5-1983

A Method for Separating Casein Micelles from Whey Proteins for Determining Casein in Milk

Robert N. Carpenter
Utah State University

A METHOD FOR SEPARATING CASEIN MICELLES FROM WHEY PROTEINS FOR DETERMINING CASEIN IN MILK

by

Robert N. Carpenter

A thesis submitted in partial fulfillment

of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

UTAH STATE UNIVERSITY

Logan, Utah

1983

ACKNOWLEDGMENTS

I would like give credit to those persons who have helped me accomplish this work.

I sincerely thank my parents who have never failed to encourage me in my endeavors. I honor them by striving to live up to their expectations.

Shelly, a dear wife and mother to our three sons, Tony, Jacob and Nathan, has been a tremendous help. She has endured many inconveniences and has always supported the decisions we make together concerning the family's welfare. Her cheerful countenance has been a great source of strength.

Rod Brown, my major professor, has counselled many hours with me on this project. He has the gift of being able to inspire and motivate others. I respectfully thank him.

Finally, I would like to thank the students and faculty in the department who have made this experience so pleasant.

Robert N. Carpenter

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

ABSTRACT

A Method for Separating Casein Micelles from Whey Proteins For Determining Casein in Milk

by

Robert N. Carpenter, Master of Science

Utah State University, 1983

Major Professor: Rodney Jay Brown

Department: Nutrition and Food Sciences

The purpose of this study was to determine if size exclusion chromatography could be used to separate casein micelles from whey proteins for a rapid, direct test to measure percent casein in milk. A size exclusion chromatography column was developed for the separation having dimensions 100 by .4 cm. Packing material selected was glycophase coated porous glass supports. A Beckman DU-8B spectrophotometer monitored the casein and whey protein peaks as they eluted and a Tektronix 4052 computer accepted data points every 4 sec, storing these on tape. Absorbances and areas of each peak were used in the evaluation of samples. Treatments of temperature, pH and calcium addition were performed on a commingled milk sample from Utah State University Dairy Laboratory. It was determined that addition of calcium and pre-warming to 40 C before injection is important for good separation. Several samples of milk from individual cows were run through the column and parameters obtained. For each sample, percent casein was measured using the standard method of acid precipitation and Kjeldahl nitrogen determination. Percent casein was then estimated

using area and absorbance of each casein peak from the elution plots of milk from individual cows. A regression line of predicted vs actual percent casein resulted in a correlation coefficient (r) of .92.

(69 Pages)

INTRODUCTION

Because of a growing cheese industry, casein is increasing in importance and the need for a rapid and accurate casein test is evident. Tests which measure percent casein in milk are time consuming and impractical for the dairy industry. Much of this need has come about as cheese plants have begun using yield formulas to calculate price paid to producers for their milk (Ernstrom, 1980). To most accurately predict cheese yield, both fat and casein percentages must be known. Since protein percentage is easily and rapidly obtained with automation (Harding, 1973), percent casein is estimated as a percentage of total protein. However, percent casein as a function of total protein is quite variable (Blake et al., 1980) so the accuracy of yield estimates is questionable.

Casein is usually separated from whey proteins before measurement can occur. Casein may then be determined as the percent protein in the casein fraction or the difference in percent protein between milk and the whey protein fraction. The purpose of this study was to determine if size exclusion chromatography (SEC) could be used to separate casein micelles from whey proteins for use in casein determinations. The benefit of separating casein micelles with SEC is that casein micelles remain in solution after separation. This allows direct protein measurement on the casein by any of a number of protein assays. Infrared spectroscopy would be ideally suited for measuring the casein fraction separated with SEC if such instruments were modified to do so. For this study, an ultraviolet (UV) spectrophotometer monitored the peaks at a wavelength of 280 nm.

REVIEW OF LITERATURE

Importance of Casein

Apart from its obvious role in cheese manufacture, casein is an ingredient in a variety of other products. It is used in paper, paint, plastics and adhesives, coffee whiteners, imitation milk, imitation cheeses, ice cream mixes and whipped toppings (Webb and Whittier, 1970). Testing for casein is economically desirable when such milk is used for its casein content.

