OBJECTIVE JUDGEMENT OF CHEESE VARIETIES BY MULTIVARIATE ANALYSIS OF HPLC PROFILES

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ABSTRACT

An objective analytical method was developed to characterize the taste profiles of five cheese varieties. Nonvolatile water extracts of Cheddar, Edam, Gouda, Swiss, and Parmesan cheeses were analyzed by high performance liquid chromatography (HPLC) with a reversed phase column. The HPLC operating conditions were determined with Mapping Super-Simplex followed by Centroid Mapping Optimization. A ternary gradient elution system was used with an Adsorbosphere C8 column to resolve a maximum number of components. The optimum solvent volume ratio was 96.8 : 1.2 : 2.0 for trifluoroacetic acid (0.1%), acetonitrile, and methanol, with a flow rate of 1.0 mL/min. Over 50.3 min this ratio was changed to 56.3 : 30.3 : 13.4.

Multivariate statistical analyses including principal component and discriminant analyses were applied to 55 peak areas from 106 cheese chromatograms. Principal component analysis reduced the dimensionality of the data from 55 to 17 principal components, which are combinations of the original variables, with a 26% loss of explained sample variation. Discriminant analysis on data from a single HPLC column was able to correctly classify cheeses by variety at a greater than 90% success rate. This grouping rate dropped to 64% when data from all four HPLC columns was combined, implicating large between column variations. A semi-trained sensory panel correctly classified cheeses by variety at a 63% rate. This objective method provides a lasting fingerprint of cheese products.

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I. INTRODUCTION

The chemical basis of taste and aroma in cheeses is not well understood. Cheese maturation is a complex process in which the curd is broken down by proteolysis, lipolysis, and other enzyme catalyzed reactions to yield a flavor and texture typical of the many varieties. Proteolysis products, including peptides and amino acids, are thought to be involved with taste and aroma directly, or as precursors in subsequent reactions. Lipolysis releases free fatty acids which may be important in subsequent flavor forming reactions (Stadhouers and Veringa, 1973).

Proteolysis is important to both textural and flavor changes in ripening cheeses. In most hard cheeses proteolysis leads to a softer and less elastic body. Microbial proteolysis is influenced by the remaining coagulant in the cheese curd, in addition to starter proteinase activity. Milk is low in free amino acids, but the starter culture's proteinases and peptidases allow them to utilize protein bound amino acids for growth (Law, 1982). Chymosin was responsible for the degradation of α_{s1}-casein and partial attack of β-casein after one month aging in Gouda cheese (Visser and de Groot-Mostert, 1977). Thus rennet proteolysis produces large and medium peptides which are in turn degraded to small peptides and amino acids by starter proteinases. These proteolysis products are involved with taste and aroma directly, or as precursors in subsequent reactions.

The importance of the nonvolatile water extractable fraction has been demonstrated in both Cheddar and Swiss cheeses. McGugan et al. (1979) and Aston and Creamer (1986) showed that the water-soluble fraction was important to Cheddar flavor intensity. This was interpreted as a direct effect of proteolysis products. Biede and Hammond (1979b) demonstrated that the small peptide fraction was responsible for the sweet and the brothy-nutty flavor in Swiss cheese.

Traditionally trained graders and sensory panels have been used to assess the degree of flavor develoment in cheeses. Due to the time involved in training a panel, and the

subjective nature of the method, a more objective analysis is desired. Pham and Nakai (1984) and Amantea (1984) successfully used reverse phase high performance liquid chromatography (RP/HPLC) and multivariate analysis to classify Cheddar cheese samples by age. Water soluble cheese extracts containing proteins, peptides, amino acids, and salts were used in the analysis.

The use of HPLC and gas liquid chromatography (GC) to measure taste and aroma compounds in foods has generated a large number of measurements, necessitating the use of pattern recognition techniques. The objectives of multivariate analysis include data reduction, grouping, determining relationships among variables, and using these relationships to predict the value of other variables.

The goals of the present research were:

1. To find an objective method to characterize the taste profiles of Cheddar, Edam, Gouda, Swiss, and Parmesan cheeses.

2. To use a computer-aided optimization procedure to determine the HPLC operating parameters.

3. To apply multivariate analyses to the HPLC peaks to characterize and separate cheese varieties.

4. To compare the analytical results with sensory data.

II. LITERATURE REVIEW

A. DEVELOPMENT OF CHEESE FLAVOR

Worldwide there may be up to 2000 varieties of cheeses but these can be related to 20 basic varieties (Kosikowski, 1985). Cheeses can be grouped according to their moisture content. Soft cheeses may have up to 80% moisture, semi-hard varieties have approximately 50% moisture, and hard cheeses contain 40% moisture (Law, 1981). The hard cheeses can be further divided into 4 groups; those with a relatively simple microflora such as Cheddar; those inoculated with mold spores and allowed to germinate such as Roquefort; the Swiss varieties that use thermophilic lactic acid bacteria as starters; and the Italian cheeses that use added lipases to develop the characteristic rancid flavor (Law, 1981). The varieties of Cheddar, Edam, Gouda, Swiss, and Parmesan can thus be classified as hard natural cheeses.

The development of cheese flavor is very complex involving a heterogeneous milk source, a changing population of microorganisms, and interactions between metabolic pathways. Many reviews on the formation of flavor and aroma compounds in cheeses have been published but the exact mechanisms are not yet understood (Law, 1982; Adda et al., 1982; Law, 1981; Forss, 1979; and Mulder, 1952).

The composition of a cheese has a great influence on its flavor. Mulder (1952) indicated that the flavor of Edam and Gouda can be related back to its constituents, fat, protein, lactose, salt, and water. Water acts as a diluting medium for all kinds of substances and contributes to texture. Salt blends with other flavors and enhances the piquant flavor of cheese. Lactose is transformed to lactic acid which gives cheese a refreshing flavor and releases aldehydes, ketones, alcohols, and esters. Breakdown products of lactic acid include acetic, butyric, and propionic acids. All of the breakdown products contribute to the general cheese taste. Proteins do not have much taste but do contribute to the texture of cheese.

Proteolytic enzymes both added and indigenous are key to the transformation of milk into a ripened cheese. Reviews of the role of proteolysis in cheese maturation are numerous (Visser, 1981; Grappin et al., 1985; Rank et al., 1985). Four proteinase categories active in cheese are the indigenous milk proteinases; the endogenous proteinases from bacterial contaminants; the milk-clotting enzymes for cheese making; and the enzymes produced by the starter cultures (Grappin et al., 1985).

The clotting of milk by proteolytic enzymes in the process of making cheese represents one of the oldest practices in food technology. Traditionally chymosin (rennin) is used in the manufacture of cheese, but due to shortages of the enzyme other sources including proteinases from microorganisms and other mammals are being used.

The enzymatic coagulation of milk consists of 3 stages. During the primary stage, the milk protein, k-casein, is attacked by proteinases, yielding 2 peptides, the glycomacropeptide which is hydrophilic, soluble and will be lost in the cheese whey, and the para-k-casein moiety, which is hydrophobic and remains on the micelle. The progressive hydrolysis of k-casein during the primary phase leads to the micelle surface charge changing from a net negative to a positive. Upon 80% destruction of the k-casein, the micelles aggregate. This aggregation is the secondary stage of the process. The tertiary stage is less clearly defined, but involves the expulsion of water from the cheese curd due to structural rearrangement and general proteolysis of the caseins in the curd (Dalgleish, 1982).

During cheesemaking, most of the milk clotting enzyme is removed in the whey. Matheson (1981) found that chymosin remaining in the curd did not decrease over a 3 month period in Cheddar cheese. Swiss cheese, due to its high heat treatment, contained no chymosin activity. Rennet is thought to cause only limited breakdown of the caseins (Visser, 1981) resulting in larger molecular weight peptides. Enzymes from the starter bacteria subsequently produce smaller peptides and free amino acids.

Lipolysis due to starter bacteria plays a limited role in the development of flavor in hard cheeses. Starter bacteria produce free fatty acids from mono- and diglycerides but not

from triglycerides. These mono- and diglycerides are most likely formed by the natural lipases of milk and the lipases of Gram-negative rods (Stadhouders and Veringa, 1973). Thus more fatty acids result from partly hydrolyzed milk fat than from fresh milk fat.

The Component Balance Theory as originally proposed by Mulder (1952) explains the flavor of cheese as a mixture of compounds balanced to yield a typical flavor. When any one component is lacking, or in excess, the flavor equilibrium is upset and an atypical cheese results. Mulder (1952) further stated that the flavor forming substances are so well balanced that it is difficult to recognize them individually.

B. CHEDDAR FLAVOR

1. Amino acids and peptides

Cheddar cheese flavor has been extensively studied for over 30 years yet the chemical basis of flavor is still mostly unknown. Free amino acids have been studied as flavor contributing compounds. Aston et al. (1983) found the level of free amino acids, measured as phosphotungstic acid-soluble amino nitrogen, strongly correlated with the degree of flavor development in control and accelerated ripened Cheddar cheeses. The levels of free amino acids increased throughout ripening in all the cheeses. These free amino acids are most likely flavor precursors rather than flavor compounds themselves.

Proteolysis products were shown to be directly responsible for Cheddar flavor intensity by McGugan et al. (1979) and Aston and Creamer (1986). Mild and aged Cheddar cheeses were separated by McGugan et al. (1979) into water-soluble, residue, and fat fractions. The nonvolatile water soluble fraction containing peptides, amino acids, and salts contributed most to Cheddar flavor intensity. The residue had no cheese flavor and the volatiles contributed to the overall Cheddar flavor but did not influence flavor intensity. Similar results were found by Aston and Creamer (1986) who further fractionated the water-soluble component. The sub-fraction containing salt, free methionine, free leucine, and

several peptides produced the most Cheddar flavor. A mixture of amino acids, salt, and calcium lactate lacked a full flavor, implicating peptides as a flavor contributor.

The role of peptides in Cheddar flavor is similar to that of free amino acids. Yet peptides may be a source of bitterness in Cheddar cheese. Czulak (1959) proposed that all bitter peptides were produced by residual rennet in cheese. Rennet has a lower optimum pH than the starter cultures enzymes, thus rennet releases peptides from the casein including bitter peptides. As the protein breakdown continues, the pH rises and bitter peptides are gradually broken down by the starter proteinases. More likely, high molecular weight nonbitter peptides caused by the rennet hydrolysis of casein are subsequently hydrolyzed by starter to bitter low molecular weight peptides (Lowrie and Lawrence, 1972). Mills and Thomas (1980) showed that cell-wall associated starter proteinases produce bitter peptides. Intracellular peptidase acitvity may then reduce these bitter peptides to non-bitter peptides (Law et al., 1976).

2. Free fatty acids

Lipolysis was shown to be the major source of volatile fatty acids in Cheddar cheese. Dulley and Grieve (1974) found that skim milk cheese produced a fatty acid level less than 20% of the control. All of the fatty acids except acetic increased at a much slower rate during aging in the skim milk cheeses versus the controls.

The role of free fatty acids in Cheddar flavor is not well accepted. Forss (1979) claimed that butyric acid was selectively hydrolyzed and was a major contributor to flavor with caproic acid playing a minor role. Law and Sharpe (1977) and Law et al.(1976) found no relationship between free fatty acid levels and flavor. Yet most researchers agree that excessive levels of free fatty acids result in rancidity. Levels 3 to 10 times higher than normal resulted in rancid defects in Cheddar flavor (Law et al., 1976). Thus the role of FFA in Cheddar flavor seems to be as a contributor to the overall background flavor.

3. Carbonyl compounds

Cheddar cheese contains many carbonyl compounds including acetaldehyde, propionaldehyde, butyraldehyde, acetone, 2-butanone, 2-pentanone, 2-heptanone, 2-nonanone, diacetyl, acetoin, and pyruvic acid (Aston and Dulley, 1982). The role of these carbonyl compounds in Cheddar flavor is unknown. Manning (1978) found ethanol and butanone levels were independent of the age of the cheese while methanol, acetone, and 2-pentanone levels increased with aging. Day et al.(1960) found no direct correlation between typical Cheddar aroma and a mixture of ten carbonyl compounds. Thus volatile carbonyls contribute to the total or overall Cheddar flavor.

4. Sulfur compounds

(a) Hydrogen sulfide (H_2S)

The first volatile sulfur compound found to relate to Cheddar flavor was hydrogen sulfide. Originally Kristoffersen and Nelson (1955) found that -SH groups and free H_2S increased up to 6 months. The cheese with the highest flavor intensity score also contained the highest level of free H_2S. Later Kristoffersen and Gould (1960) found that H_2S levels fluctuated during a 12 month aging period with no consistent trend appearing. Aston and Douglas (1983) examined volatile sulfur products in control and accelerated ripened cheeses. The levels of H_2S increased up to 6 months of age, then decreased. Manning et al. (1976) also found no correlation between H_2S and Cheddar flavor intensity, nor was the level of H_2S responsible for the sulfide flavor defect.

(b) Methanethiol (CH_3SH)

Methanethiol in Cheddar cheese is most likely produced from the decompostiion of L-methionine which is released by starter enzymes (Law and Sharpe, 1977). The importance of methanethiol to Cheddar flavor is not clear. Manning et al.(1976) found that methanethiol correlated with flavor intensity but not flavor quality in cheeses aged up to 12 months. Aston and Douglas (1983) showed that methanethiol levels increased up to 6 months of ripening

then declined. Correlations with total flavor, mature flavor and estimated age were low. Thus methanethiol is not a good indicator of flavor development.

(c) Dimethyl sulfide $(CH_3)_2S$

Dimethyl sulfide was found to be a part of Cheddar aroma by Patton et al. (1958). Good quality Cheddar aroma was thought to be directly related to the level of dimethyl sulfide. Later Aston and Douglas (1983) showed that levels of dimethyl sulfide remained constant during the aging period or decreased after 6 months of ripening. Thus dimethyl sulfide levels did not correlate with flavor development in Cheddar cheese.

C. EDAM AND GOUDA CHEESE FLAVOR

Edam and Gouda, originating from the Netherlands, are quite similar in flavor. Gouda may be ripened to produce a range of flavor intensities from mild to aged, whereas Edam is usually ripened to the mild stage. Edam contains less fat than Gouda, 24 and 28.5% (wet basis) respectively, and is therefore more firm (Campbell and Marshall, 1975 ; Kosikowski, 1977). Gouda and Edam flavors are far from understood and much research remains to be done on characterizing the cheeses.

1. Peptides and amino acids

Protein breakdown products including free amino acids were thought by Mulder (1952) to contribute to cheese flavor. Casein contains 20% amino acids with a sweet taste and 30% with a broth-like flavor. Bitter tasting amino acids are also present in cheese.

Proteolysis products resulting from the action of residual rennet, the enzymes of starter bacteria, and native milk protease were characterized by Visser (1977a,b) in Gouda cheese. Rennet was found to produce most of the soluble-N in Gouda cheese which corresponds to high and low molecular weight peptides. Very little amino-acid N was produced by rennet regardless of rennet levels (Visser, 1977b). Cheeses made with only rennet or natural milk proteases did not develop a cheese flavor, but rennet was able to

produce bitter cheeses (Visser, 1977a). Starter bacteria and milk protease produced only small amounts of soluble-N. Starter bacteria contributed significantly to the lower molecular weight (<1400) peptides and free amino acids whereas milk protease liberated these compounds in small amounts. The nonbitter starter strains produced higher levels of amino-acid N than the bitter strains (Visser, 1977b). In cheeses produced with starters, the nonbitter starters yielded characteristic Gouda flavor, whereas the bitter starters developed noticeabily less cheese flavor. Nonbitter starters were able to breakdown bitter peptides to nonbitter products (Visser, 1977a). Thus rennet influences the extent of soluble-N compounds and the starter influences the production of amino-acid N.

Amino acids themselves do not exhibit a strong smell or taste yet all hard cheeses contain the amino acids liberated from casein. Free amino acids were found to increase with ripening of Edam cheeses but not linearly due to the decomposition of free amino acids. Ali and Mulder (1961) found that a mixture of amino acids representing those found in a ripened cheese, when added to a young neutral flavored cheese, did not yield a full flavored Edam. Isoleucine produced a bitter taste, alanine and proline a sweet taste, and glutamic acid a brothy taste. Thus amino acids are thought to contribute to the basic cheese taste (Ali and Mulder, 1961).

2. Volatile compounds

Fatty acids in Gouda cheese enhance the typical flavor and give the cheese body (Mulder, 1952). Volatile fatty acids contribute to Gouda flavor and aroma but the specifics are unknown (Badings and Neeter, 1980). Gouda cheeses aged 9 to 12 months were found by Sloot and Harkes (1975) to contain bis(methylthio)methane, anethole, and a series of alkylpyrazines in the aroma. These volatile components were present in low amounts but were thought to contribute to the overall Gouda flavor.

D. SWISS CHEESE FLAVOR

There are many types of Swiss cheese but the original was produced in the Emmental river valley in Switzerland (Kosikowski, 1977). Swiss cheese is characterized by its eyes, or gas holes, and a sweet nutty flavor. Reviews of Swiss cheese manufacture and flavor have been published by Mocquot (1979) and Langsrud and Reinbold (1973a,b,c,1974). The curds are cooked at a relatively high temperature (50C) thus thermophilic starters are used, as well as propionibacteria.

Mixed cultures are added to cheese milk in the manufacture of Swiss cheese. Streptococcus thermophilus and Lactobacillus helveticus or Lactobacillus lactis produce lactic acid from lactose. Propionibacteria contribute to later cheesemaking stages (Law, 1981). After pressing and brining, the cheese is stored for 7 to 14 days in the cold room then transfered to a hot room (21-25C) to allow eye and flavor formation. The cheese is then stored at 2 to 5C for further curing for 2 to 9 months (Langsrud and Reinbold, 1973c; Kosikowski, 1977). Swiss cheese has a lower fat content, 30.5 versus 33% wet basis, and a higher protein content, 26.1 versus 25.8%, compared to Cheddar cheese (Campbell and Marshall, 1975).

1. Acids in Swiss cheese

Volatile fatty acids contribute most to Swiss cheese flavor but other fatty acids and keto acids contribute to the background flavor. Sherman (1921) was the first to show that propionibacteria caused eye formation and the characteristic sweet flavor in Swiss cheese. These bacteria metabolize lactose and lactate and yield propionic acid, acetic acid, and carbon dioxide. Cheeses with the typical sweet flavor were found to contain a higher content of volatile acids than bland cheeses (Babel and Hammer, 1939). Propionic and acetic acids themselves are not sweet but calcium and sodium propionates added to cheese lacking flavor produced the sweet note.

Good quality Swiss flavor was found to contain large amounts of acetic and propionic acid but low levels of butyric acid (Langlois and Parmelee, 1963; Krett and Stine, 1951; Harper, 1959). Flat flavored cheeses contained normal levels of acetic acid and little or no propionic and butyric acids (Krett and Stine, 1951). A burned flavor was apparent when butyric acid levels were greater than propionic acid levels.

