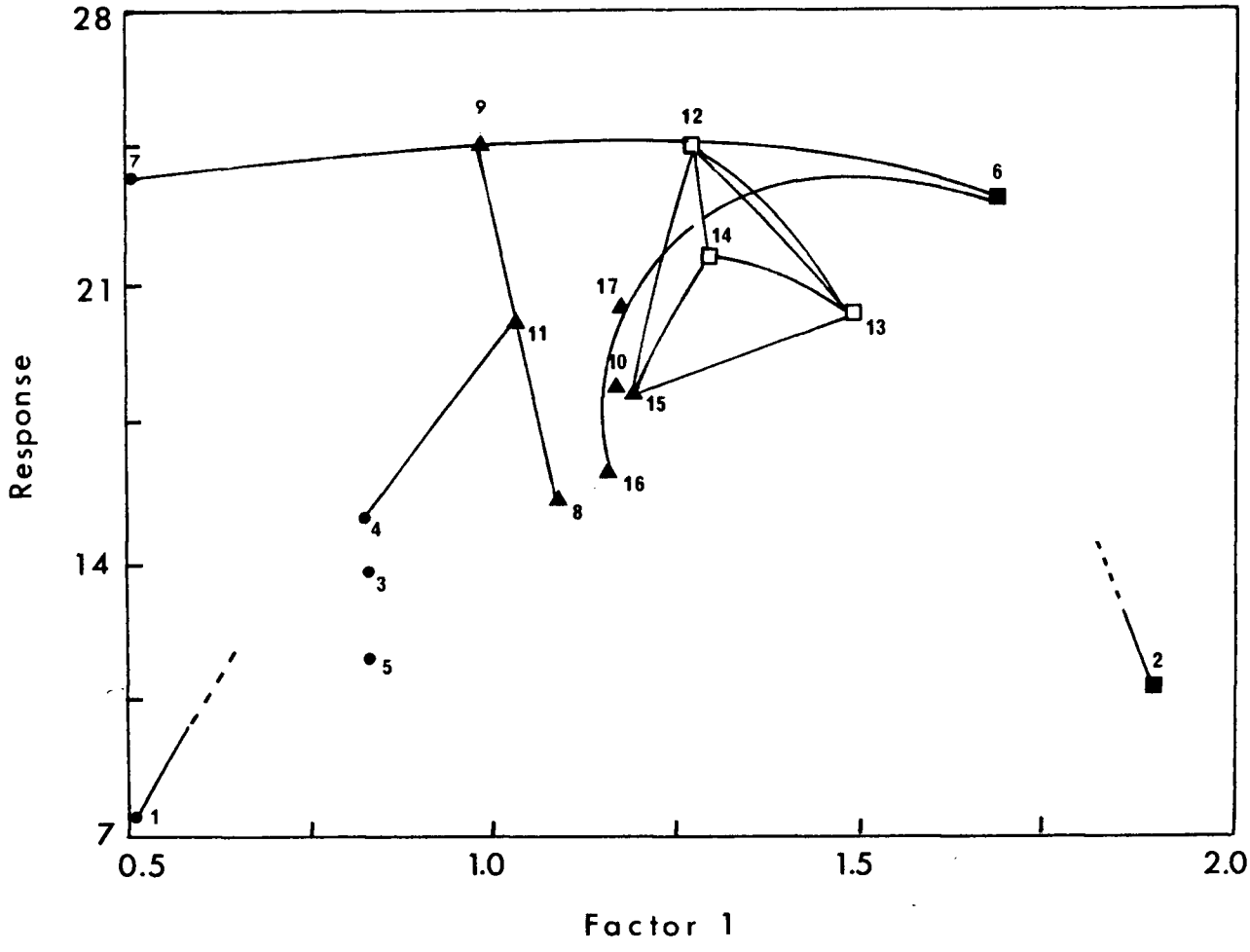


Figure 24.2 Mapping results of experiments to maximize peak resolution. Factor 1, flow rate with constraints 0.5-2.0 mL/min. -●-, group 1; -▲-, group 2; -□-, group 3; -■-, group 4.

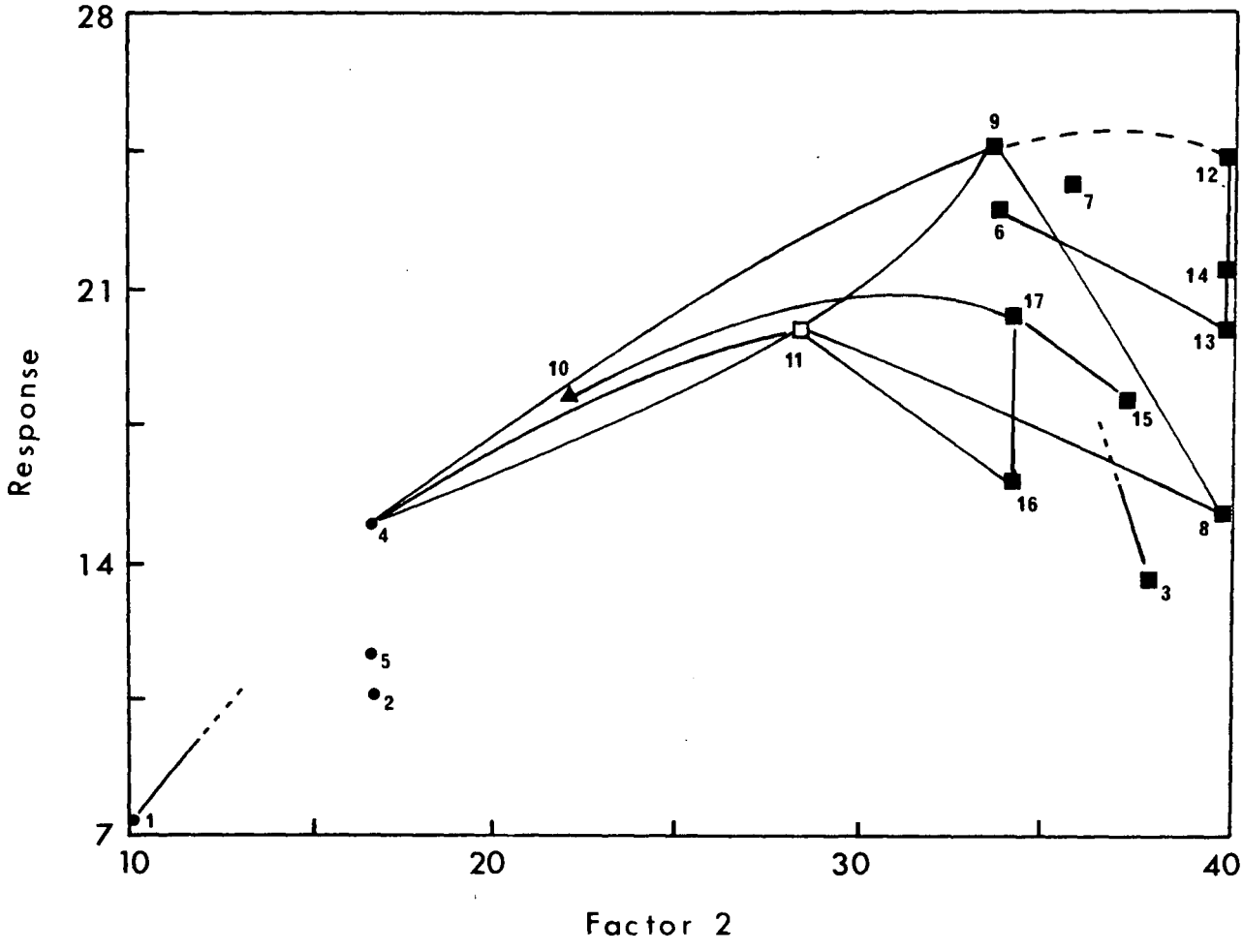


Figure 25.2 Mapping results of experiments to maximize peak resolution. Factor 2, acetonitrile concentration with constraints 10 - 40%. -●-, group 1; -▲- , group 2; -□-, group 3; -■-, group 4.

