ANALYSIS OF ORGANIC ACIDS IN CHEDDAR CHEESE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

By

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ABSTRACT

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Organic acids in natural cheese were extracted with 0.01N sulfuric acid at 60 C. Ten gram samples were slurried, followed by refrigerated centrifugation at 7000 x g. Second extraction of supernatant yielded 50 ml total volume. Final sample filtration was accomplished using Millipore Sep-Paks. The extracted acids were separated on an Aminex HPX-87H (Bio-Rad) column, using a flow rate of 0.6 ml/min., column temp. 65 C and a refractive index detector. Eluent was 0.01N sulfuric acid. Sample preparation and analysis was accomplished in 60 minutes. Acids separated and identified in Cheddar cheese included citric, lactic and other trace acids. Lactose was also separated on this column. Time-series study on Cheddar cheese ripening showed dramatic decrease in lactose during pressing with subsequent increases in lactic and citric acids. Further ripening yielded slight increases in lactic acid and stabilization of citric acid contents. Extraction and analysis of other varieties of cheese indicated additional acids including pyruvic, acetic, propionic and butyric acids. Lactic acid was the predominant acid in most cheeses analyzed.

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INTRODUCTION

Fermented milk and cheese have played a significant role in the diet of both primitive and advanced societies. Historically, they enabled populations to survive periods of famine; nutritionally, they provide elements vital to good health, making them desirable staples to the daily diet; and geographically, they lend themselves well to realistic production in many developing countries.

The evolution and world movement of fermented milk foods began many centuries before Christ, probably in the warm climate of the Mediterranean Sea Basin (Kosikowski, 1982). Many believe fermented milks and certain cheese types may have originated in parallel sequence in different countries. The first of these foods was probably discovered by accident, but once a working principle was established it must then have become easier to develop related products and thus the movement spread.

Historically, these foods were known to have been standard fare of the early Egyptians and Greeks, but it was the Romans who were credited most for spreading their own practices for making foods from fermented

milks. In their campaigns, the early Romans taught cheese making techniques to the peoples they conquered. For this reason Europe became, and remains, one of the great world centers of cheese lore, manufacture, and cuisine.

In the evolution of cheese industries in a particular area, the important guiding factors include the availability of milk, the conditions of climate, soil, pasture, and labor, and the religious and ethnic traits of a community.

Today, cheesemaking in the United States and in other leading cheese-producing countries of the world is largely a factory industry with only small amounts being made on farms for home use. Yearly production of cheese in the United States is almost two billion pounds (ARS, 1978). The state of Wisconsin is the leading cheese producer followed by New York and several other important cheese producing states. Approximately 15 percent of the annual milk production is used in making this cheese.

Since 1900, numerous studies have been conducted on the composition of milk and milk foods, as researchers have attempted to assess their nutritive value, to solve problems in the manufacture of various dairy products, and to understand the role of milk in various prepared food products. A wide array of constituents has been revealed; it is probable that others await discovery.

Rapid and accurate analyses of organic acids in cheese are just beginning to become available, due primarily to new chromatographic techniques. Organic acids in cheese can not only be qualitated, but quantitated as well providing valuable accurate organic acid profiles of cheese. Stages during ripening of cheese can now be monitored from a quality control perspective. These research efforts may also provide an analytical method by which free fatty acids and acid salts in cheese may be qualitated and quantitated. Other applications could include quantitation of products of microbial fermentations other than cheese.

LITERATURE REVIEW

Composition of Milk

Interest in the composition of milk stems largely from its use as human food. Naturally, researchers have desired to assess the nutritive value of milk in terms of the individual nutrients that it contains. Furthermore, as the manufacture of various dairy products has developed as a means of extending the use of milk as food, problems have arisen which have demanded further knowledge of the composition and properties of milk.

Previous to 1850, milk had been found to contain fat, sugar, protein, and minerals. Numerous studies have been conducted in the past century revealing the presence of a wide array of constituents in normal milk. The constituents are present in the three physical states of solution, colloidal dispersion, and emulsion. Detailed compilations of the constituents of cow's milk and their concentrations have been made by Jenness and Patten (1959) and McKenzie (1967), but the average gross composition may be summarized as follows: water, 87 percent; fat, 3.9 percent; lactose, 4.9 percent; protein, 3.5 percent; and "ash" (minerals), 0.7 percent (Watt and Merrill, 1963). Thus the chemistry of milk embraces a wide range of knowledge in physical, organic and biochemistry.

Like many biologically produced foodstuffs, milk varies considerably in composition (Palumbo, 1972). These variations are caused for the most part by the interplay of a number of factors affecting the physiology of the cow, but some variation may result from treatments following milking. Furthermore, variations in the composition of milk must be compensated in processing operations such as the manufacture of Cheddar cheese or of evaporated milk, where constant ratios among certain constituents are necessary in the finished product (Jenness and Patten, 1959). Certain constituents are necessary in the final product. The widest variation is in fat content, with protein second (Palumbo, 1972). Jenness and Patten (1959) and Corbin and Whittier (1965) have summarized the sources of variations.

(a) Inherited variations. The different breeds of dairy cattle have characteristic differences in the composition of milk they produce as well as variations among individual cows within a breed (Overman et al., 1963). It appears that the quantity of each constituent (fat, proteins, and lactose) synthesized by the mammary tissue depends to some extent on separately inherited factors.

(b) Nutrition of the cow. The feed of the cow affects the gross composition of milk only slightly, although the concentrations of such minor constituents

as vitamin A, carotene, iodine, and some trace metals are decidely affected.

(c) Seasonal variations and effect of temperature. The fat content of milk shows a pronounced seasonal trend, being higher in winter than in summer. The protein content follows the same trend. Environmental temperatures above 85 F and below 40 F tend to result in increased fat content.

(d) Age of cow. The fat content of milk tends to decline slightly with advancing age.

(e) Stage of lactation. The greatest changes occur at the beginning and end of the period.

(f) Disease. Infections of the udder (mastitis) cause a lowering of the concentrations of fat, solids-not-fat, lactose, and casein, and an increase in serum protein and chloride.

(g) Milking procedure. The fat content increases continuously during the milking process.
Finally, different analytical methods frequently influence reported values of gross composition.

Constituents of Milk

Proteins

Milk contains a number of protein components that differ in composition and properties. These proteins are generally first subdivided into two groups, the casein fraction and the whey or serum protein fraction.

The casein fraction (referred to as whole casein) is a heterogeneous group of phosphoproteins. It can be subdivided into alpha-, beta-, and kappa-caseins, based on electrophoretic mobility under standard conditions. Whole casein contains about 66 percent alpha-, 29 percent beta-, and 5 percent kappa-casein (Corbin and Whittier, 1965). Alpha-casein is itself a complex mixture of proteins with differing functional properties. It includes alpha sensitive-caseins, which are coagulated by calcium ion, and kappa-casein, which is not calcium-sensitive. Alpha sensitive-caseins are stabilized in milk by the protective action of kappa-casein (Waugh and von Hippel, 1956). The whey proteins include beta-lactoglobulin, alpha-lactalbumin, the immune globulins, blood serum albumin, a number of enzymes, proteose-peptones and some minor proteins of unknown function (Palumbo, 1972).

Casein exists in milk primarily in the form of rather large colloidal particles usually called micelles. These are complexes of protein and salt ions, principally calcium and phosphorus. The size of the casein micelles ranges from about 40 to 300 nm, although a few larger particles may be found in some milks (Shimmin and Hill, 1964). Together with the fat globules, the micelles impart to milk an opalescent or "milky" appearance due to reflection and scattering of the components of the electromagnetic spectrum

discernable by the eye.

The stability of colloidal casein micelles is very important in the manufacture and storage of milk products. To make cheese, it is necessary to destabilize the micelles. Maintenance or recovery of colloid stability is essential for the production and marketing of all beverage milk products (Palumbo, 1972).

Lipids

Milk fat is important from several points of view: economics, since it still plays the most significant role in determining the price of milk and milk products; nutrition, because it serves as a carrier for the fat-soluble vitamins; and flavor and texture, because these characteristics of milk fat greatly influence consumer acceptance of most dairy products.

Milk fat consists primarily of triglycerides of fatty acids. Jenness and Patton (1959) and Kurtz (1965) reported triglyceride content of milk lipids to be 98-99 percent, diglycerides 0.25-0.48 percent, and monoglycerides 0.016-0.038 percent. In general, milk fat is characterized by the presence of 8-12 mol percent of short-chain fatty acids that are very significant in flavors and off-flavors of milk and milk products. The simplest of these acids, butyric acid, is unique to milk fat. Fatty acid composition of milk fat is shown in Table 1.

Table 1. Fatty Acid Composition of Milk Fat [a,b]

Fatty Acid		
Common Name	Carbon Atoms: Double Bonds	Percent of Total Acids
Butyric	4:0	2.79
Caproic	6:0	2.34
Caprylic	8:0	1.06
Capric	10:0	3.04
Lauric	12:0	2.87
Myristic	14:0	8.94
Pentadecylic	15:0	0.79
Palmitic	16:0	23.80
Margaric	17:0	0.70
Stearic	18:0	13.20
Palmitoleic	16:1	1.79
Oleic	18:1	29.60
Linoleic	18:2	2.11
Linolenic	18:3	0.50
	18:2 c,t conjugated	0.63

[a]Data from Herb et al. (1962).

[b]Arbitrarily omitted from the table are those acids which constitute less than 0.5 percent of the total fatty acids. Those omitted are odd-numbered acids, branched chain acids, and acids 20-24 carbon atoms with 0-4 double bonds.

Lactose

The characteristic carbohydrate of milk is lactose, although trace amounts of glucose, galactose, and other sugars are present (Palumbo, 1972). Lactose exists in two forms, designated alpha and beta. At 20 C and rotational equilibrium, a lactose solution is composed of 62 percent beta-form and 38 percent alpha-form. The beta-form is much more soluble than the alpha-form, and therefore at temperatures below 93.5 C, it is the alpha-form that crystallizes first. As the alpha-form crystallizes out, beta-lactose shifts to the alpha-form to maintain equilibrium conditions. The alpha-lactose that crystallizes from aqueous solution is the monohydrate (one molecule of water is associated with each lactose molecule).

There are numerous chemical, physical methods to measure the amount of lactose in milk and milk products. Approximate lactose (alpha and beta) concentration by weight per liter of milk has been reported to be 45-50 g (Jenness and Patten, 1959). This represents a percentage composition of 4.5 to 5.0 percent. It should be noted that some values reported for the lactose content of milk represent lactose monohydrate, others anhydrous lactose. It is not always possible to ascertain which is meant. Anhydrous lactose = 0.95 x lactose monohydrate. Finally, researchers often use paper chromatography to isolate lactose from other

sugars. Lactose spots are cut from the chromatogram, complexed with alkaline ferricyanide reagent, and measured spectrophotometrically at 600 nm against a lactose standard curve.

Campbell and Marshall (1975) report that lactose present in the intestine stimulates growth of microorganisms that produce organic acids (especially Lactobacillus acidophilus) and synthesize many B-complex vitamins (e.g., biotin, riboflavin, nicotinic acid, and folic acid). Additionally, the high acid concentration suppresses protein putrefaction and also impedes growth of many pathogenic organisms because of their sensitivity to high acidity.

Salts

In the broadest sense, the salts of milk include all constituents except hydrogen and hydroxyl ions that are present as ions or in equilibrium with ions. Thus the term includes the proteins and trace elements of milk. However in the usual usage, the term indicates the major salt constituents that are comprised of the chlorides, phosphates, citrates, sulfates, and bicarbonates of sodium, potassium, calcium, and magnesium (Palumbo, 1972). These salts are of practical importance largely due to the result of their influence on the condition and stability of the proteins, especially the casein fraction.

In terms of solubility, some of the milk salts are

present almost entirely in the dissolved state. Others are present in amounts that exceed their solubility in the ionic environment of milk serum. These, especially calcium phosphate, exist partly in solution and partly in colloidal form.

The equilibria among the various forms of salts and ions in milk are affected by changes in temperature, pH, and ionic strength (Palumbo, 1972). They are thus partly responsible for changes in the conditions of the milk proteins during heating, souring, or concentrating of milk.

Cheese

Cheese is a complex food product consisting mainly of casein, fat, and water. Lactose is practically never present in a well-ripened hard cheese (Webb and Johnson, 1965). The percentage of fat in cheese is influenced primarily by the percentage of fat in the milk used for its manufacture. The methods of manufacture also influence considerably the composition of cheese. Although a list of the varieties of cheese manufactured in the various countries of the world would contain several hundred different names, the number of distinct types is approximately 20 (ARS, 1978).

Principles of Cheese Manufacture

Basically cheese is made by forming a curd by the action of rennin or acid on the milk and then ripening

the curd by the action of milk and microbial enzymes. This is known as natural cheese. During ripening, all components of the curd, fat, lactose, and protein are acted on to produce the flavor and texture characteristic of a particular variety of cheese.

Curd formation depends upon the coagulability of casein from milk. Casein can be coagulated by isoelectric precipitation with acid, or by destabilization of the micelle when rennet is added to split kappa casein, the stabilizing fraction of casein. As the stablizing character of kappa-casein is destroyed by rennin, the caseinate micelle becomes increasingly sensitive to calcium ions. Calcium sensitivity may in turn be affected by pH, heat treatment, colloidal phosphate, and other ions (Campbell and Marshall, 1975). At pH 5.4 or lower, a small amount of Ca++ is needed to cause precipitation following treatment with rennin. Kappa-casein stablizes alpha-and beta-caseins which, in isolated form, precipitate in the presence of calcium ions.

Fundamentals of Cheesemaking

In the simplest example of cheesemaking, raw milk is allowed to naturally sour followed by stirring to break the coagulum; heating (cooking) is used to shrink the curd and expel the whey. The cooked curd is removed and drained to obtain "green" curd, the basic component of cheese. Partial removal of whey after precipitation

of protein is the fundamental act common in the manufacture of all cheeses. The uncured curd is then ripened either loose or after pressing into a desired form or structure.

According to Palumbo (1972), the manufacture of natural cheese, regardless of the milk used or the variety desired, involves similar steps. These include:

1. Curd formation. The curd is formed by the addition of rennet extract and suitable starter cultures. Proper temperature is very important for favorable growth of the starter culture. Calcium ions (0.01 to 0.02 percent calcium chloride) are often added to increase the rate of coagulation by rennet of pasteurized milk because pasteuriation significantly slows rennet coagulation (Campbell and Marshall, 1975). The amount of rennet added, the acidity developed by the starter culture and the temperature of the milk affect the rate of whey expulsion and the rate of formation, the firmness, the elasticity, and other properties of the curd.

2. Cutting of the curd. This step facilitates curd handling and cooking. The finer the curd is cut, the higher the surface area and surface/volume ratio and the greater possible expulsion of whey. Considering that whey is more easily expelled from small curds, through which moisture has a shorter distance to travel and more surface area from which to exit, it is apparent

that curd is cut smaller in the manufacture of hard than in the manufacture of soft cheeses. Inadequate whey expulsion and drainage results in excess retention of lactose and moisture and results in sour, bitter cheese with inferior keeping quality. Soon after cutting, a thin continuous membrane forms that allows diffusion of the whey but retains the fat and caseins in the curd. Gentle, uniform cutting and handling minimize breakup of the coagulum and excessive losses of curd fines in the whey.

3. Curd cooking. Heating and agitation hasten the expulsion of whey from the curd. The curd increases in elasticity, the texture becomes more compact, and the lactic acid bacteria increase in number. Cooking at a high temperature decreases moisture content and causes the cheese to ripen less rapidly. Cooking procedures vary widely in commmercial practice, depending upon the variety of cheese. Temperatures vary from 32 to 80 C. Average cooking temperatures for blue, Cheddar, cottage, Swiss, and ricotta cheeses are 33, 39, 49, 58, and 80 C, respectively.

4. Curd drainage. In this step, the curd is permanently separated from the external whey.

5. Curd fusion. Differences in treatment at this point are important determinants of cheese characteristics. This is essentially a time step that allows further lactic acid development by the starter

and allows control of important factors such as moisture, acidity, body and texture of the particular cheese variety being made.

In terms of Cheddar cheese and its economic importance, the cheddaring process merits particular attention. After whey is drained and curds have knit together sufficiently, slabs are cut from the curd. These are turned and piled, then repiled and turned repeatedly until sufficient whey has exuded and the body becomes elastic.

To obtain desirable elasticity of cheese, acidity must be controlled within narrow limits. Elasticity is apparently related to the amount of calcium bound to casein (Campbell and Marshall, 1975). As more acid is produced in cheese, less calcium is bound. Early in cheese making the curd is tough, but as acid is produced it can be stretched considerably. However, if too much acid is produced, curd becomes brittle and crumbles when sliced.

Cheddaring is followed by milling, in which slabs of curd are reduced in size to facilitate salting, further loss of whey and to prepare the unripened cheese for hooping and pressing. Curd should be free of mechanical openings, yet be silky smooth, fibrous, plastic and firm.

6. Salting the curd. This influences the flavor, moisture, and texture of the variety, as well as

providing other benefits. Some protein is solubilized, producing conditions for better matting in varieties such as Cheddar; growth of many microorganisms capable of spoiling cheese is suppressed; lactic acid production is slowed; and some additional whey is released. Salting also assists in controlling the development of cheese body and flavor when the enzymes are released following lysis of bacterial or mold cells.

7. Pressing the curd. Pressing gives the cheese its characteristic shape and compact texture, extrudes slight amounts of whey, and completes curd knitting. Temperatures during pressing should be high enough to promote fusion, but low enough to inhibit the growth of undesirable microorganisms. Pressure should be applied slowly at first to avoid early closure of exterior holes that allow whey to drain from cheese interiors.

8. Special steps. Special treatment may be required for a particular variety. These include the addition of special cultures to the milk during cheese manufacture, addition of mold cultures to the mold-ripened cheeses, and the surface smearing of cultures on surfaced-ripened varieties. This also includes holding of ripening cheese varieties at elevated temperatures to allow eye development (carbon dioxide formation) in cheese of the Swiss variety.

9. Ripening. During the ripening period, the "green" cheese acquires the characteristic flavor and

texture of the variety. The components of the "green" cheese, lactose, fat, and protein, are converted into the various compounds of the finished cheese. The complex transformation process during ripening is carried out under controlled conditions of temperature and humidity (eg. 10 C and 85% R.H. for Cheddar cheese).

During ripening, enzymes from microorganisms and milk and those deliberately added (eg. rennin) degrade components of the curd. Lactose is rapidly converted by the microorganisms present into lactic acid. Protein is degraded by proteolysis, important to both flavor and texture development during ripening. The proteins of cheese, primarily paracaseinates provide much of the physical structure, body and texture properties. Protein is degraded to differing extents depending on the variety of cheese and its maturity. The rate and extent of proteolysis increase with increased moisture content. Ripening of Cheddar cheese causes it to lose its tough, rubbery qualities and progress toward a soft and finally almost crumbly texture as proteolysis continues.

The products of proteolysis are associated with the flavor of ripened cheese, but not specifically with the flavor of any particular variety. Products formed are polypeptides, which may undergo further breakdown, peptides of smaller molecular weight and, to a limited

extent, free amino acids. These amino acids are further converted into alcohols, aldehydes, ammonia, amines, sulfur compounds, and others by microbial action.

Hydrolysis and degradation of the lipid components of ripened cheese are important and necessary events of ripening. Milk fat is necessary for flavor development of most cheeses. Milk fat acts as a solvent for many of the flavor components can modify the flavor properties of many compounds and serves as a precursor of a variety of flavor compounds including lactones, methyl ketones, esters, alcohols, and fatty acids.

Inside the cheese, physical and chemical conditions change continually. These influence the growth of microorganisms and the activities of enzymes. In most cheeses, the oxygen supply is rapidly consumed, and the interior soon becomes anaerobic. Thus, growth in the cheese interior is limited to microorganisms that require little or no oxygen. Because lactose is metabolized to other compounds in about two weeks, the supply of carbohydrate becomes limited (Campbell and Marshall, 1975). Subsequently, the only organisms that can flourish are those that can gain energy from the metabolism of proteins, fat, and a few other compounds. Major factors that influence the rate of ripening are kinds and concentrations of enzymes, temperature, humidity, moisture in the curd, and time.

According to Kosikowski (1957), "the typical flavor

of Cheddar cheese is associated with a pleasant, slightly sweet, aromatic, walnutty sensation without any outstanding single note. In aged cheese, a bitey quality which is neither coarse nor unpleasant gives sharpness to the cheese."

Classification of Cheeses

Descriptions of cheeses number in the hundreds and many dictionaries of cheese list more than 1000 "different" cheeses by name, many of these cheeses being essentially identical. Many cheeses are named for the town or community in which they were made or for a landmark of the community. More than 800 names have been indexed by the Agricultural Research Service (1978).

It is difficult to classify the different cheeses satisfactorily in groups. Probably there are only about 18 distinct types or kinds of natural cheese. No two of these are made by the same method. The following cheeses are typical of the 18 kinds: Brick, Camembert, Cheddar, Cottage, Cream, Edam, Gouda, Hand, Limburger, Neufchatel, Parmesan, Provolone, Romano, Roquefort, Sapsago, Swiss, Trappist, and whey cheese (Mysost and Ricotta).

Such a grouping may be informative, but is imperfect and incomplete. Cheeses can be also classified by texture; and as ripened by bacteria, by

mold, by surface microorganisms, or by a combination of these, or as unripened. Following are examples of cheese classified involving differentiation based upon relative moisture content, on organisms used and ripening process:

1. Very hard ("grating" type; 13-34% moisture):
   (a) Ripened by bacteria: Parmesan and Romano.
2. Hard (34-45% moisture):
   (a) Ripened by bacteria, without eyes: Cheddar and Caciocavallo.
   (b) Ripened by bacteria, with eyes: Swiss, Emmentaler, and Gruyere.
3. Semisoft (45-46% moisture):
   (a) Ripened principally by bacteria: Brick and Munster.
   (b) Ripened by bacteria and surface microorganisms: Limburger, Port du Salut, and Trappist.
   (c) Ripened principally by blue mold in the interior: Roquefort, Gorgonzola, Blue, and Stilton.
4. Soft (47-80% moisture):
   (a) Ripened by bacteria: Liederkranz and Neufchatel.
   (b) Ripened by mold: Camembert and Brie.
   (c) Unripened (fresh): Cottage, Cream, and Ricotta.

Cheese Varieties and Descriptions

Descriptions of cheese varieties of interest are provided in the following section. These brief descriptions identify similarities and differences between the cheese types investigated. Descriptions of the cheeses were derived from the USDA Handbook 54 (ARS, 1978) and Lampert (1975).

Cheddar

Cheddar cheese is named for a village of Cheddar in Somersetshire, England where it was first made. The exact date of origin is not known, but it has been made since the latter part of the 16th century.

The first Cheddar cheese made in America was done by colonial housewives; and the first cheese factory in the United States was a Cheddar-cheese factory near Rome, Oneida County, N.Y., established in 1851 by Jesse Williams.

Cheddar is a hard cheese, ranging in color from nearly white to yellow. It is make from sweet, whole cow's milk, either raw or pasteurized. In the United States at least 75% of the Cheddar cheese is made from pasteurized milk (Lampert, 1975). "Cheddaring" is the name give to a step in making the cheese.

Cheeses are cured usually in a temperature range between 40 and 50 F, but it may be as low as 35 F or as high as 60 F. They are cured for at least 60 days, usually for 3 tO 6 months, and in some instances for as

long as a year. Yield obtained per 100 pounds of milk is between 9.5 and 11 pounds of cheese.

Analysis: Moisture, 37 to 38 percent (not more than 39 percent); fat, 32 percent (fat in solids, not less than 50 percent); protein, 25 percent; and salt, 1.4 to 1.8 percent (ARS, 1978).

Blue

Blue, Blue-mold, or Blue-veined cheese is the name for cheese of the Roquefort type. Blue-mold powder containing Penicillium roqueforti is mixed into the curd during manufacture which gives the cheese its mottled, marbled, or veined appearance. The total curing period for this cheese must be at least 60 days.

Analysis: Moisture, not more than 46 percent (usually 40 to 42 percent); fat, 29.5 to 30.5 percent (not less than 50 percent in the solids); protein, 2[illegible] to 21 percent; and salt, 4.5 to 5 percent (ARS, 1978).