Kurtz et al. (1959) disagreed with Babel and Hammer (1939) in the role of propionic acid in sweetness, by thinking propionic acid contributed a "rich" flavor to Swiss cheeses but not the sweet characteristic. Other unidentified compounds were thought to be responsible for the sweet and nutty flavors.

Butyric, propionic, and higher fatty acids were found in Swiss cheese but levels were uncorrelated with the age of the cheeses (Hintz et al., 1956). Cysteic acid, tauric, and valeric acid were found for the first time in some Swiss cheeses. Hintz et al. (1956) discovered that a good Swiss flavor required a minimum concentration of 5.0 mg propionic acid per gm cheese and 2.0 mg proline per gm cheese. Thus an interrelationship exists between different chemical classes of compounds, i.e. fatty acids and amino acids, similar results may exist in other cheese varieties (Harper, 1959). Mitchell (1981) found that levels of acetic acid, propionic acid and proline rose during a 4 month aging period. Yet a mixture of these compounds in levels typical of cheese, produced a Swiss-like flavor but was lacking some compounds of a high quality Swiss cheese.

Paulsen et al. (1980) studied the role of starter bacteria in the production of free fatty acids. Cheeses produced with S. thermophilus contained more free fatty acids of 6 to 10 chain lengths while cheeses made with P. Shermanii contained more of the 12 and 14 carbon fatty acids. Cheeses inoculated with L. helveticus released fewer fatty acids of all chain lengths. Flavor in cheeses with a single inoculum were low as was the combination of S. thermophilus and P. shermanii. Cheeses inoculated with S. thermophilus and L. helveticus were cheesy in flavor but were not characteristic of Swiss.

Keto acids were not related to the age of Swiss cheese but are present (Langsrud and Reinbold, 1973c). Bassett and Harper (1958) found pyruvic and alpha-ketoglutaric acids to be present in larger amounts while oxalsuccinic, oxalacetic and alpha-acetolactic were present in smaller amounts.

2. Misc. volatile compounds

Volatile compounds including alcohols, esters, lactones, and hydrocarbons have been identified in Swiss cheese (Langler et al., 1967; Langsrud and Reinbold, 1973c; Biede and Hammond, 1979a). Alcohols present include ethanol and 1-propanol but are in such low levels that they most likely do not contribute directly to Swiss flavor. They may form flavorful esters with fatty acids (Langsrud and Reinbold, 1973c). Aldehydes include acetaldehyde which exceeds the flavor threshold of 0.4 mg/kg (Harvey, 1960) and thus may contribute to Swiss flavor (Langsrud and Reinbold, 1973c). Esters include methyl hexanoate and ethyl butanoate in Swiss cheese and may contribute to the overall flavor. Diacetyl plays a key role in the flavor of cultured dairy products and is present in Swiss cheese. Diacetyl was found to correlate with sweetness in the water-soluble volatile fraction of Swiss cheese by Biede and Hammond (1979b). Mitchell (1981) showed that diacetyl levels were highest in fresh curd and declined after 3 weeks to a consistent level.

3. Peptides and amino acids

As in other cheese varieties, proteolysis plays a key role in the development of flavor and texture in Swiss cheese. Hintz et al. (1956) examined the levels of free amino acids in Swiss cheeses aged from 1 to 11 months. Levels of the free amino acids varied among the cheeses but cysteic acid, threonine-serine, glutamic acid, and tyrosine-phenylalanine occured in all samples. There was a trend of histidine to increase with age. Kosikowski and Dahlberg (1954) also found that stronger flavored Swiss cheeses tended to have higher levels of free amino acids. Hollywood and Doelle (1984) showed that nitrogen levels increased in a high moisture Swiss cheese aged up to 63 days.

Biede and Hammond (1979a,b) fractionated Swiss cheese into oil soluble, and water-soluble components. The nonvolatile water-soluble fraction was the sweetest and additionally contained burned, bitter, and nutty flavors. The sweet flavor was thought to be due to the interaction of small peptides and amino acids with calcium and magnesium ions. The brothy-nutty flavor was also due to small peptides. Medium peptides were resposible for the burned and bitter flavor notes.

4. Sulfur containing compounds

Sulfur containing compounds may contribute to Swiss flavor (Langsrud and Reinbold, 1973c). Dimethyl sulfide is present in concentrations of 0.056 to 0.183 ppm (Langler et al., 1967) which is above flavor thresholds and thus adds to the taste. Singh and Kristoffersen (1971) found that Swiss curd slurries ripened at 30C produced a typical Swiss flavor at 4 to 5 days but beyond this a putrid unclean flavor was formed due to excess dimethyl sulfide.

E. PARMESAN FLAVOR

The two main types of Grana cheese, Parmigiano Reggiano and Grana Padano, were originally made in the Italian valley of Po (Reinbold, 1963). The Grana class of cheeses are cooked, hard, and require long aging (Battistotti et al., 1984). Grana refers to the granular structure of the cheese (Kosikowski, 1977). The cheese is generally ripened for 2 years, or not less than 10 months in the United States, and contains not less than 32% fat (dry basis) and not more than 32% moisture (Kosikowski, 1977).

Much of the literature concerning Parmesan flavor is in Italian and the references in English are scarce. As in the other cheese varieties, proteins in Parmesan cheese are broken down releasing free amino acids. Several amino acids including asparagine, proline, glutamic acid, valine, methionine, isoleucine, tyrosine, phenylalanine, lysine, and histidine were found to increase with ripening in Grana Padano (Bianchi et al., 1974). These amino acids were found to be concentrated in granules and spots in the cheese. Tyrosine was present in high

levels in the granules followed by phenylalanine and glutamic acid. The spots contained mostly leucine and iso-leucine.

Sulfur compounds important in Cheddar flavor, including hydrogen sulfide and methanethiol, were not found in significant amounts in Parmesan cheese (Manning and Moore, 1979). Ethanol was the largest peak found by headspace analysis using a gas chromatograph.

F. REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1. Theory

Reverse phase high performance liquid chromatography (RP\HPLC) involves a nonpolar stationary phase with a polar mobile phase. The range of solvents and column types available allow a variety of compounds to be separated based on polarity. Bonded phase columns, where the stationary phase is chemically attached to a support result in weak surface energies. The nonpolar group is often an octyl or an octadecyl group. Thus attractive forces between the stationary phase, solute molecules, and the mobile phase are weak and analysis time is short (Krstulovic and Brown, 1982).

The exact mechanism of separation on RP columns is unknown, partly due to an incomplete understanding of the surface structure of the bonded phase (Krstulovic and Brown, 1982). The solvophobic, or hydrophobic, mechanism seems to be the main mechanism of solute retention (Scoble and Brown, 1983; Hearn, 1983; Hancock and Sparrow, 1983; Krstulovic and Brown, 1982). The solvophobic model explains the interaction between solute molecules and the nonpolar packing as depending on weak dispersion forces. When a solute with nonpolar regions is placed in a polar mobile phase, the subsequent hydrophobic interactions force the binding of the solute to the stationary phase. The driving force is not the nonpolar attraction between the solute and stationary phase, but the repulsion between the solute and the mobile phase (Krstulovic and Brown, 1982). An organic solvent modifier, such as methanol or acetonitrile, will reduce the surface tension of the water molecules and

thus reduce the repulsive forces between the solvent and nonpolar region of the solute. With sufficient reduction in surface tension, the solute dissolves, or is eluted, in the mobile phase (Hancock and Sparrow, 1983). The retention of a sample on a reverse phase column is thus related to its surface polarity.

Proteins were shown to adsorb on a RP column in a monolayer by Di Bussolo and Gant (1985). Proteins of differing hydrophobicity eluted in order of hydrophobicity in an organic-lean mobile phase. When 20% acetonitrile was added as an organic modifier to the mobile phase, proteins competed for the hydrophobic sites on the stationary phase. Proteins were able to displace those injected earlier of a lesser hydrophobicity, but not those of a greater hydrophobicity.

2. Applications of HPLC

Over the past decade HPLC has become an invaluable tool to biochemical analysis due in part to its speed, high resolution, excellent recovery rate, and flexibility in expermental conditions (Hearn, 1983). There are numerous articles using HPLC in the analysis of proteins and peptides, and excellent review articles by Hearn (1983) and Hancock and Sparrow (1983). These authors found that generally a mobile phase of low pH produced better separations and peak shapes. Ashoor and Knox (1985) applied water extracts of fish species to a RP column and were able to distinguish between species by the retention times of protein peaks relative to bovine serum albumin.

Whey proteins have been separated by a number of researchers using HPLC. Reverse phase columns, RP-8 and C6, were used by Diosady et al. (1980) and by Pearce (1983) respectively, while an HPLC-gel exclusion column was used by Bican and Blanc (1982) to separate whey proteins. Olieman and van den Bedem (1983) applied an HPLC gel permeation column to the detection of the glycomacropeptide in skim milk samples.

In the past four years HPLC has been applied to the detection of cheese ripening products, including peptides and amino acids (Champion and Stanley, 1982; Pham and Nakai, 1984; Polo et al., 1985; Marsili, 1985; Kaminogawa et al., 1986; Aston and Creamer, 1986).

Champion and Stanley (1982) separated 71 peptides from Cheddar cheese with a C18 RP column, some of these compounds were found to be bitter. Pham and Nakai (1984) used a RP C8 column to separate Cheddar cheeses by age based on peak areas of the chromatographic patterns. The peaks were peptides and amino acids. Aston and Creamer (1986) also studied the water soluble fraction of Cheddar cheeses with a RP-18 column. Protein breakdown products during the aging of Gouda were followed by Kaminogawa et al. (1986). Polo et al. (1985) examined the increase in free amino acids released during aging of Mahon cheese while Marsili (1985) followed amino acid production in ripening Cheddar cheese. Chang et al. (1985) applied HPLC analysis to determining levels of 3 biogenic amines in Swiss and Cheddar cheeses. Thus HPLC analysis appears to be a key to the understanding of the complexity of cheese ripening.

3. Optimization of HPLC conditions

(a) Columns

Column packings commonly used in RP/HPLC are C18, C8, CN, or phenyl (Hancock and Sparrow, 1983). A C8 packing has lower surface loadings and hydrophobicity, thus compounds are not retained as strongly as on a C18 column. Pham and Nakai (1984) compared three types of columns and found a C8 Adsorbosphere produced the best resolution and largest number of peaks with a cheese extract. Wilson et al. (1981) found comparable peptide elution patterns on a LiChrosorb C8 and C18 column.

(b) Temperature

Temperature is less important in HPLC compared to gas chromatography due mostly to the low boiling points of many of the HPLC solvents (Krstulovic and Brown, 1982). Mobile phase composition may be altered rather than temperature to control the capacity factor of compounds. Increased column temperatures will decrease the viscosity of solvents, reduce the capacity factor and increase column efficiency (Hearn, 1983; Krstulovic and Brown, 1982). Higher temperatures may also lead to solute stationary phase degradation (Hearn, 1983). Wilson et al. (1981) found no change in elution patterns of peptides over the temperature

range of 25-55C. Kolbe et al. (1985) also found no overall pattern change in the elution of 25 amino acids over the range of 25-40C, but higher temperatures produced shorter retention times compared to those at lower temperatures.

(c) Mobile phase

Snyder (1974,1978) classified numerous solvents into similar selectivity groups based on functionality. Hydrogen bonding and dipole interactions determined whether the solvents belonged to similar selectivity groups. Eight groups are arranged in triangular fashion by the degree of proton-donor, proton-acceptor, and dipole-dipole interactions they can undergo. By using solvents from the apices of the triangle, larger differences in chemical selectivity are possible (Snyder, 1978; Glajch et al., 1980).

Before the mobile phase can be optimized, a method of determining the quality of a separation must be established. The resolution of two or more peaks is influenced by the capacity factor, k', the selectivity factor, α, and the efficiency factor, N. These three terms relate to the resolution, R_S, of two peaks in the following manner (Snyder and Kirkland, 1974):

$$R_S = 1/4(\alpha-1) \cdot (\sqrt{N}) \cdot (k'/(1+k'))$$

selectivity factor — efficiency factor — capacity factor

The three terms may be varied independently in the optimization of peak separation. The capacity factor is the ratio of the retention volume of a compound to the elution volume of a nonretained compound. The selectivity, or separation factor, between two peaks is a ratio of capacity factors: k'_2 / k'_1 where k'_2 is the value of the compound with the longer retention time. Column efficiency depends on the number of theoretical plates (Krstulovic and Brown, 1982) which in turn depends on column length, particle size, and flow rate (Glajch et al., 1980). The k' value is easily altered by changing the mobile phase solvent strength, while α is changed by altering solvent composition (Glajch et al., 1980).

One of the more simple chromatographic response functions was proposed by Morgan and Deming (1975) as: $CRF = \Sigma \ln(P_i)$ where P_i represents the peak separation of the

i^{th} pair of peaks. The separation between two peaks is P = f/g where f represents the length of the depression below a straight line joining two consecutive peak maxima and g measures the height of the straight line above the baseline at the depression (Figure 1). The published list of criteria used to judge the separation of peaks is numerous; Debets (1985) in a review article listed ten different response functions. Berridge (1982) weighted the function to have maximum number of peaks as the most important factor, while D'Agostino et al. (1985) included analysis time in the function.

Simplex optimization and its various modifications has been used to determine the best set of operating conditions for many chemical analyses (Routh et al., 1977; Amantea, 1984; Berridge, 1982; Morgan and Deming, 1974). Simplex optimization starts with an initial simplex defined by n+1 vertices in the n-dimensional factor space (Morgan and Deming, 1974). The idea is to move away from the worst response towards an optimum set of experimental conditions (Nakai et al., 1984). Amantea (1984) applied Super Modified Simplex (Routh et al., 1977) with Mapping Super-Simplex (Nakai et al., 1984) to determine optimum operating parameters for HPLC analysis of cheese extracts.

G. Multivariate Analysis

Multivariate statistical techniques are becoming invaluable to the food researcher dealing with subjective-objective properties of foods. This is evident in the increasing number of articles concerned with sensory and chemical data analyses (Stungis,1976; Martens and Russwurm,1983; Bertucciolo et al.,1986). Multivariate techniques reveal the underlying structure and relationships of large tables of data, allowing an interpretation of the variables (Martens, 1983). These methods are easily carried out with the use of computer packages such as SAS (1985) and BMDP (1985).

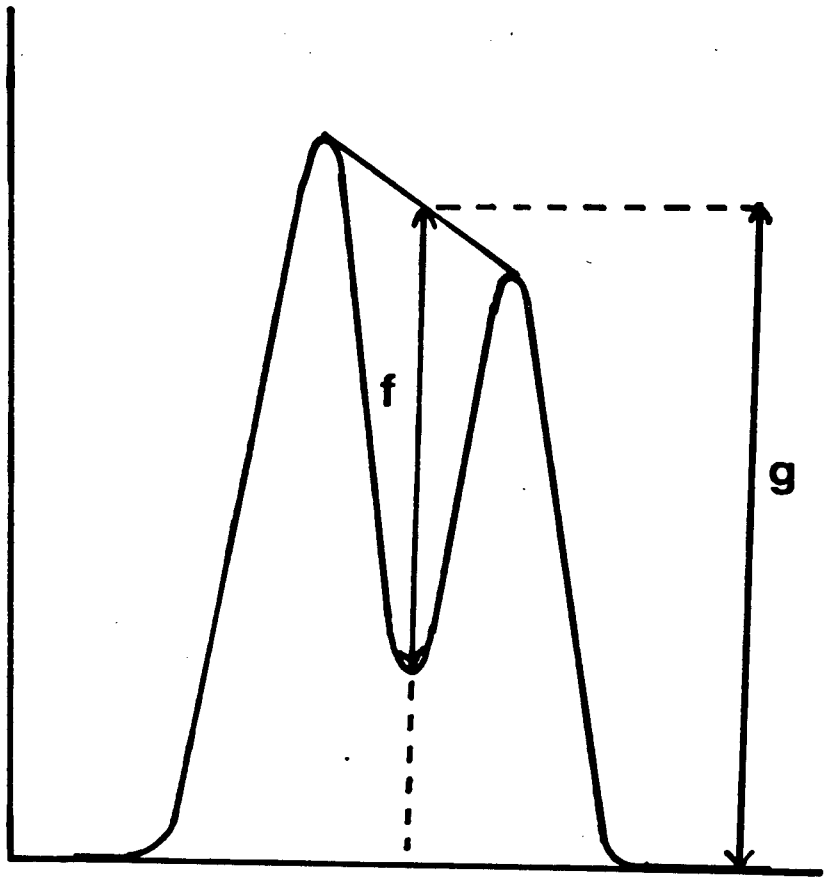

Figure 1. Method to determine peak resolution, P=f/g, adapted from Morgan and Deming (1975).

1. Principal component analysis

When a large number of variables are measured, the relationships, or covariance, between variables is soon beyond comprehension, thus a data reduction technique is essential. Principal component analysis seeks to reduce the number of variables while maintaining a majority of the original information (Dillon and Goldstein, 1984). The first principal component extracted is a linear combination of the original variables and accounts for as much of the total variance as possible. The second principal component (PC) is uncorrelated with the first PC and accounts for as much of the remaining variance not explained by the first PC. This may continue until there are as many PC's as original variables. Usually only a few principal components are necessary to describe the data or account for the sample variation (Daultrey, 1976; Dillon and Goldstein, 1984). In deciding how many components to use for data interpretation, a common rule of thumb is to retain principal components with eigenvalues greater than one (Dillon and Goldstein, 1984; Daultrey, 1976). Thus a large number of correlated variables is transformed to a few uncorrelated principal components. These components may disclose relationships that were unexpected. Principal component analysis may only be the first step in data analysis, as the PC's may be used in regression, cluster, and discriminant analyses (Johnson and Wichern, 1982).

2. Discriminant analysis

Discriminant analysis considers a data set consisting of a categorical dependent variable and a set of independent variables. The categorical variable assigns each case to pre-defined groups. The objective is to derive a function, or functions, that will discriminate among the groups. These functions can then be used to classify new cases into the groups (Dillon and Goldstein, 1984). The discriminant function minimizes the misclassification rate with linear combinations of the independent variables. The between-group variance is maximized, while the within-group variance is minimized (Dillon and Goldstein, 1984).

3. Applications

Multivariate analysis has been applied to characterizing a variety of foods including olive oil (Forina et al.,1983), frozen peas (Martens,1986), and rancid butter (Woo and Lindsay,1984). Aishima (1983) used multiple regression, discriminant, principal component, and cluster analyses to determine the relationship between gas chromatography (GC) profiles and sensory data of soy sauce. Based on 39 peak areas, 8 brands of soy sauce were classified into superior, moderate,and low quality groups. Aishima (1985) later applied similar techniques to the characterization of worcestershire sauces.