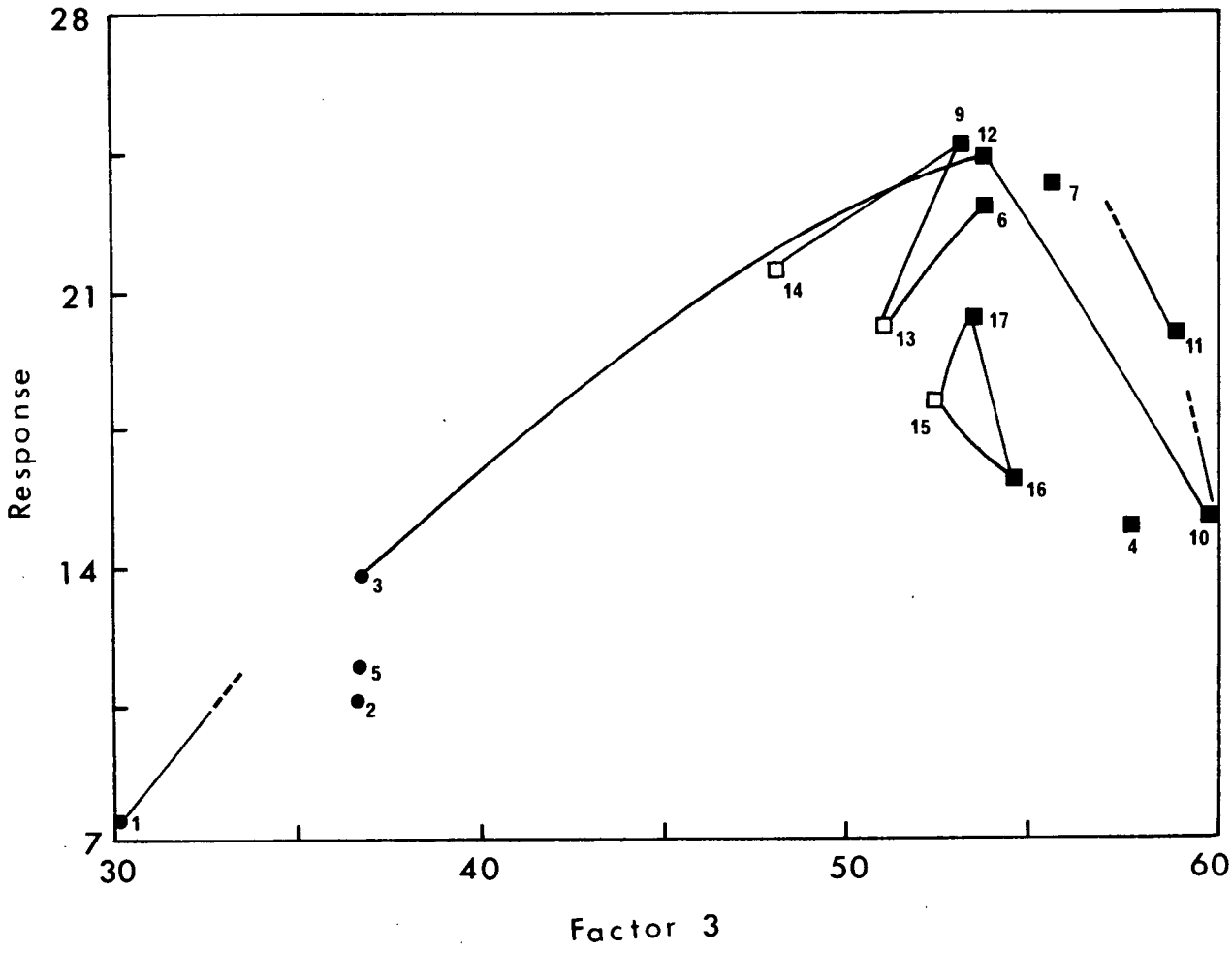


Figure 26.2 Mapping results of experiments to maximize peak resolution. Factor 3, time with constraints 30 - 60 min. -●-, group 1; -□-, group 3; -■-, group 4.

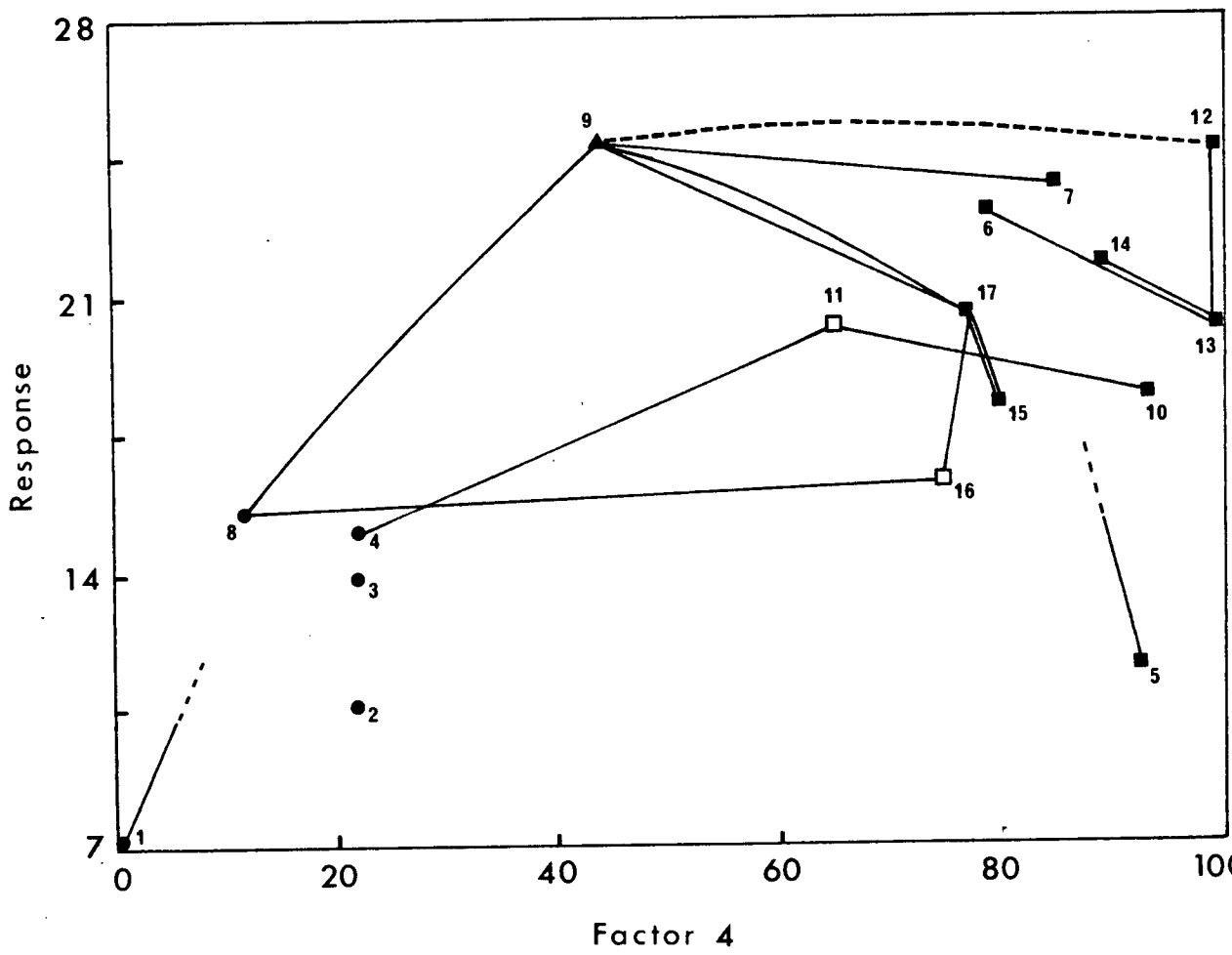


Figure 27.2 Mapping results of experiments to maximize peak resolution. Factor 4, TFA concentration 0 - 100%. -●-, group 1; -▲-, group 2; -□-, group 3; -■-, group 4.

where the optimum value for time is clearly Vertex 9 representing 56.6 min. In Figures 24.2 and 25.2, the optimum flow rate and acetonitrile concentration respectively are not as clear and may be located between Vertex 9 and 12. TFA concentration (Figure 27.2) was the least conclusive but the trend curves are toward the direction of Vertex 9.

c) Simultaneous Factor Shift

Results of the simultaneous factor shift experiments which investigates conditions around the optimum levels, in all cases produced response values less than that obtained for Vertex 9. The simultaneous shift factor program can be considered a 'fine tuning' to ensure that the true optimum is not missed. Indeed in the present study, the optimum mobile phase conditions were found to be Vertex 9 and these conditions resulted in the best HPLC profiles.

PRINCIPAL COMPONENT ANALYSIS (PCA)

The sixty HPLC chromatograms, each one representing a specific cheese sample for which each peak was converted to percent area, were analyzed by PCA. The intent of PCA was to select from each HPLC profile patterns (peaks) characteristic of the cheeses. Thus on the basis of these patterns the cheeses could be classified into groups. The HPLC data, specifically peak areas, were transformed to describe the same amount of variability in order to extract significant factors for cheese flavour contained in the 48 peaks. Eleven factors had eigenvalues larger than 1.0 as shown in Table 10.2. In other words, the eigenvalue describes the magnitude of variance of each principal component which accounts for the total variance. The larger the eigenvalue, the greater the contribution of the principal component for classification. From Table 10.2 the cumulative proportion of

Table 10.2 Eigenvalue (VP), sum of VP, and cumulative proportion in total variance (%) in a principal component analysis.