Cheese Yield Pricing

Although it has been known for many years that casein is important in the manufacture of cheese, it has not been until recent years that the price of milk was based on anything but its fat percentage (Ernstrom, 1980). The base price system of milk payment used by Federal Milk Marketing Orders gives a base value to the skim milk portion but does not account for the variability of protein content. It has been shown that under such a system, dilution of milk through breeding or herd management is economical for the producer but not for the cheese plant (Ernstrom, 1980). Breeding and herd management practices over the years have lowered fat and protein percentages of milk while increasing milk yields (Taylor and Van Horn, 1962).

Van Slyke and Price (1952) used percentages of fat and casein to predict yield in Cheddar cheese. A modification of their formula is being used in many cheese plants in the United States to calculate milk value. Other methods of payment have been devised (Brog, 1971a and 1971b; Chapman, 1974; Ladd and Dunn, 1979; Zurborg, 1978) but cheese

yield pricing seems to be the most equitable (Ernstrom, 1980). Other yield formulas have been proposed (Davis, 1965) but the Van Slyke formula has been the most widely accepted.

The Van Slyke formula is:

$$Y = \frac{(0.93\ F + C - .1)\ 1.09}{1 - W}$$

where:

Y = Kilograms of Cheddar cheese per 100 Kg milk.

F = Percent fat in the milk.

C = Percent casein in the milk.

W = Kilograms of moisture per kilogram of cheese.

The formula assumes that 93% of the fat is retained in the cheese and that all but .1% casein is retained. Nine percent of the weight of the cheese represents other components such as salt, lactic acid, whey proteins etc. The denominator, (1 - W), is the solids content per kilogram of cheese while the numerator, (.93 F + C - .1) 1.09, is kilograms of solids per 100 Kg of milk. Yield is then expressed in terms of kilograms of cheese which may be produced from 100 Kg milk.

A cheese plant using the formula will pay their producers on the basis of predicted kilograms of cheese made from 100 Kg of milk. The advantages of this system is that milk price is based on its value for making cheese. Producers gain the incentive to produce milk which has higher fat and protein content and higher cheese yield capacity. Cows should begin to be bred for the casein and fat contents of their milk as well as milk yield.

The Van Slyke formula will not predict cheese yield accurately for other cheese varieties (Kosikowski, 1968). Formulas for Swiss (Majeed, 1982), Mozzarella (Abu-Tarboush, 1982) and Cottage (Richter, 1980) cheeses have been developed.

Cheese yield pricing could be made even more equitable if percent casein were used in the formula. Moore (1983) reported an R square of .42 and standard error of the means of .12 when actual Cheddar yield was plotted against predicted Cheddar yield (Figure 1). In this case, Cheddar yield was predicted using the formula in which casein was estimated as 78% of protein.

Definitions of Nitrogen-Containing Fractions of Milk

Casein is traditionally defined as the protein which precipitates from milk at pH 4.6 and 20 C (Whitney et al., 1976). Whey proteins are those which do not precipitate under these conditions. Nitrogen contents are normally determined by Kjeldahl nitrogen determination (Association of Official Analytical Chemists, 1980). Rowland first published procedures utilizing these methods in 1938(a). Total nitrogen (TN) is the nitrogen in a milk sample before the various nitrogen-containing fractions have been separated. Casein nitrogen (CN) is the nitrogen in the separated casein fraction. Noncasein nitrogen (NCN) is the nitrogen in the whey protein fraction. Casein nitrogen may also be obtained by difference (TN minus NCN). Nonprotein nitrogen (NPN) is the nitrogen remaining in whey after all proteins have been removed. Thus, true protein nitrogen (TPN) is TN minus NPN. Protein equivalents are calculated by multiplying the nitrogen component by its respective factor. Most researchers have used 6.38 as the factor for