Multivariate analyses have been used by several groups of researchers to evaluate cheese ripening and flavor development (Pham and Nakai,1984; Rothe et al.,1982; Santa-Maria et al.,1986; Aishima and Nakai, 1987). Rothe et al.(1982) characterized the flavor of Blue cheese with 15 sensory attributes and 58 instrumental values. Based on correlations with sensory data, the instrumental measures were reduced to 20. Discriminant functions based on analytical data were able to describe the intensity of eight sensory attributes, including rancid, fruity, and stinky. Pham and Nakai (1984) and Amantea (1984) applied principal component and discriminant analyses to Cheddar cheese HPLC profiles. The cheese extracts for HPLC analysis consisted of peptides and amino acids. Amantea (1984) was able to reduce the number of HPLC peaks, necessary to classify the cheeses by age, from 48 to 8. Aishima and Nakai (1987) applied discriminant analysis of GC profiles to classify cheeses by variety. Santa-Maria et al. (1986) also used proteolysis products including total nitrogen, tyrosine, and tryptophan to characterize Manchego cheese by age. Eighteen variables were used to classify 30 cheeses as fresh, medium, or aged. Bertuccioli et al. (1986) applied PCA and partial least squares (PLS) analysis to sensory and GC data collected on Provolone cheese. Seven peaks characterized the cheese ripening while PLS analysis indicated the correlation between total aroma and the peaks.

III. MATERIALS AND METHODS

A. CHEESE SAMPLES

Commercial brands of Cheddar, Gouda, Edam, Swiss, and Parmesan cheeses were purchased from local supermarkets. In addition, samples of Cheddar cheese were received from Dairyland Foods (Burnaby, B. C.) and Canada Packers (Toronto, Ont). Swiss and Cheddar cheeses of varying ages were received from Kraft (Beaconfield, Que). All cheeses were stored at 5C.

B. SAMPLE PREPARATION

1. Water-soluble extract

Extraction of the water soluble fraction was a simplification of the method of McGugan et al. (1979). The initial separation was carried out by grating 4g of cheese and centrifuging at 27,000 X g for 30 min at 25C (Figure 2). The fat was pipetted off and discarded. The residue was transfered to a stoppered test tube and extracted with 1.0mL methanol, 1.0mL methylene chloride, and 0.6mL water. The mixture was shaken well for 15 sec and centrifuged again at 27,000 X g for 30 min at 25C. The methanol-water layer was removed and volatiles were evaporated in a Silli Therm heating module (Pearce Chemical Company, Box 117, Rockford, IL) set at a temperature of 45C under a flow of nitrogen. After 20 min of evaporation, 1mL of water was added to the extract. The evaporation was continued for a total of 60 min. The sample was diluted to a final volume of 2.0 mL and filtered through a 0.45μm Durapore HVLP Millipore filter (Bedford, MA). All cheese samples were extracted in duplicate, then combined into one final extract. The samples were frozen until analyzed. Frozen extracts were thawed at 5C and filtered through a 0.45 μm Durapore HVLP Millipore filter prior to injection into the HPLC.

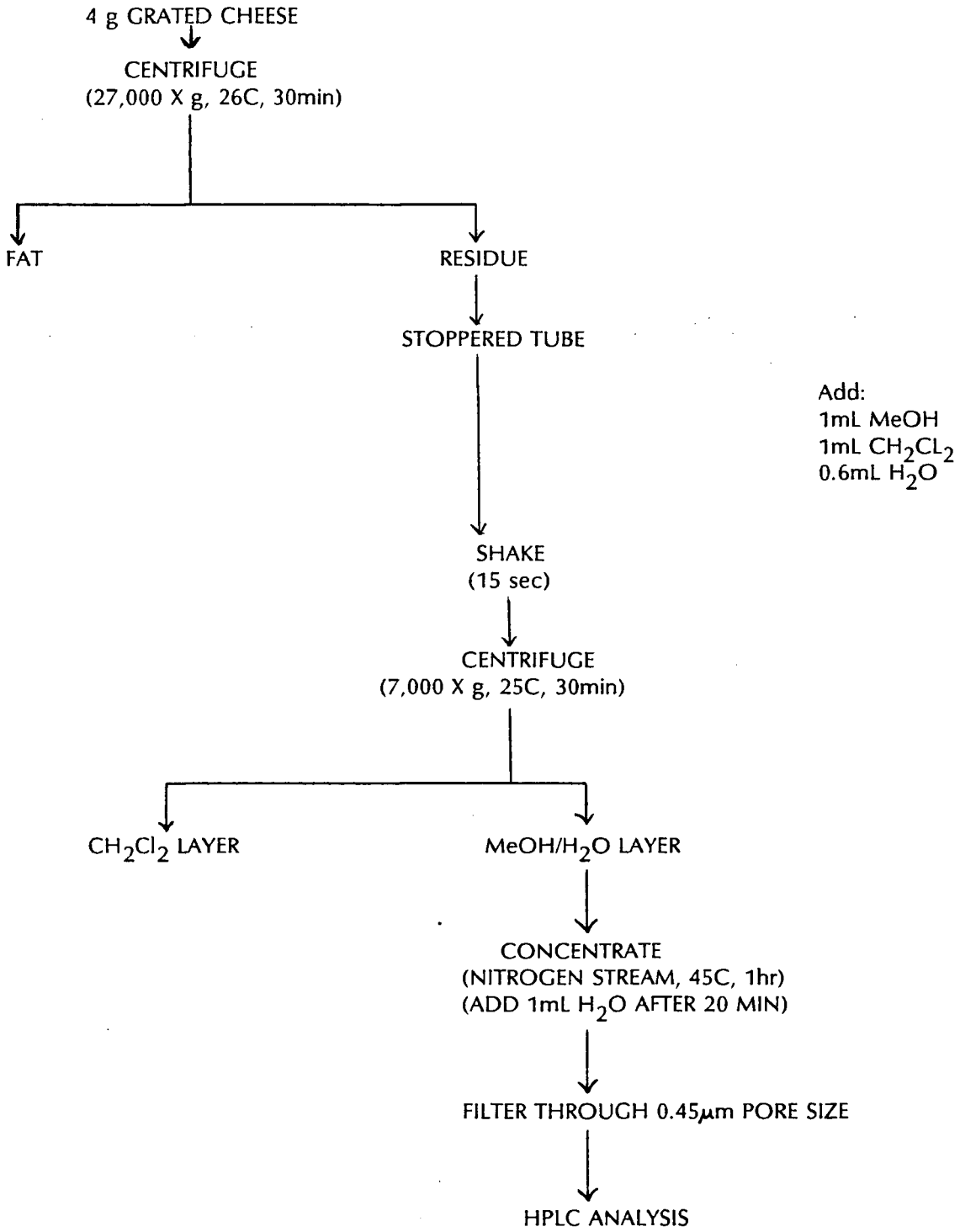

Figure 2. Extraction procedure of water-soluble cheese components for HPLC analysis.

2. pH

The final extract was measured for pH. An Accumet Model 420 digital pH meter (Fisher Scientific Co., Ottawa, Ont.) was used throughout the study. Approximately 1 mL of the final cheese extract was used to determine pH.

C. HPLC ANALYSIS

1. HPLC conditions

Chromatography was performed on a Spectra-Physics 8700 liquid chromatograph combined with a SP 8400 variable wavelength detector, and a SP 4100 computing integrator. (Spectra-Physics, Santa Clara, CA). The detector was run at a wavelength of 220 nm. A modification to the SP system was the use of a de-bubbler from Terochem Laboratories (P. O. Box 8188, Stn. F., Edmonton, Alta) placed between the ternary proportioning valve and the pump.

A reverse phase column (250 X 4.5 mm I. D.) packed with Adsorbosphere C8 (5μm) purchased from Alltech (Applied Science Labs, Deerfield, IL) was used for chromatographic analysis. The sample loop had a size of 50 μL. A guard column was placed before the Adsorbosphere C8 column. The guard column was packed with similar material as the main column. A RP-8 Spheri-10 RP-GU guard cartridge (Brownlee Labs, Santa Clara, CA.) was changed monthly during the analyses. Four HPLC columns were required to complete this study.

The reproducibility of the HPLC analysis was examined with a Swiss cheese extract. The extract was injected into the column once each day for 8 days. The standard deviation and coefficient of variation of the retention times of the major peaks were determined.

A ternary gradient system was used to elute the water soluble compounds from the column. An optimum initial solvent volume ratio of 96.8 : 1.2 : 2.0 was used for trifluoroacetic acid (0.1%), acetonitrile, and methanol. Over 50.3 min this ratio changed to

56.3 : 30.3 : 13.4. A further 30 min was necessary to gradually return to the starting elution conditions. Thus a single analysis took 80.3 min. A solvent flow rate of 1.0 mL per min was used. All HPLC runs were carried out at ambient temperature.

Methanol and acetonitrile were of HPLC grade (BDH Chemical Co., Toronto, Ont). Distilled water filtered through a Norganic cartridge (Millipore, Bedford, MA) was used to prepare the trifluoroacetic acid. All solutions were degassed for 15 min prior to use. During gradient elution, the solutions were maintained in a degassed state with a slow stream of helium.

A SP 4100 computing integrator, under BASIC control was used to calculate individual and total peak areas. A chart speed of 0.5 cm/min was used with an attenuation of 16 to record the chromatograms.

2. Internal standard

To follow the deterioration of the HPLC column and day-to-day variations, or variations within a day's run an internal standard was mixed with the cheese extracts. Several compounds were examined as possible standards, including phthalic acid, tryptophan, tyrosine, benzoic acid, p-nitroaniline, p-dimethylaminobenzaldehyde, rutin, vanillin, tannic acid, ninhydrin, and salicylaldehyde. Based on the retention time, p-dimethylaminobenzaldehyde was chosen as an internal standard. A stock solution of 100 ppm p-dimethylaminobenzaldehyde in acetonitrile was prepared. The solution was filtered through a 0.45μm Durapore HVLP Millipore filter. The standard stock solution was stored in the dark at 10C. A cheese extract was mixed with 20 ppm of the standard prior to analysis.

The purity of the p-dimethylaminobenzaldehyde was checked by thin layer chromatography (TLC) and by HPLC. Two solvent systems were used to elute the compound for TLC:

1. 95% benzene-5% methanol
2. chloroform

The p-dimethylaminobenzaldehyde sample was prepared by dissolving approximately 0.01 g in a few drops of methylene chloride. Three concentrations of the sample were spotted on each of two TLC plates pre-coated with Silica Gel 60 (BDH Chemicals, Toronto, Ont.). The spots were allowed to dry and the plates were eluted for 3 hrs in their respective chambers. The plates were checked under uv illumination and stained in an iodine chamber for 15 min.

The purity of the p-dimethylaminobenzaldehyde standard was examined secondly by the ternary gradient elution on the HPLC. A sample of 10 μL of the stock solution in acetonitrile, was injected into the HPLC.

D. OPTIMIZATION METHODS

A blend of the new Mapping Super-Simplex optimization (new MSO) method (Nakai and Kaneko,1985) with the Centroid Mapping Optimization method (Aishima and Nakai, 1986) was used to determine the best conditions for HPLC resolution. The idea is to move away from the worst response towards an optimum set of conditions.

Peak resolution was measured by the method of Morgan and Deming (1975) during the optimization procedure . Peak separation P_i of the i^{th} pair of peaks in a chromatogram is defined as:

$$P_i = f/g$$

where f represents the depth of the depression below a straight line joining two adjacent peak maxima, and g represents the height of the straight line above the baseline at the depression (Figure 1). The sum of P_i is the resolution or response used in the optimization procedure.

E. SENSORY EVALUATION

All cheese samples were evaluated by a semi-trained taste panel. The members were initially screened for their ability to distinguish the four basic tastes, sweet, sour, salty, and bitter. The panel consisted of 7 university students, who were present during the time of the

study and who liked cheese. A minimum of 5 panelists rated the cheeses. Taste panels were held twice a week.

Training consisted of familiarizing the panelists with the 5 varieties of cheeses being evaluated. Discussion occured among the panelists as to intensity scores for the four parameters being evaluated.

Cheese samples were prepared in a standard manner. The outer portions of a block of cheese were removed and cubes of approximately 2 cm^3 were cut. The cheeses were held at room temperature for 30 min prior to sensory evaluation.

At each session, 4 or 6 cheese samples were evaluated under red lights to eliminate color differences. Cheeses were identified as Cheddar, Edam, Gouda, Swiss, or Parmesan and rated on a scale from 1 to 5 for taste intensity, preference, and bitterness. Taste intensity ranged from a 1 for very low to a 5 for very strong. Preference ranged from a 1 for dislike very much to a 5 for like very much. Bitterness ranged from a 1 for none present to a 5 for strongly bitter.

F. STATISTICAL ANALYSIS

1. HPLC data

Each peak on the chromatogram, measured as area, was used for analysis. The internal standard peak, p-dimethylaminobenzaldehyde, was used to normalize the remaining peaks for each sample. Multivariate analyses including, principal component and discriminant analyses, were used to interpret the HPLC data. Statistical packages used were SAS programs of STEPDISC, DISCRIM, AND CANDISC (SAS Institute Inc, Cary, NC). An Amdahl 470 V/8 computer was used to perform the analyses. In multivariate analysis each peak from a chromatogram was considered a variable for a given cheese sample . Thus HPLC profiles with p peaks for each of n cheese samples, can be thought of as p variables for n cases or observations.

(a) Principal component analysis

The main goal of principal component analysis is to reduce the total sample data variance. The variance of the original data matrix was explained by "p" components, but often much of this variability can be explained by a smaller number "m" of the principal components. The principal components consist of linear combinations of the original variables and are uncorrelated. Principal components with eigenvalues greater than one were used in discriminant analysis.

(b) Discriminant analysis

Discriminant analysis deals with separating distinct sets of observations and subsequently allocating new observations to the defined groups. In addition, to representing the data with discriminant functions, discriminant analysis can be used to reduce the p-dimensional data space to 2 or 3 dimensions. The number of HPLC peaks "p" represent the dimensionality of the data. Plots of the means of the reduced linear combinations, or canonical variables, more easily show the relationships between the groups. Discriminant analysis was performed with both the principal components and the original peak areas.

2. Sensory data

The frequency of correctly identifying cheeses by variety was determined by SAS FREQ. The parameters of correct choice of variety, taste intensity, preference, and bitterness, were evaluated by multivariate analysis of variance using SAS GLM (SAS, 1985).

IV. RESULTS AND DISCUSSION

A. SAMPLE PREPARATION

A total of 106 cheese samples were purchased from local supermarkets or received from three Canadian cheese manufacturers. The cheeses were from ten different countries with unknown histories for the most part. The breakdown by cheese variety was: 40 Cheddars, 21 Edams, 28 Goudas, 13 Swiss, and 4 Parmesans. The Cheddars and a few Goudas were labeled by age, but the remaining cheeses were not identified as to their length of ripening. Fifteen of the Cheddars analyzed on the first column were from an accelerated ripening study using enzymes and elevated temperatures.

The extraction of the water soluble components was a modification of the long tedious procedure of McGugan et al. (1979). Pham and Nakai (1984) and Amantea (1984) modified the procedure for a smaller sample size. McGugan et al. (1979) designed the extraction steps to completly separate the fat from the non-fat fractions, thus there were many repetitions of the solvent extraction step. Methylene chloride, methanol and water were used to separate the volatile from the non-volatile fractions. The goal of the current study was to develop a quick extraction that could be used for quality control purposes by cheese manufacturers. Thus a rapid single step extraction was used (Figure 2).

The water-soluble fraction indicates changes that occur due to proteolysis during cheese ripening (Rank et al.,1985; Noomen,1977; Kuchroo and Fox,1982a,b). Noomen (1977) developed a water-soluble extraction method adjusted to the conditions of the cheese. Kuchroo and Fox (1982 a,b) showed the water-soluble fraction to be a mixture of peptides produced by enzymatic action of the coagulant, starter, and other bacteria. Large peptides from casein were not present in the water extract as demonstrated by gel electrophoresis (Kuchroo and Fox, 1982a). Pham and Nakai (1984) determined that water soluble extracts of Cheddar cheese were protein breakdown products based on a positive Ninhydrin reaction.

The inclusion of methanol and water in the extraction procedure selects for more hydrophobic compounds versus water alone (Rank et al.,1985). Harwarkar and Elliott (1971) used a cholorform-methanol-water extraction of Cheddar cheese. Rank et al.(1985) found the only difference between a water extraction and the Harwarkar and Elliott method was a slight increase in the peptide patterns at an absorbance of 280nm. Aston and Creamer (1986) also noted minor differences in HPLC patterns between the McGugan et al. (1979) method and their water extraction.

The Durapore filter used to remove particles larger than 0.45μm had no effect on the extracts. This was demonstrated by analyzing filtered and unfiltered HPLC grade methanol. There was no extra peak with the filtered methanol, indicating the purity of the filter paper.

The internal standard, p-dimethylaminobenzaldehyde, was added to the water-methanol extract prior to HPLC analysis rather than during the extraction procedure. This was necessary as otherwise the standard was discarded with the methylene chloride layer.

B. HPLC CONDITIONS

An Adsorbosphere C8 reversed phase column was used to elute the non-volatile cheese components, since Pham and Nakai (1984) found this column produced the largest number of peaks and best resolution with Cheddar extracts. Amantea (1984) also used this C8 column to elute Cheddar cheese compounds with a ternary gradient. An RP-8 guard cartridge was used between the injection port and the analytical column. The guard column prolongs the life of the C8 column by removing contaminants, highly retained solutes and particulate matter that may be in solvents (Anon, 1986). The guard cartridge was replaced at 2 to 4 week intervals.

The reproducibility of the HPLC profiles was determined over a 15 day period with a Swiss cheese extract. Nine major peaks were selected from the chromatographic profiles. Means, standard deviations, and coefficients of variation of the nine peaks for 8 injections

areshown in Table 1. The results indicate less variability between runs early and late in the elution pattern, and a general increase in variability during the middle of the elution.

Representative chromatograms for the five cheese varieties, Cheddar, Edam, Gouda, Swiss, and Parmesan, are shown in Figures 3-7 . The internal standard peak in all cheeses was the latest eluted large peak. The purity of the p-dimethylaminobenzaldehyde was demonstrated by the elution of one peak with HPLC analysis and one spot by TLC. The standard eluted late in the run and thus did not interfer with any cheese peaks of interest.

A ternary gradient was used to elute a variety of components from the HPLC column. Methanol, acetonitrile, and water are each from different Snyder (1978) selectivity groups, and thus would allow different compounds, based on their ability to undergo hydrogen bonding and dipole interactions, to be separated. Trifluoroacetic acid (TFA) acts as an ion-pairing agent. At a low pH of 2.0, the basic side chains of amino acids are fullly charged and associate with the oppositely charged TFA anions (Acharya et al.,1983; Hancock and Sparrow,1983). Trifluoroacetic acid is one of the most commonly used anions and allows a greater recovery of larger relatively nonpolar peptides (Hearn, 1983). The presence of TFA increased the retention times of histidine-containing tryptic peptides by making them more hydrophobic (Acharya et al.,1983). This ion pairing effect resulted in improved peak shapes (Hearn,1983). Amantea (1984) used 0.1% TFA to improve the resolution of peptides and amino acids in Cheddar cheese.