Principal Component	Eigen Value	Sum of VP	Cumulative Proportion
1	17.39	17.39	36.2
2	5.54	22.93	47.8
3	5.27	28.20	54.6
4	3.04	31.24	60.9
5	2.47	33.71	66.0
6	2.38	36.09	71.0
7	2.09	38.18	75.4
8	1.80	39.98	79.1
9	1.60	41.58	82.5
10	1.32	42.90	85.2
11	1.19	44.09	87.7
12	0.94	45.03	89.7
13	0.82	45.85	91.4

total variance up to the eleventh principal component was 87.7%. Therefore, total variance contained in the 48 peaks could be contracted in 11 principal components with 12.3% loss of variance. Factor loading for the major five principal components and communality are shown in Appendix I. Peaks with large factor loadings indicate close association with the principal components. As shown, peaks 1, 19, 28, 36, 39 and 41 had the largest factor loadings for the first principal component whereas peaks 3, 8, 9 and 33 were largest for the second principal component. Visual examination of these most discriminating peaks on the HPLC chromatograms were difficult to interpret. Moreover, the intent of principal component analysis as a pattern recognition technique is to graphically illustrate the data. However, when this was attempted, distinct age classification into groups based on the principal components was not obtained. Figure 28.2, a plot of Factor loading 1 vs. Factor loading 2 did not separate the cheese samples. Additional plots of Factor 1 vs. Factor 3 and Factor 2 vs. Factor 3 although not shown also resulted in poor separation of the cheeses. A possible explanation is that the principal components are classifying the cheeses based on something other than age. Aishima (1979) suggested that the factor loadings for each principal component lacked discriminating information which may have occurred in the present study for age classification.

An attempt was made to examine the relationship between sensory score of the cheese and principal components from the HPLC profiles. Plots of the first and second principal components (Appendix I) vs. sensory score could not separate the cheese samples based on age classification. It is reasonable to assume that based on the HPLC data provided, principal component analysis is extracting discriminating factors, but factors which are not significant for age classification.

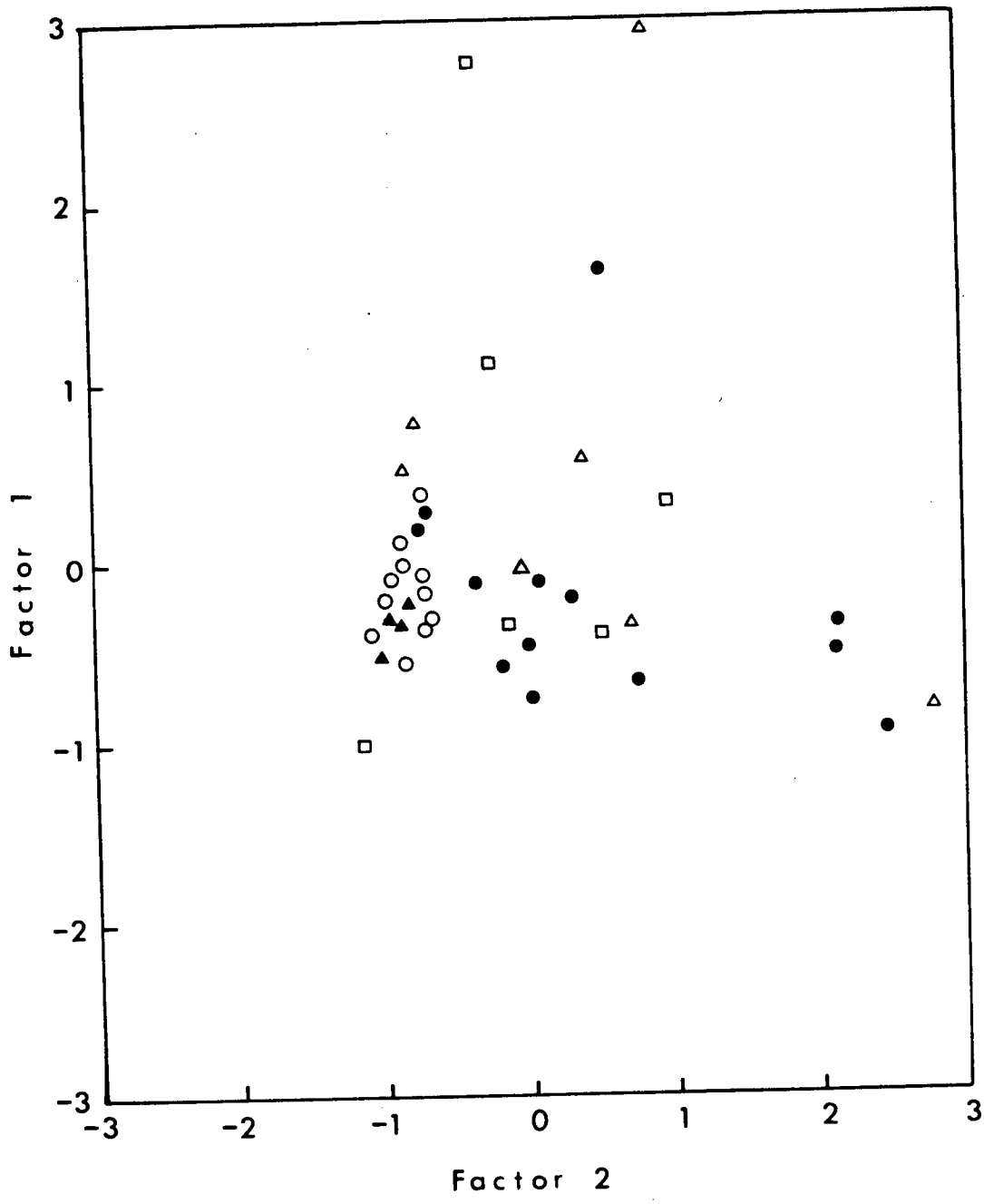


Figure 28.2 Principal component plot of factor 1 vs factor 2. X-aged - △ ; old - □ ; medium - ● ; mild - ○ ; young - ▲.

REGRESSION ON PRINCIPAL COMPONENTS

The transformed HPLC data were analyzed by multiple regression analysis, in order to determine which of the principal components was important for age discrimination. Appendix II shows the correlation between principal components (PC) and the dependent variable. The first PC with a correlation of -0.79978 best describes the relationship. The index of the PC's being entered show that PCs no 1, 10, 3, 15, 29 and 21 resulted in a r^2 value of 0.8130. In the regression equation the degrees of freedom for the F-value are p and N - p - 1 where p is the number of components in the regression (principal components) and N is the number of cases (cheese samples). This is an overall test of significance for the regression equation. The F-value component to enter is a test of the significance of the coefficient of this component only and the degrees of freedom for this F-value are 1 and N - p - 1. Clearly, the degrees of freedom after the first three PC is (1, 37) and F = 7.42. Therefore, after the third PC the F-values are no longer significant. Thus, the PC's 1, 10 and 3 best describe the age of the cheese. The eigenvalues for the factor loadings for PC one through ten are shown in Appendix III. The HPLC peaks with high eigenvalue were considered significant in describing the total variance and used for the basis for the selection in describing the classification. However, from the plots it was evident that classification was not based on age.

STEPWISE LINEAR DISCRIMINANT ANALYSIS

Using the optimized gradient elution 48 peaks were separated from the water-extract. The percent area for each peak was calculated and the flavour score, grade and age assessed as previously discussed. The peak

areas and age classification were used in the discriminant analysis (BMDP:7M program). F-values of 4.000 and 3.996 to-enter and to-remove respectively were used. The particular variable(s) (peak) that adds the most or least to the separation of the groups is entered into the discriminant function. The variable (peak) with the highest F-to-enter is the variable that discriminates best between groups and was found to be the 32nd peak labelled (ff) in Figures 17.2 to 21.2. The next variable having the highest F-to-enter was entered and the procedure continued in a stepwise fashion. From the 48 peaks obtained the statistical procedure was able to reduce this number to 8 significant peaks resulting in 100% correct classification. The significant peaks in the following order of importance were: ff, p, nn, v, q, o, k, e, and f. Peak ff was later removed from the discriminant analysis because of a F-value of 3.687.

Figure 29.2 shows a canonical plot of the group means. The first canonical variable is the linear combination of variables entered that best discriminates among the groups; and as shown, good classification was obtained. Figure 30.2, a canonical plot of each individual cheese sample was able to separate the cheese into distinct groups. However, the mild and medium category were not clearly separated. This was not surprising in that with the sensory evaluation significant differences between these two groups was not found ($P > 0.05$).