Fontinella

Fontinella is the trade name of a cooked-curd, whole milk, semisoft to hard, slightly yellow cheese with a delicate, nutty flavor and a pleasing aroma. Like Fontina, it is an Italian-type cheese made by a method similar to that used in making Gruyere. Curing is usually for at least 2 months. It may have a few small, round eyes.

Analysis (Fontina): Moisture, not more than 42 percent (usually 38 percent); fat, 28 to 31.5 percent

(not less than 50 percent in the solids) (ARS, 1978).

Gorgonzola

Gorgonzola, known also as Stracchino di Gorgonzola, is the principle blue-green veined cheese of Italy. It is a semi-hard, rennet curd, mold-ripened cheese, which is similar to Roquefort. A classic Gorgonzola is made from goat's milk. The curing period is at least 90 days, frequently is 6 months, and may be a year.

Analysis: Moisture, not more than 42 percent (usually 35 to 38 percent); fat, 31 to 33 percent (not less than 50 percent of the solids); protein, 24 to 26 percent; and salt, 3 to 4 percent (ARS, 1978).

Jarlsberg

Jarlsberg is a creamy white, Swiss cheese made in Norway. It has a smooth texture with small holes and a mild somewhat bland flavor, but pleasing. Jarlsberg is made with part skimmed milk.

Analysis: See Swiss.

Liederkranz

Liederkranz is the trade name of a soft, surface-ripened cheese that is made in Ohio from whole milk. It is similar to a very mild Limburger in body, flavor, aroma, and type of ripening. Curing temperature is 45 F for about 3 or 4 weeks. Surface-growing microorganisms produce progressive ripening from the surface inward. The cheeses, which are rather perishable, are wrapped in tinfoil for marketing.

Analysis: Moisture, 54 percent; fat, 24.2 percent (fat in solids, not more than 50 percent); protein, 16.8 percent; and ash, 3.9 percent (ARS, 1978).

Limburger

Limburger was first made in the province of Luttich, Belgium and is named for the town of Limburg, where originally much of it was marketed. It is a semisoft, surface-ripened cheese with a characteristic strong flavor and aroma. Ripening takes about two months in a cool room with a high humidity.

Analysis: Moisture, not more than 50 percent (usually 43 to 48 percent); fat, 26.5 to 29.5 percent (not less than 50 percent in the solids); protein, 20 to 24 percent; ash, 4.8 percent; and salt, 1.6 to 3.2 percent (ARS, 1978).

Parmesan

Parmesan, as the cheese is called outside of Italy, is a Grana cheese made from partly skimmed milk by a method very similar to that followed for Swiss cheese. It is salted by soaking the cheese in brine for two to three weeks. The curing process may take from 14 months to 3 years. Fully cured Parmesan is very hard and keeps almost indefinitely.

Analysis: Moisture, 30 percent (not more than 32 percent); fat, 28 percent (not less than 32 percent in the solids) (ARS, 1978).

Provolone

Provolone is an Italian "pasta filata" (plastic curd) cheese. It is light in color, mellow, smooth, cuts without crumbling, and has an agreeable flavor. Provolone is an excellent table cheese after it has cured for 6 to 9 months.

Analysis (Domestic Provolone): Moisture, not more than 45 percent (usually 37 to 43.5 percent); fat, 25 to 33 percent (fat in the solids, at least 45 percent and usually 47 percent); and salt, 2 to 4 percent (ARS, 1978).

Romano

Romano is similar to Parmesan and other Grana cheeses. It is sometimes called Incanestrato and is one of the most popular of the very hard Italian cheeses. The cheese has a characteristic sharp flavor due to enzyme activity during a ripening period of up to one year.

Analysis: Moisture, not more than 34 percent (usually 32 percent); fat in the solids, not less than 38 percent; and salt, 5 to 6 percent (ARS, 1978).

Swiss

Swiss cheese is also called "Emmentaler" cheese, after the Emmental (valley of the Emme), a valley in the Canton of Berne, Switzerland. Swiss is a large, hard, pressed-curd cheese with an elastic body and a mild, nut-like, sweetish flavor. The presence of holes or

"eyes" is a characteristic of this cheese that develop in the curd as the cheese ripens. Ripening requires about six months. The typical flavor of a good Swiss cheese continues to develop for several months after the eyes have formed and it is associated with the accumulation of traces of acetic and propionic acids (Lampert, 1975). A good Swiss cheese has comparatively few eyes, which range from one-half to one inch in diameter. A cheese with many small holes, or one with only large eyes near the surface, has not undergone proper development.

Analysis (Domestic Swiss): Moisture, 39.4 percent (not more than 41 percent); fat, 27.5 percent (not less than 43 percent in the solids); protein, 27.4 percent; and salt, 1 to 1.6 percent (ARS, 1978).

Cheese Fermentations

A fermentation, as defined by Gale (1948), is the process leading to the anaerobic breakdown of carbohydrates. Major compounds other than carbohydrates, such as organic acids, proteins, and fats, are fermentable in the broader view that a fermentation is an energy-yielding, oxidation-reduction process.

Milk fermentations generally cause the breakdown of lactose to lactic acid through mechanisms initiated by streptococci and lactobacilli bacteria. In addition,

interesting parallel or post-fermentation reactions occur to produce distinctive fermented milk foods. For example, in ripened cheese these secondary fermentations occur with peptones, peptides, amino acids, and fatty acids (Kosikowski, 1982).

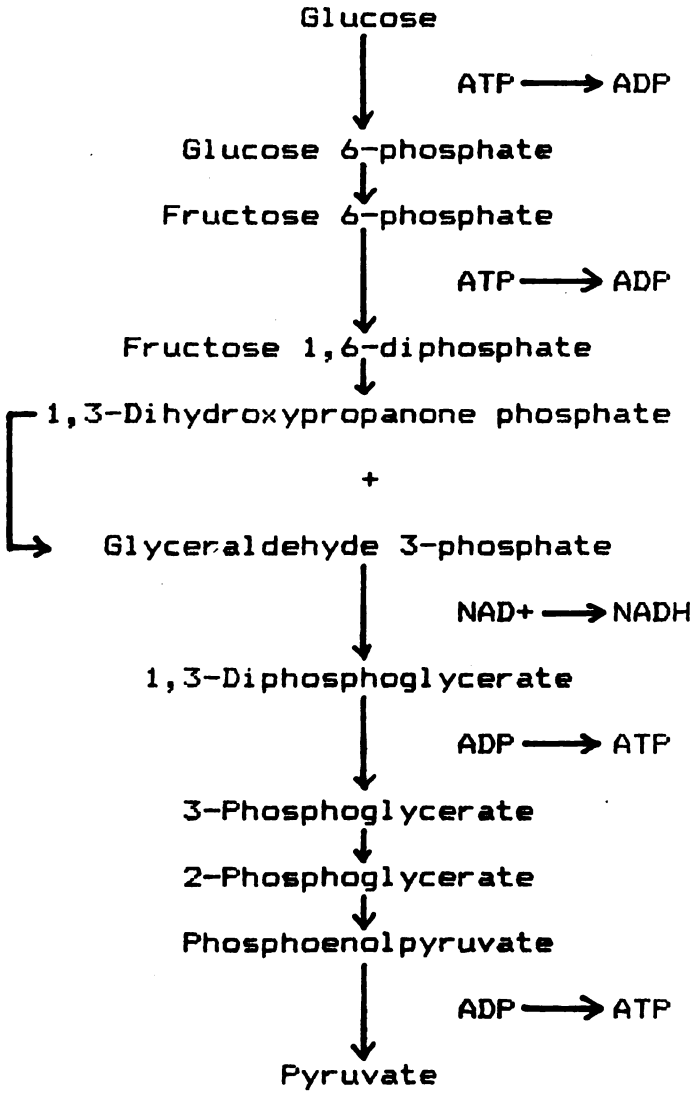
Lactic Acid Fermentation

Lactic acid and flavor and aroma compounds of several types are produced by a variety of cultures used in dairy products. The lactic acid bacteria are gram positive, nonspore-forming, nonmotile, and almost always catalase-free. The homofermentative types produce lactic acid from sugar in yields ranging from 80-90%, and small quantities of other products (Leviton and Marth, 1965). Included among these types are species of the genus Streptococcus. Streptococcus species produce dextro-lactic acid in concentrations up to one percent (Foster et al., 1961).

The heterofermentative lactic acid bacteria ferment glucose to form carbon dioxide, alcohol, and acetic acid, in addition to lactic acid. Comprising this group are species of Lactobacillus, which produce inactive (dl) lactic acid, and species of Leuconostoc, which usually develop alcohol, carbon dioxide, and limited amounts of lactic and acetic acids. Levo-lactic acid is always produced, and simultaneously dextro-lactic acid is sometimes formed (Leviton and Marth, 1965).

The metabolic route followed in the production of

lactic acid by homolactic bacteria is believed to parallel that followed in glycolysis (ie. Embden-Meyerhof mechanism). This would mean that the lactic acid fermentation follows the pathway of the alcohol fermentation up to the point at which pyruvic acid is produced. The sequence of steps involved in the mechanism of glycolysis is as follows (Roberts and Caserio, 1977):



The lactic acid fermentation diverges at this point. Lacking pyruvic apocarboxylase but possessing lactic apodehydrogenase, the lactic acid bacteria utilize reduced coenzyme I for the reduction of pyruvic to lactic acid (Meister, 1972). In contrast, however, with respect to the heterolactics generally, no unique pathway exists. The major homofermentative reaction equation for the lactic acid fermentation is as follows (Lehninger, 1979):

$$C_6H_{12}O_6 + 2P_i + 2ADP \longrightarrow 2CH_3{*}CHOH{*}COOH + 2ATP + 2H_2O$$

Glucose Lactic Acid

The lactic acid formed is a non-volatile, 3-carbon chain, alpha-hydroxypropionic acid. It is an odorless, colorless, and slightly viscous liquid; miscible in water, alcohol, and ether in all proportions. A characteristic of lactic acid is its sharp, prickly taste.

Propionic Acid Fermentation

The propionic acid bacteria are gram positive, nonspore-forming, nonmotile rods. Like the homolactics, they grow under anaerobic and microaerophilic conditions. They ferment lactic acid, carbohydrates, and polyalcohols with the formation of propionic and acetic acids and carbon dioxide (Leviton and Marth, 1965). They are usually strongly catalase positive.

In the manufacture of certain types of cheese, such as Swiss and Gruyere, starter microorganisms convert the

lactose of milk into lactic acid, and it is only after this conversion is practically complete that the propionic bacteria (eg. Propionibacterium shermanii) enter the fermentation and dissimilate lactic acid. This fermentation reaction is as follows (Kosikowski, 1982):

$$3C_6H_{12}O_6 \longrightarrow 6CH_3{*}CHOH{*}COOH$$

Glucose Lactic Acid

$$3CH_3{*}CHOH{*}COOH \longrightarrow 2CH_3{*}CH_2{*}COOH + CH_3{*}COOH + CH_2 + H_2O$$

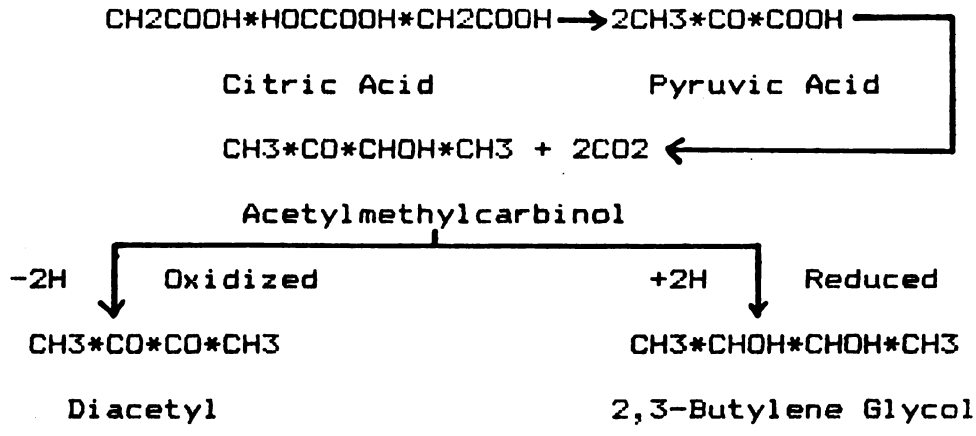
Lactic Acid Propionic Acid Acetic Acid

Citric Acid Fermentation

About 0.2 percent citric acid exists in fresh milk (Kosikowski, 1982). Citrate (expressed as citric acid) is fermented by Leuconostoc dextranicum or Leuconostoc citrovorum to give diacetyl, acetylmethylcarbinol, 2,3-butylene glycol, carbon dioxide, acetic acid, and propionic acid as the principle products (Foster et al., 1961). Traces of alcohols, aldehydes, and similiar compounds are formed. Fermentation of the citrate usually is so complete that all the citric acid present in milk is utilized.

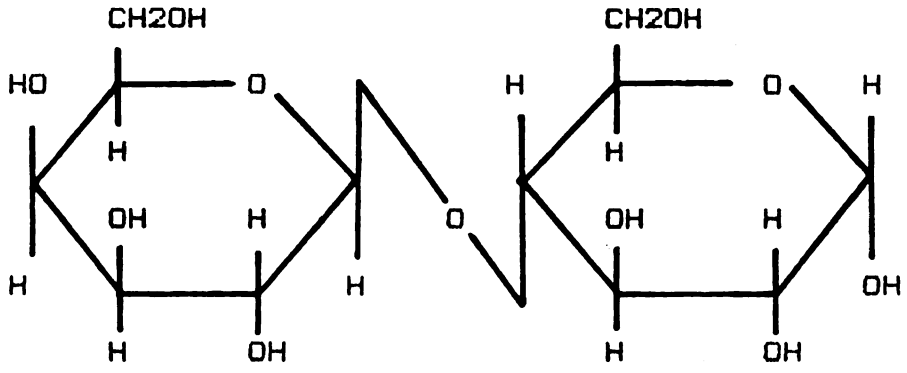
Of the products formed in the fermentation of citrate, diacetyl contributes the most to flavor and aroma. Acetic and propionic acids are also important in this respect. All strains of these organisms ferment glucose and fructose with the production of considerable lactic acid, as well as acetic acid, ethyl alcohol and

carbon dioxide. The Leuconostocs produce primarily the D(-)-lactic acid although small amounts of L(+)-lactic acid may be produced by some cultures. The citric acid fermentation reaction is as follows (Kosikowski, 1982):



Lactose and Lactase

Lactose, the chief source of energy in milk for microbial metabolism, is a dissaccharide consisting of glucose and galactose. Strictly speaking, lactose is alpha-D-glucopyranose-1,4-beta-D-galactopyranose. In bacterial metabolism, lactose is first hydrolyzed to glucose and galactose. The enzyme which brings this about is lactase or more properly, beta-galactosidase. Galactose produced is converted to glucose by means of a second microbial enzyme, galactose isomerase. The dissaccharide, beta-lactose, whose monosaccharide units are bound together with a beta-glycosidic bond is shown as follows (Baker and Allen, 1978):



Beta-D-Galactose Beta-D-Glucose

Cheddar Cheese Microbiology

Starter Culture

During manufacture of Cheddar cheese microbial activity and acid formation are very important. The organisms responsible for acid production are lactic streptococci, either Streptococcus lactis or Streptococcus cremoris. In the United States, cheese makers usually employ a mixed culture consisting of one or more strains of Streptococcus lactis in association with the citrate-fermenting species of Leuconostoc (Foster et al., 1961). The lactic streptococci, however, ordinarily make up more than 90 percent of the population of the culture. In the manufacture of Cheddar cheese, the acid production by Streptococcus lactis and Streptococcus cremoris is the important factor; flavor and aroma production by Leuconostoc citrovorum or Leuconostoc dextranicum usually is not considered important. Carbon dioxide and other fermentation end-products produced by these last two

organisms may actually be detrimental, under some circumstances, in cheese making of naturally ripened cheese. Of the lactose fermented by the culture under consideration, over 90 percent is usually converted to L(+)-lactic acid (Foster et al., 1961).

Because of its importance in bringing about the desired changes during Cheddar cheese manufacture, a great deal of attention has been paid to acid development and its measurement. It is routine procedure to adjust the timing of the various steps in making by results of acid determinations. It has been shown that acid development is most rapid during the interval between dipping and milling the curd (Brown and Price, 1934).

The starter organisms continue to grow and produce acid until the lactose is completely used up. Normally this occurs within a few days after manufacture (Foster et al., 1961). It is apparent, then, that the final acidity reached in the cheese is determined by the amount of sugar left in the curd during making and this is regulated in turn by the moisture content. Excessive moisture in fresh curd will contain too much lactose and will most likely become too sour. Conversely, curd that is too dry will not contain enough lactose to yield the correct amount of acid.

Changes During Ripening

Freshly made Cheddar cheese has a mildly acidic

flavor and a slight aroma. Body changes in the first 2 to 4 weeks result in a reduction in firmness and elasticity due to conversion of part of the protein to water-soluble compounds, mainly proteoses and peptones. The decrease in protein and the corresponding increase in water-soluble derivatives continue and are most rapid during the first 10 weeks (Foster et al., 1961). Further hydrolysis to amino acids occurs over the entire ripening period.

The development of typical Cheddar cheese flavor occurs more slowly than do changes in body. A considerable number of volatile and soluble compounds have been found in cheese at different stages of ripening. Lactic acid, lactates, and sodium chloride have been found to account for much of the flavor of mild Cheddar. Appreciable quantities of acetic and n-butyric acids and traces of diacetyl also have been found in young cheese. As the cheese ages its odor becomes more noticeable, and flavors become sharper. Coincidentially there is an increase in volatile fatty acids, particularly acetic, n-butyric, caproic, caprylic, and capric (Foster et al., 1961). Small amounts of alcohol and esters of fatty acids have been found in highly-flavored Cheddar cheese.

The mode of origin of some of these compounds is not entirely clear. Lactose fermentation is the source of lactic acid and may give rise, in addition, to the

diacetyl, acetic acid and n-butyric acid found in young cheese. The further increase in acetic acid may result from lactate decomposition or deamination of glycine, but the additional n-butyric, caproic, caprylic and capric acids most likely result from fat hydrolysis (Peterson et al., 1949).

The chemical reactions that occur during ripening of Cheddar cheese cause a slow but steady increase in the pH. Brown and Price (1934) obtained the following average values for several lots of good quality:

Age of Cheese	pH
3 days	5.05
7 days	5.06
49 days	5.13
9 months	5.32
24 months	5.58

The microorganisms in Cheddar cheese undergo a regular succession during ripening. The lactic streptococci from the starter reach maximum numbers, usually a few billion per gram, soon after making is finished (Foster et al., 1961). For the remainder of the ripening period their numbers decrease, at which time lactobacilli usually can be detected in Cheddar cheese. They increase gradually until finally they may outnumber the starter organisms. Lactobacillus casei and Lactobacillus plantarum are the species most often found in normal cheese. It is believed these organisms

as well as the starter bacteria release enzymes that can hydrolyze casein. Also, some cultures release intracellular lipolytic enzymes that can liberate free fatty acids from butterfat, thought to be important in flavor development (Peterson and Johnson, 1949). Hence these organisms have been assigned an important role in Cheddar ripening.

Acid Theory

Tradition reserves the use of the name "acid" for substances that transfer protons measurably to water. Thus the carboxylic acids stand out from alkynes, halides, alcohols, and simple aldehydes and ketones in giving water solutions that are "acidic" to indicator papers or pH meters as the result of proton transfers from the carboxyl groups (Robert and Caserio, 1977):

$$RCO_2H + H_2O \rightleftharpoons RCO_2^- + H_3O^+$$

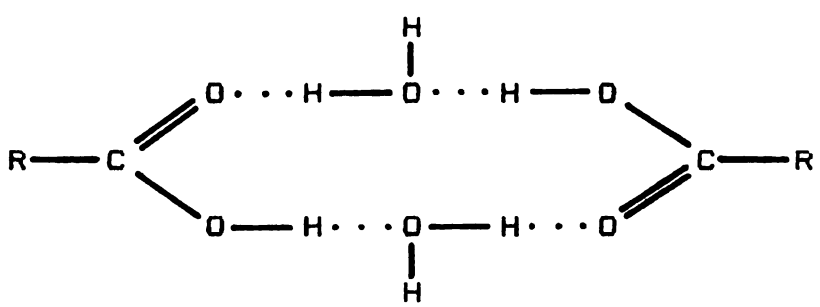
Even so, carboxylic acids are not very strong acids and, in a 1M water solution, a typical carboxylic acid is converted to ions to the extent of only about 0.5%.

Many carboxylic acids and their derivatives have trivial names and are often referred to as "fatty acids." This term applies best to the naturally occurring straight-chain saturated and unsaturated aliphatic acids, which, as esters are constituents of fats, waxes, and oils of plants and animals. Palmitic, stearic, oleic, and linoleic acids are the most abundant

of these acids. They occur as glycerides, which are esters of the trihydric alcohol, 1,2,3-propanetriol (glycerol). Representative carboxylic acids and their corresponding structures are listed as follows:

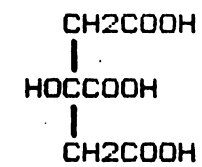
Acid	Structure
Methanoic (formic)	HCO2H
Ethanoic (acetic)	CH3CO2H
Propanoic (propionic)	CH3CH2CO2H
Butanoic (butyric)	CH3CH2CH2CO2H
Hexadecanoic (palmitic)	CH3(CH2)14CO2H
Octadecanoic (stearic)	CH3(CH2)16CO2H

Carboxylic acids show a high degree of association through hydrogen bonding. It is this hydrogen bonding that is responsible for the high water solubility of the simple carboxylic acids with less than five carbons; water can solvate the carbonyl group through hydrogen bonds (Roberts and Casario, 1977). Nonetheless, as the chain length of the hydrocarbon residue R increases, the solubility decreases markedly, because the proportion of polar to nonpolar groups becomes smaller. Solvation of carboxylic acid by water through hydrogen bonding is shown as follows:



Acids in which there are two carboxyl groups separated by a chain of more than five carbon atoms (n>5) for the most part do not have exceptional properties, and the carboxyl groups behave more or less independently of one another. However, when the carboxyl groups are closer together the possibilities for interaction increase. In this case, the inductive effect of one carboxyl group is expected to enhance the activity of the other.

Additional types of carboxylic acids exist including tricarboxylic acids, hydroxycarboxylic acids, and aromatic acids. Examples of tricarboxylic acids and hydroxycarboxylic acids of interest to this study include citric and lactic acid, respectively. Formulas of these acids are as follows:



Citric Acid

Lactic Acid

Chromatography

Chromatography permits the separation and partial description of unreported substances whose presence is unknown or unsuspected. It is an indispensible exploratory method in all sciences dealing with chemical substances and their reactions. It is among the most selective and the most widely applicable separatory

techniques yet devised (Synder and Kirkland, 1974).

The overall process of chromatography is a differential migration phenomenon. The separation of the components of mixtures depends upon their differential penetration into the porous sorbent. This migration is produced by a nonselective driving force, the flow of the wash liquid. The differential migration results from a selective resistive action, namely, the selective sorption of the components of the mixture.

Therefore, chromatography is a method of analysis in which flow of solvent or gas promotes the separation of substances by differential migration from a narrow initial zone in a porous sorptive medium (Synder and Kirkland, 1974). Gas chromatography and liquid chromatography are major subdivisions of chromatography. Column and paper chromatography are subdivisions of liquid chromatography based upon the form of the sorption system. Liquid chromatography may be divided further with respect to the sorption phenomená upon which the separations are based. Examples are ion exchange, size exclusion, partition, and adsorption. Gas chromatography falls into two major categories, partition and adsorption. The relationships described are illustrated in Figure 1.

Liquid Chromatography (HPLC)

Liquid chromatography was first developed by M.S. Tswett, a Russian botanist, for the separation and

isolation of plant pigments in 1903 (Yost et al., 1980). Tswett coined the name from the Greek words meaning "color writing." He separated colored bands (pigments) using powdered chalk columns. In 1930, the method was revitalized in Germany where Kuhn and Lederer further developed column liquid chromatography. More advancements in theory and "hardware" technology over the past century have made modern liquid chromatography one of the most powerful analytical techniques available in research today.