C. OPTIMIZATION

Optimization has become necessary in analytical procedures such as HPLC and GC where many parameters are important to the analysis and may interact. The trial and error or one-factor-at-a-time approaches require numerous experiments and may vary from one operator to another in the results. When a number of factors are necessary to an analysis, computer optimization is a must. Advantages of computerized optimization include improved

Table 1. Reproducibility of HPLC peak elutions from a Swiss cheese extract, n=8.

Retention Time (min)		
Mean	Standard Deviation	Coefficient of Variation
3.82	0.072	1.9
4.80	0.084	1.8
8.10	0.850	10.5
8.79	0.772	8.8
11.03	0.936	8.5
17.74	0.937	5.3
27.74	1.300	4.7
32.56	0.928	2.8
36.89	1.401	3.8

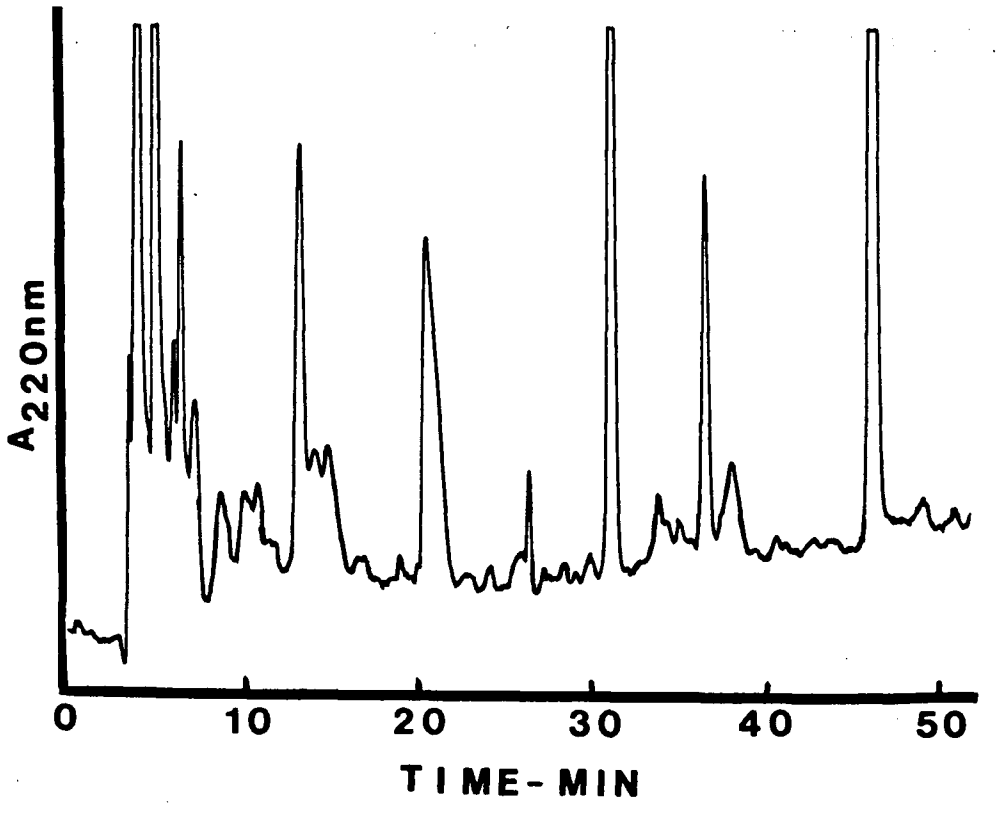

Figure 3. Representative HPLC profile of water-soluble components of Cheddar cheese.

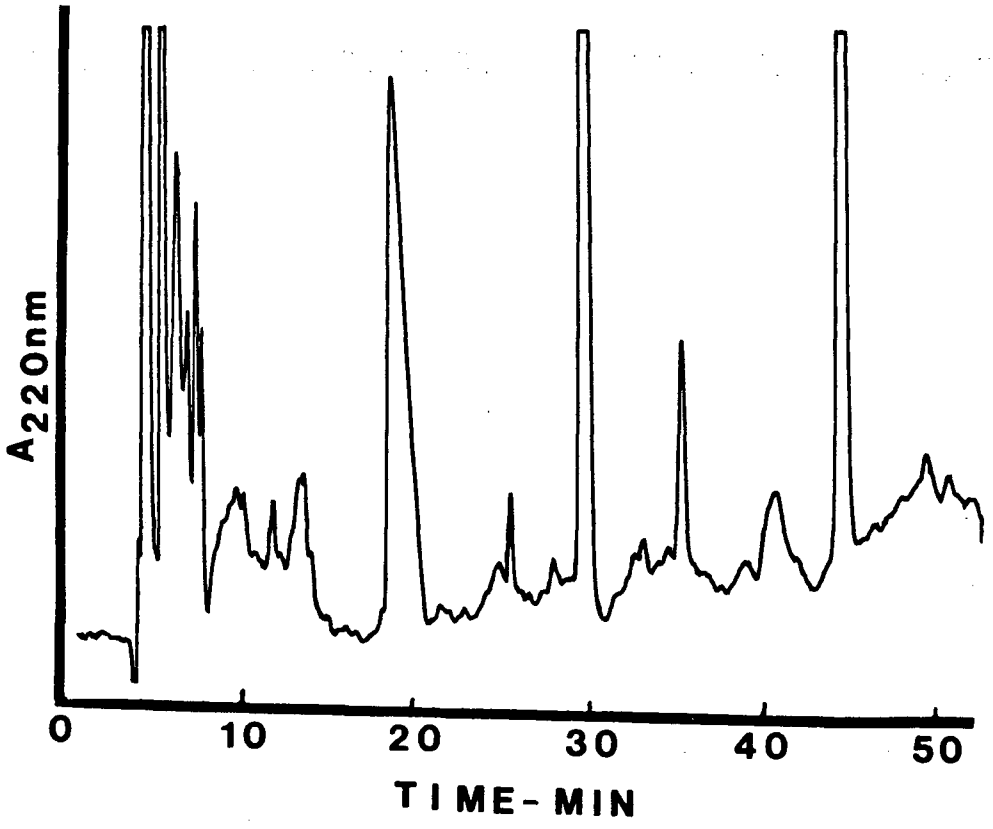

Figure 4. Representative HPLC profile of water-soluble components of Edam cheese.

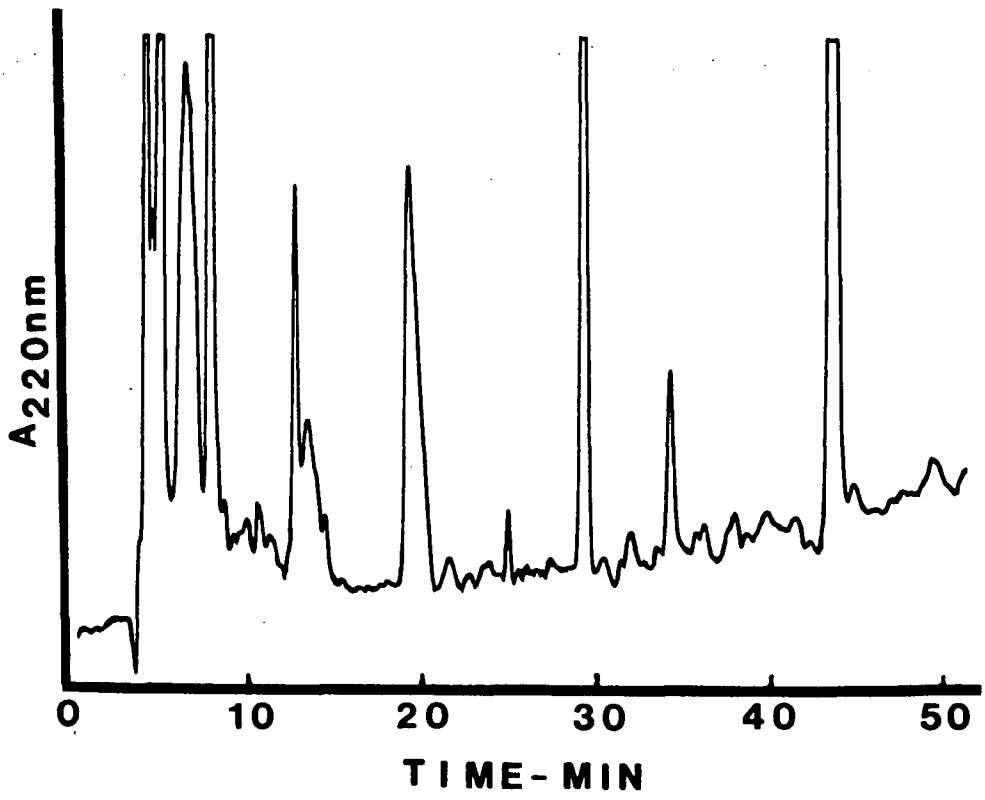

Figure 5. Representative HPLC profile of water-soluble components of Gouda cheese.

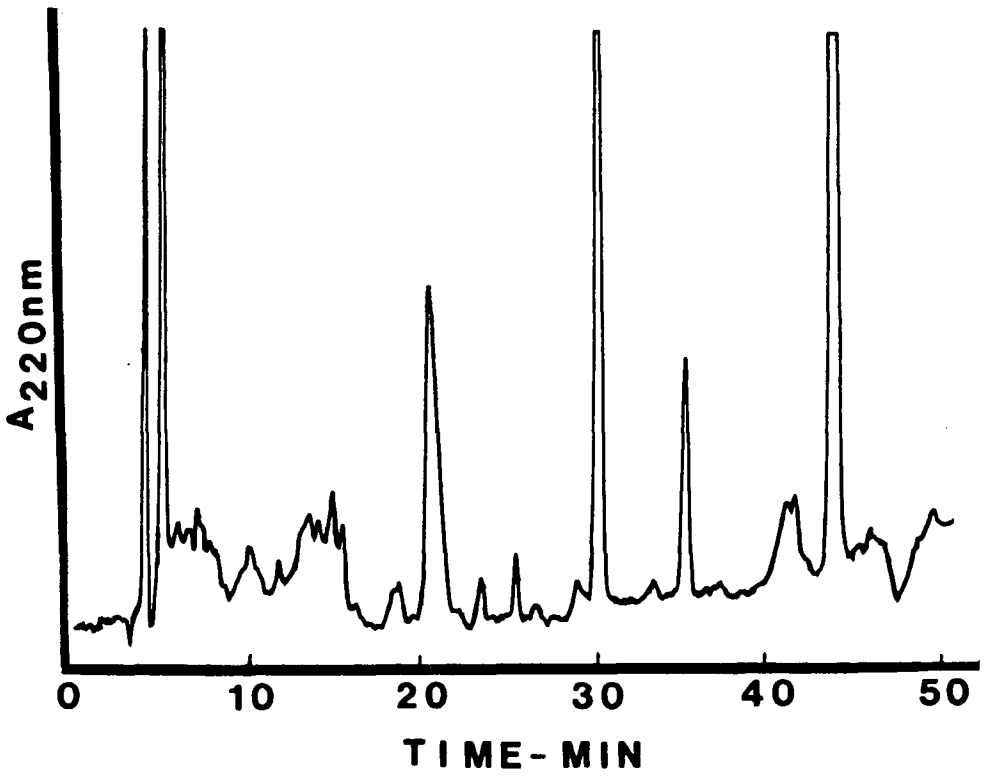

Figure 6. Representative HPLC profile of water-soluble components of Swiss cheese.

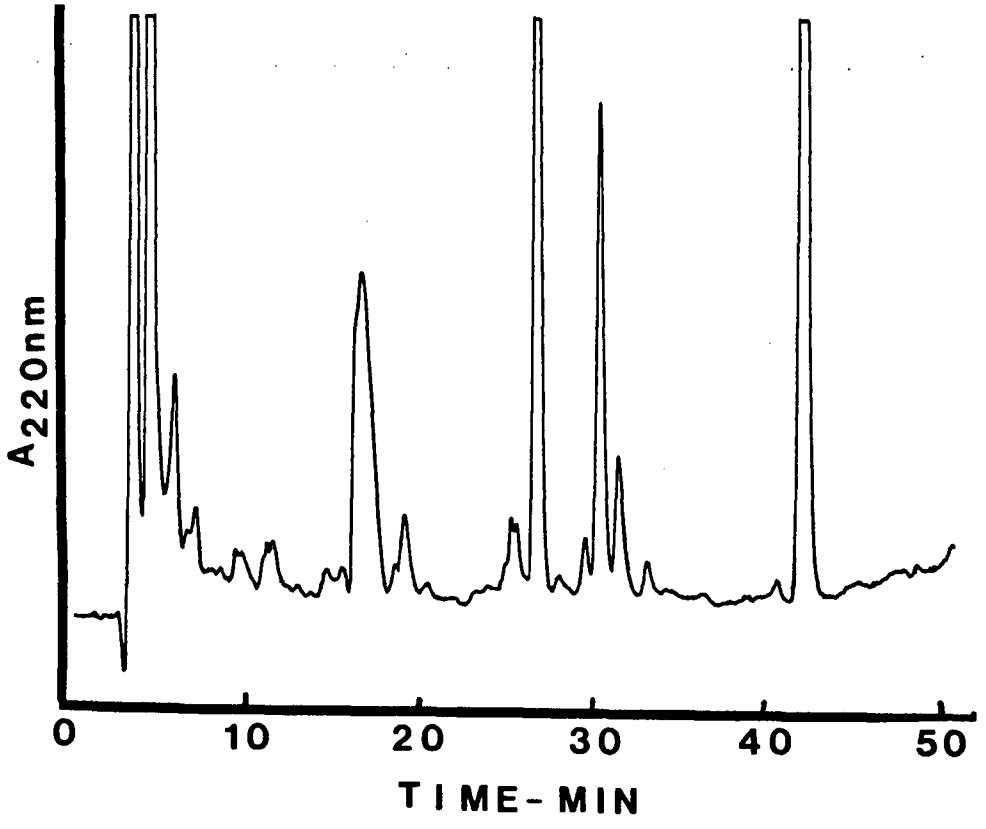

Figure 7. Representative HPLC profile of water-soluble components of Parmesan cheese.

research efficiency, while mapping the results graphically shows the response surface (Nakai et al.,1984)

The selection of the ternary gradient elution conditions were determined by the new Mapping Simplex Optimizataion (MSO) of Nakai and Kaneko (1985) and The Centroid Mapping Optimization (CMO) procedures of Aishima and Nakai (1986). A drawback to the Centroid procedure is the possibility of reaching a local optimum rather than the overall optimum if the initial factor ranges are set too narrow. Thus the new MSO procedure was used to narrow the ranges of the factors. The initial factor ranges were selected based on the optimum solvent conditions of Amantea (1984), where the organic modifier varied from 6% to 37% (Table 2). Ten experiments were carried out followed by graphing of the resolution responses against the factor ranges. The trend towards the optimum allowed the selection of new narrower factor ranges that were used with CMO to rapidly find the optimum HPLC parameters.

A total of 24 experiments, including the 10 from MSO, were necessary to reach the optimum HPLC separation. Mappings of the evolution towards the optimum for the five factors is shown in Figures 8-12. CMO indicates which points are to be connected, showing the trend towards the optimum. The figures indicate experimental conditions corresponding to vertex 20 as the optimum with a response of 21.825. For factor 5, time of HPLC run, vertex 20 corresponded to 67.4 min which was higher than most of the other vertices, with a range of 45 to 55 min (Figure 12). The additional time of elution may have produced more peaks than a shorter run, and thus showed a higher response. Vertex 24 showed the second highest response of 21.147, and corresponded to a more reasonable elution time of 50.3 min. Vertex 20 ranged from 2.0% to 26.2% acetonitrile while vertex 24 ranged from 1.2% to 30.3%; the methanol concentrations were similar. Thus vertex 20 had a shallower gradient than vertex 24. Vertex 24 also had 5% more organic modifier by the end of the run and therefore would be expected to elute more hydrophobic compounds than vertex 20.

Table 2. Starting factor ranges for the optimization of HPLC operating parameters by Mapping Simplex Optimization (MSO) and Centroid Mapping Optimization (CMO).

	FACTOR	STARTING RANGES MSO	CMO
F_1:	Initial methanol conc	0-10%	1-5%
F_2:	Final methanol conc.	20-50%	25-35%
F_3:	Initial acetonitrile conc.	0-10%	1-3%
F_4:	Final acetonitrile conc.	10-20%	11-14%
F_5:	Time of HPLC run	40-70 min	45-55min

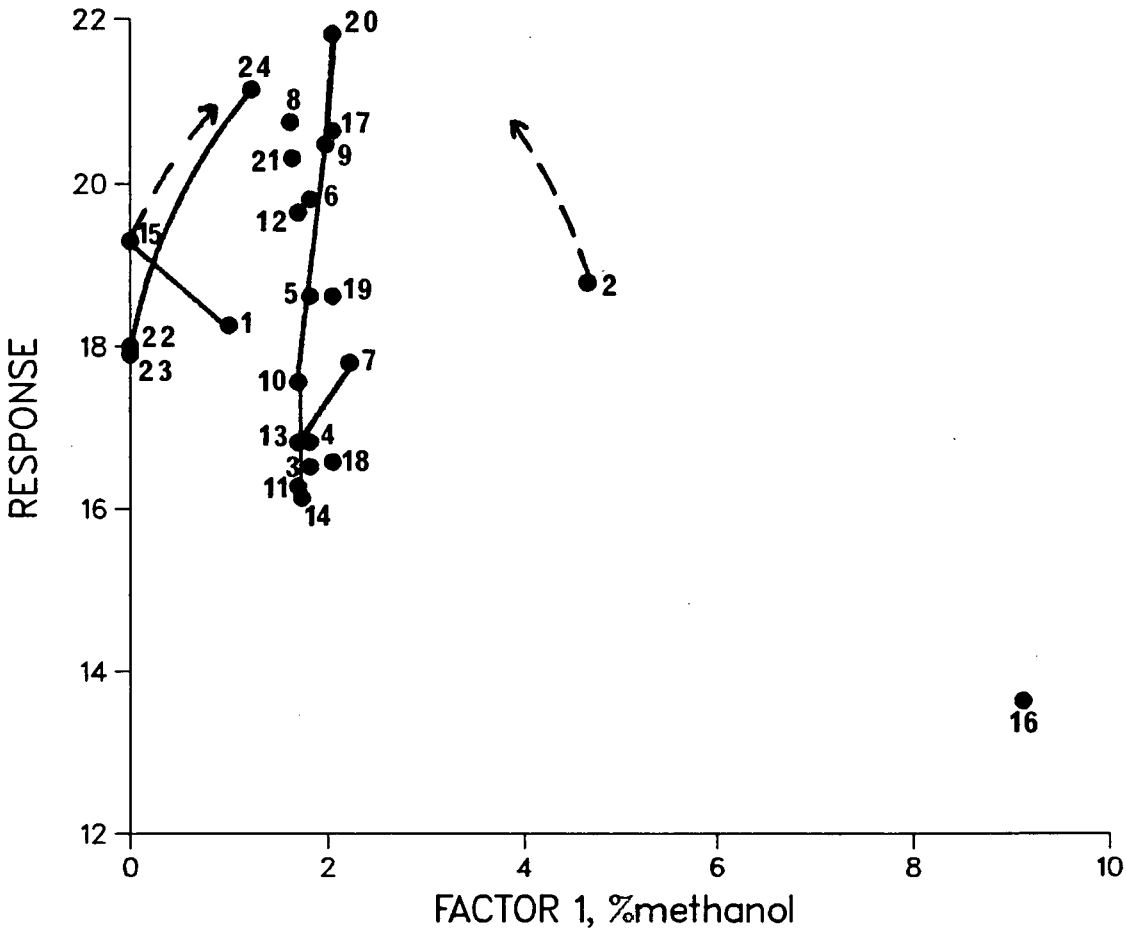

Figure 8. Mapping responses of experiments to optimize peak resolution. Factor 1, initial methanol concentration.