The plot (Figure 30.2) shows that cheese aging is a complex dynamic process evident by the almost linear progression of the group classifications with X-aged separated from the others. This continuous function along the X-axis was also found by Pham and Nakai (1984).

Based on the data provided, SLDA calculates discriminant functions which are the linear combinations of the eight significant peaks and used

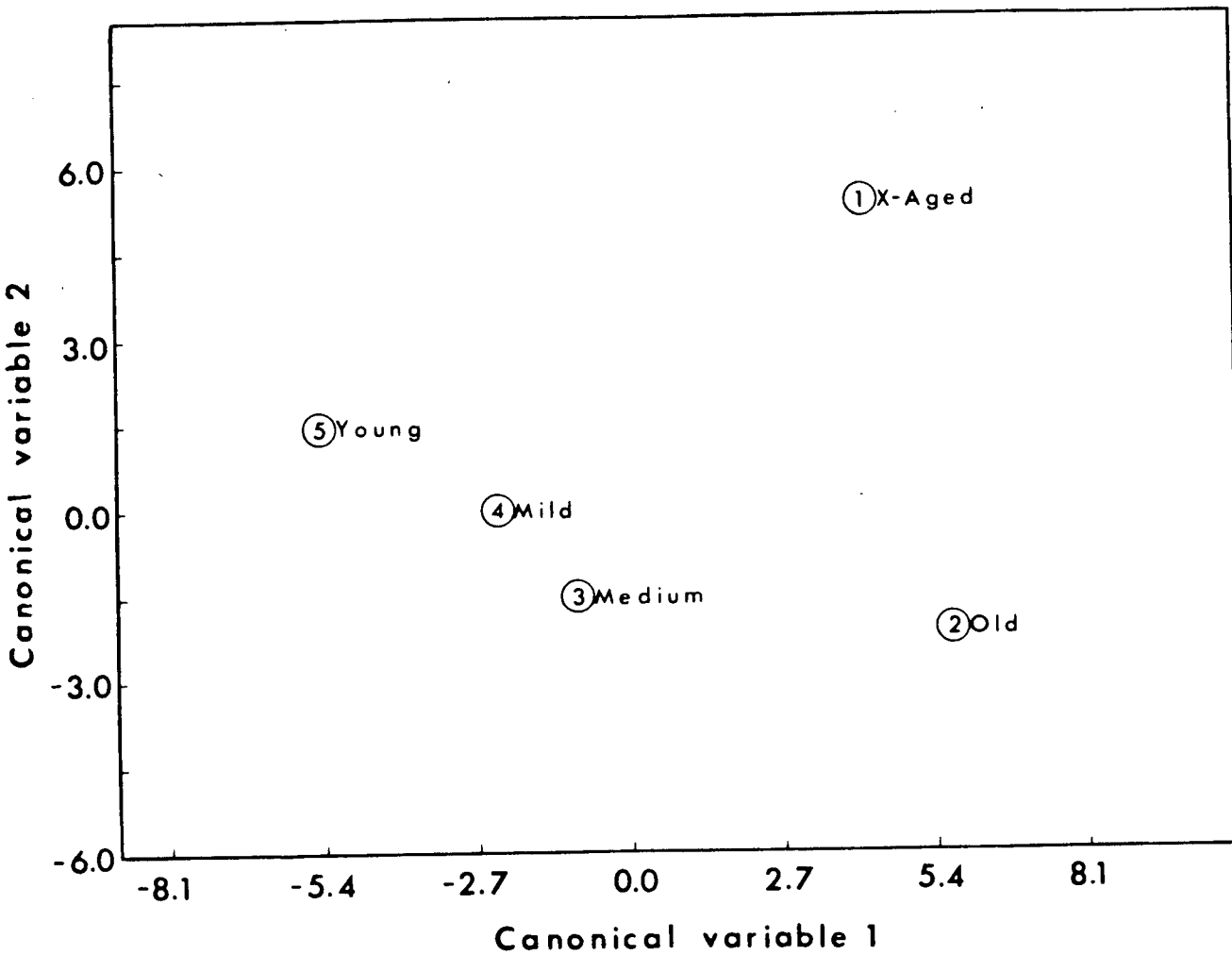


Figure 29.2 Canonical plot of the group means for the 60 cheese samples.

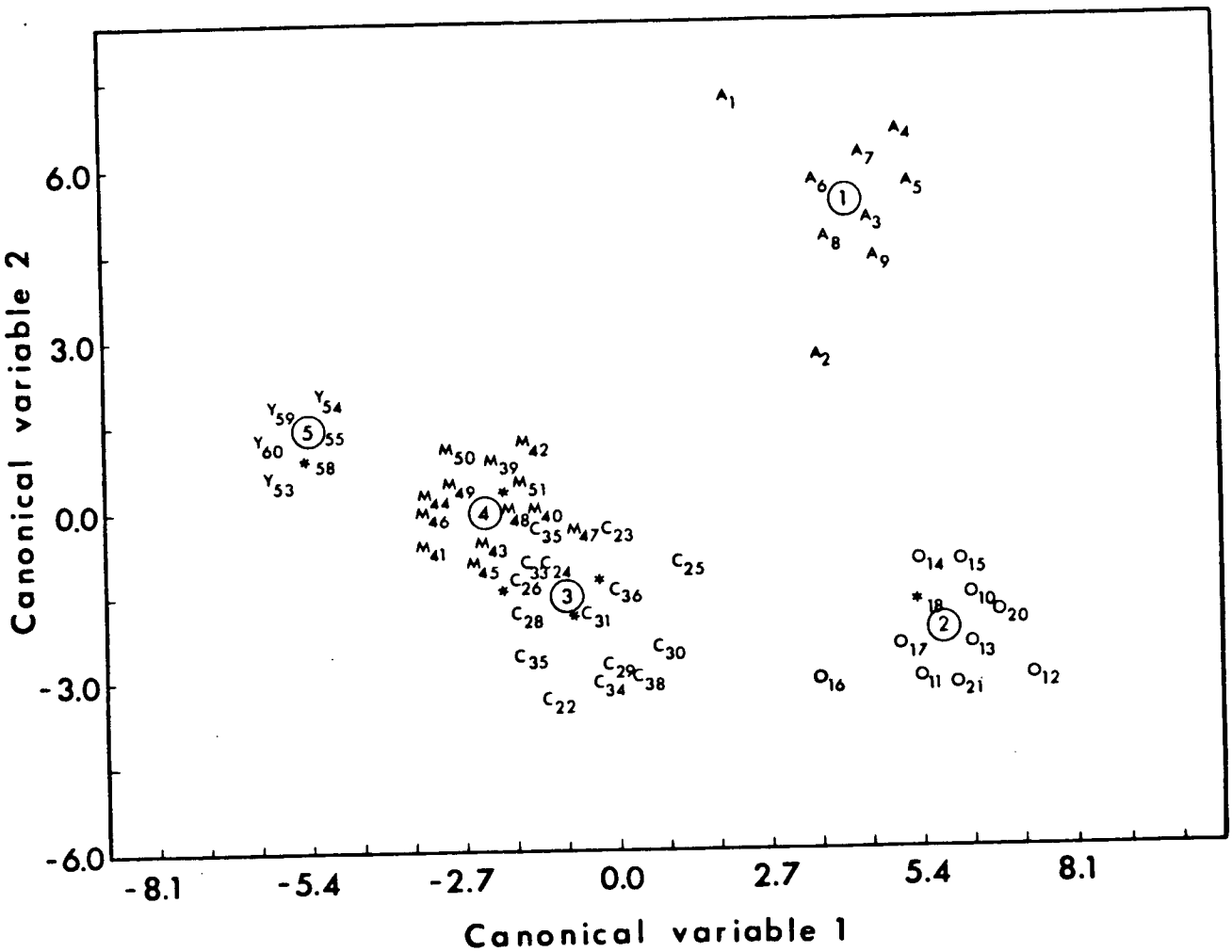


Figure 30.2 Canonical plot of the 60 cheese samples. A, X-aged; O, old; C, medium; M, mild; Y, young. Overlap indicated by asterisk.

for classification of the groups. The discriminant functions were calculated as follows for the five groups:

X-Aged: 1.16(e) - 4.34(f) + 3.55(k) + 0.16(o) + 5.55(p) + 1.50(q) + 0.20(v) - 1.08(nn) - 62.53

Old: -0.94(e) - 2.82(f) + 3.98(k) + 0.44(o) - 5.99(p) + 1.71(q) + 5.89(v) - 2.99(nn) - 75.42

Medium: -1.02(e) - 0.13(f) + 0.17(k) + 0.25(o) - 2.93(p) - 0.73(q) + 2.45(v) - 1.53(nn) - 19.56

Mild: -0.21(e) - 0.92(f) + 0.93(k) + 0.14(o) - 1.81(p) + 0.58(q) + 1.84(v) - 1.04(nn) - 9.25

Young: 0.52(e) - 0.51(f) + 0.12(k) + 0.0(o) + 0.57(p) + 0.07(q) - 0.34(v) - 0.17(nn) - 2.81

Four cheese samples which were assessed as old by the sensory panel were handled as if there were unknown samples. By substituting the peak areas (e, f, k, o, p, q, v, and nn) into each of the classification functions as shown above yields a discriminant score. The largest discriminant score is therefore the group to which the unknown samples are assigned. A graphic representation is shown in Figure 31.2 with the correct classification of the unknown samples into the old group. Indeed as suggested by Pham and Nakai (1984) an improvement in RP/HPLC resolution as found in the present study results in better discriminant analysis.