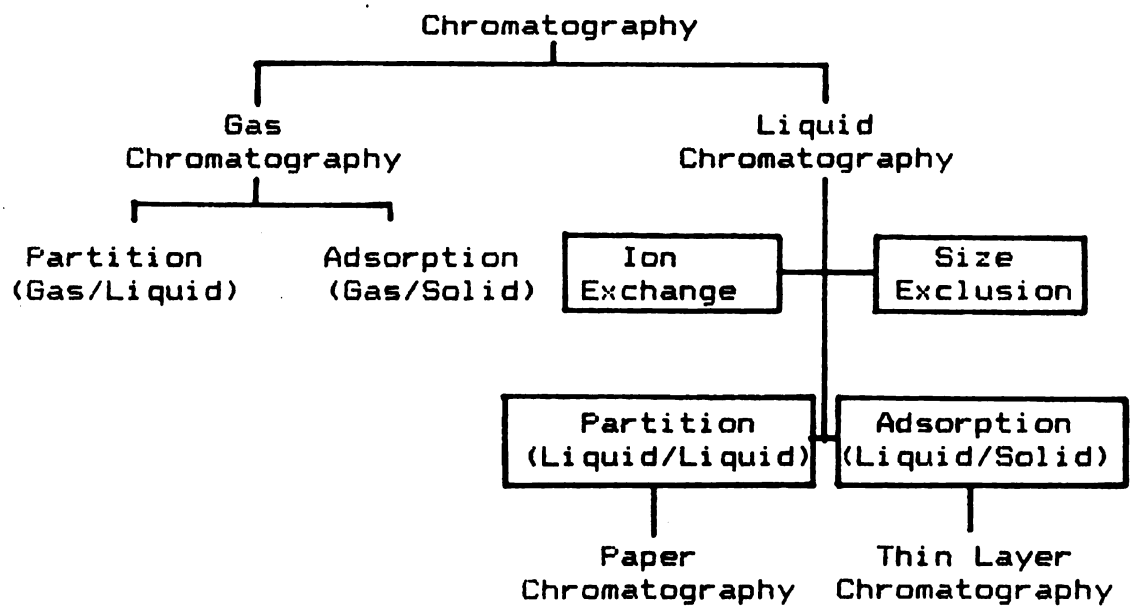


Figure 1. The branches of chromatography (Johnson and Stevenson, 1978).

As modern liquid chromatography systems developed, the use of small diameter (2-5 mm), reusable columns operated under relatively high inlet pressures and controlled flow of the mobile phase resulted. The

controlled flow of the mobile phase was achieved with the help of high pressure pumps and hence, the acronym HPLC was coined meaning high pressure liquid chromatography. Later, chromatographers recognized that high pressure was not primarily responsible for the improved performance using such a system and began referring to the technique as high performance liquid chromatography - still permitting the use of the acronym HPLC.

In general a modern liquid chromatography system consists of several basic components. Such a system includes a pump to propel the mobile phase; a device for sample introduction; a column containing the stationary phase; and a detector to determine what separation has taken place and provide data permitting the qualitative and quantitative evaluation of the results. This can simply be accomplished by recording the response of the detector in the form of a chromatogram (a response vs time curve) and/or with the help of a data handling device. Figure 2 presents a functional schematic of a general liquid chromatography system.

According to Yost et al. (1980), the basis of the separation process in liquid chromatography is: sample molecules introduced into the operating system are carried by the mobile phase through the bed of stationary phase. During this travel, the individual sample molecules are retarded by the stationary phase,

depending on the interaction between the individual sample components, the mobile, and the stationary phases. This retardation is selective which means with a given mobile/stationary phase system, the amount of retardation will be different for each sample component.

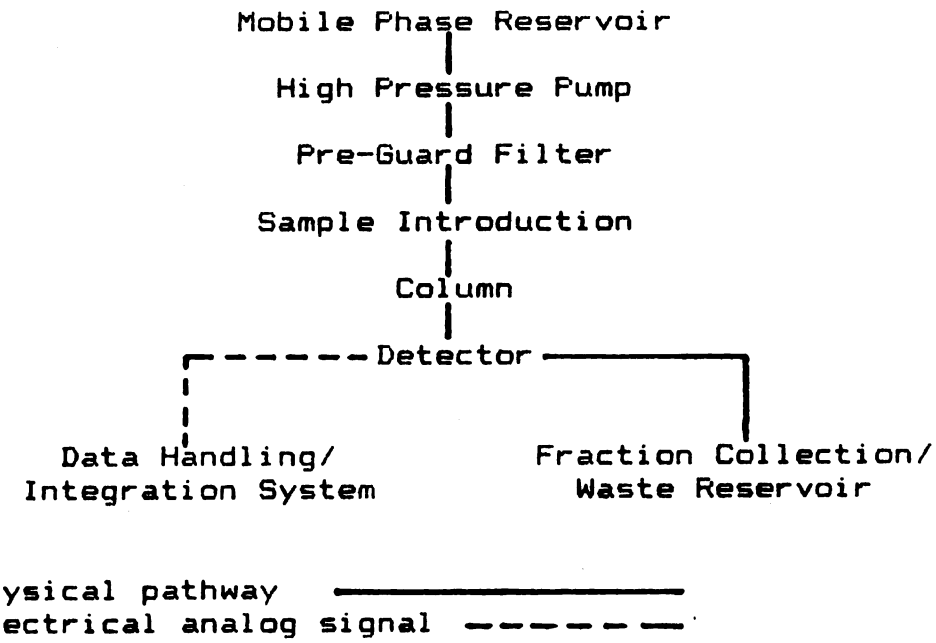


Figure 2. Functional schematic of a modern liquid chromatographic (HPLC) system.

Liquid column chromatography can be further specified into four different modes upon which the nature of the stationary phase and the separation process can be based. As previously indicated, these four modes are adsorption, partition, ion exchange, and size exclusion chromatography. Each division will be discussed as follows:

* In adsorption chromatography - the stationary phase is an adsorbent and the separation is based on repeated adsorption - desorption steps.

* In partition chromatography - the separation is not based on adsorption, but rather partition between the mobile and stationary phases.

* In ion exchange chromatography - the stationary bed has an ionically charged surface of opposite charge to the sample. The mobile phase is an aqueous buffer where pH and polarity are used to control elution time from the column.

* In size exclusion chromatography - the column is filled with material of specific controlled pore sizes and the sample is filtered according to molecular size differences.

Concerning the first two modes, it is sometimes not equivocal whether the dominant process is adsorption or partition or both. For this reason, two or more modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography. These differences are described as follows:

* In normal phase chromatography - the stationary bed is strongly polar in nature and the mobile phase is nonpolar. Polar samples are thus retained on the column longer than less polar materials.

* In reversed-phase chromatography - the exact inverse of normal phase chromatography occurs. The

stationary bed is nonpolar in nature while the mobile phase is a polar liquid. Thus, the more nonpolar the material is, the longer it will be retained.

According to Guiochon (1980), the goal of the analyst is to separate the components of a mixture under such convenient conditions that a satisifactory quantitative analysis can be obtained at a reasonable cost, or that the compounds can be easily identified. In using HPLC, one's attempt toward optimal resolution and efficiency of the sample mixture is denoted by band profiles which resemble Gaussian peaks. The resolution between two Gaussian peaks is given by the following equation:

$$R = 2 (TR2 - TR1) / (W1 + W2)$$

where TRi and Wi are the retention times and base width of peak i.

Once the chromatographic system has been selected, the problem reduces to the design of the column having the required resolution power while minimizing analysis time and possibly inlet pressure. Factors which influence this decision affect the relationships between analysis time, flow velocity, and pressue, and between resolving power and flow velocity.

Finally, differential migration or the movement of individual compounds through the column depends upon the equilibrium distribution of each compound between stationary and moving phases (Synder and Kirkland,

1974). Therefore, differential migration is determined by those experimental variables which affect this distribution: the composition of the moving phase, the composition of the stationary phase, and the separation temperature. When one wants to change the differential migration to improve separation, then one of these three variables must be changed.

In both normal-phase and reverse-phase chromatography the effect of increasing temperature is to decrease retention, increase column efficiency, improve sample capacity, and increase column permeability (i.e., decrease pressure drop). The increase in efficiency results from an increase in the diffusion coefficient of the solute by decreasing mobile-phase viscosity, thereby aiding in the rate of mass transfer (Majors, 1980). For the same reason, the reduction in viscosity causes a lowering of column head pressure. Retention is usually decreased and sample capacity increased since higher temperatures improve solubility in the mobile phase (Majors, 1980).

Detectors based on refractive index are widely used in LC and often considered only second in popularity to detectors based upon ultraviolet (UV) absorption. This device continuously monitors the difference in refractive index (RI) between a reference mobile phase and the mobile phase containing the sample as it elutes from the column. Response to all solutes under the

proper operating conditions is possible using this general purpose detector.

The commercially available RI detectors operate on two different principles. The first is the Fresnel refractometer. This device is based on Fresnel's law of reflection, which states that the percentages of light reflected at a glass/liquid interface varies with the angle of incidence and the refractive indices of the two phases. The second type of RI detector is the deflection device. This device focuses light on a position-sensitive photodetector, which produces an electrical signal proportional to the position of the light. The output signal is then amplified and relayed to a potentiometric recorder. This deflection system has the advantage of a wide range of linearity. Waters Associates uses this type of mechanism in its RI detectors. In summary, characteristics of refractive index detectors are listed as follows (Snyder and Kirkland, 1974):

(a) Excellent versatility - any solute can be detected

(b) Moderate sensitivity for solutes

(c) Generally not useful for trace analyses

(d) Efficient heat exchanger required

(e) Relatively insensitive to carrier flow changes

(f) Sensitive to temperature changes

(g) Reliable, fairly easy to operate

(h) Difficult to use with gradient elution

(i) Nondestructive

The choice of a given liquid for use as the mobile phase in liquid chromatography is governed by many considerations: cost, purity, viscosity (or boiling point), ability to dissolve a given sample, compatibility with the detector and column in use, solvent strength, and solvent selectivity. Each of these aspects is important in choosing a solvent. When considering water for use as the solvent, it is very important that it has been specially purified and degassed, either by application of vacuum or heat or by ultrasonification. Failure to degas solvents, particularly protic solvents, may lead to air bubble formation in the detection cell, with consequent disruption of the chromatogram (Macrae, 1980). Poor quality water may cause ghosting (ie. undesired peaks due to prior injection "bleeding"), especially when a gradient is run (Przybytek, 1980). These "ghost" peaks can be a problem in trace analysis but can be eliminated by washing the column with several blank gradients at a faster gradient rate than is used for the chromatographic method (Di Bussolo, 1984).

Ion Moderated Partition (IMP) HPLC

The technique of ion moderated partition (IMP) chromatography in HPLC refers to the use of ion exchange resins for the separation of polar nonionic compounds.

IMP allows a variety of partition-type separations without many of the disadvantages attendant on the use of bonded-phase silica materials (Jupille et al., 1981).

Bio-Rad Laboratories has recently carried out work in which ion exchange resin columns have been applied to applications commonly considered for reversed-phase packings. Their evidence suggests that the fundamental partition process responsible for separation is moderated by the ionic groups bound to the resin and by the choice of counterion. The commercial application of their results has been the development of columns packed with strong cation exchangers, which are a sulfonated polystyrenedivinylbenzene copolymer. (Figure 3). This type of material may be considered as a solid-state surfactant; it bears groups that are capable of both polar (the sulfonic acid moiety) and nonpolar (the aromatic backbone) interaction. According to Jupille et al. (1981), the types of sample/packing interactions that occur can be characterized as: ion exchange, ion exclusion, normal-phase partition, size exclusion, ligand exchange and reversed-phase partition. These separation mechanisms are described briefly as follows:

* Ion exchange - simple ion exchange applies primarily to inorganic ions.

* Ion exclusion - based on the inability of mobile ions of like charge to penetrate the resin bead (which carries a fixed charge).

* Normal-phase partition - based on the distribution of sample between intraparticle (bound) water and a less polar mobile phase.

* Size exclusion - based on the physical exclusion from the intraparticle volume of molecules too large to penetrate the effective pore structure of the resin.

* Ligand exchange - involves the interaction of the sample with a counterion ionically bound to the resin.

* Reversed-phase partition - involves the distribution of sample molecules between a polar (usually aqueous) mobile phase and nonpolar (aromatic) resin backbone.

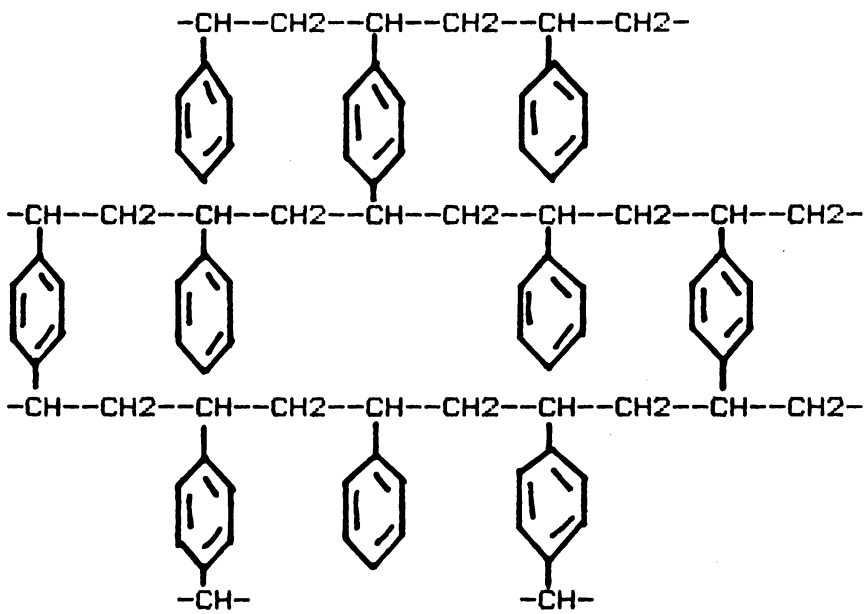
The literature suggests that these previously described sample/packing interactions are thought to be the primary mechanism influencing separation for major classes of compounds as follows:

Separation Mechanism	Compound Class
Normal-phase partition	Carbohydrates
Size exclusion	Oligosaccharides
Ligand exchange	Alpha and beta enomers
Reversed-phase partition	Aliphatic alcohols
Ion exclusion	Organic acids

However, secondary selectivity does tend to influence the sample/packing primary separation mechanism as in the case of organic acids where the reverse-phase partition mechanism seems to impact upon separation (Jupille et al., 1981).

Styrene

Divinylbenzene



Crosslinked styrene-divinylbenzene copolymer

Figure 3. Structure of polystyrenedivinylbenzene ion exchange resin.

Carbohydrate Analysis By HPLC

Simple sugars, mono- and disaccharides, can readily be analyzed by HPLC without derivatization to more volatile trimethylsilyl derivatives as required in gas chromatography. As a result, the speed of analysis and precision is improved. In such analysis, the most commonly used detector is the differential

refractometer, although it is possible to use ultraviolet detection at 190-200 nm (Macrae, 1980). However, HPLC does not provide the answer to all sugar analyses in foodstuffs, as some complex sugars are not readily separated by this technique, for example sucrose and lactose (Richmond, 1982).

HPLC has been used for the analysis of sugars in many commodities, including milk products (Euber and Brunner, 1979), fruits (Richmond et al., 1981), candy (Tallman, 1980), white wine and corn syrup (Cummings, 1979).

Organic Acids Analysis By HPLC

A considerable number of organic acids occur in foods and they are often major constituents of plant foods. More than 36 acids are reported to occur in fruits (Hulme, 1970). In some foods, these acids often influence flavor, stability and keeping quality and have been proposed as an index of maturity, ripeness or spoilage in some foods. Certain organic acids (eg. citric, fumaric, lactic, malic and tartaric) are commonly added to foods as acidulants or flavor modifiers. Organic acids may also be produced during fermentations or other processing operations.

A number of chromatographic methods have been developed for determining organic acids in biological samples. Numerous applications or adaptions of partition chromatography on silica gel and anion

exchange chromatography have been reported (Palmer and List, 1973) in addition to gas chromatography techniques (Harvey et al., 1979; Ledford, 1968). However, these are slow and cumbersome procedures, at least in relation to recent developments in high efficiency liquid chromatography (Kirkland, 1971). These previous methods using silica packing materials in liquid chromatographic conditions were limited due to characteristics of silica, being unstable at high pH values and eventually dissolved by aqueous media even at slightly acid pH (Woo and Benson, 1983).

Despite earlier limitations, liquid chromatography using recently available high efficiency ion-exchange polymers has become the method of choice among investigators interested in organic acid determination. According to Woo and Benson (1983), columns packed with ion-exchange polymers offer many important advantages. These are as follows:

(1) Polymers are known to exhibit exceptional chemical stability and many are not degraded by aqueous media at any value of pH.

(2) In most cases, only isocratic (single solvent) elution with dilute sulfuric acid is required to achieve excellent separation.

(3) No elaborate sample preparation is required.

In contrast to wet chemical methods and gas chromatography, liquid chromatographs using polymeric

stationary phases can provide very fast analysis times and result in high sensitivity detection of solute components. Other advantages of polymers over conventional silica-based packings are their inertness to various salts present in conglomerate sample mixtures.

Currently there are several polymeric columns commercially available for the separation of organic acids. Two such popular columns are the HPX-87H Organic Acids Column supplied by Bio-Rad Laboratories and the ORH-801 Organic Acids Column supplied by Interaction Chemicals, Inc. Both of these columns are packed with strong cation exchange resin.

As previously described, multiple mechanisms seem to be involved in separation using these new microporous sulfonated polystyrenedivinylbenzene resins. The principal mechanism is ion-exchange (Woo and Benson, 1984).

Generally, the elution order of a randomly chosen group of organic acids correlates with their associated ionization constants, pKa. However, other separation mechanisms, especially partitioning or reverse-phase, are also observed due to interaction between the hydrophobic aromatic backbone of the resin particle and the nonpolar groups present in the solute.

Since the eluent usually choosen for these polymeric columns is dilute sulfuric, phosphoric, or

perchloric acid in the pH range of 2 to 2.5, the acid continuously regenerates the column with hydrogen ions that also form a Donnan layer. Donnan membrane equilibria is a principle that can be applied in the separation of nonelectrolytes from electrolytes or for the separation of weak electrolytes form strong electrolytes. According to Woo and Benson (1984), this results in stronger ionized solutes with lower pKa constants to be in their conjugate base form at the pH, and will be excluded from the resin matrix of the column, thereby eluting early. The weaker acids will penetrate the Donnan layer and interact with the resin matix. Their elution times depend upon the degree of nonpolar attraction to the resin.

HPLC applications using polymeric columns for organic acid determination are many. According to Bio-Rad Laboratories (n.d.), use of their Aminex HPX-87H Organic Acids Column can be used to monitor the microbiological process in dairy products, wines, and canned foods as well as determine organic acids in foodstuffs such vinegar, soy sauce, and beer. Other applications may include determination organic acids in biological fluids such as alpha-ketocarboxylic acids in urine, quantitative analysis of organic acids in pharmaceutical preparations, and industrial formulations consisting of organic compounds including aldehydes, carboxylic acids, and alcohols. Guerrant et al. (1982),

resolved a mixture of 25 short-chained fatty acids from anaerobic bacteria by HPLC using a HPX-87H column. Previously, Turkelson and Richards (1978) separated citric acid cycle acids by liquid chromatography using a earlier version of the present polymeric column.

Due to rapid advances in HPLC technology, expansion of its capabilities and methodologies have made it a useful, versatile tool. In terms of the food industry, it can be presented as a useful analytical technique where accurate knowledge for control of important cost-control factors is important. A specific area which has and can be well served through the use of HPLC is the dairy industry. A wide range of both analytical and quality control applications involving many different HPLC techniques can provide the dairy industry with meaningful information concerning its raw materials, processes, and products (Morawski, 1984). Conventional methods of analysis for many dairy products are time-consuming and yield very general data regarding the samples. For example, the determination of volatile fatty acids in cheese is very labor-intensive, yielding a single composite value for cheese acidity expressed as mililiters N/10 lactic acid per 100 grams of cheese (Kosikowsky and Dahlberg, 1946). HPLC can replace many of these traditional techniques and provide rapid results which are much more specific for the components of interest.

Recently, several researchers have investigated the organic acid composition of cheese. Most researchers have employed HPLC as their analytical method of analysis, although gas chromatography (Harvey et al., 1981; Ledford, 1968) and radioisotope tracer techniques have been employed (Lin et al., 1979). Lin et al. (1979) investigated the chemistry of cheese ripening dealing primarily with manifestations of lipolysis and proteolysis and changes in the resulting products. Carbohydrate changes were followed during ripening of Cheddar curd and reported concentration changes in lactose and glucose, lactic acid, pyruvic and alpha-ketoglutaric acids, diacetyl, acetaldehyde, and free fatty and related acids. Harvey et al. (1981) quantitated sugars as trimethylsilyl derivatives and nonvolatile water-soluble organic acids as methyl esters in extracts from commercial Cheddar cheese. Organic acids detected included lactic and succinic acids. Lactic acids amounted to at least 1% by weight in all lots of cheese analyzed, whereas succinic acid was in only low concentrations. Ledford (1968) quantitated acetic, propionic, and butyric acids in milk and ripened cheeses. Two varieties of cheese were analyzed but not identified as to cheese type. Researchers employing the use of HPLC in their investigations separated organic acids on polymeric columns. Most notable is Marsili et al. (1981) who quantitated organic acids in dairy

products using a simple isocratic HPLC technique. Organic acids quantitated included: orotic, citric, pyruvate, lactic, uric, formic, acetic, propionic, butyric, and hippuric acids. Cheddar cheese was reported to contain predominately lactic acid and traces of orotic, pyruvic, uric, acetic, and possibly propionic. Detection was by UV at 220 and 275 nm. Finally, Ashoor and Welty (1984) analyzed dairy products for lactic acid content by HPLC. Detection was by UV at 210 nm. Lactic acid content (g/100g) reported for Swiss and Blue cheese was 0.671 and 1.275, respectively.

Other studies on cheese composition and organic acid content include Keen and Walker's (1974) study on the determination of acetic, propionic, and butyric acids in cheese. Isothermal GLC was used to determine acid concentrations. Acetic acid production was determined to be very similar in Cheddar cheese manufactured using whole and skim milk (Dulley and Grieve, 1974). Emmental cheese was characterized by high propionic and fairly high acetic and butyric acid contents, Camembert by a low content of volatile acids, predominately butyric, Munster cheese by a high content of branched acids, Tomme and Reblochon cheese by a high acetic acid content (Kuzdzal-Savoie and Kuzdzal, 1971). Determinations were done also by GLC. Base and Dolezalek (1972) examined Emmental cheeses and reported the predominate organic acids as being acetic,

propionic, and butyric acid. Free volatile acids were estimated by steam distillation of cheese followed by analysis by GLC. Changes in the free organic acid composition of Camembert cheese during a 114-day period were studied by Tsuyuki and Abe (1982). The three main acids were citric, malic and glycolic, both at the start of ripening and after 24 days; citric, glycolic and lactic after 17 days; citric, malic and propionic after 79 days; and citric, acetic and propionic after 114 days. Contents of free organic acids increased during ripening, with the exception of citric, malic and pyroglutamic acids, which decreased. Partition chromatography on silica gel was used for the separations. Finally, Dolezalek et al. (1981) examined lactose fermentation during cheese production and reported that complete fermentation of glucose and galactose into lactic acid occurred between 1 to 6 days depending on cheese variety. In all cases, glucose and galactose were not detected at any stage using borate complexes on anion exchangers for separation and by quantification of saccharides in the effluent.

EXPERIMENT

Apparatus

High Performance Liquid Chromatography studies were performed on a Waters Associates (Milford, Mass., USA) chromatograph comprising the following modules:

(1) M-45 solvent delivery system with a flow rate of 0.6 ml/min.

(2) U6K septumless injector.

(3) Model R401 differential refractive index detector.

(4) Model 730 data module printer, plotter integration system.

Column

A 300 mm x 7.8 mm i.d. HPLC Organic Acid Analysis Column (Bio-Rad Laboratories, Richmond, CA) was used. The column contains Aminex HPX-87H, a strong cation exchange resin which separates organic acids by ion exclusion and partition chromatography. Specific composition of packing material is 8% cross-linked styrenedivinylbenzene cationic resin, sulfonated acid type in the Ca++ form with a mean particule size of 9 µm. An in-line Bio-Rad Micro-Guard filter guard column was fitted prior to the analytical column containing an ion exculsion disposable cartridge of similiar resin composition.