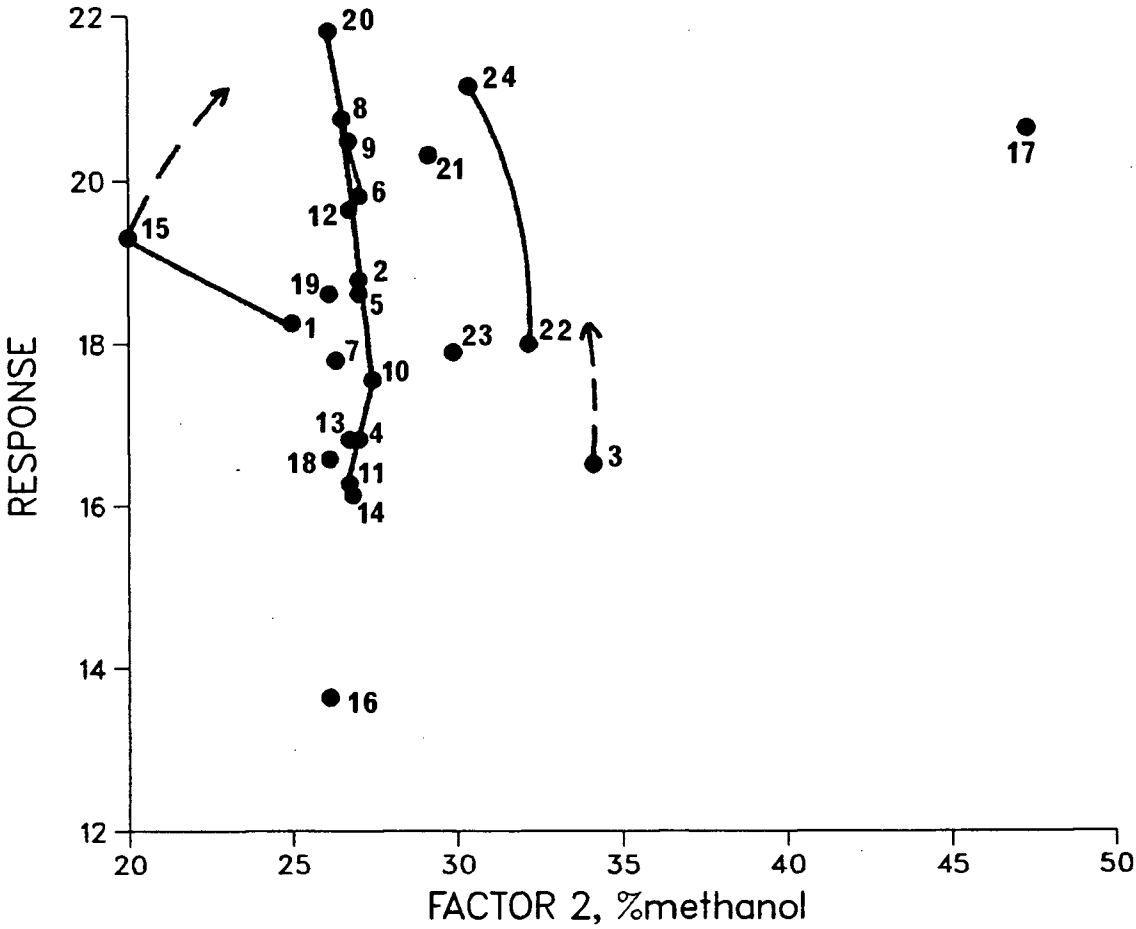

Figure 9. Mapping responses of experiments to optimize peak resolution. Factor 2, final methanol concentration.

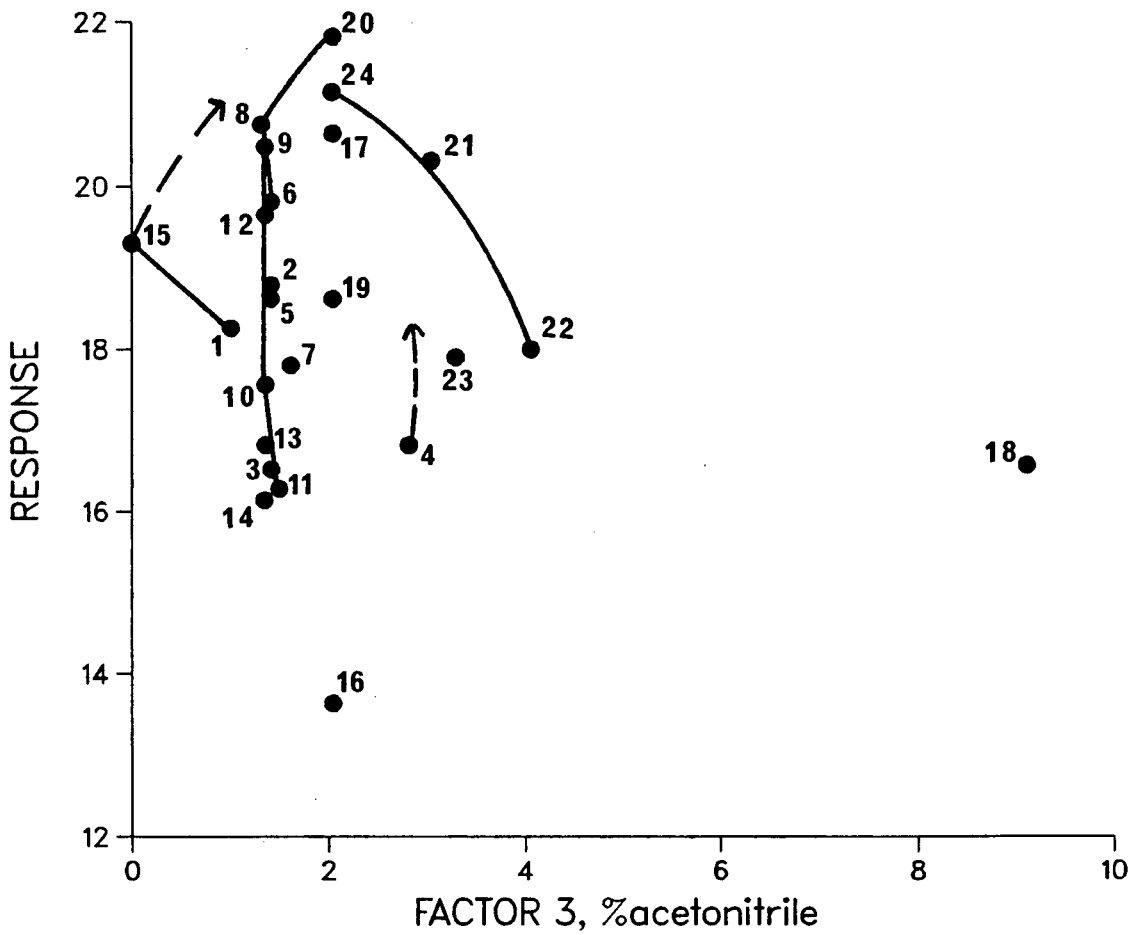

Figure 10. Mapping responses of experiments to optimize peak resolution. Factor 3, initial acetonitrile concentration.

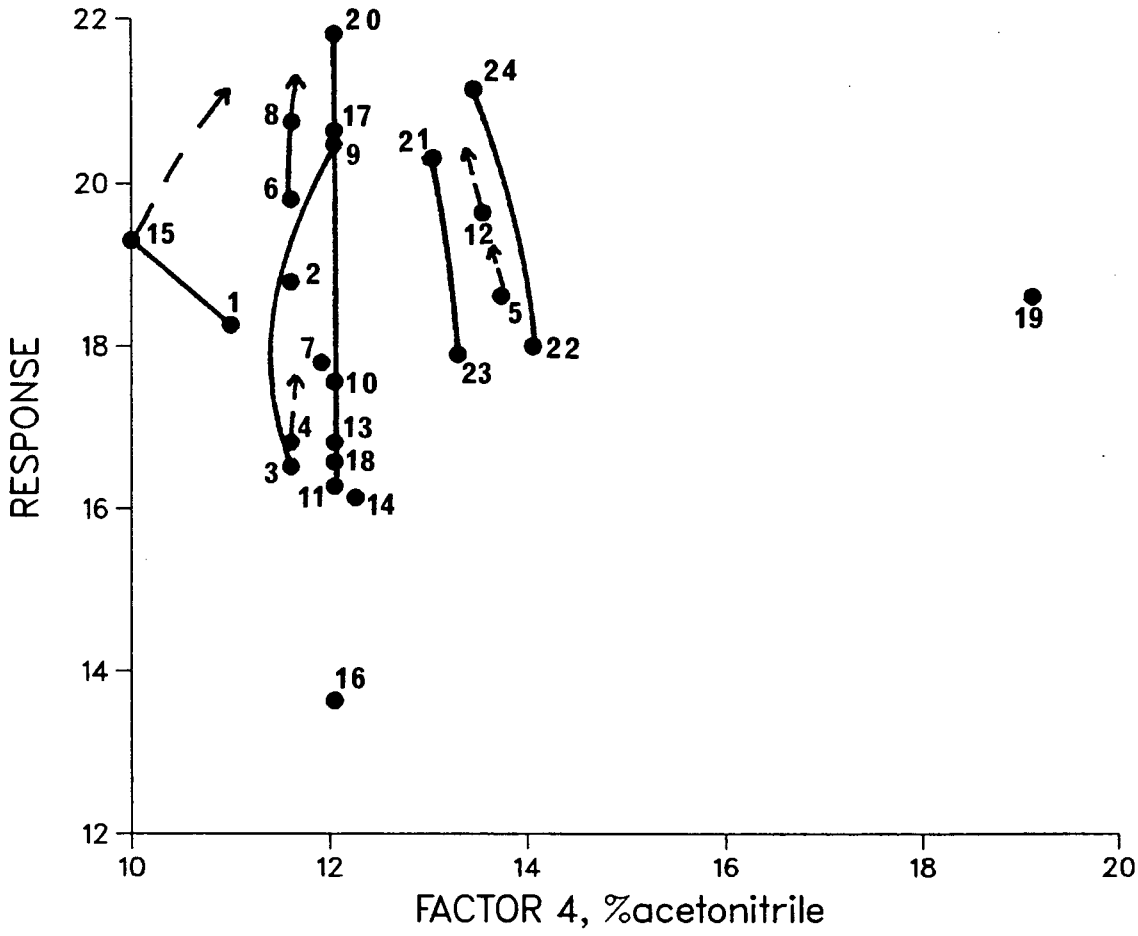

Figure 11. Mapping responses of experiments to optimize peak resolution. Factor 4, final acetonitrile concentration.

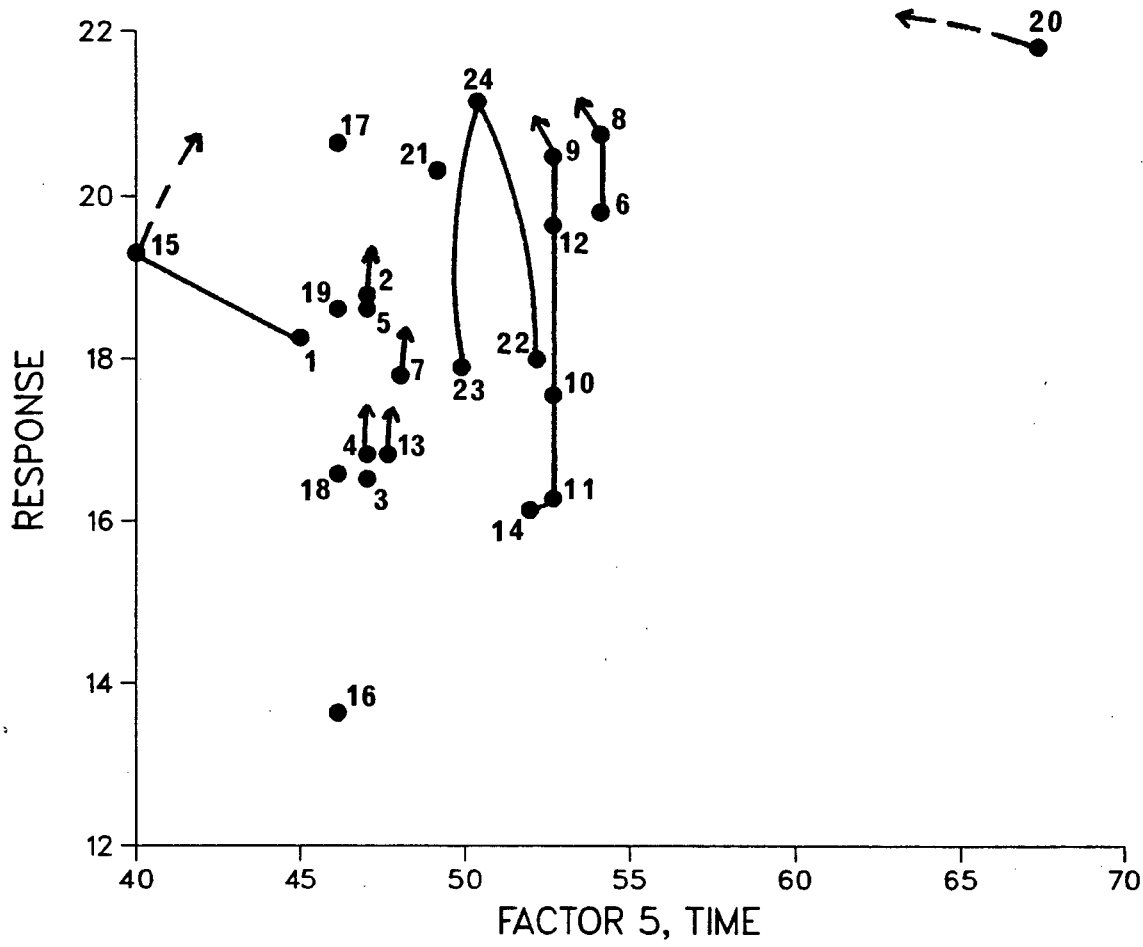

Figure 12. Mapping responses of experiments to optimize peak resolution. Factor 5, time of HPLC run.

Optimization produced a 55% improvement from the worst response to the best. The number of peaks separated increased 17%. Vertex 24 was chosen as the optimum, which corresponded to a solvent volume ratio of: 96.8 : 1.2 : 2.0 for TFA (0.1%), acetonitrile, and methanol. Over 50.3 min this ratio was changed to 56.3 : 30.3 : 13.4. An additional 30 min was used to bring the solvent ratio back to the initial ratio, thus one cheese extract was analyzed in 80.3 min.

D. HPLC STATISTICAL ANALYSES

Peak areas from each cheese profile were used for statistical analysis. Each profile was examined for the presence of 55 peaks. These 55 peaks were selected after examining relative retention times, in relation to the internal standard, of many varietal profiles. Several of the peaks were often absent in the chromatograms. To moderate or normalize large differences in peak areas among the 55 peaks, each peak area was divided by the area of the internal standard peak. The data was checked for multivariate normality and a $\log_e$ transformation of the peak areas improved the fit.

1. Principal component analysis

Principal component analysis (PCA) seeks to reduce the total sample data variance (Johnson and Wichern, 1982). The data matrix in this study can be considered a 106 by 55 matrix, corresponding to 55 variables, or HPLC peaks, for each of 106 cheese samples. Therefore the variance of this data matrix was explained by 55 components corresponding to the 55 peaks. Principal component analysis of the HPLC data resulted in 17 components with eigenvalues greater than 1.0, which is a common statistical cutoff point (Daultrey, 1976; Dillon and Goldstein, 1984). The principal components consist of a few linear combinations of the original variables and are uncorrelated. The 17 components explained 74% of the total sample variation (Table 3). Thus the dimensionality of the data was reduced from 55 to 17 with a 26% loss of explained variation. The proportion of variance explained by any one principal component was not greater than 13% and most were only 2 to 4%. This is fairly low

Table 3. Eigenvalue, proportion of variance explained, and cumulative proportion of total variance, in a principal component analysis using chromatographic data.

PRINCIPAL COMPONENT	EIGENVALUE	PROP. VAR. EXPLAINED	CUMULATIVE VARIANCE
Prin 1	7.0521	0.1306	0.1306
Prin 2	5.5089	0.1020	0.2326
Prin 3	3.7983	0.0703	0.3029
Prin 4	2.8967	0.0536	0.3566
Prin 5	2.3333	0.0432	0.3998
Prin 6	2.1928	0.0406	0.4404
Prin 7	2.0181	0.0374	0.4778
Prin 8	1.7948	0.0332	0.5110
Prin 9	1.7381	0.0322	0.5432
Prin 10	1.5777	0.0292	0.5724
Prin 11	1.5161	0.0281	0.6005
Prin 12	1.4827	0.0274	0.6280
Prin 13	1.3879	0.0257	0.6536
Prin 14	1.2045	0.0223	0.6760
Prin 15	1.1714	0.0217	0.6977
Prin 16	1.1178	0.0207	0.7184
Prin 17	1.0920	0.0202	0.7386

and indicates why the individual peak loadings on the components were relatively uniform. The peaks are unknown and the components did not load heavily on any one or several peaks, thus the loadings are shown in Appendix 1.

A second principal component analysis included the additional data of pH of the extracts, HPLC column number, and country of origin of the cheese samples. In this case 18 components had eigenvalues greater than 1.0 and these explained 75% of the total sample variation. Since HPLC column number and country of origin were indicated as categorical measurements, these two variables were eliminated from the analyses. The 55 peak areas with pH yielded again 18 eigenvalues that explained 75% of the variation. The proportion of variance explained by the eigenvalues and loadings on the principal components were similar to HPLC peak data alone and therefore are not shown.