The HPLC profile (peak areas) of the water-soluble extract of a downgraded acid and fruity cheese sample was subjected to SLDA. The technique clearly classified the samples into the correct age group but was unable to discriminant between the first grade and the two downgraded cheese samples. The result was not surprising in that both these defects are not associated with the water-extractable portion of the cheese.

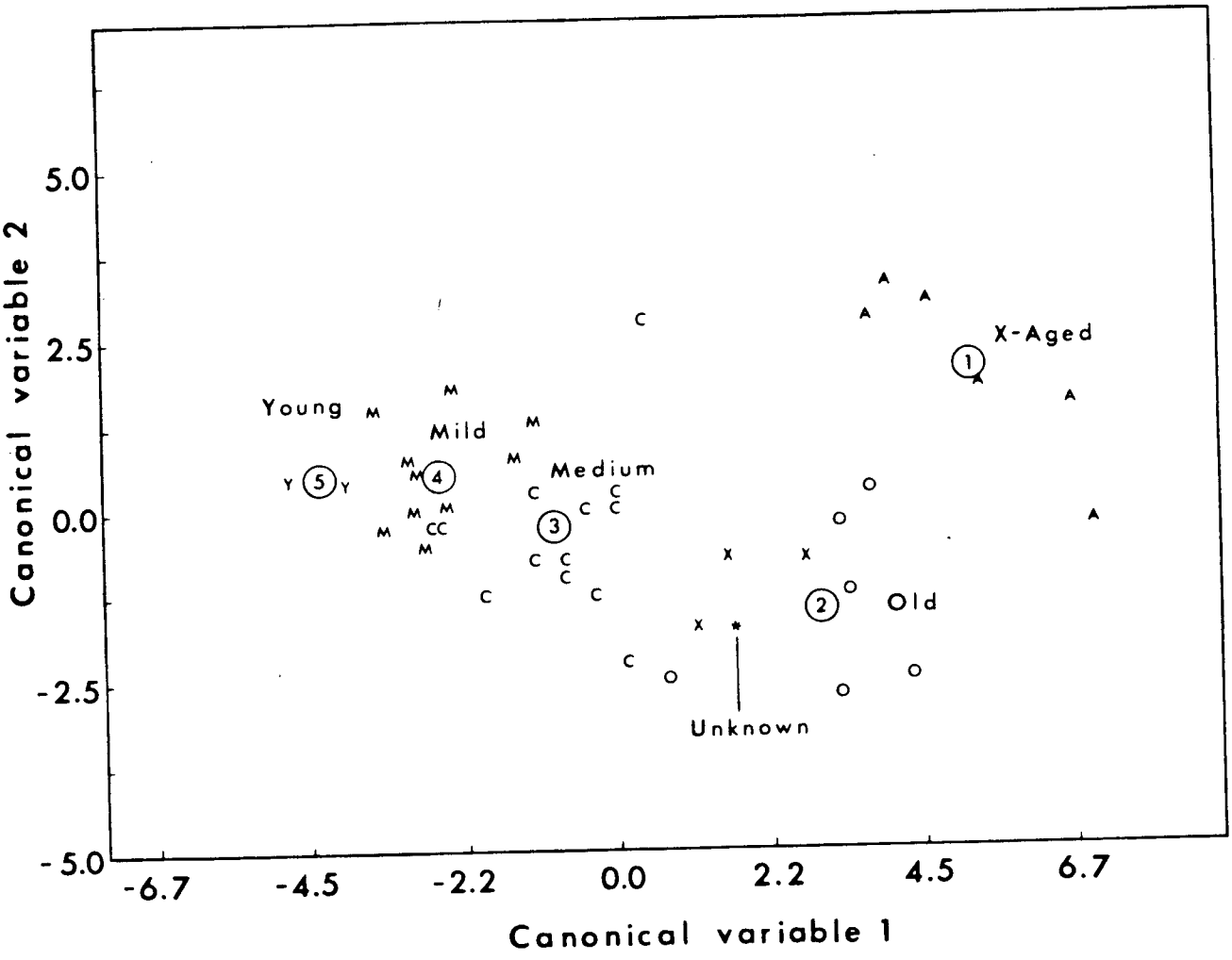


Figure 31.2 Canonical plot of the cheese samples showing location of the unknown samples, X. Overlap indicated by asterisk.

Fruitiness was found to be mainly due to the esters ethyl butyrate and ethyl hexanoate (Aston and Dulley, 1982; Forss, 1979) while acidity is determined by pH and lactic acid. However, when the statistical technique was applied to bitter cheeses with a typical HPLC profile as shown in Figure 22.2 two distinct classifications were obtained (Figure 32.2). Peak q was the variable that was found to discriminate best between groups and a new set of discriminant functions were calculated. Although good separation was obtained between first grade and downgraded bitter cheese samples the statistical techique resulted in only 88.1% correct classifcation for the six groups. Aishima (1979) in his study of the aroma quality of soy sauce using discriminant analysis suggested that complete discrimination of all groups on the basis of two axes on the canonical plot is difficult.

AMINO ACID ANALYSIS

Figure 33.2 shows a typical amino acid profile for the water-soluble extract from a X-aged cheese sample. Profiles from young, mild, medium and old all had similar patterns. The results of the amino acid analysis (Table 11.2) showed no significant difference ($P > 0.05$) among the cheese samples including the bitter and acid sample. Gooda *et al.* (1983) using the extraction procedure of Harwalkar and Elliot (1970) followed the changes in free amino acids and peptides during the ripening of Cheddar cheese from milk treated with lactase and found the accumulation of free amino acids was proportional to the ripening period. The amount of water-soluble peptides and amino acids increased during proteolysis