Reagents

The mobile phase and sample extracting solvent used 0.01N sulfuric acid prepared by diluting reagent grade sulfuric acid with reversed-osmosis, ion-exchanged water. Approximate water purity was greater than 2 megohm resistance.

Sample Preparation

Organic acids in milk and natural cheese were extracted with 25 ml 0.01N sulfuric acid at 60 C, based upon a modified technique of Marsili et al. (1981) and Kosikowski's (1946) classical direct-distillation method. Ten gram samples weighed on a top-loading mettler balance were ground in a 12 oz. blender jar (not milk samples), slurried with a glass stirring rod (not milk samples), and shaken for 2 min. in a 50 ml Nalgene polycarbonate centrifuge tube. Centrifugation followed in an IEC high-speed refrigerated centrifuge (Model HR-1) at 7000 x G to 0 C in 5 minutes. The supernatant was transferred to a 50 ml volumetric flask, requiring minimal further sample clean-up. Second extraction of pellet prepared as previously described without blender step yielded 50 ml total volume. Final sample filtration was accomplished using Millipore Sep-Paks (C18 cartridge, reverse phase). The extracted organic acid samples were stored frozen until injected into the chromatograph.

Standards Preparation

Aqueous solutions containing analytical grade carbohydrate and organic acids (Anspec, Ann Arbor, MI) were prepared with reverse-osmosis, ion-exchanged water. Purity of organic acids ranged from 98+% to 99.6+%. All standards were accurately weighed using a Mettler balance to 1 x 10-4 grams. Three standard stock solutions were prepared; two lactose solutions and one organic acids solution. Standard identities, composition, and concentrations are as follows:

Stock No.	Identity	Composition	Concentration
1	100001	Lactose	1.4560 mg/ml
2	200001	Lactose	0.7450 mg/ml
3	300001	Citric Acid	0.3750 mg/ml
3	300002	Pyruvic Acid	0.9960 mg/ml
3	300003	Lactic Acid	1.0000 mg/ml
3	300004	Acetic Acid	1.1960 mg/ml
3	300005	Propionic Acid	1.0460 mg/ml
3	300006	Butyric Acid	1.0390 mg/ml

Standard stock no.1 was used to quantitate the higher lactose concentration in milk whereas standard stock solutions nos.2 and 3 were used to quantitate constituents in cheese. In all cases, the standards were always prepared at concentrations similiar to the expected concentration of the samples to minimize any linearity deviations. The standards were kept frozen before being brought to ambient temperature for

injection into the chromatograph.

Calibration

The aqueous standard stock solutions for lactose and organic acids were calibrated into the data module and checked for linear detector response. This was accomplished by injecting into the chromatograph standard stock solutions at full strength and corresponding diluted preparations. Standard stock no.3 was analyzed for organic acids linear detector response at 100% and 40% strength. Lactose standard stock solution no.2 was similarly chromatographed at 100% and 50% strength in addition to full strength lactose solution no.1. Constant injection volumes for organic acids and lactose standards were used for all calibration analyses corresponding to respective sample analyses. In all cases the resulting peak heights were determined and plotted against concentration. The slopes of these lines are the response factors for each constituent of interest; the linear correlation coefficients and y-intercepts provide a measure of analytical precision. Quantitation was based on the external standard method.

Chromatography

Analyses were performed on a commercial resin-based column designated "HPX-87H Organic Acids," the column

being heated to 65 C in a water jacketed column during all analyses. The mobile phase was 0.01N sulfuric acid prepared in bulk. Smaller working volumes (approx. 450 ml) of solvent were heated to 50 C and degassed by sonication under vacuum treatment prior to use in order to prevent recorder disturbances caused by air bubbles. Solvent treatment was complete once boiling of solvent was detected (normally 1-2 minutes under described conditions). It was also an advantage to maintain the mobile phase at elevated temperatures (47 C) by standing the reservoir in the sonicator waterbath to prevent re-absorption of oxygen. Small bore stainless tubing (dia. 0.23 mm; 0.009 inches) was used between the column and the detector to minimize the formation of gas bubbles. The injection volumes of samples were maintained at 30 μl for cheese samples and 5 μl for milk samples. A flow rate of 0.6 ml/min. was used. Detection of the eluate was made using differential refractive index at an attenuation of 4X. An integrator was used to calculate the results by external standard methodology. The use of automatic data reduction was not an essential part of the analysis. Sample preparation and anaylsis can be accomplished in 60 minutes.

Recovery Study

The recovery of lactose and organic acids added to Cheddar cheese was evaluated to determine the accuracy

of the test. Cheddar cheese was analyzed in duplicate before and after the addition of known amounts of lactose and organic acids.

RESULTS AND DISCUSSION

Standard Chromatograms

Initially, aqueous standards of lactose and individual organic acids were chromatographed separately to determine the retention times of each constituent. Lactose and mixtures of aqueous acids were then chromatographed and chromatographic conditions were altered to maximize resolution of peaks. The effects of changing pH of the eluent and temperature on the resolution of organic acids in wine for Aminex HPX-87H resin have been previously reported (Bio-Rad, 1979). Changes in chromatographic conditions were made based on this work as well as that of Marsili et al. (1981).

Initial chromatography was performed with several normalities of mobile phase. Eluent effect on separation of an acid standard solution was observed using the following normalities of sulfuric acid: 0.0000N (water), 0.0010N, 0.0025N, 0.0050N, 0.0075N, 0.0100N, 0.0250N, 0.0500N, 0.1000N, and 0.1500N sulfuric acid. Representative chromatograms depicting these observed effects for 0.0025N, 0.0050N, and 0.0100N sulfuric acid are shown in Figures 4, 5, and 6, respectively. Using 0.0100N sulfuric acid mobile phase permitted best separation and resolution of acid standard solution. Mobile phase normalities greater than 0.01N sulfuric acid did not improve separation;

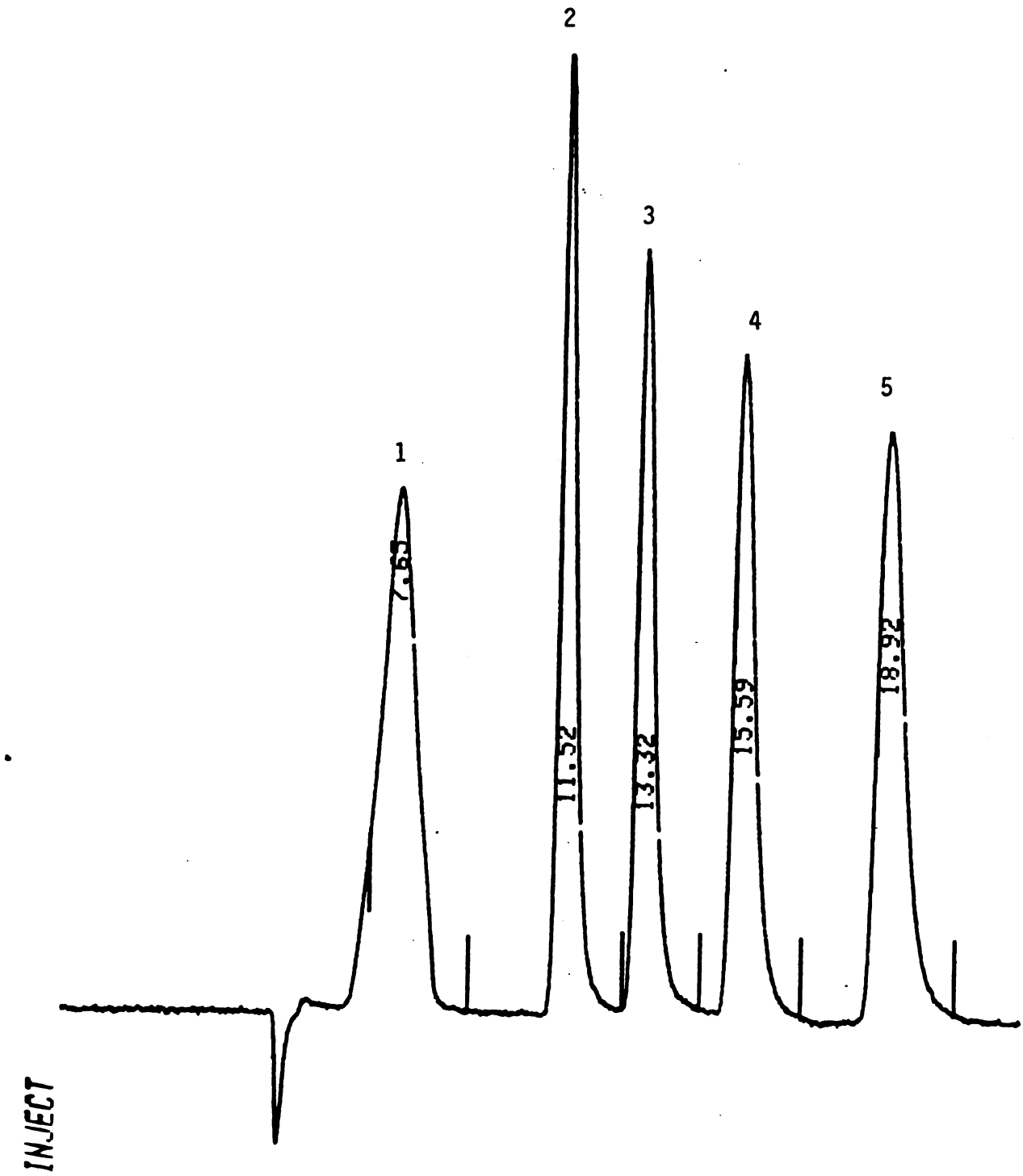


Figure 4. Chromatogram of an aqueous organic acid standard solution containing the following acids: (1) citric, (2) pyruvic, (3) lactic, (4) acetic, (5) propionic, and (6) butyric. Chromatographic conditions: 30 µl injected, 0.0025N sulfuric acid mobile phase, 65 C, 0.7 ml/min. flow rate.

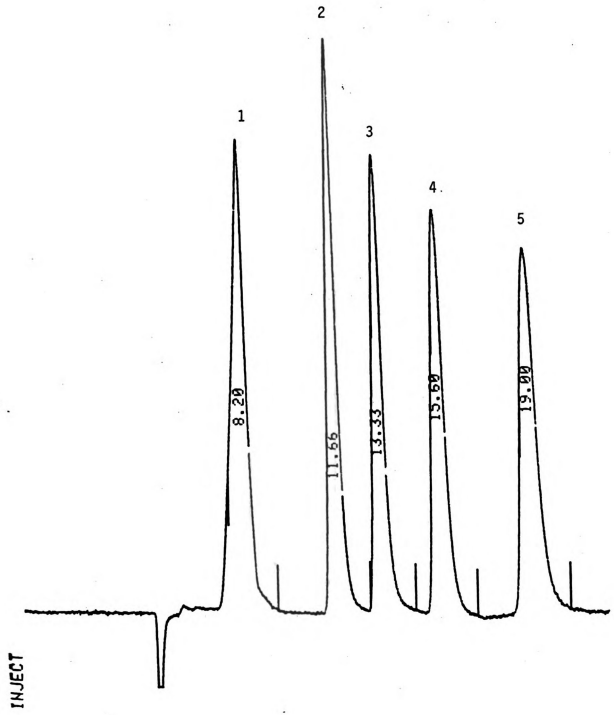


Figure 5. Chromatogram of an aqueous organic acid standard solution. Chromatographic conditions same as Figure 4, except 0.0050N sulfuric acid mobile phase.

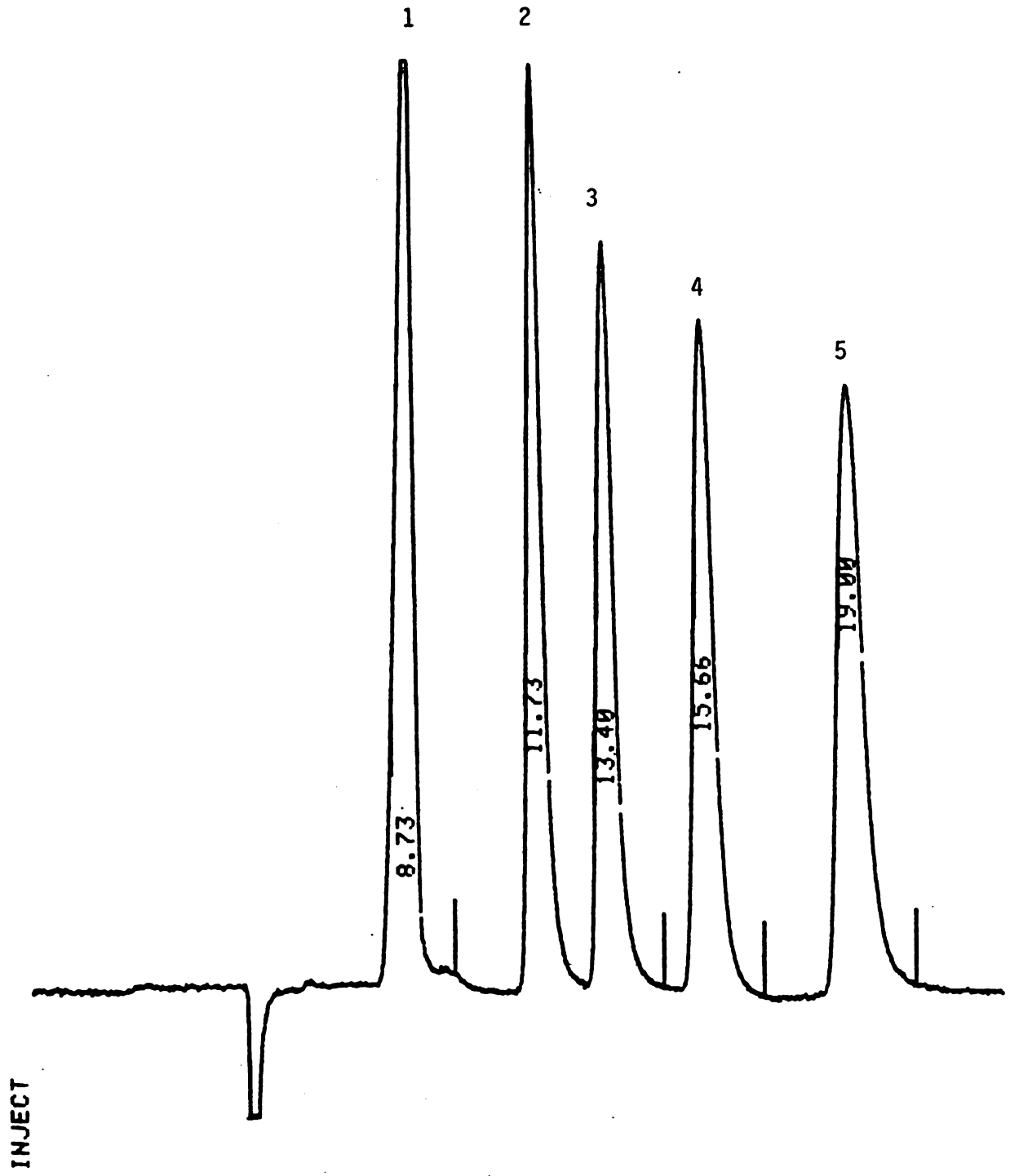


Figure 6. Chromatogram of an aqueous organic acid standard solution. Chromatographic conditions same as Figure 4, except 0.0100N sulfuric acid mobile phase.

quality of separation gradually decreased with higher normality eluent. This can be accounted for due to the degree of swelling of the column resin.

Ion exchange resins swell depending on the pH, the ionic strength of the buffers used, and the nature of the counter-ion (Pharmacia, 1983). At low ionic strength, repulsion between groups carrying the same charge on the matrix is maximal, and swelling is at its greatest. In terms of pH dependence, the degree of dissociation and hence the extent to which an ion exchange resin is charged is dependent on the pH. Repulsion between charged groups is greatest at pH values where the resin is fully dissociated, and decreases at pH values close to the pK of the charged groups. Thus, as the normality of the mobile phase increased, the resin swelling decreased with a coinciding decrease in the separation and resolution of the acid standard solution.

Chromatography was also performed at several different temperatures of the column. Column temperature effect on separation of an acid standard solution was observed at the following temperatures: 25, 30, 35, 40, 45, 50, 55, 60, and 65 C. In all cases, the acid standard solution separation and resolution increased with increasing column temperature. This can be attributed to increased efficiency of the column and decreased pressure drop due to temperature. Effects of

this nature are typical of polymeric columns (Johnson and Stevenson, 1978). The highest rated recommended column temperature by the manufacturer was 65 C.

Finally, flow rate effects on separation of an acid standard solution was observed. Flow rates of 0.4 ml/min. to 1.0 ml/min., incremented by rate increases of 0.1 ml/min. were employed. Acid standard solution separation and resolution was best accomplished with reasonable acid peak retention times with a flow rate of 0.6 ml/min.

In summary, the choice of 0.01N sulfuric acid mobile phase, 65 C column temperature, and 0.6 ml/min. flow rate, provided acceptable resolution for the lactose and organic acids found in milk and natural cheese. An aqueous standard chromatogram without annotation containing a mixture of lactose and organic acids is illustrated in Figure 7, chromatographed under operating conditions as described. Previous chromatograms (Figures 4, 5, and 6) have included all annotation by integrating data module. In all cases, optimization criteria for chromatographic separation of standards have involved only a single parameter of interest being modified while all other variables remained constant.

CONDITIONS

SAMPLE SIZE:	30 µl
SOLVENT:	0.01N Sulfuric Acid
TEMPERATURE:	65 C
FLOW RATE:	0.6 ml/min.
PEAKS:	A. Lactose B. Citric Acid C. Pyruvic Acid D. Lactic Acid E. Acetic Acid F. Propionic Acid G. Butyric Acid H. Unknown S. Solvent Front

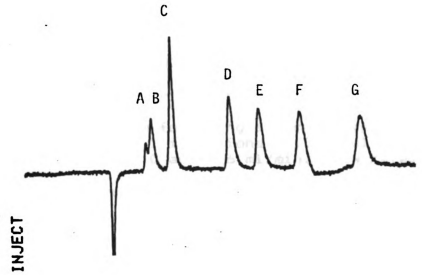


Figure 7. Chromatogram of an aqueous standard solution containing lactose and organic acids.

Standard Calibration Curves

The calibration curve data are presented in Table 2 and clearly indicate excellent linear responses over wide concentration ranges of lactose and organic acids.

Table 2. Calibration curve data for aqueous calibration standards.*

Constituent Standard	Calibration Curve Data		
	Slope+ (x10,000)	y-intercept (x10,000)	Correlation Coefficient
Lactose	44.9246	0.2018	0.9998
Citric Acid	41.2744	-0.4529	0.9965
Pyruvic Acid	38.6628	-0.7068	0.9986
Lactic Acid	22.0002	-0.2240	0.9996
Acetic Acid	15.1294	-0.1563	0.9997
Propionic Acid	16.0882	-0.1510	0.9997
Butyric Acid	14.5807	-0.1947	0.9993

* Data apply to the following chromatographic conditions: 30 l injections, 0.01N sulfuric acid mobile phase, 65 C and 0.6 ml/min. flow rate.

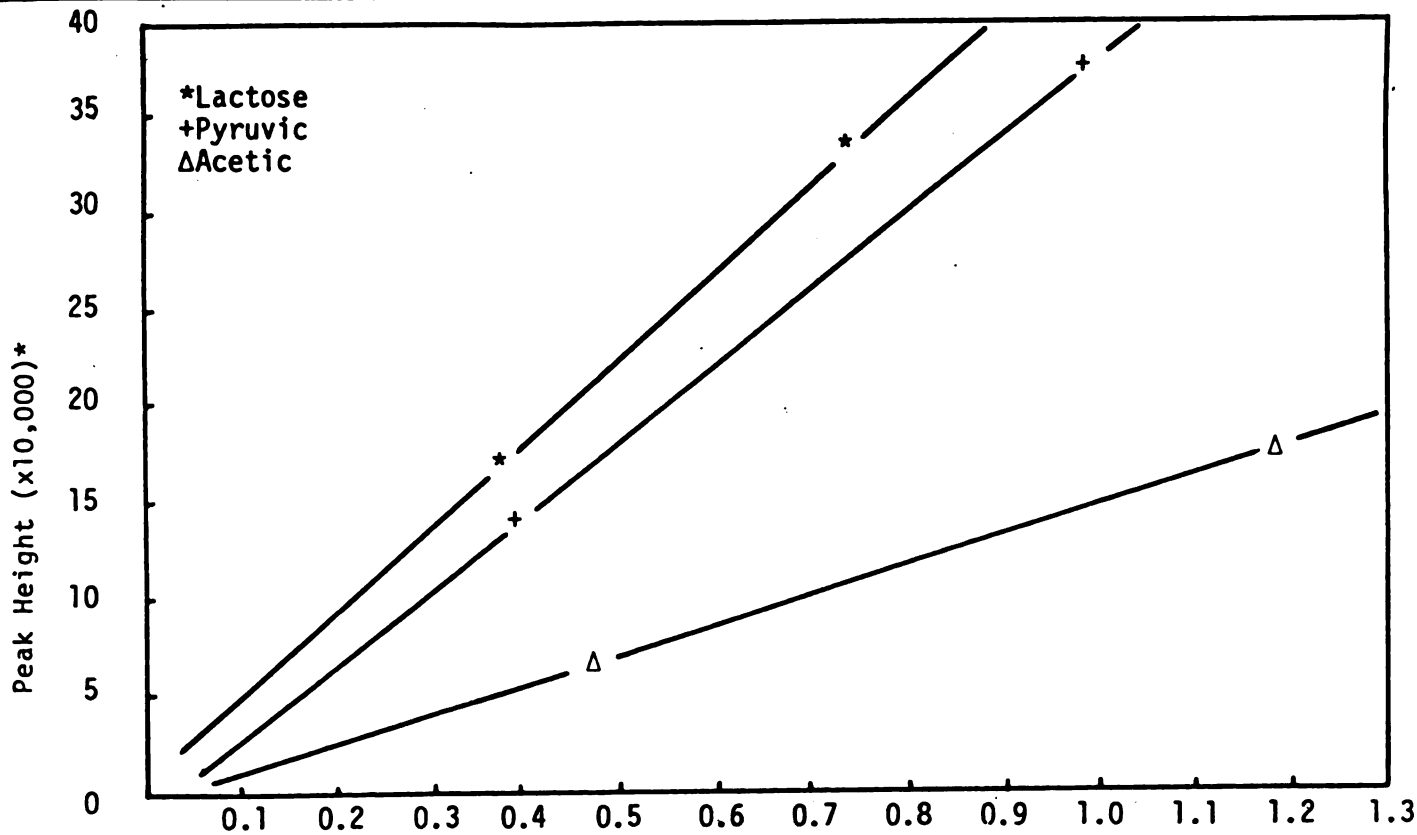
\+ Slope is calculated as peak height per 1 mg/ml.