2. Discriminant analysis

Discriminant analysis deals with separating distinct sets of observations and subsequently allocating new observations to the defined groups. Linear combinations of the data can reduce the dimensionality to 2 or 3 dimensions, that more easily show the relationships between the cheeses, this is shown in a plot of the canonical variables.

Factors that must be considered for an optimum classification are prior probabilities of group membership and the cost of misclassifying a case. If one class contains a larger population than another, the prior probabilities for classification should be weighted accordingly. Similarly if certain misclassifications represent a more serious error than others, the cost associated with these misclassifications should be considered (Johnson and Wichern, 1982).

Prior probabilities for classification as any one variety were set proportional to the sample size for each variety. This was necessary since the class sizes ranged from 40 Cheddars to 4 Parmesans. Misclassification costs were not considered, as one type of error was not worse than another.

Discriminant analysis is flexible allowing the use of the original variables for calculating the functions, or the use of principal components after such an analysis. All of the predictor variables may be used in determining the discriminant function. If there are a large number of variables, a stepwise selection method can reduce the number to a few significant variables necessary for classification. Significance levels are used to add to or eliminate predictor variables from the discriminant function.

The results from discriminant analysis can be visually represented by a canonical plot. This dimension reduction technique shows with 2 or 3 axes the groupings of the cases. Canonical variables are linear combinations of the original variables that have the highest multiple correlations with the groups, or best indicate differences between the groups (SAS, 1985). Canonical variables are uncorrelated with each other.

Discriminant analysis was firstly performed on the 17 principal components and yielded a total percent correct classification rate of 64%, with a high of 85% for Cheddar and a low of 25% for Parmesan (Table 4). A plot of the first and second canonical variables (Figure 13) indicates the grouping of cheese samples by variety. Cheddar forms a fairly distinct group which correlates with its 85% classification rate. The remaining varieties of Edam, Gouda, Swiss, and Parmesan, are mixed and do not form unique groups.

Discriminant functions can be derived for each cheese variety and these can be used to classify unknown samples. The general form of the function for Cheddar is:

Cheddar: a_1(prin 1) + a_2(prin 2) + ... a_{17}(prin 17)-constant

where prin 1....prin 17 are eigenvalues of the principal components for Cheddar, and a_1....a_{17} are coefficients of the function. The values for the coefficients for each cheese variety are shown in Table 5. Thus the actual discriminant function for Cheddar cheese would be:

Cheddar: 0.56(prin 1)+0.016(prin 2)-0.93(prin 3)-0.45(prin 4)-0.22(prin 5)
+0.41(prin 6)-0.26(prin 7)-0.27(prin 8)-0.095(prin 9)-0.24(prin10)
+0.018(prin11)-0.30(prin12)-0.031(prin13)-0.19(prin14)-0.06(prin15)
+0.084(prin16)+0.18(prin17)-1.55.

Table 4. Percent correct classification rate of cheese variety by discriminant and sensory analysis.

DESCRIPTION OF ANALYSIS[a]	CHEESE VARIETY[b] CHEDDAR	EDAM	GOUDA	SWISS	PARM	TOTAL
17 PC	85	52	64	31	25	64
17 PC,eq	72	57	54	77	100	72
18 PC+3var	90	57	75	50	50	73
18 PC+pH	80	57	71	67	25	71
8 peaks	82	48	57	42	50	63
Column 1	100	100	100	100	NA	100
Column 2	100	100	100	NA	NA	100
Column 3	100	100	89	88	NA	93
Column 4	100	60	100	NA	100	93
SENSORY	86	42	43	74	76	63

a. PC =principal components; equal=equal prior probability;

3 var = pH, country of origin, column number.

b. N. A., not analyzed on the column.

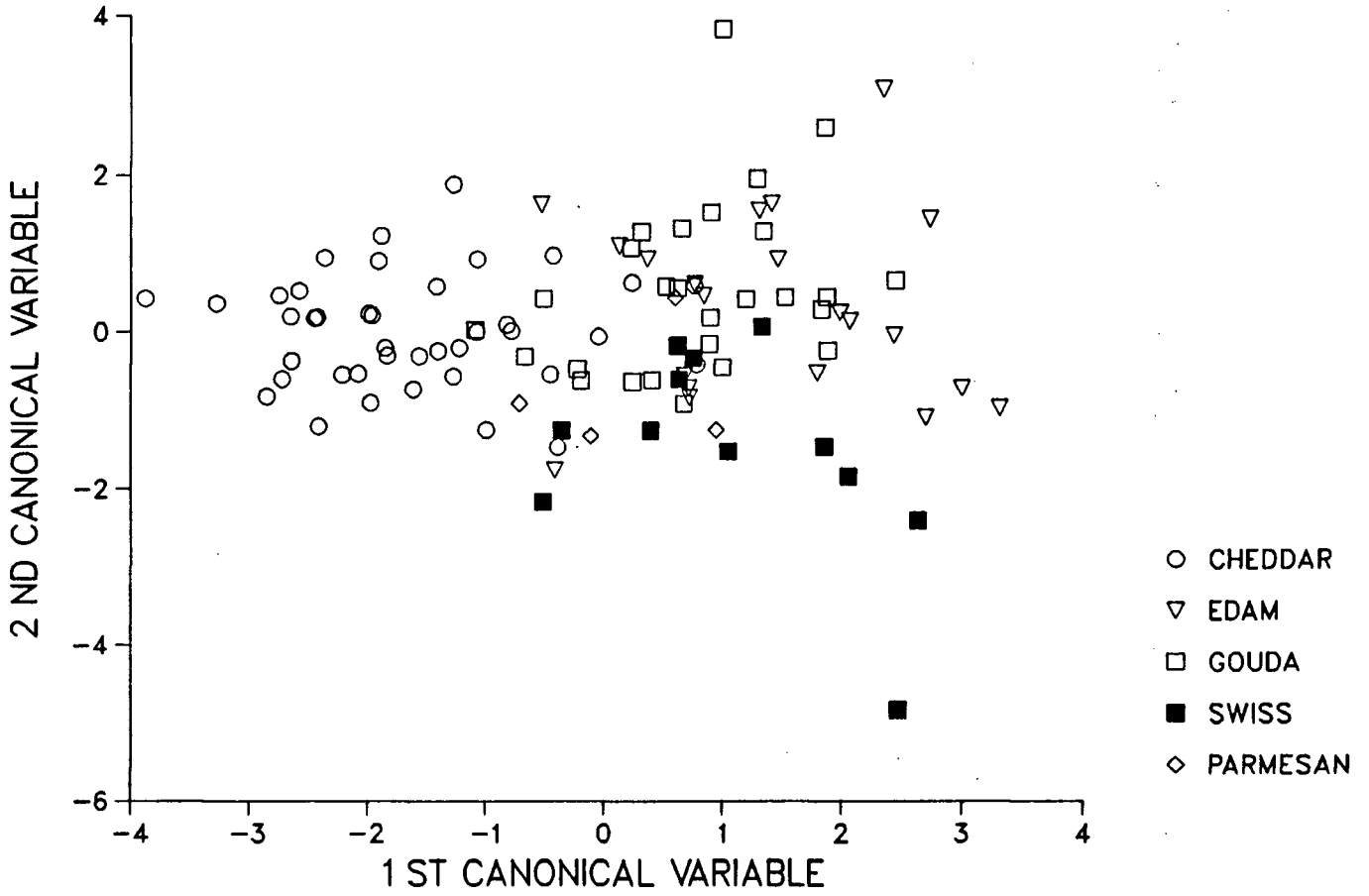

Figure 13. Canonical plot of 106 cheese samples grouped by variety using proportional prior probabilities.

Table 5. Coefficients of discriminant functions from principal components for separation of 106 cheeses by variety.

VARIABLE	CHEDDAR	EDAM	GOUDA	SWISS	PARMESAN
Constant	-1.551	-2.453	-1.881	-2.960	-5.675
Prin 1	0.559	-0.296	-0.097	-0.704	-1.069
Prin 2	0.016	0.374	-0.116	-0.517	0.371
Prin 3	-0.933	0.656	0.876	-0.038	-0.123
Prin 4	-0.446	0.276	0.553	-0.165	-0.323
Prin 5	-0.222	0.412	0.219	-0.450	-0.013
Prin 6	0.408	-0.529	-0.427	0.415	0.340
Prin 7	-0.258	0.454	-0.090	0.323	-0.229
Prin 8	-0.268	0.211	0.201	0.144	-0.305
Prin 9	-0.095	-0.086	0.101	0.330	-0.377
Prin 10	-0.236	0.534	-0.185	0.424	-0.534
Prin 11	0.018	0.162	-0.181	-0.193	0.867
Prin 12	-0.303	0.549	0.105	-0.160	-0.073
Prin 13	-0.031	-0.021	0.068	-0.401	1.248
Prin 14	-0.186	0.243	0.059	0.022	0.108
Prin 15	0.061	0.362	-0.319	-0.024	-0.194
Prin 16	0.084	-0.002	-0.102	-0.204	0.550
Prin 17	0.182	0.510	-0.291	-0.485	-0.892

Similar functions can be obtained with the remaining varieties. An unknown cheese sample could be classified by variety by substituting values for the principal components in each equation for the 5 varieties. The unknown sample would be allocated to the variety with the highest discriminant function.

If prior probabilities were set equally, an overall classification rate of 72% was obtained (Table 4). This increase was due to a large improvement in the classification rates for Parmesan and Swiss cheeses, which were weighted much higher in the equal probability analysis. Cheddar and Gouda were grouped at lower rates, while Edam was the same. Thus sample size may alter the results significantly.

Discriminant analysis of the 18 PC's from peak areas, pH, and the 2 categorical data produced an overall 73% correct classification rate. The 18 PC's resulting from peak areas and pH yielded a 71% correct discrimination rate (Table 4).

Discriminant analysis may also be performed on the original HPLC peaks. A stepwise discriminant analysis was carried out with the 55 peaks where a significance level to enter and stay in the function was 0.05. The analysis added nine peaks to the function and removed one before stopping. A summary table showing the peak selection at each step can be seen in Table 6. The discriminant function for Cheddar cheese is:

$$\text{Cheddar: } 19.75(X_6) + 26.59(X_{28}) - 0.37(X_9) + 11.55(X_{10}) + 69.87(X_{50}) + 0.010(X_{39}) - 1.38(X_{21}) + 3.36(X_{15}) - 10.35.$$

Similar functions can be derived from the data in Table 7 for the remaining 4 varieties. Thus to classify an unknown cheese sample, the peak areas would be substituted into the functions and the variety with the highest score would be the identity of the unknown sample. The overall classification rate using the eight peaks was 63% with a high of 82% for Cheddar, and a low of 42% for Swiss (Table 4). This rate is comparable to that using the 17 principal components.

Discriminant analyses thus far indicated a similar or slightly higher rate of cheese variety classification than that of the sensory panel. Table 4 shows an overall 63% correct

Table 6. Summary table for peaks entered in stepwise discriminant analysis.

STEP	VARIABLE ENTERED	VARIABLE REMOVED	F STATISTIC
1	X6		6.986
2	X28		6.202
3	X9		4.978
4	X10		4.703
5	X50		3.711
6	X39		3.060
7	X17		2.697
8	X21		2.518
9	X15		3.186
10		X17	2.296

Table 7. Coefficients for discriminant functions from eight HPLC peaks.

VARIABLE	CHEDDAR	EDAM	GOUDA	SWISS	PARMESAN
Constant	-10.349	- 7.762	- 8.533	- 6.425	-10.966
X6	19.753	11.209	15.545	10.869	17.873
X28	26.588	12.571	7.313	4.670	13.101
X9	-0.371	29.500	-0.105	9.842	7.089
X10	11.550	11.702	20.103	4.725	6.810
X50	69.870	42.805	76.940	27.781	21.612
X39	0.010	0.172	-0.701	5.686	3.377
X21	-1.378	-32.250	-5.132	-13.882	127.163
X15	3.364	13.610	4.054	6.558	-16.393

classification rate by the taste panel, with a high of 86% for Cheddar and a low of 42 and 43% for Edam and Gouda respectively.

A variable refered to as HPLC column number, mentioned previously, was in reference to the four HPLC columns that were used to analyze the 106 cheese extracts. The columms were all the same type and from the same manufacturer, but ordered at varying times of the year. Throughout the life-time of a column, the chromatographic pattern was compressed and the peak resolution decreased. This was shown by the elution pattern of the test mixture that came with each column, and the retention time of the internal standard peak.

To determine if column-to-column differences existed, principal component and discriminant analyses of cheese variety were performed separately on samples from each HPLC column. For each column, 14 or 15 principal components were able to explain from 88 to 99% of the total sample variation (Tables 8 to 11). Each principal component explained a larger amount of the sample variation than with the earlier combined data analysis. The component loadings were again relatively low for most of the HPLC peaks and are not shown.

Discriminant analysis by column of the PC's yielded a total correct varietal classification of 93 to 100% (Table 4). Columns one and two grouped cheeses by variety at a 100% rate. This clear separation by variety can be seen in the canonical plots in Figures 14 to 17. Parmesan was only analyzed on column 4 and Swiss on columns one and three.

E. SENSORY DATA

Each cheese sample was tasted by 5 to 7 semi-trained judges. The panelists rated the cheeses on a scale from 1 to 5 for taste intensity, preference, and bitterness. The judges also identified the cheese by variety. These results should not be generalized to the public at large, as they reflect the tastes of this single panel. Another panel of judges may indicate differing results, thus the data is not absolute. It was difficult to "train" the panelists since cheeses labeled as a specific variety varied tremendously in the type of taste. Domestic

Table 8. Eigenvalue, proportion of variance explained, and cumulative proportion of total variance, in a principal component analysis using chromatographic data from column 1.

PRINCIPAL COMPONENT	EIGENVALUE	PROP. VAR. EXPLAINED	CUMULATIVE VARIANCE
Prin 1	9.1690	0.1698	0.1698
Prin 2	6.5973	0.1222	0.2920
Prin 3	5.7970	0.1074	0.3993
Prin 4	4.0034	0.0741	0.4734
Prin 5	3.6187	0.0670	0.5405
Prin 6	3.2443	0.0601	0.6006
Prin 7	2.5710	0.0476	0.6482
Prin 8	2.1203	0.0392	0.6874
Prin 9	1.9767	0.0366	0.7240
Prin 10	1.8894	0.0350	0.7590
Prin 11	1.8416	0.0341	0.7931
Prin 12	1.4855	0.0275	0.8206
Prin 13	1.2708	0.0235	0.8442
Prin 14	1.0894	0.0202	0.8643
Prin 15	1.0545	0.0195	0.8839

Table 9. Eigenvalue, proportion of variance explained, and cumulative proportion of total variance, in a principal component analysis using chromatographic data from column 2.

PRINCIPAL COMPONENT	EIGENVALUE	PROP. VAR. EXPLAINED	CUMULATIVE VARIANCE
Prin 1	7.4347	0.1377	0.1377
Prin 2	6.6257	0.1227	0.2604
Prin 3	6.2795	0.1163	0.3767
Prin 4	5.6778	0.1051	0.4818
Prin 5	4.9778	0.0922	0.5734
Prin 6	4.1152	0.0762	0.6502
Prin 7	3.5952	0.0666	0.7168
Prin 8	3.1557	0.0584	0.7752
Prin 9	2.6337	0.0488	0.8234
Prin 10	2.4810	0.0459	0.8699
Prin 11	1.9786	0.0366	0.9066
Prin 12	1.7696	0.0328	0.9393
Prin 13	1.3892	0.0257	0.9651
Prin 14	1.1513	0.0213	0.9864

Table 10. Eigenvalue, proportion of variance explained, and cumulative proportion of total variance in a principal component analysis using chromatographic data from column 3.

PRINCIPAL COMPONENT	EIGENVALUE	PROP. VAR. EXPLAINED	CUMULATIVE VARIANCE
Prin 1	9.2034	0.1704	0.1704
Prin 2	8.0748	0.1495	0.3200
Prin 3	5.7410	0.1063	0.4263
Prin 4	4.4100	0.0817	0.5079
Prin 5	3.2848	0.0608	0.5688
Prin 6	2.6238	0.0459	0.6174
Prin 7	2.3639	0.0438	0.6611
Prin 8	2.1926	0.0406	0.7017
Prin 9	2.1783	0.0404	0.7421
Prin 10	1.9920	0.0369	0.7790
Prin 11	1.6539	0.0306	0.8096
Prin 12	1.6045	0.0297	0.8393
Prin 13	1.2973	0.0240	0.8633
Prin 14	1.1711	0.0217	0.8850
Prin 15	1.1253	0.0208	0.9059

Table 11. Eigenvalue, proportion of variance explained, and cumulative proportion of total variance, in a principal component analysis using chromatographic data from column 4.