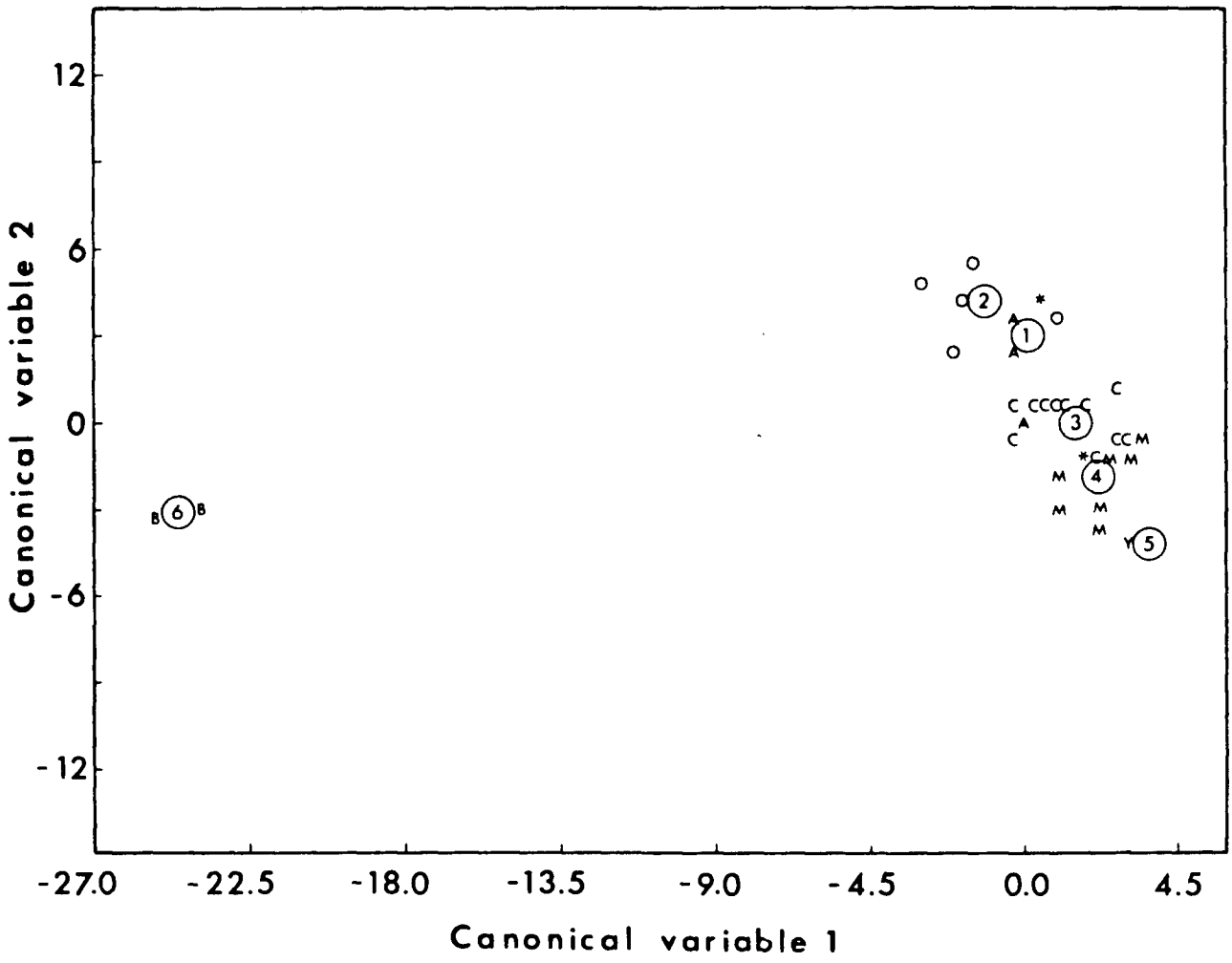


Figure 32.2 Canonical plot of the cheese samples showing location of the bitter samples. A, X-aged; O, old; C, medium; M, mild; Y, young; B, bitter. Overlap indicated by asterisk.

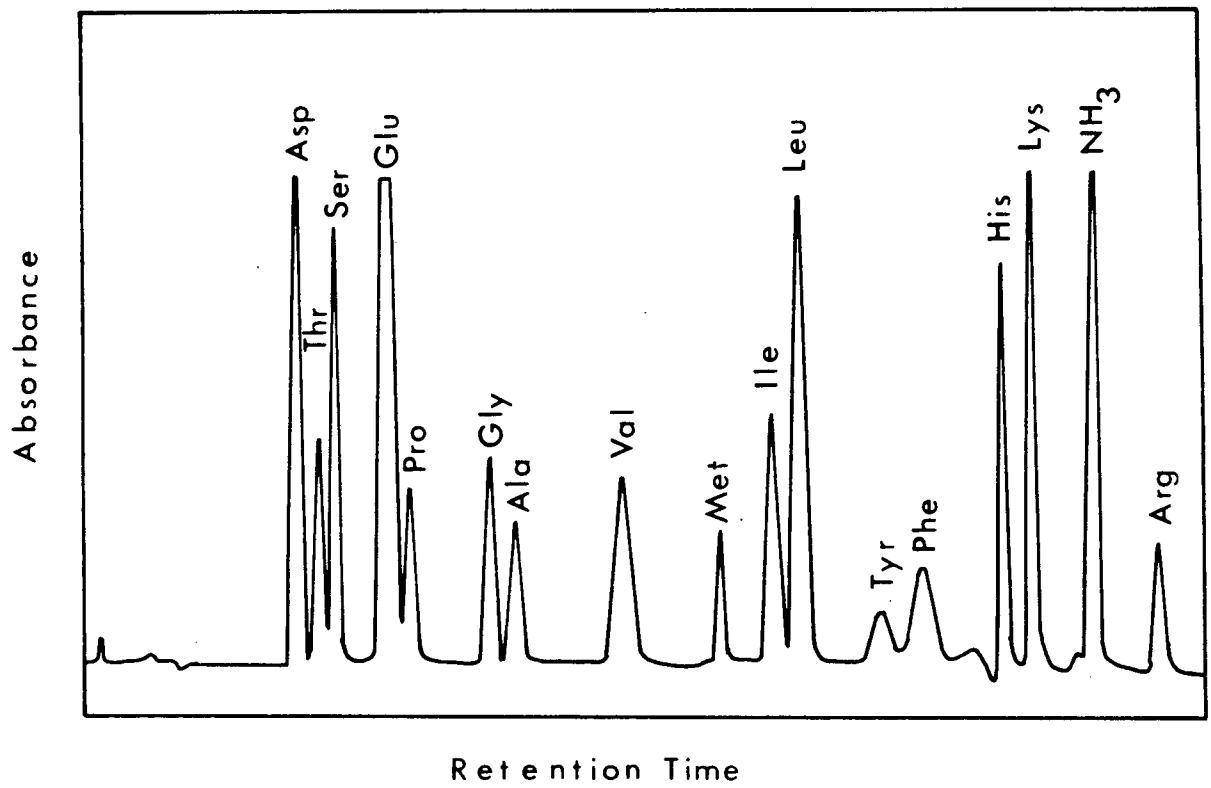


Figure 33.2 Amino acid profile of water-extract from an X-aged cheese sample.

Amino Acid	Percentage of Total N						
	Young	Mild	Medium	Old	X-Aged	Old (bitter)	X-Aged (acid)
ASP	7.0	6.7	5.7	7.1	7.3	7.0	6.9
THR	3.5	3.3	3.1	3.2	2.6	3.0	3.2
SER	4.7	4.9	4.3	4.4	4.3	5.2	4.4
GLU	21.6	23.6	24.6	22.7	22.3	23.3	22.7
PRO	9.4	9.0	8.7	8.8	8.3	8.2	9.2
GLY	1.7	1.5	1.9	1.7	1.9	1.8	1.6
ALA	2.0	2.0	3.3	2.0	1.9	1.9	2.0
CYS	0.0	0.0	0.0	0.0	0.0	0.0	0.0
VAL	5.6	5.8	6.3	5.5	5.5	5.7	5.6
MET	2.0	2.4	2.3	2.1	1.9	2.2	2.0
ILE	4.9	5.0	5.6	4.8	4.7	5.1	4.8
LEU	9.4	9.0	9.6	9.2	10.3	9.4	9.4
TYR	3.2	2.7	0.9	3.1	3.1	2.8	3.5
PHE	6.3	6.6	5.5	6.5	5.9	5.7	6.5
HIS	4.5	5.2	6.0	4.5	6.0	4.5	4.5
LYS	9.4	9.4	10.9	9.6	9.9	9.8	9.2
ARG	4.6	4.0	1.4	4.7	4.2	4.4	4.6

Table 11.2 Amino acid analysis of the total water-soluble extract from various cheeses.

(Table 9.2) and could be used as an indication of degree of maturity of ripening cheese as has been done by others (Ney, 1971; Nieuwoudt, 1977). Law and Sharpe (1977) considered amino acids to be intermediate products in the production of certain aroma compounds. Therefore, there appears to be considerable confusion in the literature on the role of free amino acids during ripening and their subsequent contribution to Cheddar cheese flavour. Although the amino acid composition of the water-extractable fraction was the same for the different ages of cheese (Table 14.2) the respective HPLC profiles were characteristic for each age category suggesting the importance of the peptides.

ANALYSIS OF THE HPLC FRACTIONS

Fractions f, o, ff and rr from a X-aged bitter sample (Figure 22.2) were collected after repeated injections: Homogeneity of each fraction after HPLC analysis indicated freedom from other interfering peaks. Further work is required on the amino acid composition of these fractions thereby relating peptide hydrophobicity and subsequent retention on the HPLC column. Moreover, the importance of the statistical pattern recognition techniques in selecting significant peaks characteristic of Cheddar cheese taste and in particular bitterness should be assessed.

CONCLUSIONS

Cheese aging is complex and the agents responsible for Cheddar cheese flavour are still largely unknown. However, the non-volatile water extractable fraction has been shown to be important for flavour intensity.

1) As the cheese ages protein degradation products accumulate in the water-soluble fraction.

2) The extraction procedure removed soluble N components from the methylene chloride fat fraction.

3) No significant difference was found for cheese flavour assessment between the trained sensory panel and the Federal graders.

4) Analysis of the water extracts from more than 60 Cheddar cheese samples by RP/HPLC on a C_8 Adsorbosphere column using an optimized ternary gradient system yielded more than 45 peaks.

5) The systematic optimization of the mobile phase for selectivity resulted in well resolved HPLC profiles. The elution patterns differed for the various age categories of cheese.

6) Graphic representation of the data using mapping super simplex clearly located the optimum experimental condition and was confirmed by simultaneous factor shift.