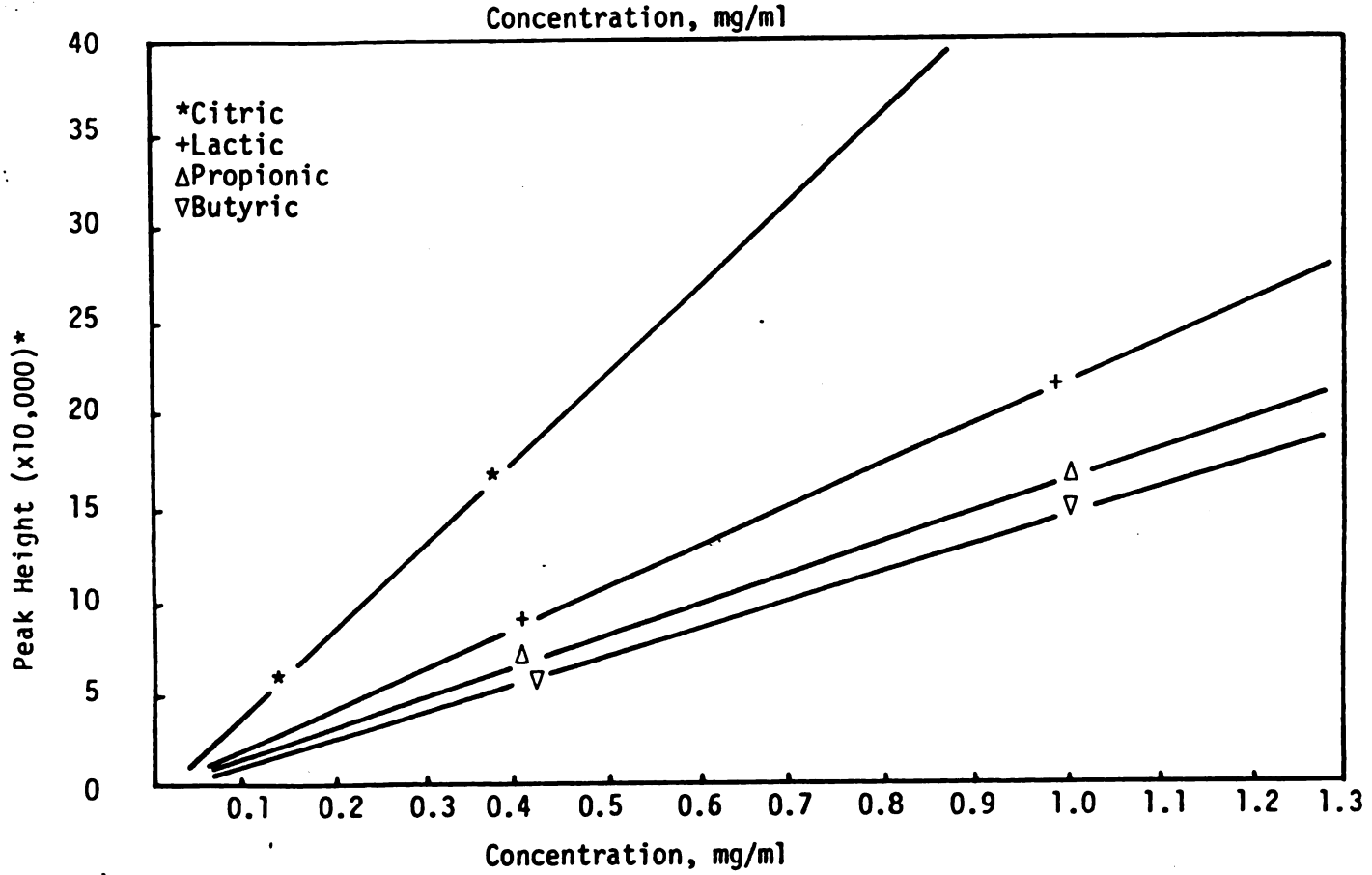
The actual calibration curves for lactose and each acid are illustrated in Figure 8. Calibration curve data are based on peak height integration of lactose standard stock solutions nos. 1 and 2, and organic acid standard stock solution no. 3. Actual chromatograms showing data module integration annotation of lactose and acid standard stock solutions are illustrated in Figures 9 and 10, respectively. Data module integration parameters are listed as follows:

00	CURRENT	DATE
01	CURRENT	TIME
02	0.40	CHART SPEED, CM/MIN
03	OFF	PLOT
04	OFF	PEN 2
05	10	PEN 1 ZERO
06	90	PEN 2 ZERO
07	OFF	AUTO ZERO
08	LC	GPC/LC
09	OFF	CALIB
20	OFF	AUTO PARAMETER
21	15	PEAK WIDTH
22	1500.00	NOISE REJECTION
23	100	AREA REJECTION
24	25.00	RUN STOP

Elution order and corresponding retention times for lactose and acid standards are listed as follows:

Standard	Retention Time (min)
Lactose	8.20
Citric Acid	8.42
Pyruvic Acid	9.92
Lactic Acid	13.67
Acetic Acid	15.65
Propionic Acid	18.35
Butyric Acid	22.32





*Peak Height = input level of detector (analog signal)

Figure 8. Calibration curves for aqueous standards. Chromatographic conditions same as Figure 7.

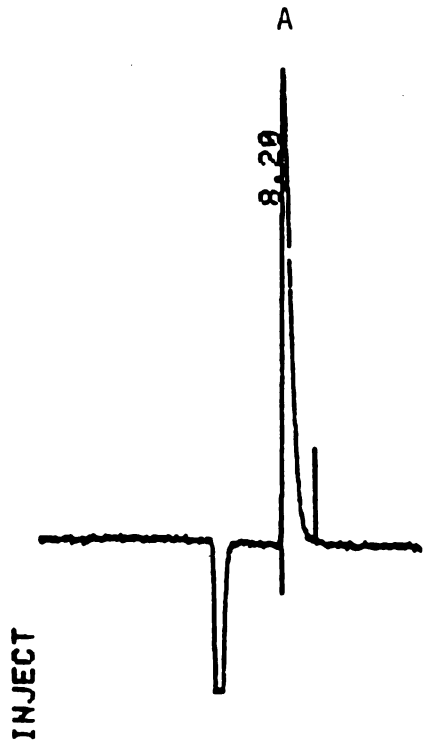


Figure 9. Chromatogram of aqueous standard solution containing lactose. Chromatographic conditions same as Figure 7.

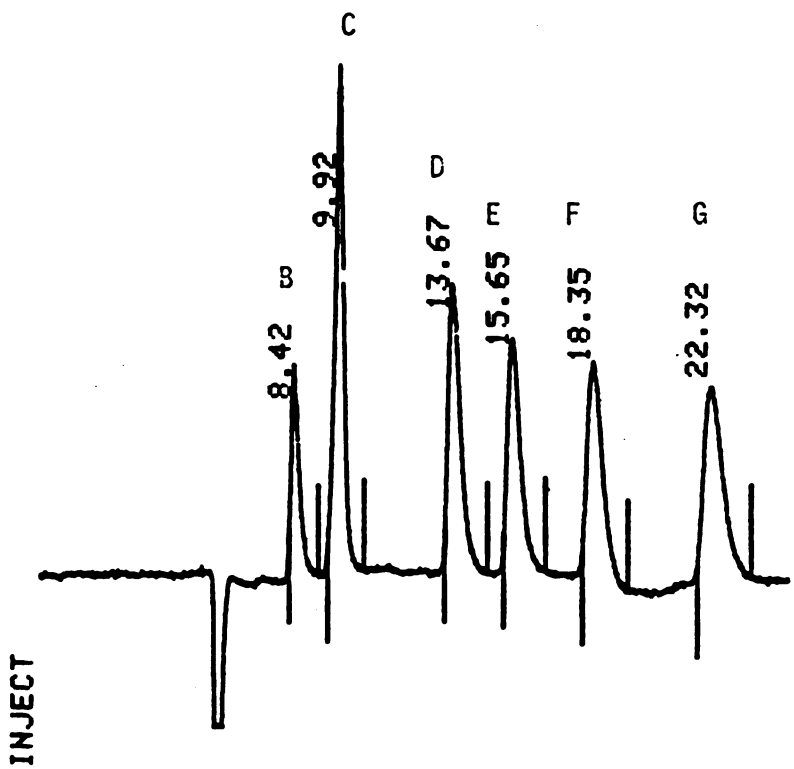


Figure 10. Chromatogram of aqueous standard solution containing organic acids. Chromatographic conditions same as Figure 7.

Additional standards investigated and chromatographed are listed as follows:

Standard	Retention Time (min)
Orotic Acid	8.25
Formic Acid	14.42
Uric Acid	14.60
Diacetyl	17.45

These compounds were evaluated for possible identity of unknown peaks in cheese sample chromatograms. No correlation between unknown peak retention times and these compounds existed.

A chromatogram of reverse-osmosis, ion-exchanged water shows identity of negative peak which exists in most chromatograms. (See Figure 11).

Method Development

Development of a procedure to extract the organic acids from natural cheese was modeled after Kosikowski's (1946) rapid direct-distillation method for determining the volatile fatty acids of cheese and a methodology developed by Marsili et al. (1981) to analyze organic acids in dairy products by HPLC. The procedure developed and previously described is the result of this effort. It represents an improvement over both past methodologies.

Due to the physical properties of detection by refractive index, a different acid extracting solvent

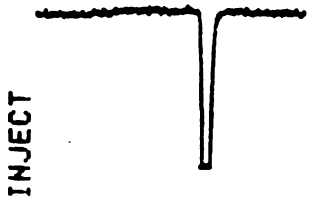


Figure 11. Chromatogram of reverse-osmosis, ion-exchanged water. Chromatographic conditions same as Figure 7.

was required. Acetonitrile (80% with water) used by Marsili et al. (1981), tends to co-elute with organic acids of interest and masks detector response. Detection by UV would eliminate this problem but not enable detection of lactose as desired in this study. The following solvents were considered as candidates for extracting acids from cheese:

0.01N	Sulfuric Acid
10%	Sulfuric Acid
80%	Ethanol
80%/20%	Acetonitrile:Water

Chromatograms showing results of these solvents used to extract organic acids from Cheddar cheese are shown in Figures 12, 13, 14, and 15, respectively. Conditions of cheese preparation and chromatography were identical. Cheese extracted with 0.01N sulfuric acid produced the most desirable results in terms of constituent separation, resolution, solvent safety, and compatibility with mobile phase. Selection of identical solution for both acid extraction and mobile phase use enables lowest possible effluent interference and disturbance of detector.

Other factors investigated during development of the method included consideration of different sample sizes (e.g., 5, 10, and 15 gram), effect of extracting solvent temperature on cheese preparation (cool/hot), amount of extracting solvent used per step, number of

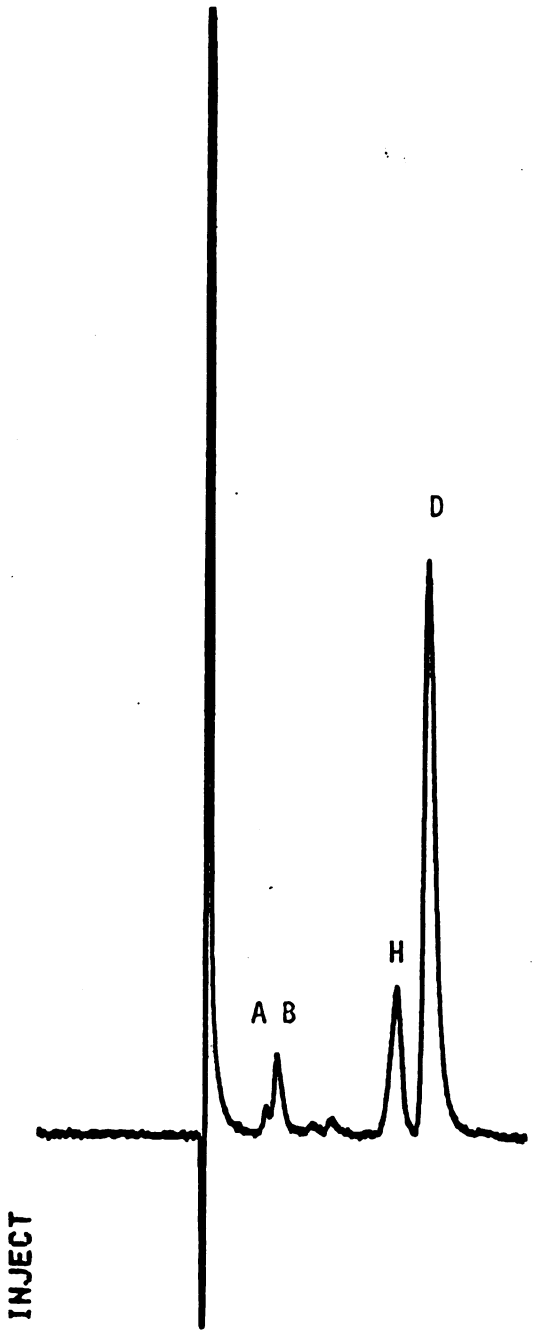


Figure 12. Chromatogram of Cheddar cheese sample extracted with 0.01N sulfuric acid. Chromatographic conditions same as Figure 7, except 15 µl injection volume.

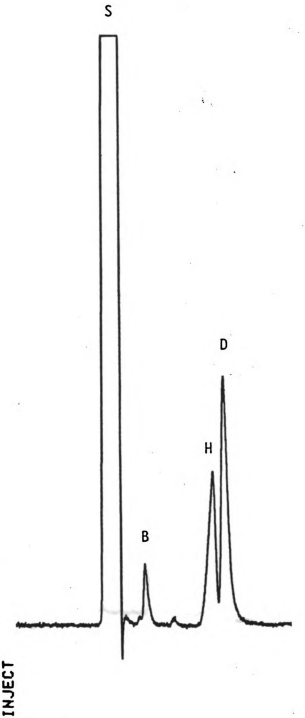


Figure 13. Chromatogram of Cheddar cheese sample extracted with 10% sulfuric acid solution. Chromatographic conditions same as Figure 7, except 15 µl injection volume.

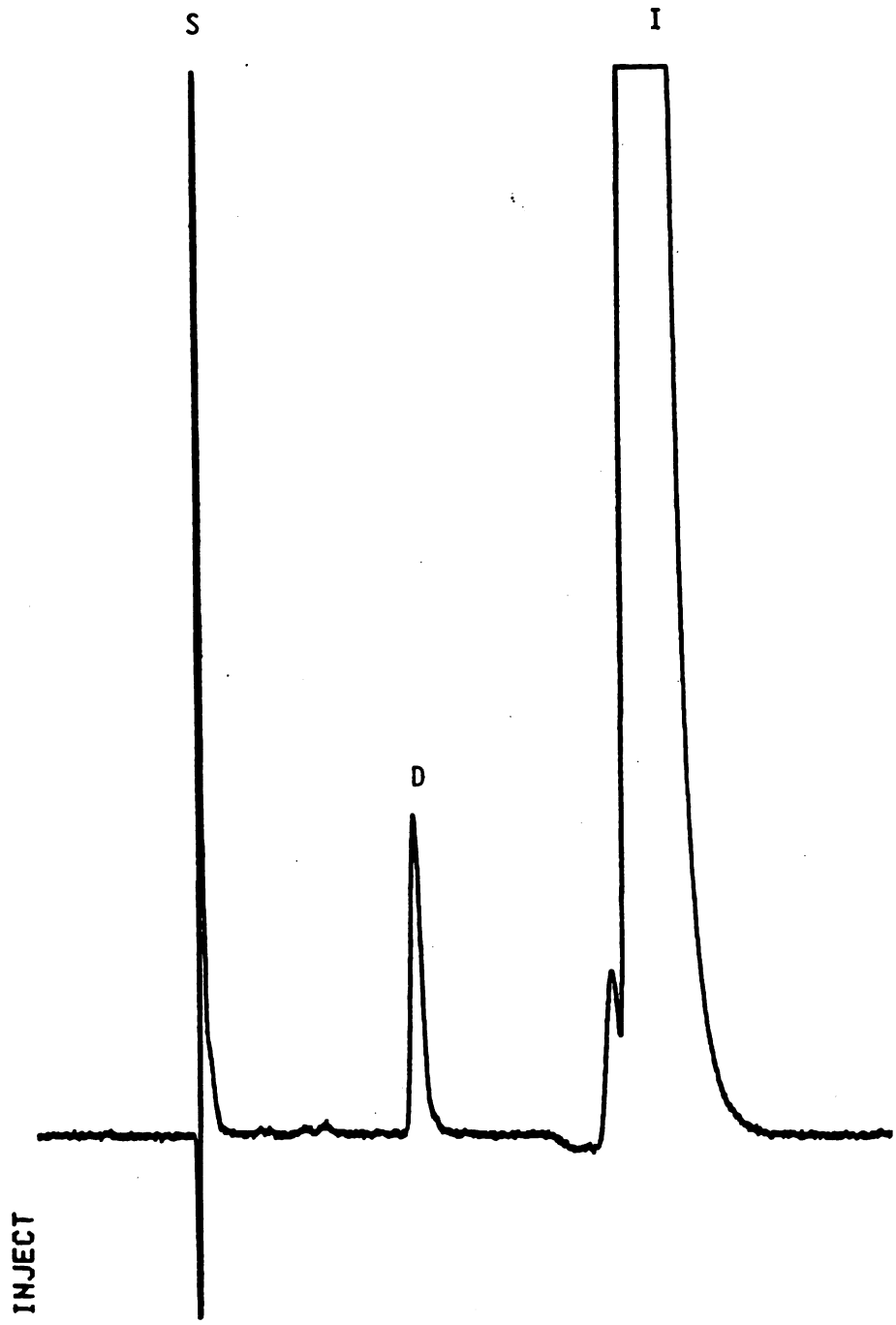


Figure 14. Chromatogram of Cheddar cheese sample extracted with 80% ethanol. Chromatographic conditions same as Figure 7, except 15 µl injection volume. Additional peak: (I) ethanol.

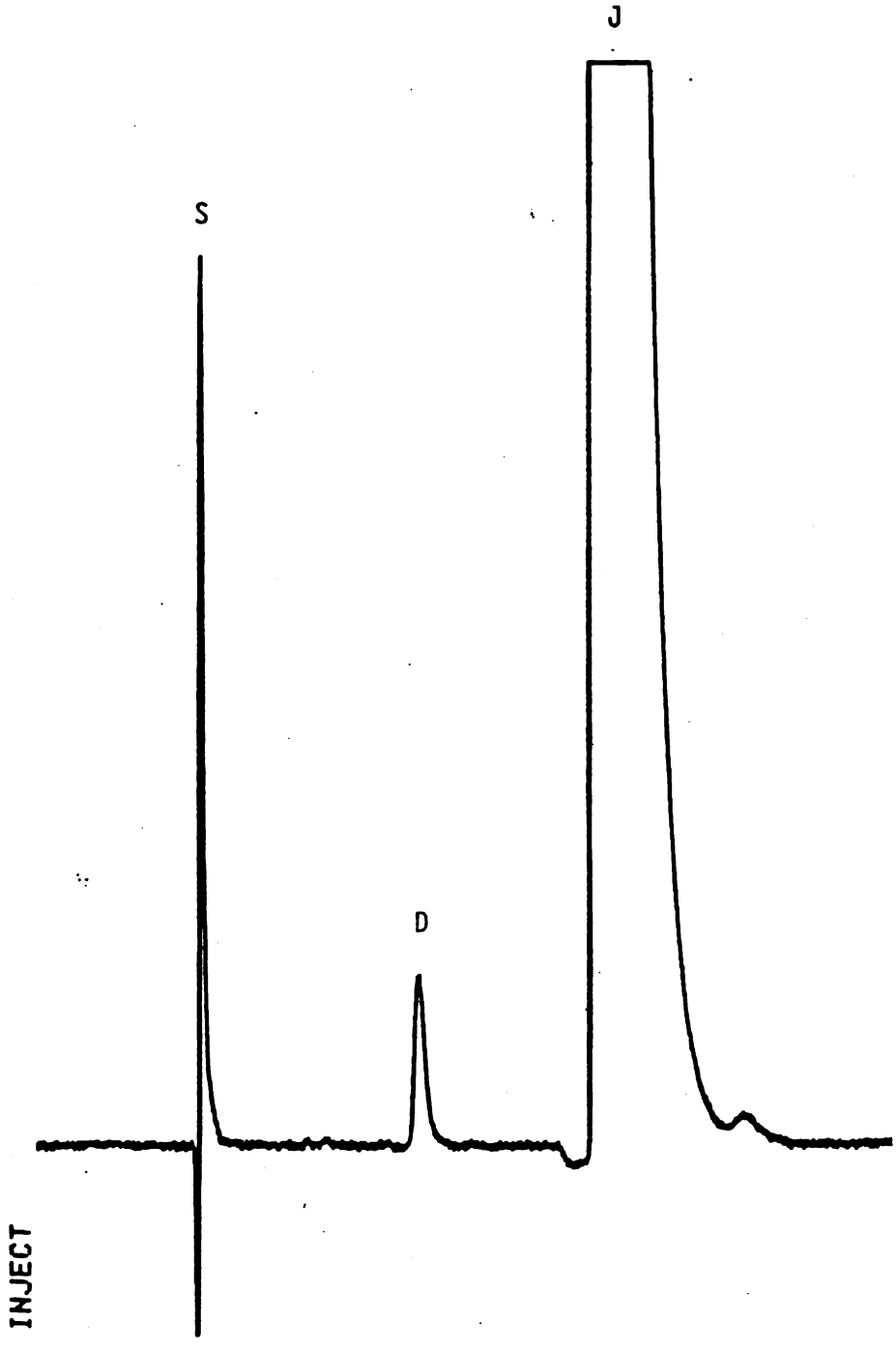


Figure 15. Chromatogram of Cheddar cheese sample extracted with 80% actonitrile:water solution. Chromatographic conditions same as Figure 7, except 15µl injection volume. Additional peak: (J) acetonitrile.

extracting steps, and time of shaking. Due to size restrictions and limitations on available equipment, a 10 gram sample extracted twice with 25 ml portions of solvent provided the best results. Heating the extracting solvent facilitated cheese preparation and dispersion of fat during shaking. A two minute shake period was found to be adequate. Also a three step extraction process did not provide significant additional amounts of analyzed constituents of interest. The two step procedure did provide improved results than those obtained in Cheddar cheese by Marsili et al. (1981).

Finally, the effect of freezing stock cheese samples before extracting acids was investigated. Investigation suggested that freezing properly protected samples had no effect on organic acid content. Chromatograms of poorly wrapped samples, however, did show slight variations, possibly due to moisture loss from the cheese and/or sampling error. In addition, analysis of the resulting fat layer during refrigerated centrifugation during cheese extraction revealed no peaks when chromatographed under existing operating conditions. Also, no significant peaks were detected in the filtered retentive material obtained from the Sep-Paks used to filter the final supernatant before chromatographing.

Time-Series Ripening Study

Two blocks (approx. wt., 40 lbs) of newly made "green" Cheddar cheese were obtained from Michigan State University's Dairy Plant and Lake-to-Lake Dairy, Wisconsin. The Wisconsin cheese was identified as "Pasteurized Colored Cheddar Cheese, Wisconsin Factory Number 0398." This cheese was from vat 74, produced on January 24, 1984. Each of these cheese blocks was cut into smaller 1-2 lb pieces, vacuum packaged in Cryovac bags and incubated at 10 C during the duration of the study.

Chromatograms showing time-lapsed developments during ripening for each cheese follows. Chromatograms show constituent profiles of lactose and organic acids in these cheeses and in milk used to manufacture the MSU Cheddar cheese. Stages of ripening include: unpressed cheese curd, 24-hr pressed cheese curd, 3-day, 8-day, 15-day, 30-day, and 60-day chromatograms of aged MSU Cheddar cheese. Chromatograms of Wisconsin Cheddar cheese show stages of ripening at: 8-days, 11-days, 16-days, 23-days, 38-days, and 68-days.

In the analysis of the Cheddar cheeses, histograms are shown to provide "at-a-glance" changes in lactose and organic acids concentration which occurred in the cheeses during ripening. Constituent percentage concentrations calculated from chromatograms are multiplied by a value of 5 to account for sample

dilution during extraction. During extraction, 10.0 gram milk and cheese samples were diluted to a total volume of 50 ml with extracting solvent. This step is required because the data module is calibrated in units of 1 mg/ml. Final manipulation of data requires adjustment to compensate for dilutions of sample that took place in the handling and preparation prior to analysis by HPLC.

A sample calculation for determining lactic acid percentage composition for 60-day aged MSU Cheddar cheese is shown as follows:

Chromatogram organic acid unadjusted value:

Lactic acid = 3.1490 mg/ml

Acid value unit conversion:

3.1490 mg/ml x 1g/1000ml = 0.0031 g/ml

Acid value correction for sample dilution:

0.0031 g/ml x 5 = 0.0157 g/ml

Acid value correction for percent recovery:

0.0157 g/ml x 1/.971 = 0.0162 g/ml

Adjusted acid value reported as percent:

0.0162 g/ml x 100 = 1.62% Lactic acid

Analyses of Cheddar cheeses showed a similar trend in terms of compositional changes during ripening. Decrease of lactose in MSU Cheddar was very rapid with no detection of lactose after 3 days of ripening.

Lactose depletion in Wisconsin Cheddar was less rapid and was not detected after 23 days of ripening. In both cheeses, the concentration of lactose in cheese was very low soon after manufacture. Appearance of lactose in later cheese samples could be due to sampling error. Poor quality cheeses have been reported to contain pockets of lactose (Campbell and Marshall, 1975). According to Foster et al. (1961), lactose is normally completely metabolized in about two weeks after manufacture.