PRINCIPAL COMPONENT	EIGENVALUE	PROP. VAR. EXPLAINED	CUMULATIVE VARIANCE
Prin 1	10.2611	0.1900	0.1900
Prin 2	7.0130	0.1299	0.3199
Prin 3	4.9030	0.0908	0.4107
Prin 4	4.5140	0.0836	0.4943
Prin 5	3.6005	0.0667	0.5610
Prin 6	3.1023	0.0574	0.6184
Prin 7	2.4305	0.0450	0.6634
Prin 8	2.2988	0.0425	0.7060
Prin 9	2.2297	0.0413	0.7473
Prin 10	1.6892	0.0313	0.7786
Prin 11	1.5680	0.0290	0.8076
Prin 12	1.4294	0.0265	0.8340
Prin 13	1.2770	0.0236	0.8577
Prin 14	1.2529	0.0232	0.8809

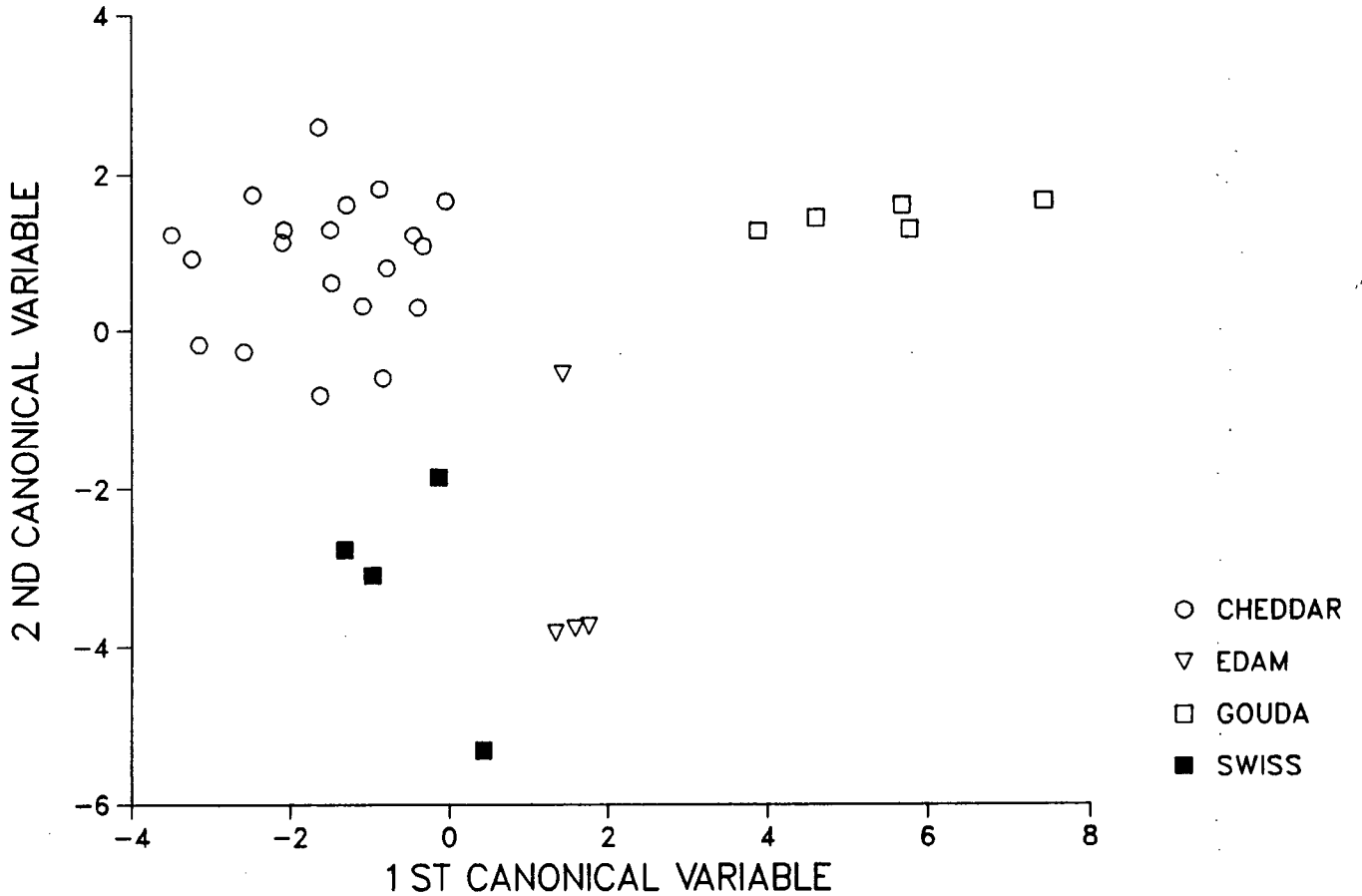

Figure 14. Canonical plot of cheese samples from column one grouped by variety.

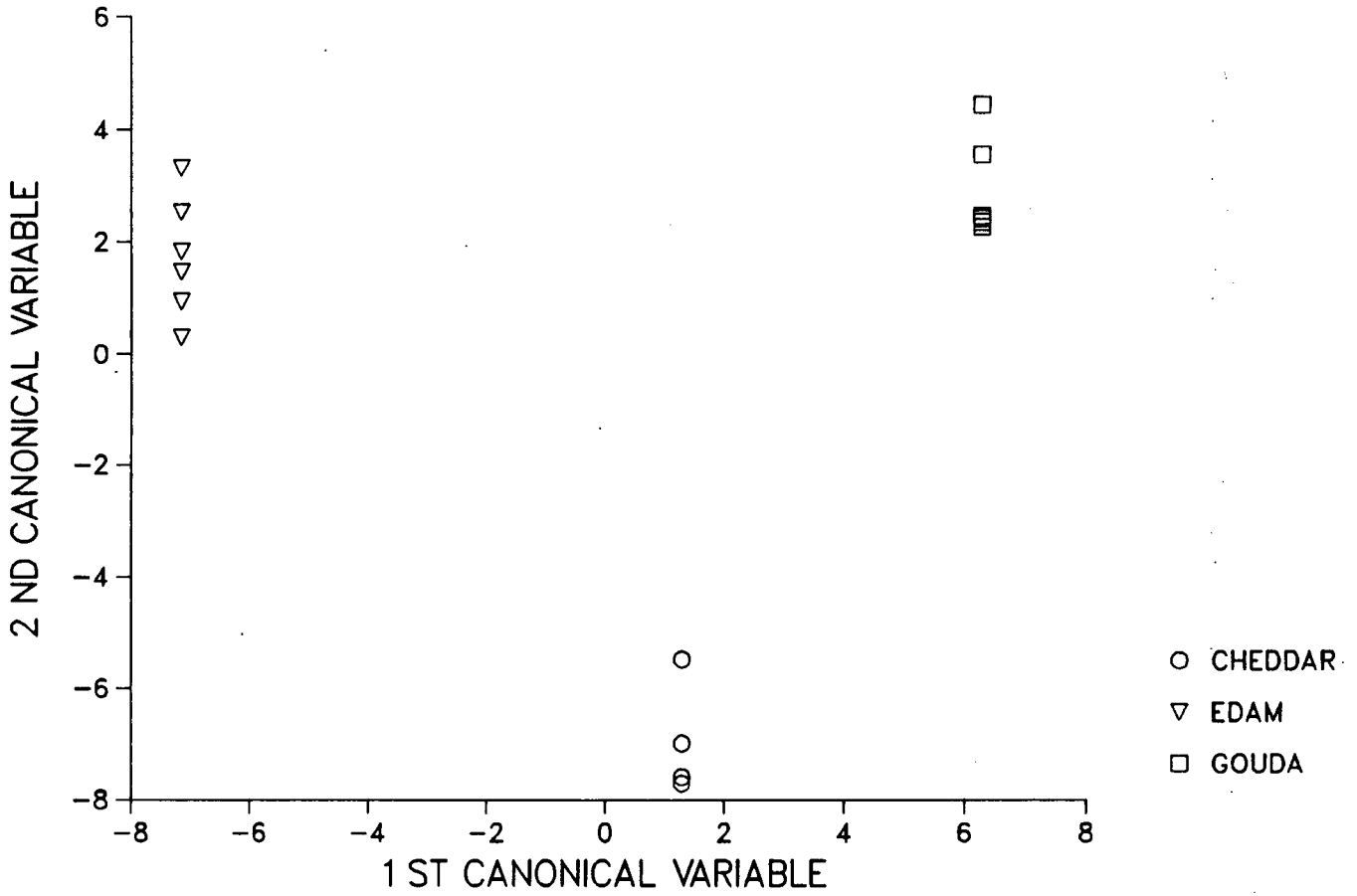

Figure 15. Canonical plot of cheese samples from column two grouped by variety.

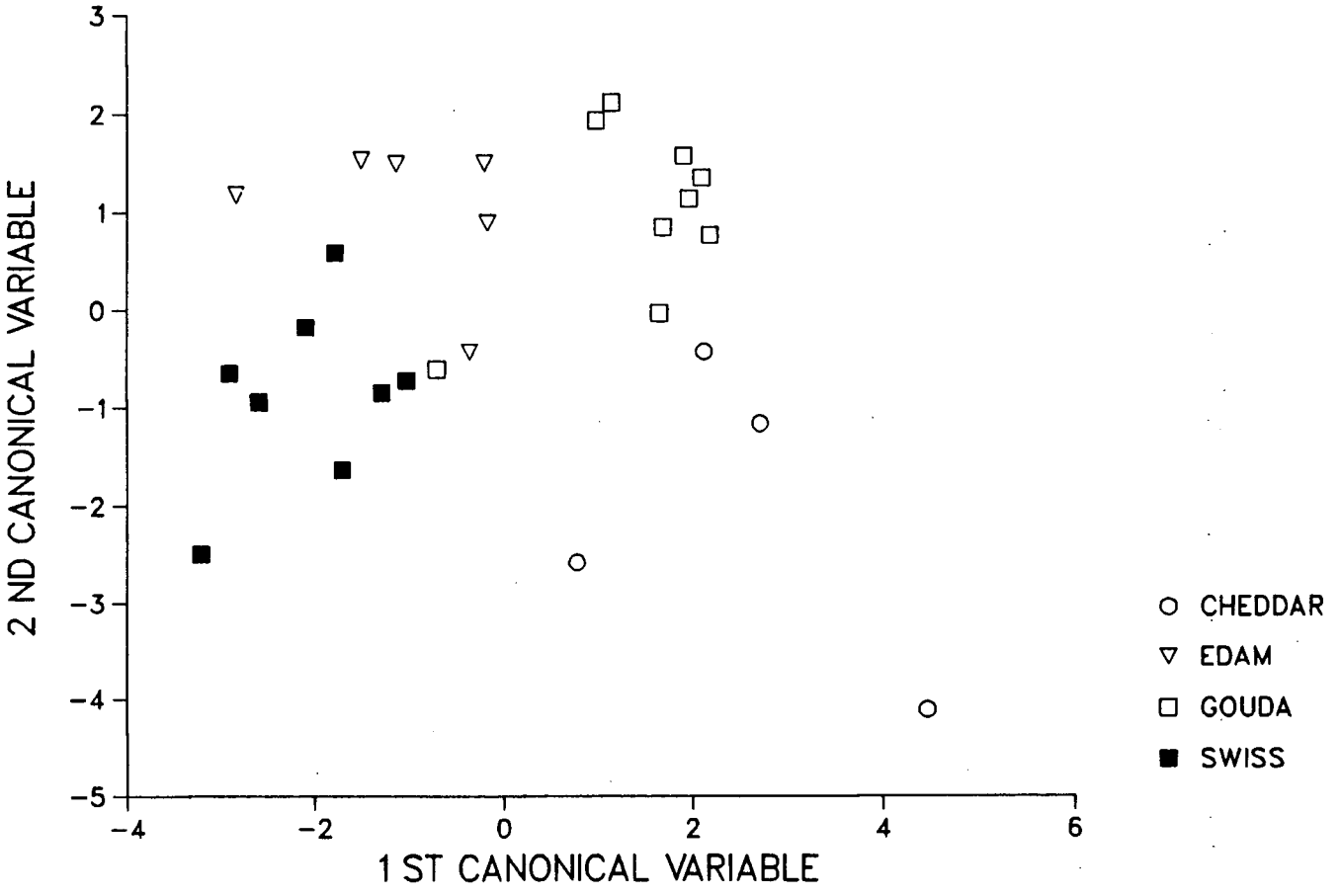

Figure 16. Canonical plot of cheese samples from column three grouped by variety.

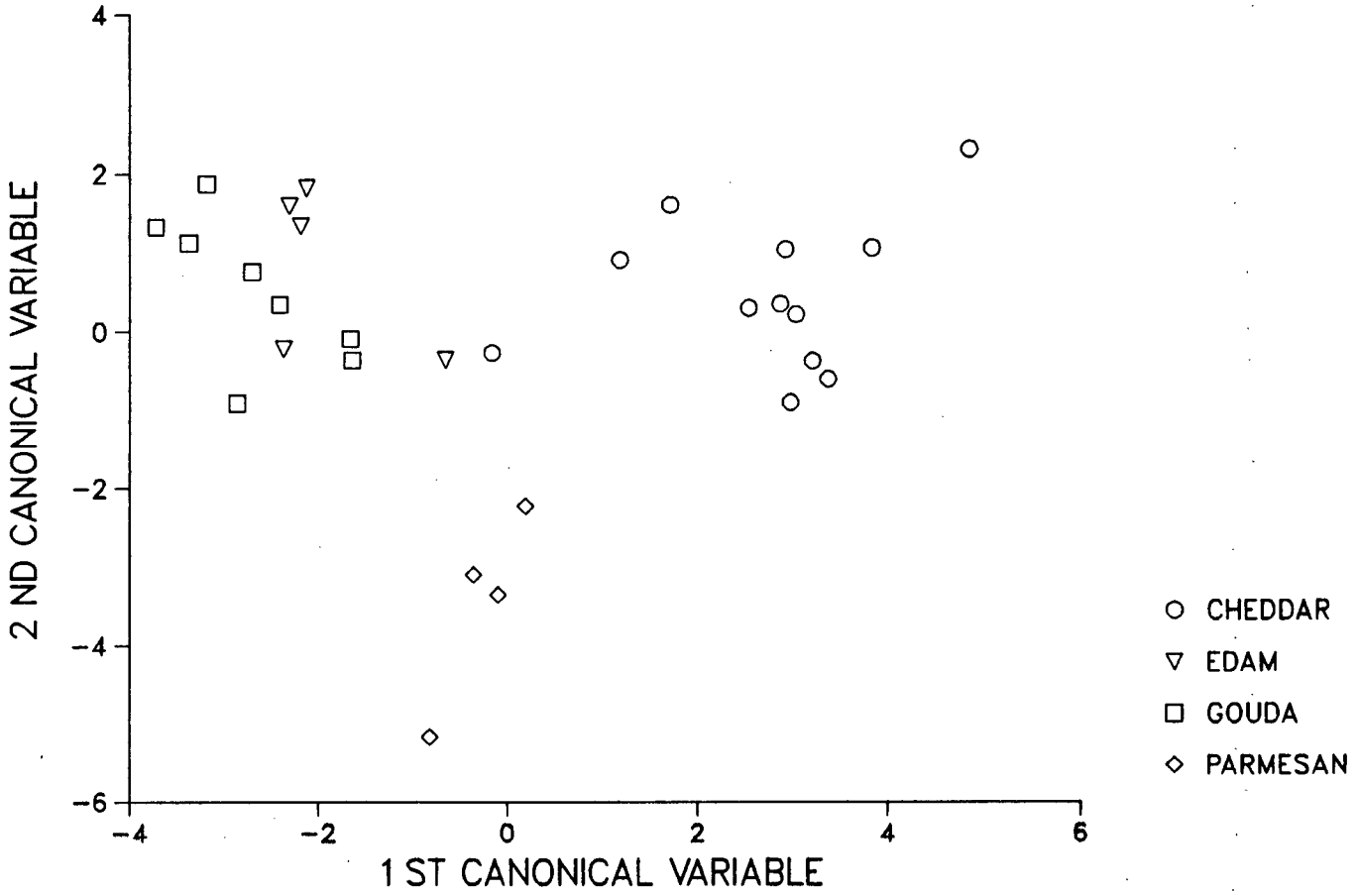

Figure 17. Canonical plot of cheese samples from column four grouped by variety.

versus imported cheeses, of a given variety, account for some of the diversity. The panelists were familiar with Cheddar cheese taste but varied in their exposure to the varieties of Edam, Gouda, Swiss, and Parmesan. At times they expressed frustration in the identification of cheeses by variety. When this occured, samples of each cheese variety were made available for varietal confirmation.

The overall percent correct classification rate by sensory analysis was 63%. The classification rate by variety was: Cheddar 86%, Edam 42%, Gouda 43%, Swiss 74% and Parmesan 76% (Table 4).

The four parameters measured by the taste panel were analyzed by a multivariate analysis of variance (MANOVA). Univariate test statistics showed a highly significant ($\alpha = 0.0001$) difference between cheeses for taste intensity, preference, and correct choice of variety (1=yes, 2=no) but no difference for bitterness. Waller-Duncan k-ratio t-tests of the means indicated Parmesan taste intensities were higher than the remaining 4 varieties; Edam had a lower taste intensity than the other varieties; and Cheddar, Gouda, and Swiss cheeses were perceived at an equal level of taste intensity. Means for preference scores showed that Cheddar, Edam, and Gouda were equally liked, while Swiss was preferred less, and Parmesan even less than Swiss. Means for the correct choice of identification revealed that Edam and Gouda were identified less often than Cheddar, Swiss, or Parmesan.

The main objective of the sensory results was to obtain a classification rate by cheese variety. A second goal was to use the taste intensity ratings to evaluate the age of the cheeses, but since few of the cheeses themselves were marketed by degree of ripening, this became impossible. The parameters of preference and bitterness were recorded to see if they influenced the selection by variety. In general the stronger flavored cheeses, Swiss and Parmesan, were preferred less than the milder varieties of Cheddar, Edam, and Gouda. This follows the general trend in America where Cheddar is the most popular cheese consumed. Battistotti et al. (1980) indicated that 80% of the cheese made in America is the Cheddar type.

F. MODIFIED EXTRACTION PROCEDURE

The relatively short life span of the HPLC columns in this study is of concern. The extraction method could be lengthened to produce an extract with less interfering compounds, or a correlation factor must be determined to correct for column-to-column variation. An objective of this study was to develop a quick simple extraction procedure that could be used for quality monitoring by cheese manufacturers. Thus an extended extraction procedure to extend the HPLC column life would be counter productive.

Lipid material in the cheese extracts may be responsible for the rapid deterioration of the columns. A second solvent extraction step and the use of a C8 extraction tube was used to clean up the extracts (Figure 18). The evaporation of methanol with nitrogen was eliminated in the modified procedure to save time, but a precipitate formed in the extracts. The extracts were refrigerated overnight and filtered through a 0.45 μm filter to remove the precipitate prior to analysis. The decrease in peak resolution and shortening of the retention times still occured. These modifications were only examined with a medium Cheddar cheese.

Rather than lengthen the extraction procedure, the column variability could be monitored and corrected. A standard cheese extract run on each new HPLC column would indicate differences and allow subsequent modifications in the detection or interpretation of peaks. The use of two internal standards such as alanine and methionine would also allow better detection of the column's condition and of any changes in retention times.