7) Statistical pattern recognition techniques were used to interpret the peaks in the HPLC profiles and correctly classified the cheese samples into distinct groups.

8) Based on the calculated discriminant functions unknown samples were correctly classified according to their discriminant score.

9) Bitter cheese samples were clearly identified by their typical HPLC profile showing strongly adsorbed hydrophobic peptides eluting only at the high concentration of organic solvent.

10) Amino acid analysis of the water extract for the different categories of cheeses showed no relationship between the total amino acids and the intensity of Cheddar cheese flavour.

Further work is currently underway to determine the distribution of amino acids from peaks discriminated as being important to Cheddar taste with particular emphasis on bitter peptides. Moreover, the multivariate data technique analyzes the entire HPLC profile, thus has significant advantages over visual interpretation which may, by its subjective nature, overlook important variables.

This study proposes a chemical index to measure cheese maturation. However, incorporation of additional chemical and physical data into the discriminant analysis would provide complete objective assessment of overall Cheddar cheese quality thus allowing interpretation of many different variables simultaneously.

Appendix 1 Factor loading and communality matrix.

Peak No.	Principal Components					Cumulative proportion of total variance
	1	2	3	4	5	
1	0.796	-0.044	0.077	-0.441	-0.016	0.362430
2	0.281	0.123	0.702	-0.343	0.132	0.477758
3	0.152	0.833	0.243	-0.124	-0.207	0.545785
4	-0.179	0.278	-0.264	-0.141	-0.514	0.609057
5	0.170	0.535	0.366	0.539	0.033	0.660511
6	0.214	0.254	0.728	0.222	0.063	0.710010
7	0.076	0.483	0.299	0.151	-0.199	0.753719
8	0.089	0.842	-0.038	0.096	0.046	0.791335
9	0.048	0.736	0.496	-0.188	-0.073	0.824826
10	0.221	0.654	-0.133	0.086	-0.094	0.852286
11	0.088	0.653	0.530	-0.132	0.067	0.877159
12	0.143	0.344	0.776	0.250	0.061	0.896808
13	0.191	0.564	0.355	0.088	0.090	0.913942
14	0.361	-0.027	0.139	0.740	0.029	0.928255
15	0.504	0.156	0.628	0.186	0.095	0.942308
16	0.014	0.518	0.522	-0.003	-0.064	0.952797
17	0.307	0.323	0.149	0.171	0.727	0.962063
18	0.089	-0.113	0.812	0.144	0.424	0.968440
19	0.740	0.388	0.224	0.238	0.093	0.974073
20	0.528	-0.067	0.032	0.146	0.556	0.978997
21	0.339	0.137	0.482	0.383	0.430	0.982891
22	0.016	0.232	0.155	0.118	0.062	0.986122
23	0.064	0.187	0.123	0.090	0.128	0.988729
24	0.072	0.180	0.283	-0.028	-0.052	0.991084
25	0.336	-0.078	0.096	0.681	0.117	0.992781
26	0.041	-0.056	0.152	-0.115	0.853	0.994129
27	0.582	0.148	0.403	0.203	0.414	0.995265
28	0.827	0.295	0.210	0.222	0.068	0.996384
29	0.477	0.481	0.041	-0.028	0.417	0.997237
30	0.444	0.418	0.249	0.471	0,257	0.998070
31	0.503	-0.165	0.273	0.136	0.354	0.998685
32	0.487	0.325	0.729	0.139	0.153	0.999033
33	0.004	0.794	0.155	0.097	0.278	0.999331
34	0.572	0.637	0.221	0.040	0.009	0.999591
35	0.678	-0.140	0.163	0.408	0.113	0.999781
36	0.900	-0.011	0.002	0.319	0.077	0.999896
37	0.710	0.183	0.338	0.203	0.424	0.999955
38	-0.054	0.053	0.028	0.055	0.310	1.000000
39	0.828	0.296	0.096	0.156	0.116	1.000000
40	0.596	0.156	0.213	0.528	0.086	1.000000
41	0.755	0.017	0.123	0.087	0.112	1.000000
42	0.361	0.458	0.116	0.362	0.043	1.000000
43	0.003	0.145	-0.013	0.132	0.055	1.000000
44	0.674	0.294	0.272	0.006	0.018	1.000000
45	-0.001	0.089	-0.043	0.145	0.049	1.000000
46	0.260	0.038	0.107	0.053	0.095	1.000000
47	0.327	-0.011	0.090	0.578	0.098	1.000000
48	0.074	0.049	0.046	-0.015	-0.102	1.000000

Appendix II Table showing correlation between principal components and dependent variable, regression coefficients of principal components and coefficients of variables obtained from regression on principal components.

CORRELATION BETWEEN PRINCIPAL COMPONENTS AND DEPENDENT VARIABLE

-0.79978	-0.13799	0.21714	0.03238	-0.12266	-0.00462	-0.05299	0.03828	0.02153	-0.24620
0.08900	-0.05801	-0.03547	0.00707	-0.15881	-0.04982	-0.11507	0.09496	0.12816	0.06871
-0.14154	0.13993	-0.04092	-0.00929	-0.02526	0.12266	0.05347	-0.02159	-0.14257	-0.02104
-0.00224	0.07190	0.01222	0.10696	-0.06297	0.00822	-0.03201	0.01627	-0.00523	

REGRESSION COEFFICIENTS OF PRINCIPAL COMPONENTS

CONSTANT (MEAN OF Y)	COMPONENTS								
2.86273	-0.25097	-0.07230	0.14921	0.02395	-0.09400	-0.00374	-0.04459	0.03459	0.02000	-0.24620
	0.09598	-0.06435	-0.04341	0.00928	-0.22342	-0.07473	-0.18390	0.16459	0.23514	0.13776
	-0.29875	0.30165	-0.09825	-0.02515	-0.06935	0.36995	0.17514	-0.08220	-0.59235	-0.09154
	-0.01026	0.35189	0.06686	0.63352	-0.41128	0.06189	-0.30109	0.16703	-0.05469	

COEFFICIENTS OF VARIABLES OBTAINED FROM REGRESSION ON PRINCIPAL COMPONENTS

INDEX OF COMPONENTS ENTERING	RESIDUAL SUM OF SQUARES	F-VALUES REGRESSION MODEL	F-VALUES COMPONENT TO ENTER	R2	CONSTANT	VARIABLES 1 A	2 B	3 C	4 D	5 E	6 F
1	26.68069	86.98	86.98	0.6396	4.2694	-0.0024	-0.0020	-0.0018	0.0012	-0.0047	-0.0048
10	22.19292	56.07	9.71	0.7003	4.4650	-0.0039	0.0022	-0.0021	-0.0018	-0.0072	-0.0087
3	18.70187	46.36	8.77	0.7474	4.4494	-0.0036	0.0018	0.0011	-0.0021	-0.0013	-0.0103
15	16.83458	39.08	5.10	0.7726	4.4364	-0.0040	0.0036	-0.0017	-0.0005	0.0018	-0.0105
29	15.32969	34.47	4.42	0.7930	4.8315	-0.0072	0.0019	0.0018	-0.0133	0.0020	-0.0153
21	13.84647	31.88	4.71	0.8130	4.6810	-0.0029	0.0035	0.0023	-0.0030	0.0166	-0.0208
22	12.39673	30.55	5.03	0.8326	4.7456	-0.0020	-0.0004	0.0083	-0.0022	0.0028	-0.0158
2	10.98685	30.13	5.39	0.8516	4.7577	-0.0011	-0.0006	0.0071	-0.0036	0.0027	-0.0164
19	9.77070	29.97	5.10	0.8680	4.6963	0.0009	0.0011	0.0069	-0.0002	0.0090	-0.0136
5	8.65679	30.21	5.15	0.8831	4.6967	0.0002	-0.0007	0.0057	-0.0008	0.0090	-0.0139
26	7.54291	31.26	5.76	0.8981	4.6427	0.0020	-0.0001	0.0004	0.0053	-0.0156	-0.0104
17	6.56262	32.56	5.68	0.9114	4.6931	0.0024	-0.0021	-0.0004	0.0006	-0.0051	-0.0120
34	5.71551	34.02	5.48	0.9228	4.8916	-0.0066	-0.0028	-0.0007	0.0023	0.0007	-0.0097
18	5.04780	35.15	4.76	0.9318	4.9301	-0.0086	-0.0044	-0.0011	0.0083	0.0006	-0.0129
11	4.46135	36.39	4.60	0.9397	4.9129	-0.0081	-0.0041	-0.0017	0.0086	0.0029	-0.0125
32	4.07859	36.45	3.19	0.9449	4.9424	-0.0086	-0.0060	-0.0036	0.0046	-0.0006	0.0008
20	3.72901	36.60	3.09	0.9496	4.9388	-0.0073	-0.0055	-0.0055	0.0082	-0.0006	0.0019
35	3.43540	36.54	2.73	0.9536	4.8348	-0.0047	-0.0002	-0.0005	0.0092	0.0010	-0.0068
12	3.18626	36.28	2.42	0.9570	4.8774	-0.0041	-0.0011	-0.0002	0.0064	0.0001	-0.0083
27	2.97455	35.84	2.14	0.9598	4.8083	-0.0019	0.0000	-0.0010	0.0096	0.0043	-0.0066
7	2.76669	35.57	2.18	0.9626	4.8036	-0.0010	0.0009	-0.0008	0.0091	0.0026	-0.0069
16	2.58291	35.21	1.99	0.9651	4.8374	-0.0015	0.0017	-0.0001	0.0078	0.0037	-0.0089
23	2.45893	34.17	1.36	0.9668	4.8391	-0.0023	0.0029	-0.0016	0.0085	0.0044	-0.0070
8	2.35042	33.04	1.20	0.9683	4.8564	-0.0027	0.0031	-0.0012	0.0072	0.0058	-0.0061
13	2.25727	31.80	1.03	0.9695	4.8629	-0.0026	0.0021	-0.0013	0.0075	0.0078	-0.0051