Development of lactic acid was rapid throughout manufacture and increased significantly during ripening for cheeses analyzed. Citric acid decreased slightly throughout the entire study once detected. Lactic acid was the predominant organic acid detected during all phases of ripening. Trace amounts of orotic, pyruvic, uric, acetic, and propionic acids were not detected as by Marsili et al. (1981). The material comprising the "unknown" peak eluting prior to lactic acid is believed to be that of an insoluble salt. The other detected trace peaks were not identified at this time.

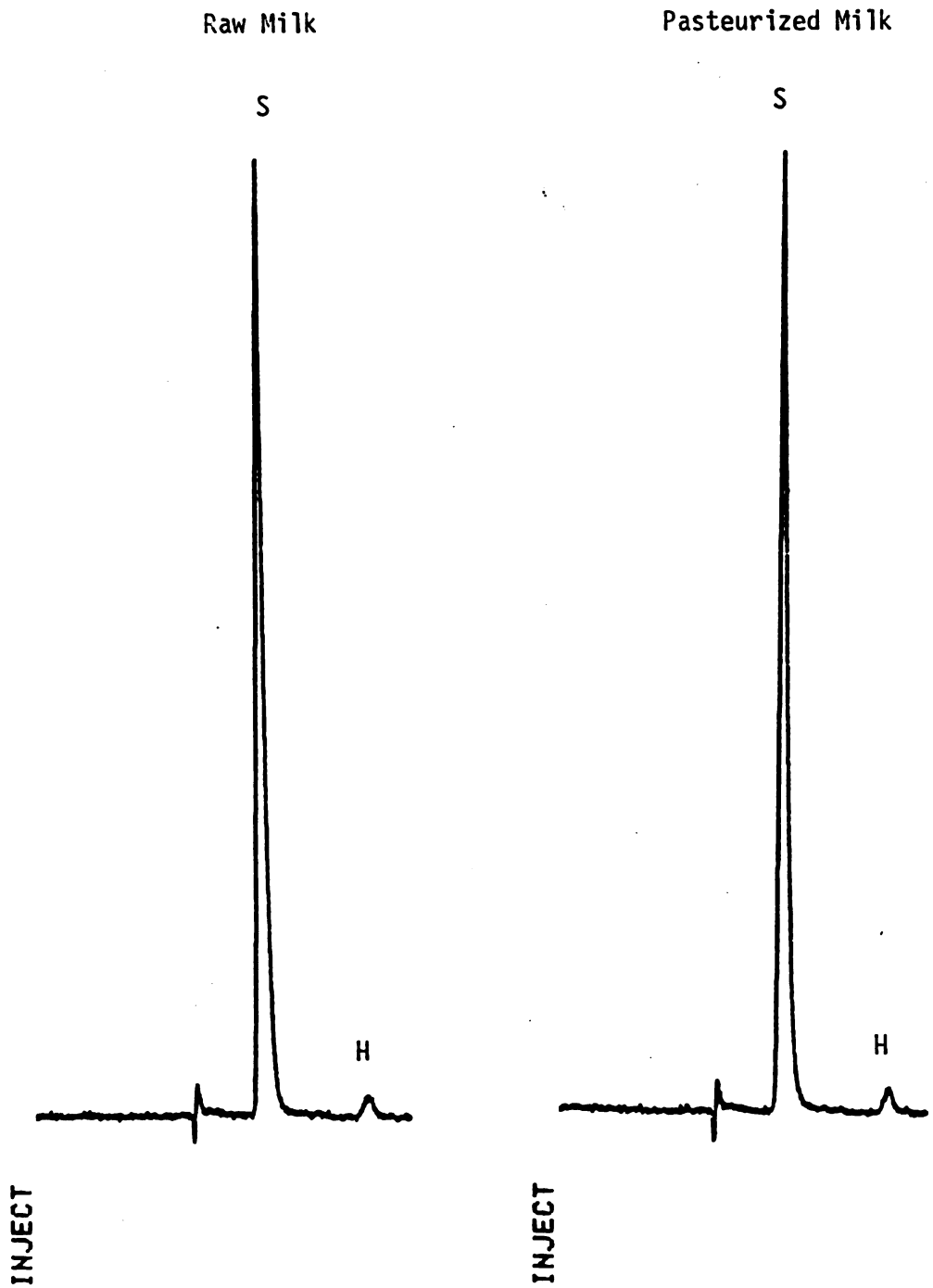


Figure 16. Chromatograms raw and pasteurized milk for manufacture of MSU Cheddar cheese. Chromatographic conditions same as Figure 7, except 5 µl injection volume.

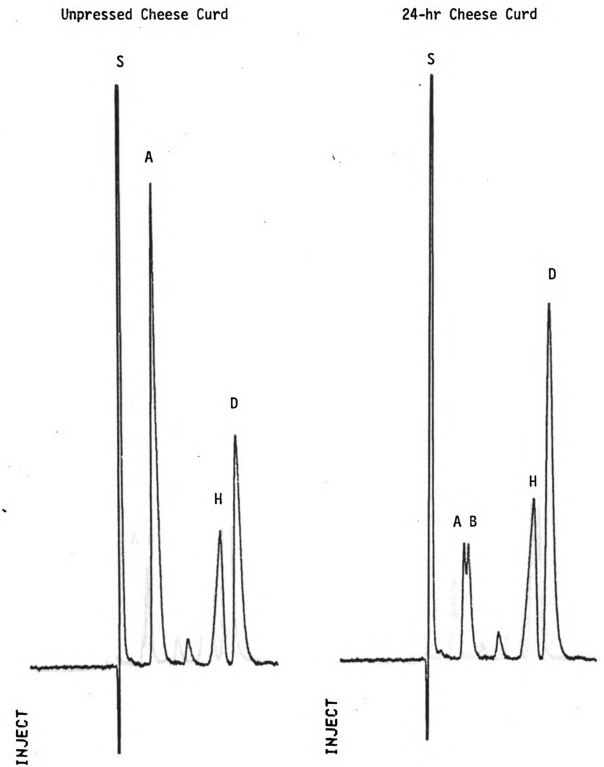


Figure 17. Chromatograms unpressed cheese curd and 24-hour pressed cheese curd, MSU Cheddar cheese. Chromatographic conditions same as Figure 7.

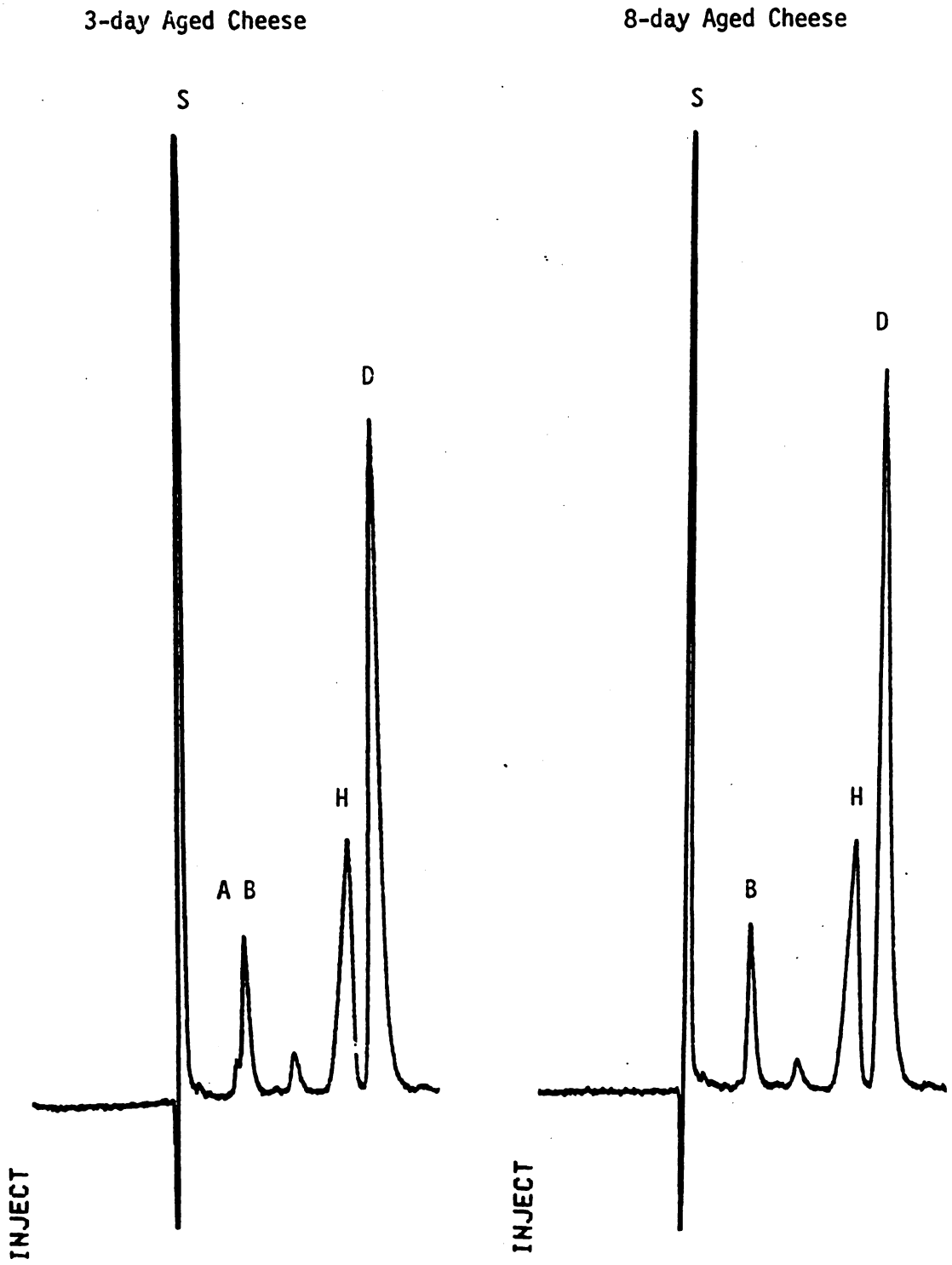


Figure 18. Chromatograms 3-day and 8-day aged MSU Cheddar cheese. Chromatographic conditions same as Figure 7.

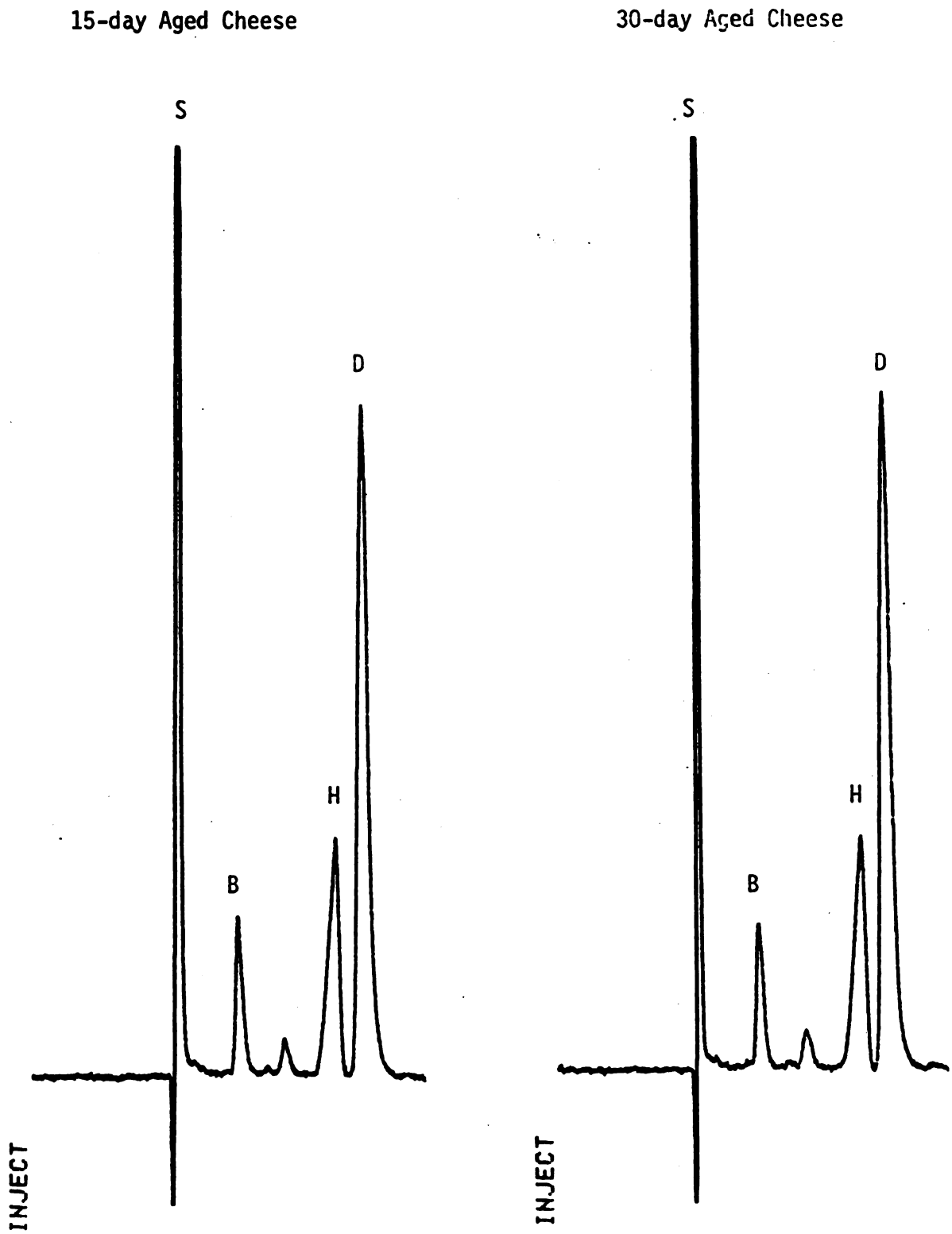


Figure 19. Chromatograms 15-day and 30-day aged MSU Cheddar cheese. Chromatographic conditions same as Figure 7.

60-day Aged Cheese

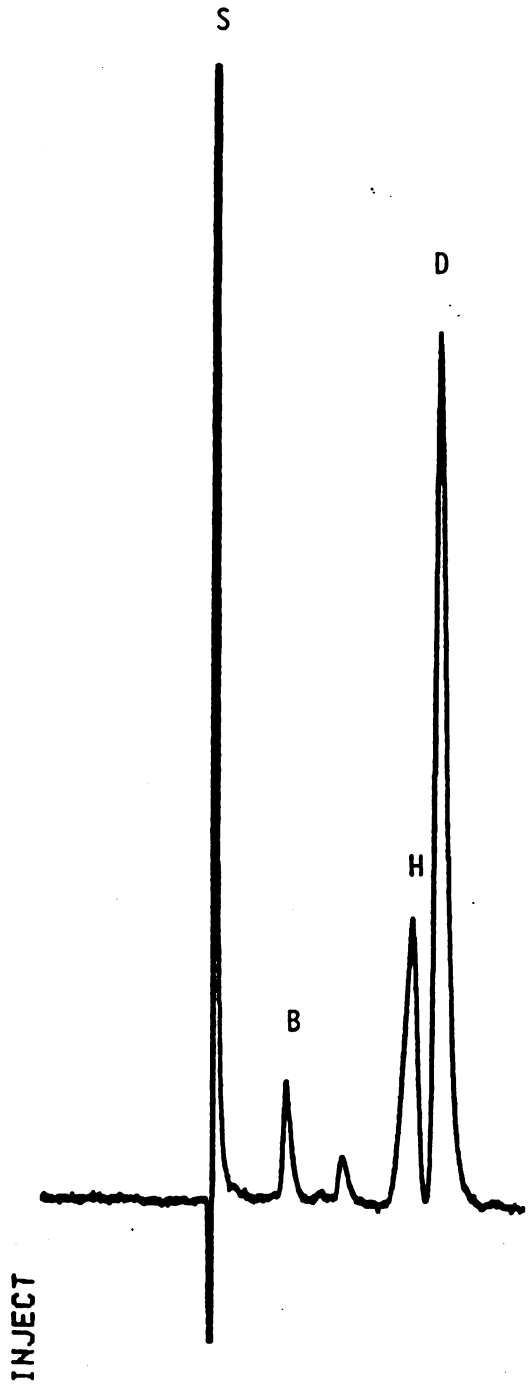


Figure 20. Chromatograms 60-day aged MSU Cheddar cheese. Chromatographic conditions same as Figure 7.

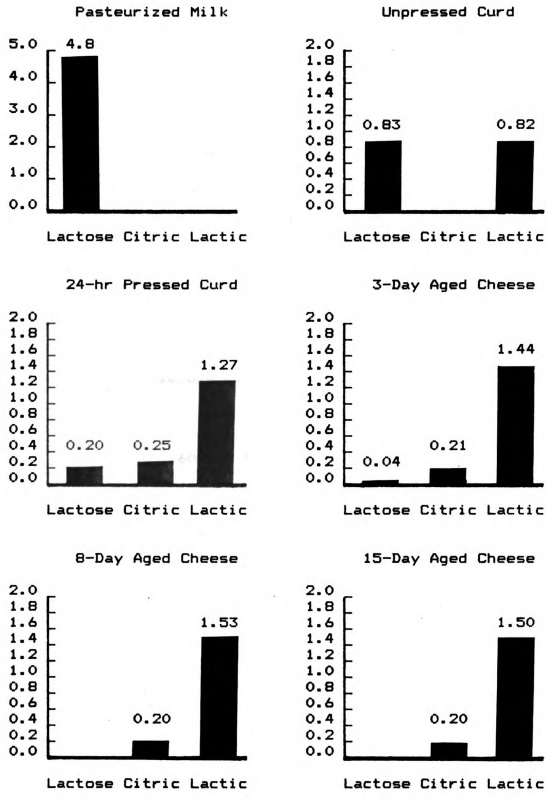


Figure 21. Summary histograms of time-series MSU Cheddar cheese ripening study. Lactose and acid values corrected for percent recovery.

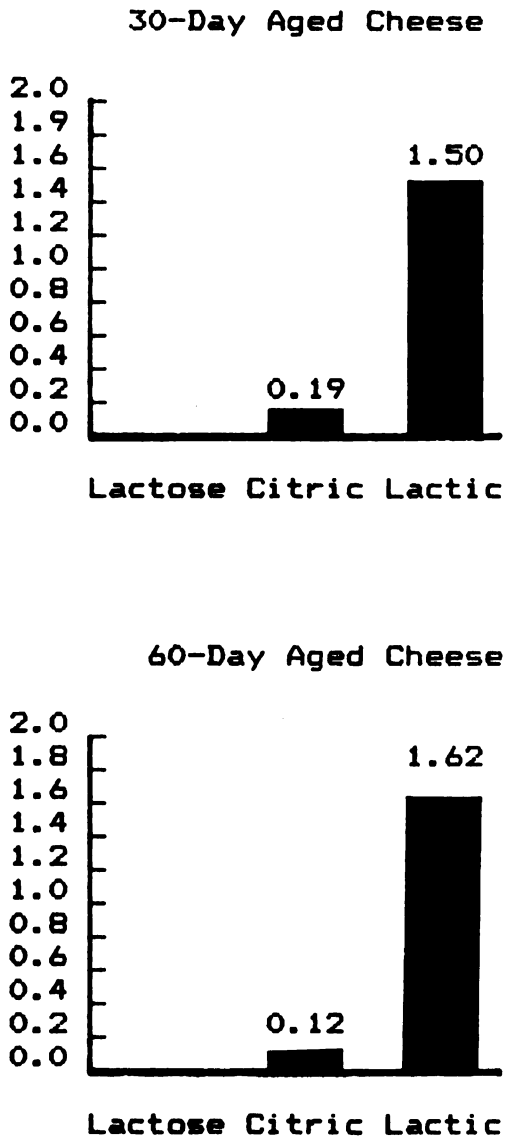


Figure 22. Summary histograms of time-series MSU Cheddar cheese ripening study. Acid values corrected for percent recovery.

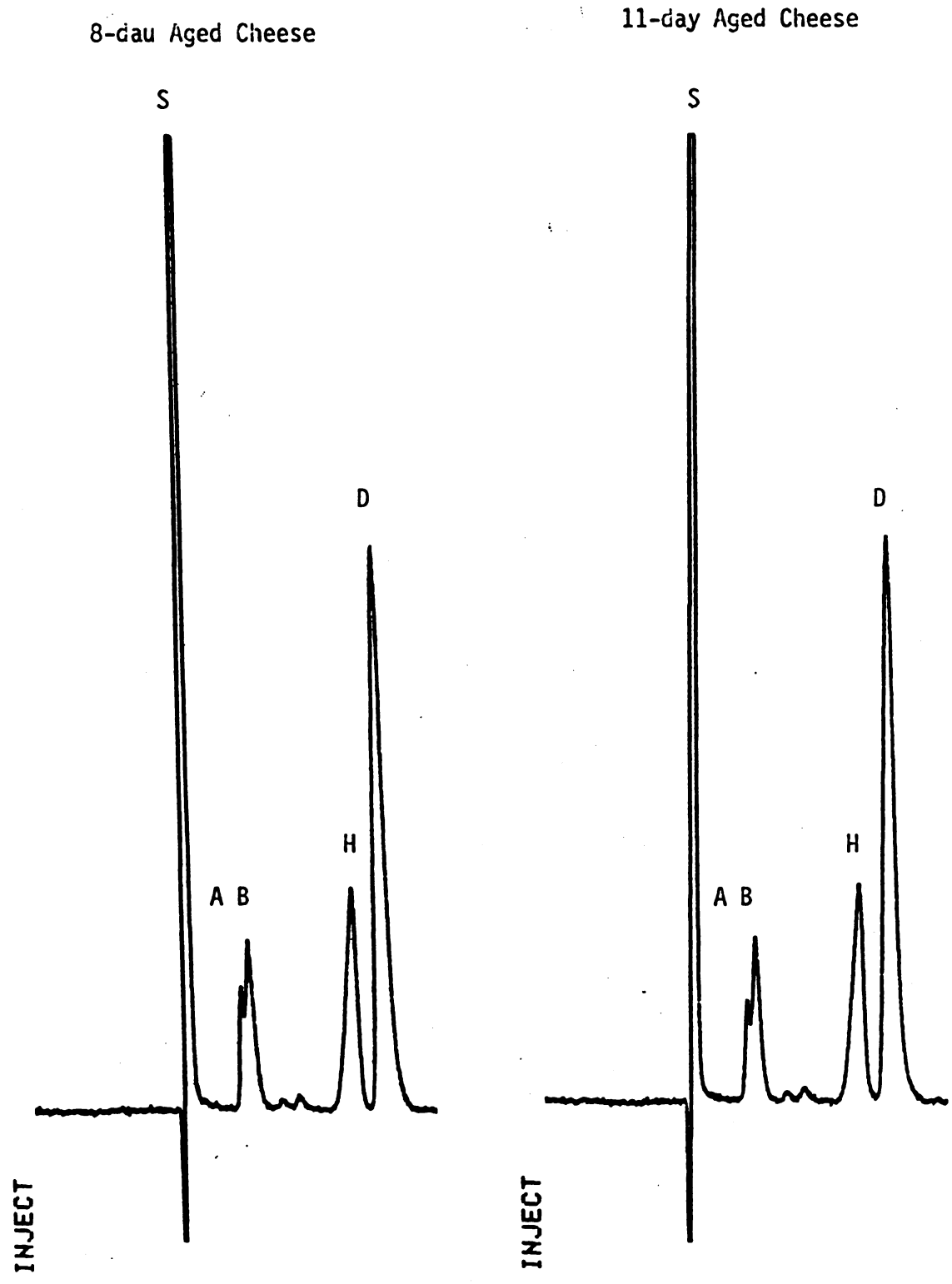


Figure 23. Chromatograms 8-day and 11-day aged Wisconsin Cheddar cheese. Chromatographic conditions same as Figure 7.