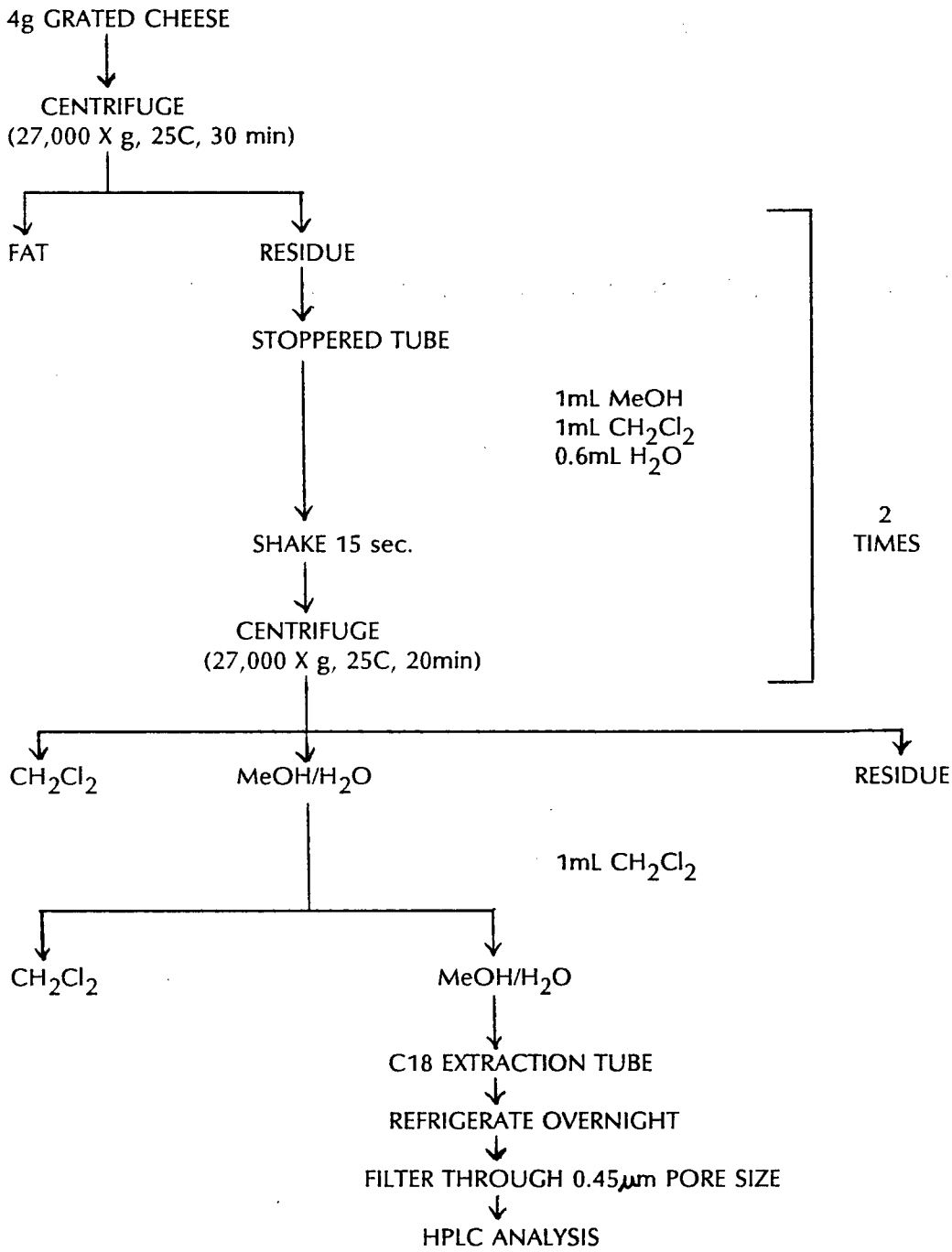

Figure 18. Modified extraction procedure of water-soluble cheese components for HPLC analysis.

V. CONCLUSIONS

Cheese ripening is a complex process in which both aroma and taste contribute to the overall flavor. Volatiles, such as free fatty acids and carbonyl compounds, are important to aroma, whereas protein breakdown products, such as peptides and amino acids, have been shown to be responsible for cheese taste. These proteolysis products are found in the nonvolatile water soluble fraction of cheeses.

In this study, water soluble extracts of over 100 cheeses representing five varieties were analyzed with a reversed phase high performance liquid chromatograph (RP/HPLC). A quick single step extraction that could be used by cheese manufacturers was developed. The nonvolatile fraction was collected in a water-methanol layer while the fat soluble components, were eliminated in a methylene chloride layer. The simplicity of the extraction most likely allowed some lipid material in the water-soluble fraction, which shortened the column life, as four HPLC columns were required to analyze the cheeses.

The conditions used to elute the taste compounds were determined by a combination of Mapping Super-Simplex and Centroid Mapping Optimization . Optimization resulted in a ternary gradient elution, using trifluoroacetic acid (0.1%), methanol, and acetonitrile, which yielded a maximum number of resolved peaks consisting of a range of compounds of differing polarity.

Multivariate statistical analyses were performed on 55 peaks from each chromatographic profile in order to characterize the cheese varieties of Cheddar, Edam, Gouda, Swiss, and Parmesan. Principal component analysis was successful in reducing the dimensionality of the data from 55 peaks to 17 principal components consisting of linear combinations of the peaks. Since the identity of the individual peaks are unknown, an interpretation of the results is difficult. Discriminant analysis performed on both the 17

principal components and the original peaks yielded a 64% correct classification rate by cheese variety. These data were comparable to the 63% rate from a semi-trained taste panel.

Column variability was a problem that must be resolved. Chromatographic data analyzed by a single HPLC column was able to discriminate by cheese variety at a greater than 90% rate. A standard cheese extract should be analyzed on each new column to check for variation. Cheeses used in this study were both Canadian and imported and thus represented a range of flavors and histories within each variety. An individual cheese producer would unlikely encounter the diversity found here. Therefore this method would be useful to register a fingerprint of each cheese production lot. Once a history of the HPLC profiles was established by a cheese manufacturer, problems or variations from day-to-day would become apparent.

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Appendix 1. Eigenvectors for principal component analysis of chromatographic data

PRINCIPAL COMPONENT ANALYSIS

EIGENVECTORS

	PRIN1	PRIN2	PRIN3	PRIN4	PRIN5	PRIN6	PRIN7	PRIN8	PRIN9	PRIN10
X3	0.041453	0.063780	0.177279	0.054334	0.159709	-.182430	0.132599	0.103067	0.022933	0.080488
X4	-.016825	0.180610	0.166300	-.113506	0.018937	0.055313	-.009363	-.061670	0.086822	-.314731
X5	-.078775	0.030137	0.077552	0.115387	0.142937	0.062544	0.284600	-.012806	0.034790	0.091747
X6	0.057280	0.140509	-.086986	-.272346	-.009888	0.134026	-.104186	-.077896	0.166322	-.105987
X7	-.015826	0.102451	0.226204	0.002566	-.026635	0.124829	0.168121	-.073949	-.025082	-.081798
X8	0.144363	0.123993	0.228795	-.049784	-.029328	-.341959	-.067880	-.049980	0.187681	0.034299
X9	-.059996	0.058342	0.144232	-.045557	0.000391	-.125385	0.276742	0.141764	-.132784	0.119666
X10	0.079847	0.158743	0.267655	0.145221	-.096194	-.275833	-.030618	0.055449	0.023935	0.026434
X11	0.054314	0.082001	0.201728	0.169383	-.177211	-.062053	-.087745	-.123406	0.192988	-.003879
X12	0.157758	0.102753	0.044342	0.065576	-.329861	-.071972	-.068545	0.071591	0.032896	-.065543
X13	0.039829	0.193441	0.078254	0.205796	0.004429	0.218920	-.119512	-.002155	-.089042	0.161708
X14	0.171820	0.186918	-.069913	0.020432	-.138936	0.092614	-.112520	0.024917	-.105931	0.120386
X15	0.158092	0.232743	-.045551	-.005374	0.081710	0.161975	0.120509	0.154473	0.021721	0.148827
X16	0.033001	0.207794	0.081439	0.028614	-.027545	0.047096	0.054021	0.000544	-.045271	0.005050
X17	0.019397	0.248937	-.002394	0.128722	-.040718	-.100971	-.007503	0.206913	0.151908	-.033924
X18	0.048241	0.253005	-.110345	0.293709	0.020743	0.093245	-.041671	0.021458	-.052492	0.033716
X19	-.005376	0.201028	-.045685	0.249423	0.134718	0.105552	-.051357	0.122922	-.156304	-.043759
X20	0.108107	0.214759	-.048675	0.066471	-.005000	-.059574	-.139564	0.040117	0.203380	-.091037
X21	0.056392	0.207656	-.082495	0.242375	-.000267	0.113965	-.005783	0.009012	-.067938	0.075246
X22	0.136025	-.064517	-.076586	-.088395	-.040362	-.210026	-.231068	-.054682	-.036726	0.121768
X23	-.010173	0.286212	0.108772	-.233865	-.064792	0.183168	0.100575	-.007433	0.147990	-.174126
X24	0.082902	0.197459	-.015140	0.075218	0.155791	0.040003	0.046993	-.264668	0.025486	0.059236
X25	0.039367	0.121180	0.062081	-.113786	0.153803	0.163730	0.102583	-.167443	0.005051	0.168723
X26	0.147773	0.149716	-.052867	-.239986	-.070559	-.023120	0.067524	0.122782	-.113344	0.128662
X27	0.200932	0.086383	-.254304	0.025950	-.057036	0.015496	0.069252	0.038413	-.000058	-.099609
X28	0.209144	-.017531	-.254071	-.068188	-.045229	-.031372	-.056340	0.006978	-.047614	0.065204
X29	0.128315	0.026361	-.246543	0.132475	-.065741	-.087885	0.109185	-.091972	-.012010	-.045076
X30	0.161366	0.026900	0.183039	-.097106	0.148337	-.090950	-.226437	0.173028	-.214886	0.114082
X31	0.165457	-.015405	0.053342	-.069556	0.188236	-.093079	-.024222	0.035292	-.084621	-.108429
X32	0.045224	0.184606	0.055175	-.354889	-.061004	0.179018	0.001981	-.038181	0.101935	-.181656
X33	0.231603	-.076538	-.132118	0.121377	-.052838	-.013551	0.008500	-.185884	0.096797	0.009289
X34	0.272855	-.008831	-.203440	-.132763	-.002561	-.089772	0.038721	-.063119	0.065470	-.074933
X35	0.188221	-.059565	0.040991	-.031308	0.277212	-.161463	-.070922	-.134908	0.225239	0.087259
X36	0.195744	0.057505	0.035600	-.124785	0.136206	-.142955	0.144949	0.125952	-.211783	-.202388
X37	0.226626	-.006825	-.051879	-.016233	0.227239	0.075839	0.021098	0.121586	-.133529	-.088729
X38	0.206396	-.059492	-.192836	0.024273	-.075278	0.019137	0.110595	-.206613	0.102926	0.078859
X39	0.053437	0.025982	0.089337	-.082670	-.107017	0.159205	0.230821	-.128758	0.171157	0.409991
X40	0.101460	-.033275	0.076793	-.112650	-.316206	0.042556	0.075041	0.110641	-.023118	0.428241
X41	0.140563	-.043054	0.132469	-.182957	-.265073	-.045937	0.025444	0.196309	-.300824	0.039812
X42	0.029172	0.049447	0.060009	0.049374	0.121734	0.030405	-.398403	-.241953	-.267483	0.130878
X43	0.051069	0.015729	0.088349	-.192529	0.234742	0.058424	0.054730	-.340896	-.235585	0.149879
X44	0.193662	0.028946	0.199860	0.015068	0.211998	0.018333	-.092481	0.004406	0.051467	-.096701
X45	0.184222	-.050359	0.089190	0.012328	0.074335	-.198997	0.074021	-.121058	0.248779	0.113875
X46	0.167096	-.179538	0.030983	0.177734	0.029544	0.076702	0.191644	0.005429	0.062242	-.033210
X47	0.112215	-.047658	0.097407	0.174731	-.111134	-.102125	0.259089	-.166535	-.134858	-.179910
X48	0.207311	-.081033	0.016224	0.058802	-.041970	0.017661	0.317176	-.097711	-.231028	-.180477
X49	0.227639	-.119097	0.134086	0.176186	0.004164	0.117227	-.009155	0.089545	-.047772	-.052556
X50	0.087551	-.119566	-.059323	0.050895	0.144395	0.194612	0.020853	0.341029	0.261000	0.019848
X51	0.171023	-.094240	-.154598	-.120691	0.056579	0.094222	-.037991	0.222933	0.050611	0.017636
X52	0.022572	-.132863	0.106970	0.021780	0.239245	0.002178	0.085320	0.271815	0.162496	0.114619
X54	0.153658	-.168726	0.172577	0.014394	0.066621	0.289772	-.132813	-.005594	-.003198	0.000854
X55	0.126133	-.180899	0.199305	0.065582	-.133469	0.221639	-.011164	-.105230	-.012978	-.143557
X56	0.127167	-.183057	0.173698	0.030951	-.081032	0.241456	-.152573	0.031940	0.155584	-.000762
X57	0.133741	-.151732	0.178754	0.045367	-.208169	0.097195	-.128325	-.016210	-.033016	-.111320

PRINCIPAL COMPONENT ANALYSIS

EIGENVECTORS

	PRIN11	PRIN12	PRIN13	PRIN14	PRIN15	PRIN16	PRIN17	PRIN18	PRIN19	PRIN20
X3	-.074568	-.316535	0.008659	-.072506	0.189066	0.337488	0.314909	0.023118	-.234374	-.021472
X4	0.063692	-.081006	0.136673	0.106733	-.191628	-.054697	-.124602	0.297049	0.203534	-.140946
X5	-.105176	0.029014	-.348016	-.085602	-.095366	0.213093	0.071485	0.177907	-.052234	-.242596
X6	-.026077	-.205148	0.129105	0.029019	-.058246	0.017585	-.027007	0.337167	-.347832	0.091699
X7	-.034150	0.164203	0.070853	0.354208	0.013841	0.281630	0.199355	-.207803	0.278778	-.087742
X8	0.034471	0.055882	-.015620	0.010538	-.134377	-.052039	-.004911	0.058082	-.038881	-.019693
X9	0.099197	0.288668	0.183029	0.088275	0.100706	-.120855	0.153567	0.106718	-.154036	0.335541
X10	0.069418	0.104288	-.014151	-.005800	0.146754	0.036234	-.015438	0.164075	-.006328	-.079409
X11	-.208970	0.019981	0.159097	0.216132	0.144854	-.264015	-.047206	0.017664	-.123474	-.060220
X12	-.298334	0.016754	0.071149	-.087747	0.012869	-.166686	0.199592	-.020841	0.131897	-.165145
X13	-.272120	-.007177	-.216266	0.137127	-.185079	0.024593	0.029524	0.070105	-.054283	0.022683
X14	-.219061	0.165017	-.154421	0.095950	-.157470	-.092041	-.026843	-.102160	-.201208	-.085623
X15	0.095469	-.163806	-.112678	0.086446	0.076300	-.031243	-.107927	-.044019	0.036420	0.005202
X16	0.271084	0.112101	-.262488	-.121366	-.172723	-.231786	0.292353	0.190187	0.131900	0.035524
X17	0.110154	-.022222	-.102440	-.103402	0.326511	0.089127	-.047447	-.149825	-.106070	0.095098
X18	0.115003	-.048605	0.039455	0.164323	-.121225	-.068381	0.064365	0.069436	-.061269	0.089682
X19	0.253398	-.285732	0.129758	-.037921	-.040128	0.011401	0.036090	-.122484	0.136402	-.043969
X20	0.027514	0.089774	0.001796	-.201855	0.271987	0.044222	-.318278	0.101698	0.081037	-.071067
X21	0.170877	-.041899	0.355627	-.016897	-.157821	0.012492	-.059635	-.034854	-.080395	0.172014
X22	0.258100	-.099747	-.058580	0.090622	-.158285	0.120793	0.124249	0.106561	0.062429	-.127777
X23	-.045875	0.050749	-.070102	-.040697	0.031765	0.058156	0.052236	-.112109	0.046951	0.062684
X24	-.015350	0.329182	0.012823	-.061244	-.003342	0.238065	-.152580	-.005183	0.156558	0.166115
X25	0.061204	0.026506	0.446025	-.128133	0.150627	-.045328	0.007770	-.159097	-.011659	-.377658
X26	0.201563	0.258683	0.021667	-.198472	-.007083	0.022253	-.180572	0.127161	0.035116	-.066635
X27	0.040277	0.050336	-.053109	0.090342	-.086053	-.078107	0.129581	-.110422	-.058874	-.093934
X28	0.003887	-.081087	-.055709	0.126400	0.210944	0.008462	0.089477	0.144485	0.249397	0.054862
X29	-.062867	-.057461	0.074185	-.044056	0.212908	-.038008	0.331568	0.233199	0.293775	-.061319
X30	-.142273	0.009924	-.045429	0.160391	0.089266	0.152078	-.025834	0.112287	0.044636	0.145111
X31	-.091181	-.169757	-.015383	0.368224	0.010566	-.132196	-.267973	-.066720	0.170761	-.090537
X32	-.080584	-.035796	-.054270	0.016213	-.006526	0.130893	0.188297	-.125268	-.000794	0.160683
X33	-.024347	0.063542	0.185933	0.086977	-.164144	0.292455	0.002427	-.104021	-.095351	-.015610
X34	0.002072	0.019969	-.050606	-.043624	0.011632	0.097242	0.103330	-.035480	-.053256	-.200778
X35	0.064321	0.123624	-.068495	0.049021	0.032938	-.105107	0.031738	-.211451	0.053397	0.133776
X36	0.078451	-.103859	-.115676	0.074844	-.061584	-.037508	-.143584	-.196617	-.016625	0.105333
X37	-.102476	0.126308	-.145768	-.137896	0.089615	-.271066	-.025671	-.105948	-.108159	-.122930
X38	0.112867	0.040050	-.056046	0.245498	0.124889	0.120064	-.071056	0.139638	-.216941	0.177305
X39	0.039994	-.274185	-.147593	0.063450	0.058842	-.115577	-.138145	-.036651	0.154928	0.039345
X40	-.088567	-.186919	0.072713	-.112143	-.022590	-.001789	-.130642	-.019990	0.086834	-.019548
X41	0.036449	0.074079	0.165741	-.048311	-.187051	0.192381	-.034365	-.031035	-.009088	-.052262
X42	-.061595	0.067968	-.004650	-.216560	0.047455	0.059577	0.046824	-.047576	0.120443	0.036083
X43	-.071163	0.032310	0.075264	0.032205	0.169876	-.229525	0.148671	0.098495	-.068657	0.014121
X44	-.112110	-.219376	0.089333	-.243283	-.130059	0.083261	0.037504	0.085257	-.019247	0.020248
X45	0.068138	-.077629	0.002287	-.177139	-.399681	-.131867	0.097191	-.208299	-.004362	0.102136
X46	-.058546	0.159860	-.033419	-.057662	-.094476	0.210790	-.226615	0.172236	0.141456	-.084386
X47	-.189180	-.139883	0.059695	-.164009	-.033474	-.104328	-.078812	0.049351	0.181639	0.368466
X48	0.059424	-.051148	0.003921	-.070897	0.033726	-.106253	-.016065	0.059709	-.191041	-.172722
X49	-.067393	-.004800	0.040847	0.010615	0.118899	0.057632	0.010117	-.102035	-.180509	-.062585
X50	-.154417	0.219761	0.160725	-.063642	-.089292	-.105030	0.114013	0.040412	0.003150	0.071459
X51	-.182536	-.033768	0.140853	-.068217	0.015431	0.056283	0.103265	0.035206	0.204599	0.277275
X52	0.077493	0.051329	0.129761	0.226410	-.052463	-.093802	0.019444	0.173814	0.115326	-.130532
X54	0.101167	0.024395	-.021744	-.034599	0.016735	0.001079	0.040018	0.300402	-.084097	0.028636
X55	0.129010	-.069365	-.118669	-.119985	0.109641	-.002654	-.127714	-.012651	0.003131	0.127081
X56	0.247140	0.017288	-.099962	-.070236	0.120940	-.039384	0.150935	-.152413	0.090063	-.007767
X57	0.285373	0.064941	0.005715	0.172797	0.088148	-.005468	0.112318	-.055382	-.053776	0.018979