Table showing correlation between principal components and dependent variable, regression coefficients of principal components and coefficients of variables obtained from regression on principal components.

Appendix III Eigenvalues for principal components 1 through 10 for each peak obtained from the HPLC profile.

Component Peaks	Eigenvalues for Principal Components									
	1	2	3	4	5	6	7	8	9	10
1 A	0.1169	-0.1603	0.0246	0.1176	0.0853	0.1350	-0.2431	-0.1574	0.3945	0.0694
2 B	0.1104	0.0244	-0.0407	-0.2202	0.2734	0.0921	-0.2607	0.0675	0.0225	-0.2354
3 C	0.0968	0.2272	0.2945	0.0584	0.1766	-0.0646	-0.0545	0.1659	0.0923	0.0174
4 D	-0.0411	0.1603	-0.0191	0.0852	0.0576	-0.2380	0.1111	-0.3087	-0.1759	0.1043
5 E	0.1183	0.0056	0.2461	-0.0530	-0.0022	0.0767	0.2343	0.2635	-0.0467	0.0617
6 F	0.1742	0.0755	-0.0974	0.0604	0.0294	0.1954	0.0692	0.2551	0.0688	0.1461
7 G	0.0976	0.2114	0.0388	-0.1746	0.0235	0.1024	0.2672	-0.2235	-0.0305	-0.1415
8 H	0.0919	0.2247	0.2481	0.0089	0.0057	-0.2470	0.0381	0.0090	-0.0212	0.0503
9 I	0.1408	0.2872	-0.0505	0.0451	0.1823	0.0912	-0.0476	-0.0570	-0.0206	-0.0434
10 J	0.1050	0.2138	-0.0615	0.2219	-0.0482	-0.1700	0.0963	-0.2279	0.0069	0.0987
11 K	0.1461	0.2414	0.0281	-0.1820	0.1812	0.0157	0.0204	-0.0622	-0.0369	0.0097
12 L	0.1398	0.1442	-0.2880	0.2628	0.0747	0.0615	0.0105	0.0886	-0.0247	-0.0029
13 M	0.1429	0.1419	0.1578	-0.1628	0.0163	0.1954	0.0516	-0.1567	-0.2263	-0.1156
14 N	0.1203	-0.1011	-0.0125	-0.0522	-0.1968	-0.2318	0.2777	0.2245	0.0605	-0.1599
15 O	0.1958	-0.0112	-0.0024	-0.0028	0.0504	0.2124	0.1005	0.1106	0.0835	0.1117
16 P	0.1501	0.2549	-0.1357	0.0846	0.0725	0.0256	-0.0098	0.0739	0.0321	-0.1564
17 Q	0.1419	0.0303	-0.1287	-0.0028	0.0829	-0.1887	0.0832	-0.1072	-0.1988	0.4098
18 R	0.1374	-0.0803	-0.1584	-0.3129	0.1247	0.1646	0.0179	0.2339	0.0072	0.1099
19 S	0.1958	-0.0787	0.0580	0.1778	0.0775	-0.0760	-0.0047	-0.0348	0.1188	0.0623
20 T	0.1375	-0.1769	-0.1351	-0.0602	0.0204	-0.2027	0.0291	-0.0662	0.0729	0.2533
21 U	0.1875	-0.0621	-0.1784	-0.1559	0.0856	-0.1372	0.1044	0.0835	-0.1292	-0.0527
22 V	0.0895	0.1627	-0.0998	0.1119	-0.1820	0.0352	0.0436	0.3174	0.0967	-0.0670
23 W	0.1068	0.0700	-0.1250	-0.2125	-0.1245	0.0171	0.0659	-0.1691	0.4527	-0.0322
24 X	0.1120	0.1469	-0.2222	-0.1778	-0.0225	-0.1017	-0.0157	-0.2686	0.3050	-0.1911
25 Y	0.1466	-0.0516	-0.1637	-0.0448	-0.2810	-0.0745	0.2812	-0.0353	-0.0582	-0.2007
26 Z	0.0941	-0.0329	-0.2312	-0.2364	-0.1098	-0.0731	-0.2866	0.0296	-0.2653	0.2752
27 AA	0.1920	-0.1712	-0.0159	-0.0729	0.1770	-0.0550	-0.0671	0.0128	-0.1257	-0.0055
28 BB	0.2111	-0.0923	0.0258	0.2128	0.0482	-0.0971	-0.0643	-0.0246	0.0792	-0.0989
29 CC	0.1923	0.0122	0.0162	-0.0367	-0.0610	-0.0936	-0.2132	-0.0771	0.0047	-0.0097
30 DD	0.1970	0.0039	0.0302	0.0182	-0.0931	-0.2280	-0.0077	0.1731	-0.0151	-0.1628
31 EE	0.1363	-0.2047	-0.1101	-0.0559	-0.0169	-0.0332	-0.1597	0.0689	-0.1351	-0.1252
32 FF	0.2175	0.0407	-0.1049	0.0896	0.1681	0.1098	0.0631	0.1214	0.0732	0.0929
33 GG	0.1318	0.2071	0.1319	-0.1517	-0.0225	-0.2229	-0.1173	0.0186	0.0218	0.0514
34 HH	0.1702	0.0556	0.3179	0.0421	-0.0136	0.0775	-0.1711	-0.0212	-0.1555	-0.0702
35 II	0.1361	-0.2544	0.0358	-0.0665	0.0217	-0.0335	0.1170	-0.0682	-0.0493	-0.1947
36 JJ	0.1676	-0.2397	0.1073	0.1492	-0.0455	-0.1296	0.0216	-0.0555	0.0558	-0.0917
37 KK	0.2159	-0.1299	-0.0317	-0.0329	0.1216	-0.1031	-0.0186	-0.0770	-0.1195	0.0960
38 LL	0.0587	0.0889	-0.0083	-0.2346	-0.2902	0.2821	0.0973	-0.1207	-0.0787	0.1651
39 MM	0.1905	-0.1245	0.2139	0.0480	-0.0132	0.0286	0.0438	-0.1380	0.0942	0.1334
40 NN	0.1493	-0.1720	0.1682	-0.0024	0.0281	0.2001	0.3045	-0.1123	-0.0596	0.0273
41 OO	0.1386	-0.2165	0.1549	-0.0001	0.0047	0.1881	0.0279	-0.2002	0.0651	0.1165
42 PP	0.1553	0.0818	0.2036	0.0620	-0.2851	0.0197	0.0067	0.0843	-0.0525	0.0705
43 QQ	0.0525	0.0612	0.0991	-0.0882	-0.3813	-0.0808	-0.2482	0.1188	-0.0103	0.1122
44 RR	0.1872	0.0053	0.0544	0.1483	-0.0929	0.0123	-0.2437	-0.0104	-0.0380	-0.0782
45 SS	0.0264	0.0447	0.0877	-0.2197	-0.2239	-0.0695	-0.0801	0.0342	0.3194	0.1984
46 TT	0.0942	-0.0187	0.0214	-0.0666	-0.1984	0.1510	-0.2406	-0.1757	-0.1953	-0.2949
47 UU	0.1386	-0.0051	-0.2374	0.2625	-0.2075	0.0842	0.0029	-0.0738	-0.1133	-0.1184
48 VV	0.0696	0.0891	-0.1388	0.2587	-0.1981	0.3140	-0.0700	-0.0749	-0.0584	0.1925

Eigenvalues for principal components 1 through 10 for each peak obtained from the HPLC profile.

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