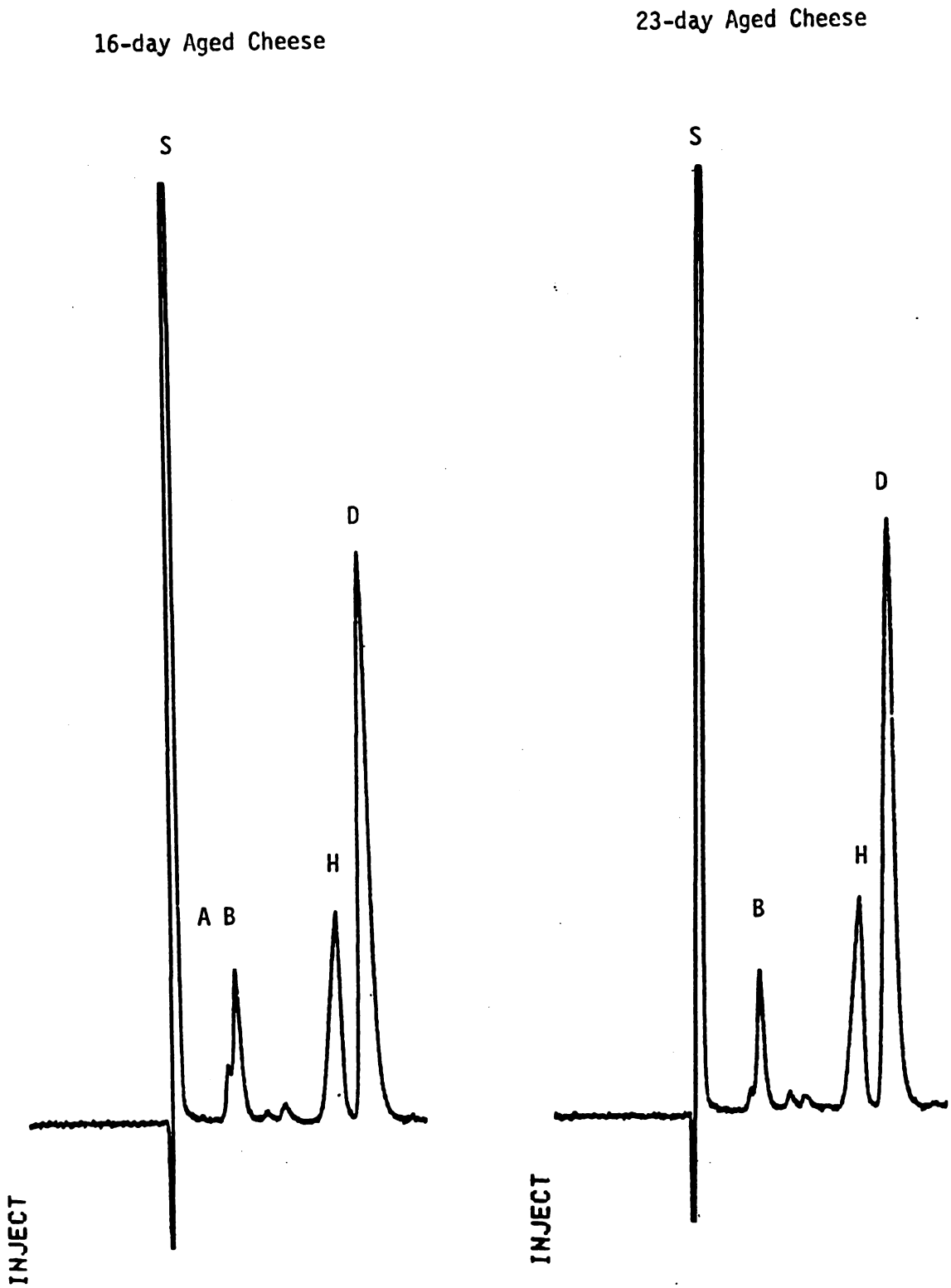


Figure 24. Chromatograms 16-day and 23-day aged Wisconsin Cheddar cheese. Chromatographic conditions same as Figure 7.

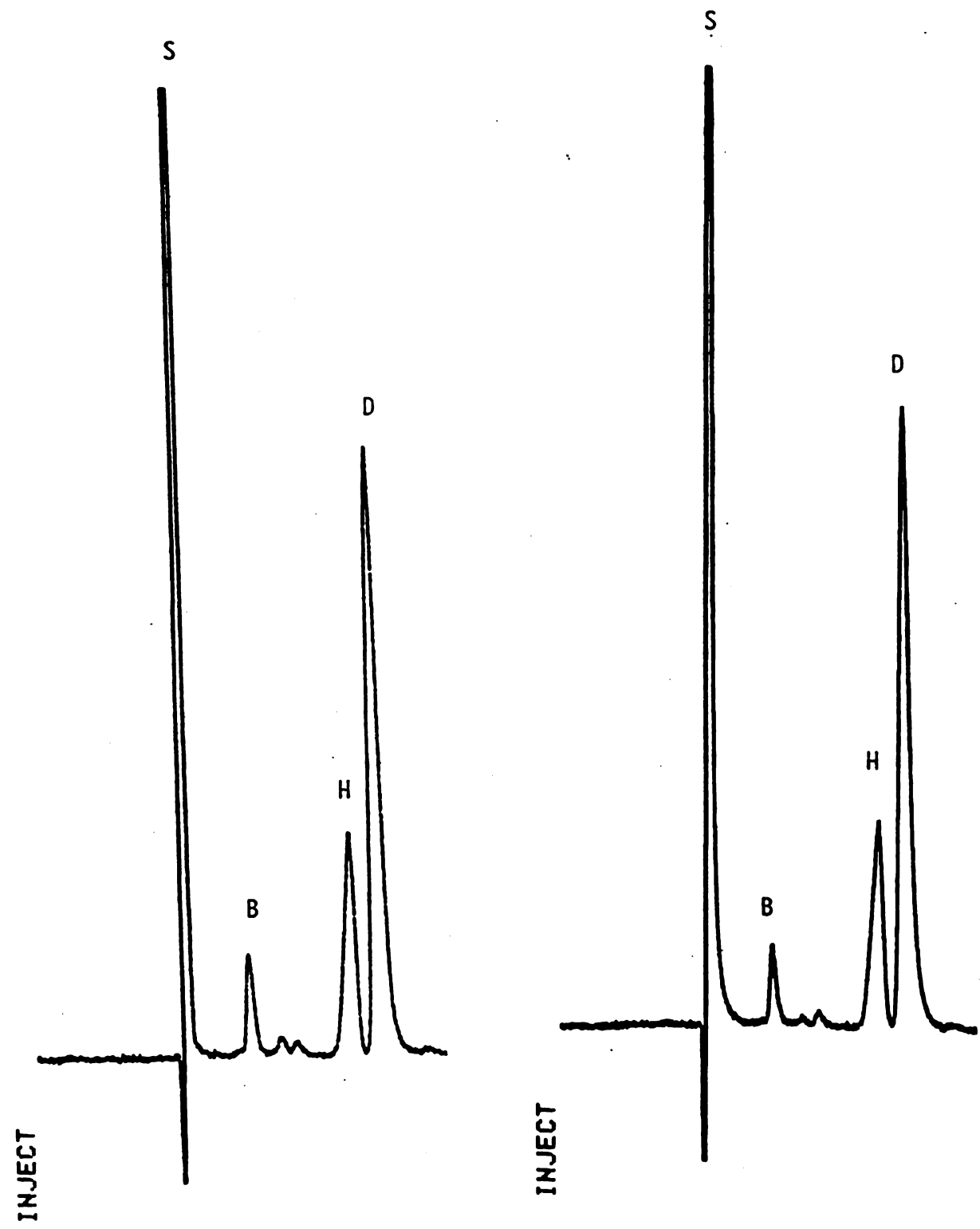


Figure 25. Chromatograms 38-day and 68-day aged Wisconsin Cheddar cheese. Chromatographic conditions same as Figure 7.

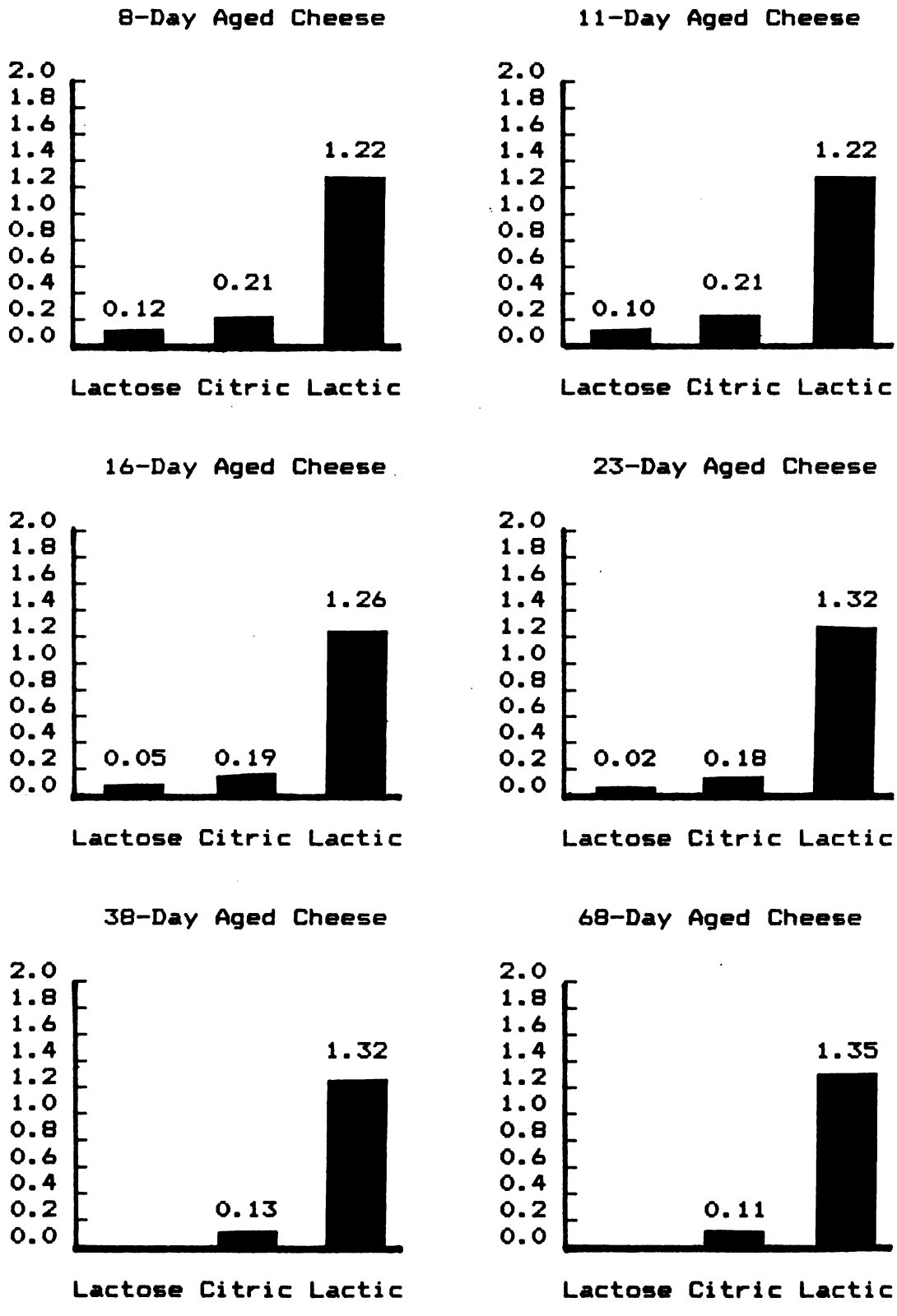


Figure 26. Summary histograms of time-series Wisconsin Cheddar cheese ripening study. Lactose and acid values corrected for percent recovery.

Examination of Market Cheeses

Several varieties of cheese were purchased from retail stores in the East Lansing market area. These cheese varieties have been described in detail previously. Chromatograms showing constituent profiles follow. Cheeses analyzed include: Mild Cheddar, Sharp Cheddar, Extra Sharp Cheddar, Raw Milk Cheddar, Blue, Fontinella, Gorgonzola, Jarlsberg, Liederkranz, Limburger, Parmesan, Provolone, Romano, and Swiss. Extraction and analysis of these cheese varieties indicated additional acids beyond citric and lactic, including pyruvic, acetic, propionic, and butyric acids. Concentration of the acids identified in these cheeses is reported in Table 3. Lactic acid was predominant in most cheeses analyzed. In Limburger and Liederkranz cheeses, acetic acid was the predominant acid with no lactic acid being detected in the Liederkranz sample. A trace amount of lactose was detected in Provolone indicating possible sampling error and/or poor uniform quality of the cheese.

Table 3. Concentration percentages of organic acids identified in market cheese varieties analyzed by HPLC.*

Cheese Variety	Constituent Concentration, %					
	Citric	Pyruvic	Lactic	Acetic	Propionic	Butyric
M Cheddar	0.11	----	1.35	----	----	----
S Cheddar	0.12	----	1.32	----	----	----
X-S Cheddar	0.11	----	1.31	----	----	----
R-M Cheddar	0.09	----	1.40	----	----	----
Blue	----	0.02	0.66	Trace	----	----
Fontinella	Trace	----	1.25	Trace	Trace	Trace
Gorgonzola	----	Trace	1.33	Trace	0.27	Trace
Jarlsberg	----	0.02	0.89	0.18	0.35	Trace
Liederkranz	----	----	----	0.06	Trace	----
Limburger	----	----	Trace	0.25	0.08	Trace
Parmesan	Trace	----	1.11	0.12	Trace	0.18
Provolone	0.14	----	1.06	Trace	Trace	Trace
Romano	0.05	----	1.56	Trace	0.19	0.51
Swiss	----	Trace	0.84	0.18	0.22	Trace

*Values corrected for percent recovery.

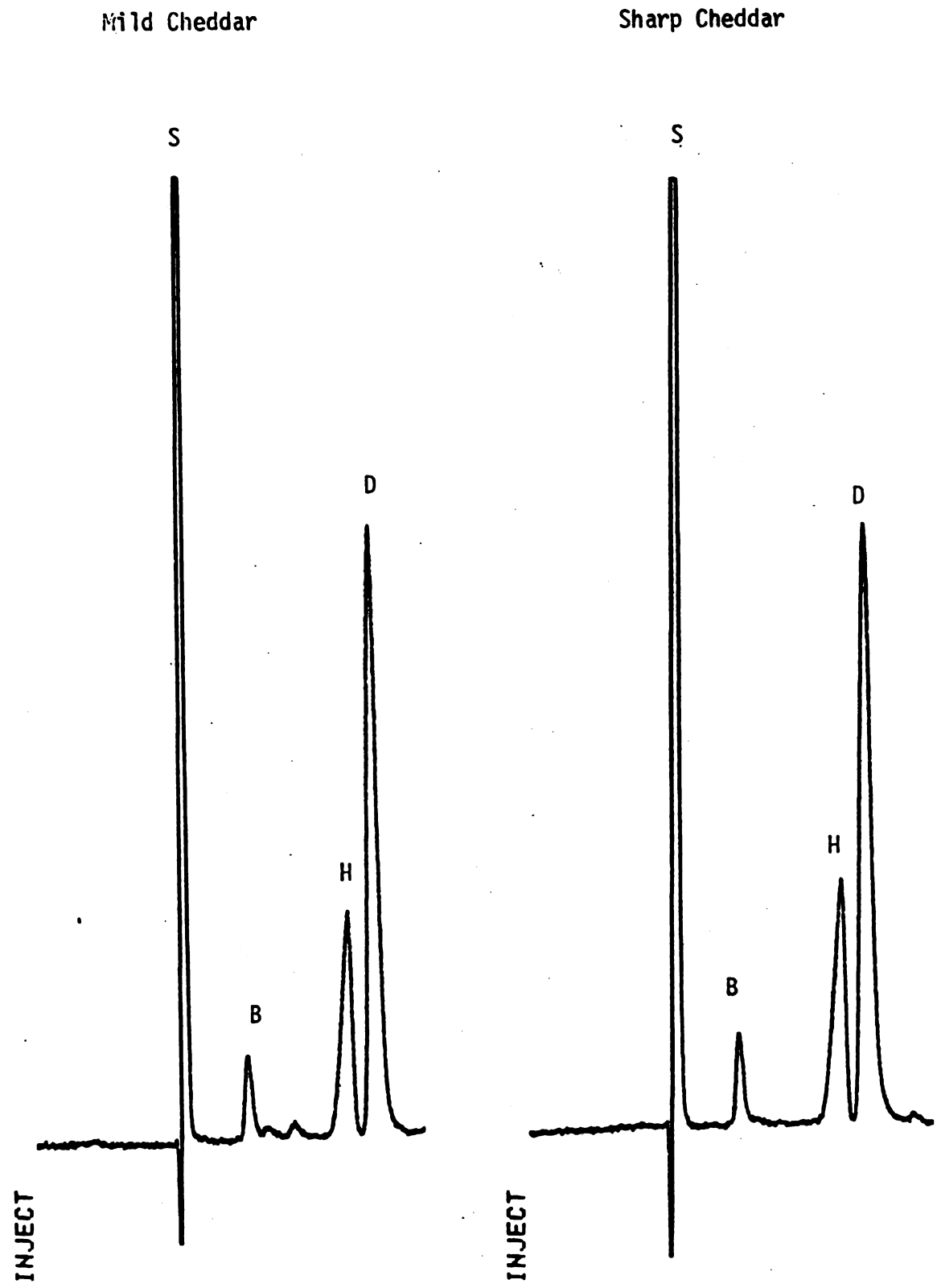


Figure 27. Chromatograms mild and sharp Cheddar market cheeses. Chromatographic conditions same as Figure 7.

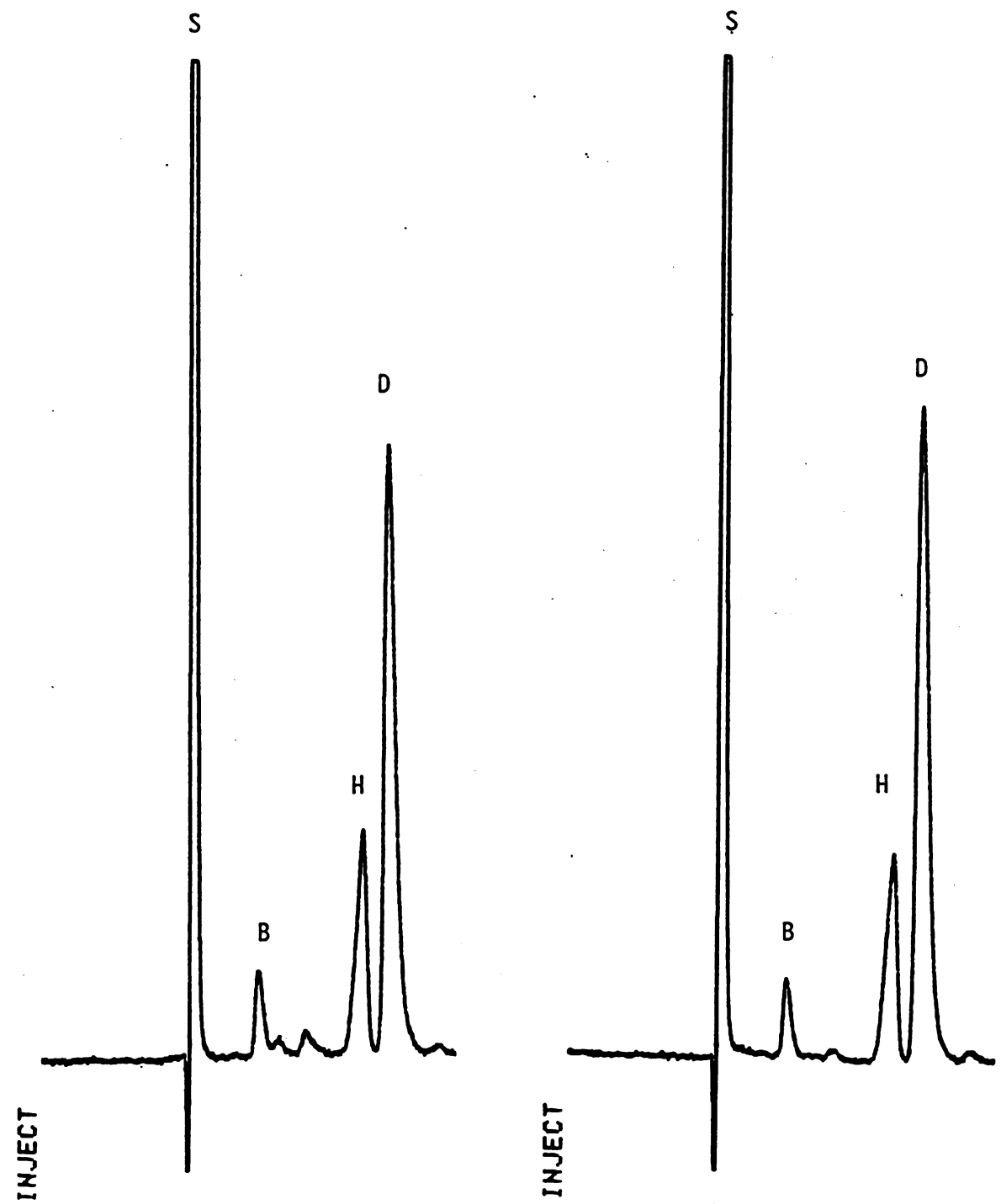


Figure 28. Chromatograms extra sharp and raw milk Cheddar market cheeses. Chromatographic conditions same as Figure 7.

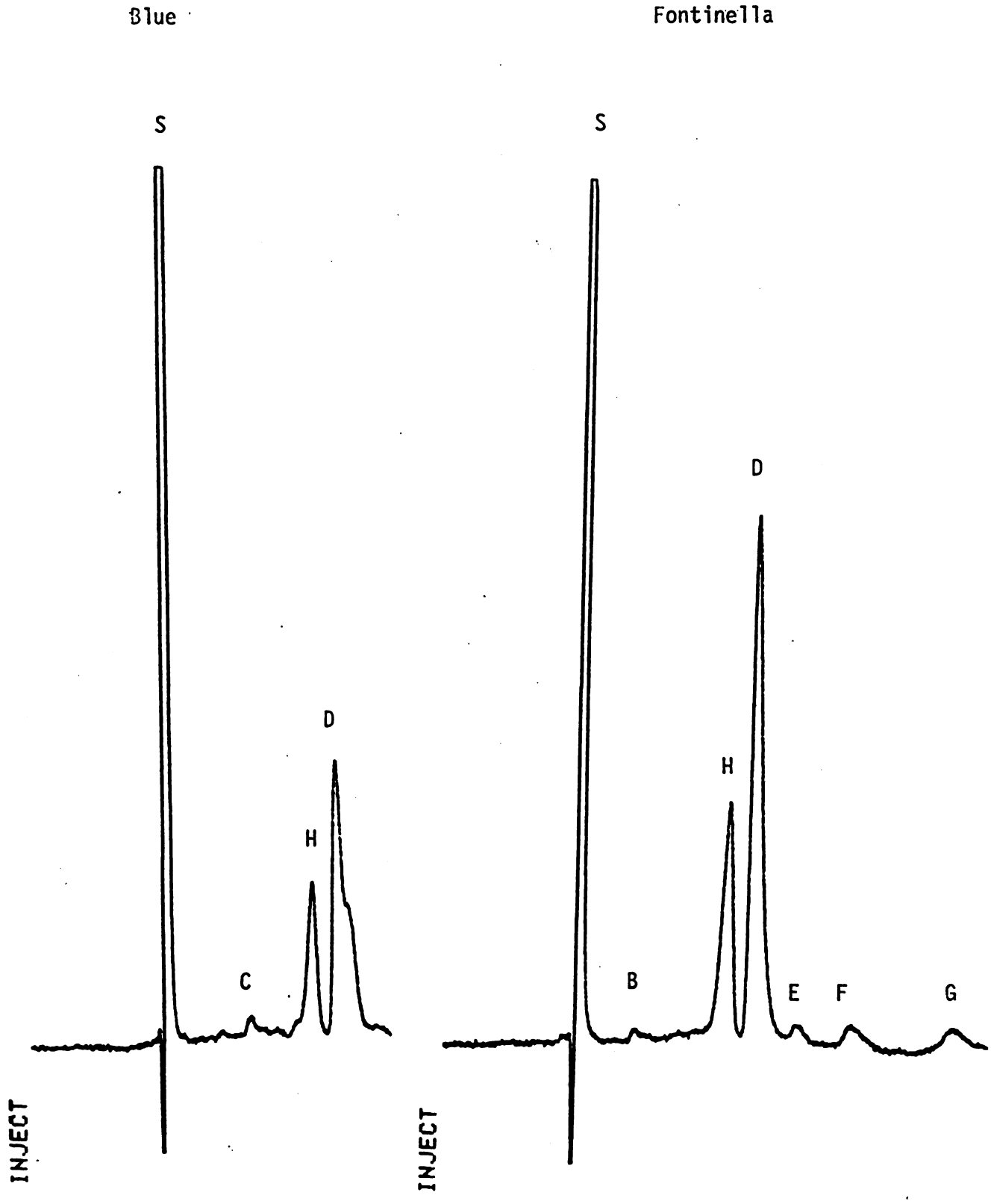


Figure 29. Chromatograms Blue and Fontinella market cheeses. Chromatographic conditions same as Figure 7.

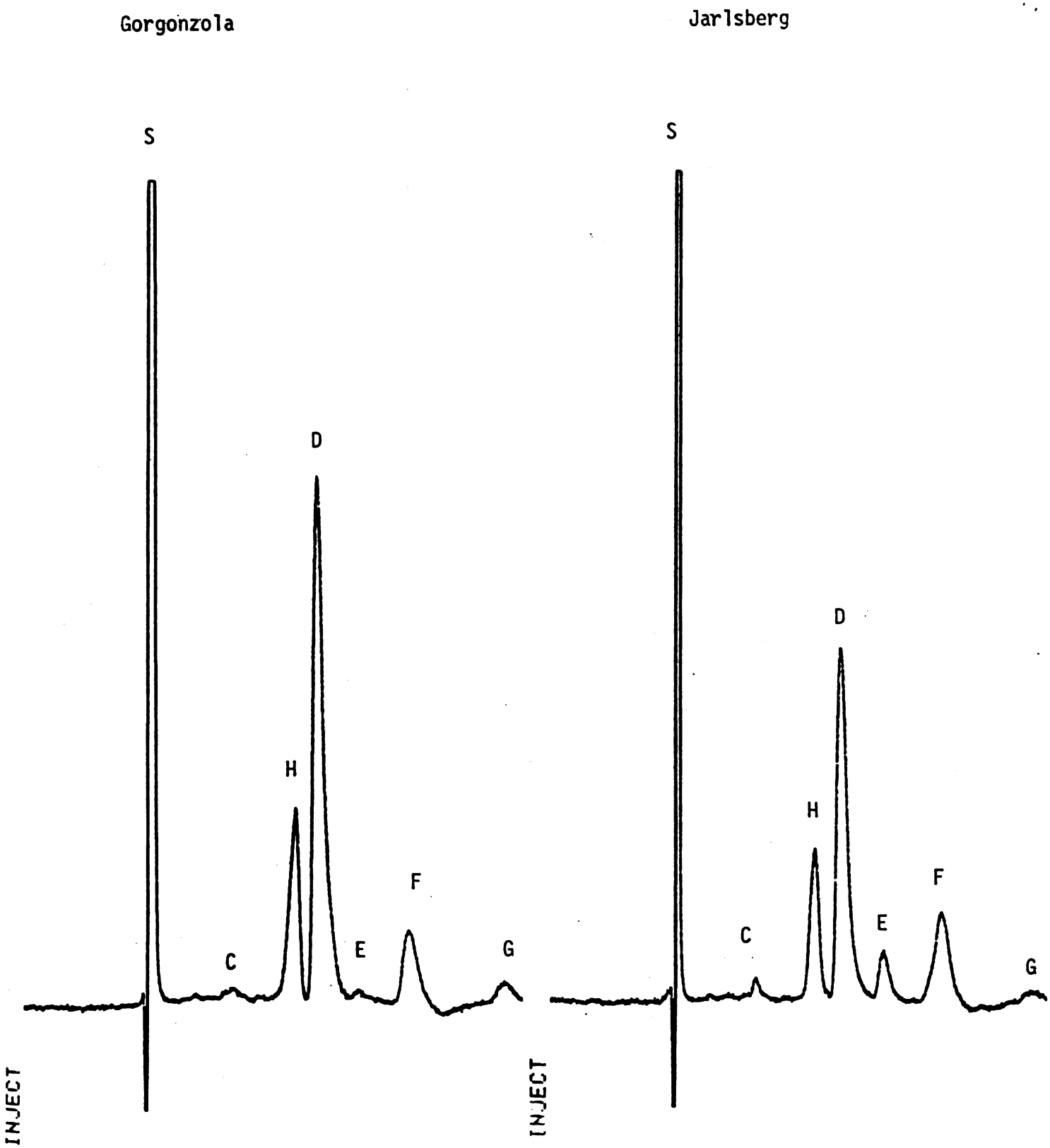


Figure 30. Chromatograms Gorgonzola and Jarlsberg market cheeses. Chromatographic conditions same as Figure 7.

Liederkranz

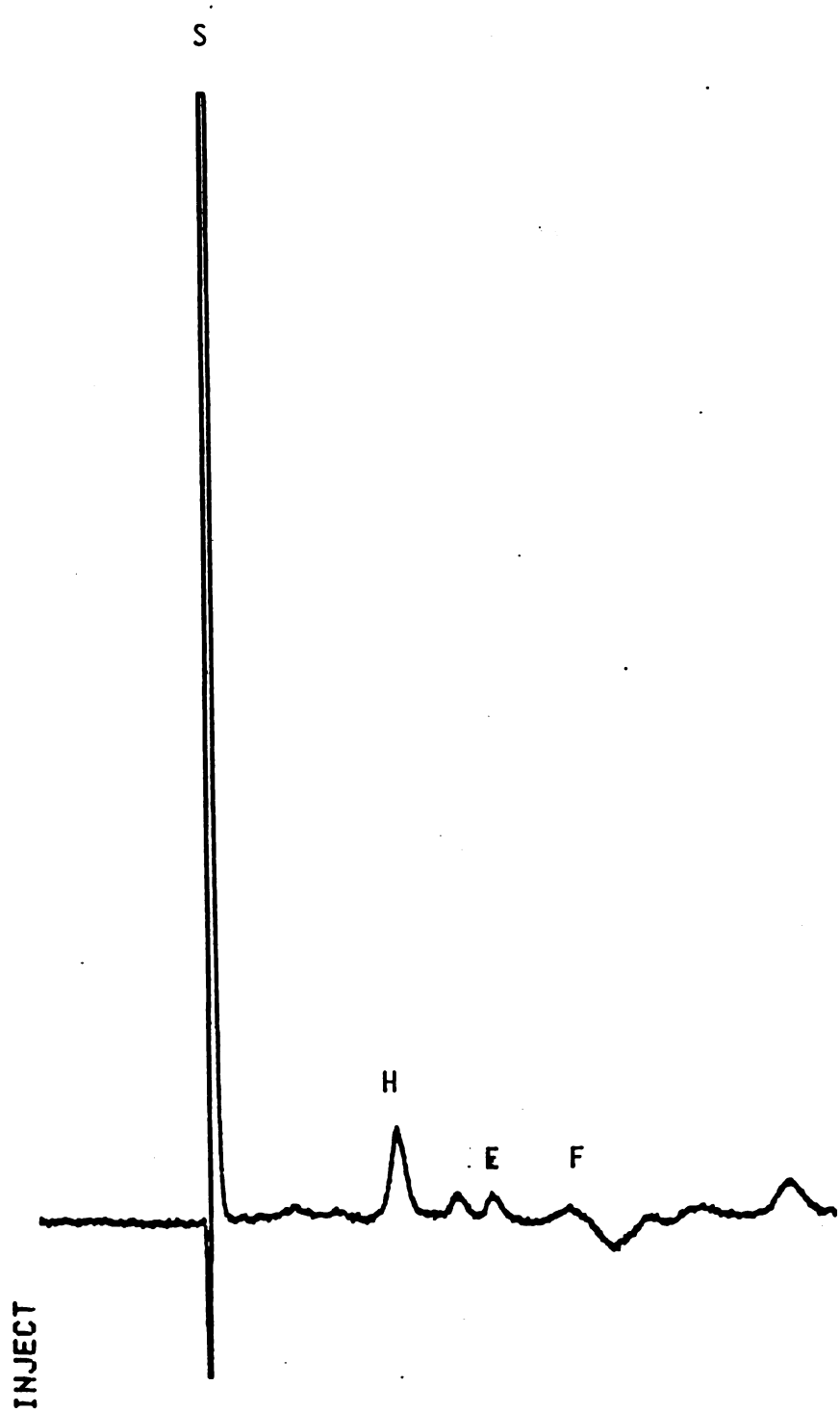


Figure 31. Chromatogram Liederkranz market cheese. Chromatographic conditions same as Figure 7.

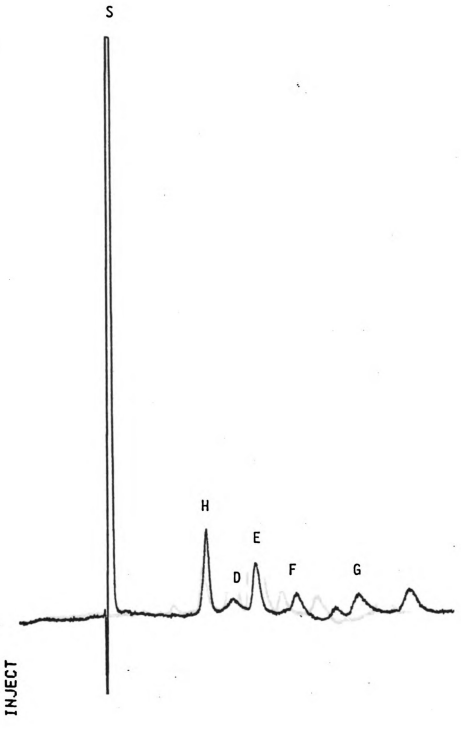


Figure 32. Chromatogram Limburger market cheese. Chromatographic conditions same as Figure 7.

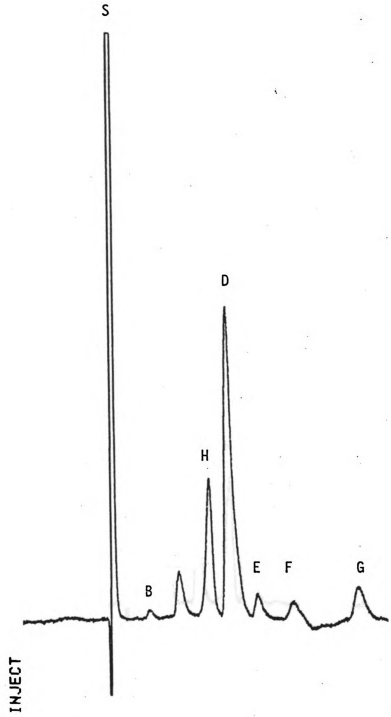


Figure 33. Chromatogram Parmesan market cheese. Chromatographic conditions same as Figure 7.

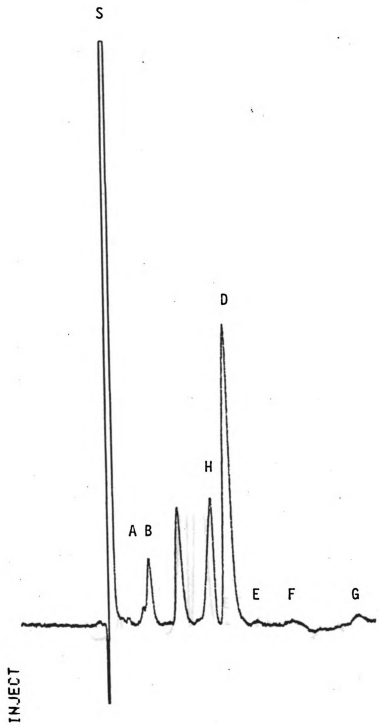


Figure 34. Chromatogram Provolone market cheese. Chromatographic conditions same as Figure 7.

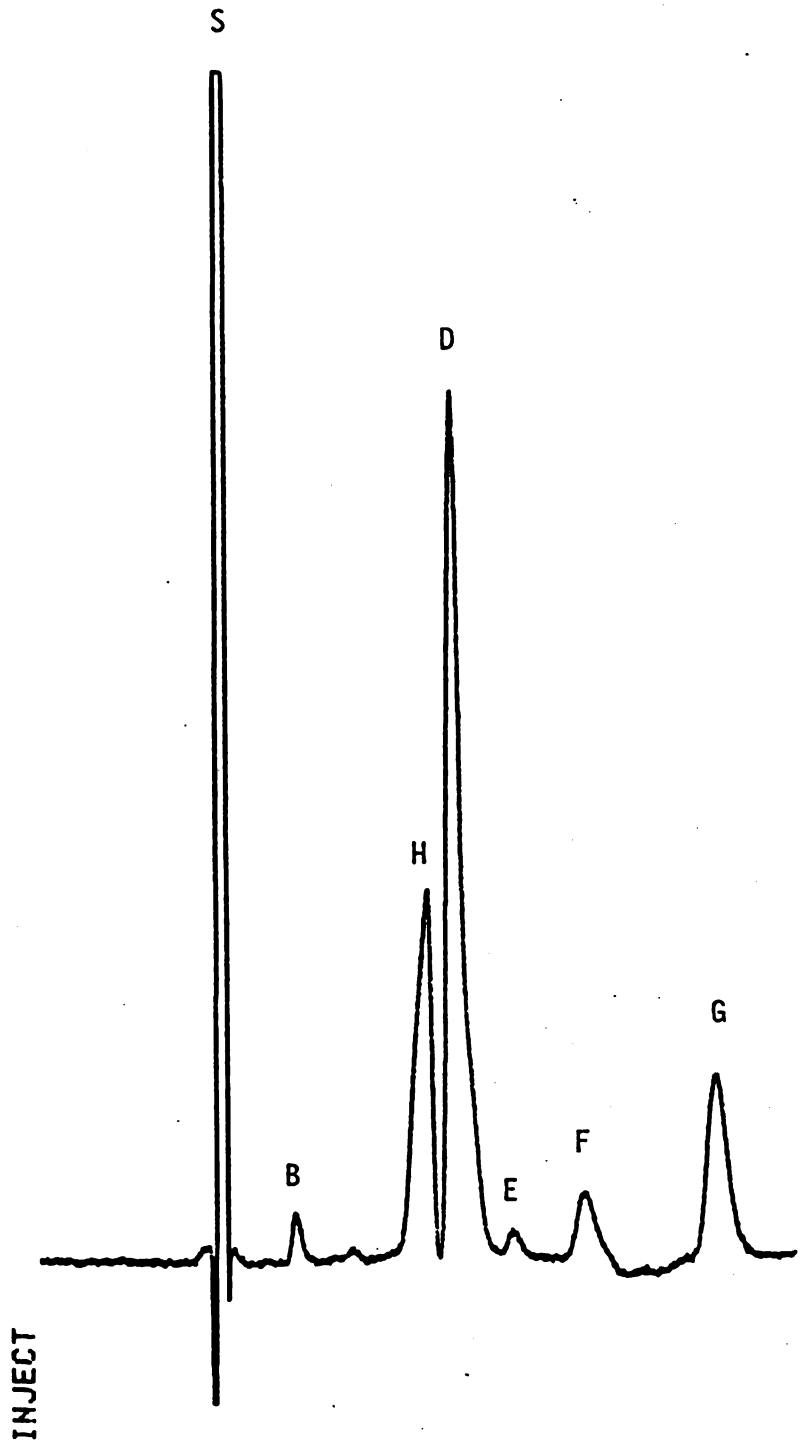


Figure 35. Chromatogram Romano market cheese. Chromatographic conditions same as Figure 7.

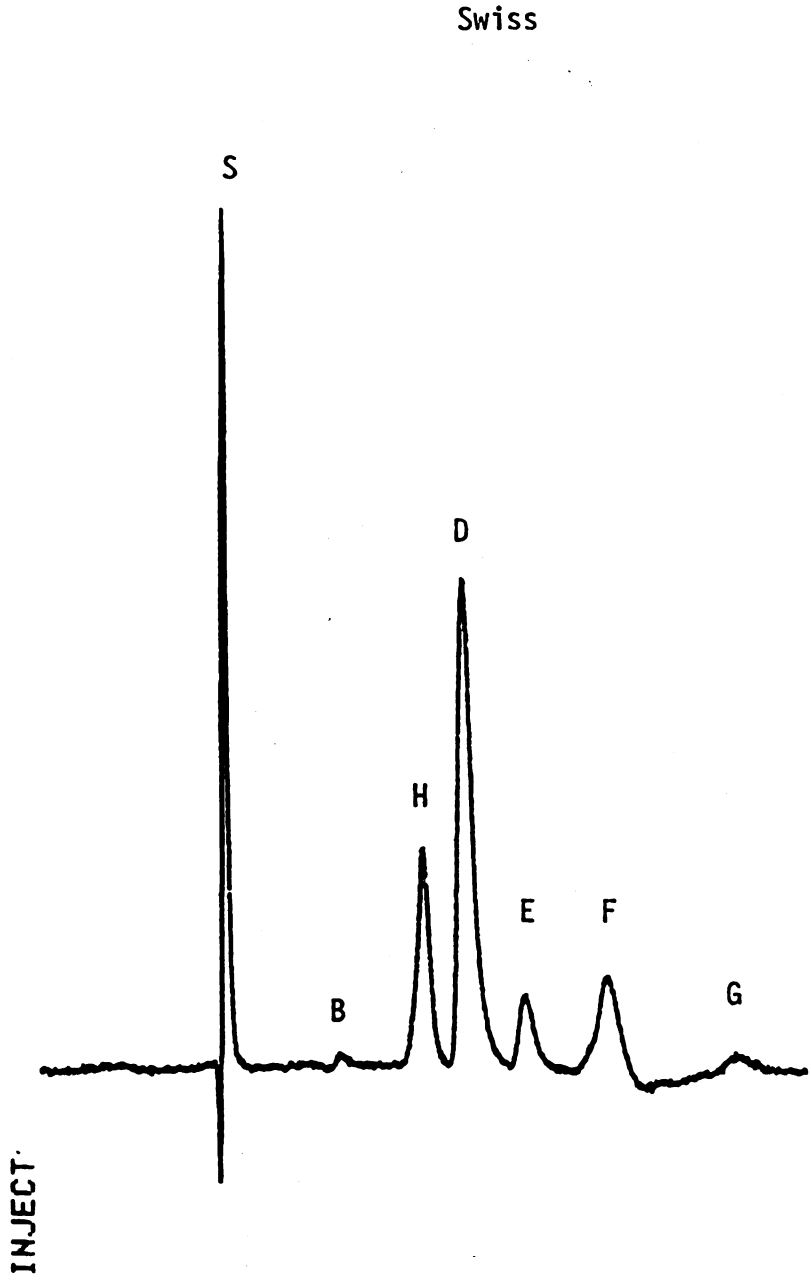


Figure 36. Chromatogram Swiss market cheese. Chromatographic conditions same as Figure 7.

Recovery Study

The average recoveries of lactose and organic acid standards added to Cheddar cheese were over 91% except for citric acid. The average percent recovery for citric was 85.2%. Recovery data are presented in Table 4. In the study by Marsili et al. (1981) on organic acids in dairy products, their recoveries of organic acids added to whole milk were over 90% for all acids except for butyric. The average percent recovery for butyric was 85.6%.

Table 4. Recovery study of lactose and organic acids added to Cheddar cheese.

Constituent	Amount in Cheese mg/ml	Added mg/ml	Found mg/ml	% Recovery
Lactose	------	0.08683	0.083800 ±0.001660	96.5
Citric	0.196155 ±0.006840	0.17840	0.319090 ±0.004580	85.2
Pyruvic	------	0.48326	0.467745 ±0.015645	96.8
Lactic	2.596330 ±0.010430	0.50578	3.011045 ±0.021105	97.1
Acetic	------	0.59700	0.566750 ±0.014890	94.9
Propionic	------	0.54664	0.499205 ±0.002905	91.3
Butyric	------	0.52383	0.492210 ±0.002800	94.0
Total	2.79249 ±0.01727	2.92176	5.439870 ±0.026690	95.2

CONCLUSION

The procedure for isolation, separation and identification of organic acids described in this thesis is well suited to analysis of natural cheese and other applicable systems. Advantages of this method are: (a) a total analysis time of less than 1 hour per sample is required; (b) RI detector sensitivity to lactose and organic acids is high; (c) linear calibration curves for aqueous standards were observed over a broad concentration range; (d) simple isocratic elution is used; (e) over 91% recoveries of lactose and organic acids added to cheese were observed for every standard except citric.

Organic acids detected and identified in Cheddar and market cheese samples were consistent with those reported in the general literature as previously reviewed. Acids separated and identified in Cheddar cheese included citric, lactic, and other possible trace acids. Both the MSU dairy plant Cheddar and the Wisconsin Cheddar had similar constituent chromatographic profiles. The time-series study on Cheddar cheese showed a dramatic decrease in lactose during pressing with subsequent increases in lactic and citric acids. Further ripening of cheeses yielded a significant increase in lactic acid and a slight decrease in citric acid concentration. Residual lactose

in the MSU Cheddar was not detected after 3-days of ripening, whereas slight traces of lactose were detected in the Wisconsin Cheddar 2 to 3 weeks after manufacture.

Application of the extraction method and analysis by HPLC to the market cheese varieties indicated additional acids present in the samples. Organic acids detected and quantified other than citric and lactic acids, included: pyruvate, acetic, propionic, and butyric acids. Lactic acid was the predominant acid in most of the cheeses analyzed.

The methodology prescribed to analyze organic acids and lactose in natural cheese is relatively simple and rapid. In addition, the Bio-Rad HPX-87H column used in the study performed well with no deterioration of separation capabilities being noticed. The column exhibits a great deal of flexibility in terms of conditions of use and can be cleaned with 40% acetonitrile in 0.01N sulfuric acid by gentle backwashing. Similarly, the column ion exchange resins can be regenerated with 0.05N sulfuric acid.

One potentially useful application of the extraction and HPLC procedure is to supply data to microbiological studies by quantitating bacterial metabolites. Production of citric, pyruvic, lactic, acetic, propionic, and butyric acids is easily monitored by this technique. Application of this method with eluent modifications could also easily achieve

separation and quantification of free fatty acid and acid salts contained in cheese. Other applications could include qualitation and quantitation of products of microbial fermentations other than cheese.

APPENDIX

APPENDIX

HPLC Determination of Organic Acids in Dairy Products (Marsili et al., 1981):

Method Summary. A simple isocratic HPLC technique is used to quantitate organic acids in dairy products. An Aminex HPX-87H column at 65 C, 0.0090N sulfuric acid mobile phase and UV detection at 220 and 275 nm is utilized.

Procedure. Five-gram of sample, 5.0 ml distilled water, and 20 ml of acetonitrile is added to a 50 ml glass centrifuge tube, shaken for 1 min., centrifuged at 7000 x g for 5 min. The supernatant is injected into a 10 l loop with a 5 ml syringe fitted with a Swinney syringe filter holder (Gelman Sciences, Inc., Ann Arbor, Mich.) containing 0.2 m Teflon membrane filters.

Determination of Volatile Fatty Acids in Cheese - Direct Distillation Method (Kosikowski, 1946):

Method Summary. A fixed weight of cheese, solubilized in warm, 10 percent sulfuric acid is mixed with magnesium sulphate and water, and steam distilled. The steam which comes over contains volatile fatty acids in a concentration directly related to their solubility and volatility. The volatile acids in the condensed aqueous solution are titrated against a standard sodium hydroxide solution.

Procedure. A 10-gram portion of cheese is well ground in a mortar with warm 10 percent sulfuric acid (50-55 C). The ground cheese mixture is washed quantitatively from the mortar into an 800 ml Kjeldahl flask with a total of 50 ml of sulfuric acid solution including both grinding and washing. A glass beads and 35 grams of magnesium sulphate is added to the Kjeldahl flask. The entire mixture is well shaken and 230 ml of distilled water at 25 C is added. The acid-cheese mixture is refluxed over a low flame for 3 to 5 minutes to drive off carbon dioxide. After slight cooling, 20 ml of freshly boiled distilled water at 25 C is used to rinse down the refluxer. The flask is placed on a Kjeldahl

distillation apparatus and 280 ml of distillate collected. The distillate is removed for alkali titration using N/10 NaOH and phenophathalein.

A funnel fitted with No.2 Whatman filter paper is inserted into a small Erlenmeyer flask, and placed under the condensing pipe to catch the insoluble acids at the end of distillation. The condenser pipes are washed with 25 ml of neutral alcohol, which passes through the filter paper into a separate glass flask. The alcohol is then titrated.

The sum of the separate titrations of the water solution and the alcohol rinse is the total volatile acidity of the cheese.

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