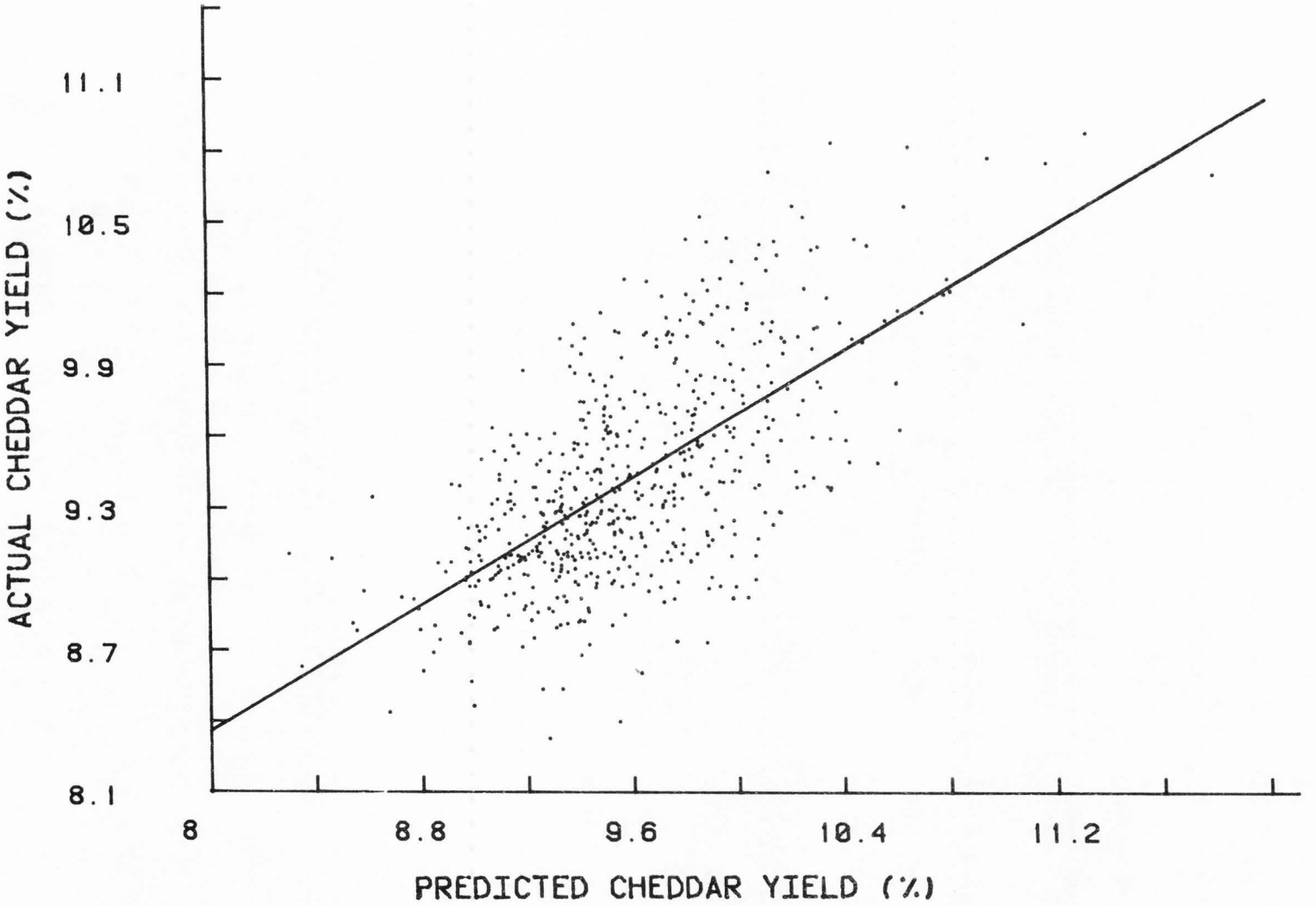


Figure 1. Predicted versus actual cheese yield (Moore, 1983).

all milk components but others have been more precise (Jennes, 1970). Casein number is defined as casein nitrogen as a percent of total nitrogen (Rowland, 1938b). I would like to define "true casein number" as casein nitrogen as related to true protein nitrogen. In many cases true casein number is less variable than casein number because variations in NPN have been subtracted.

Variability of Casein Number

Casein in general varies in the same direction as total protein in milk (Larson et al., 1956; Waite et al., 1956), tending to make casein number relatively constant in commingled milk. However, any factor which influences either percent casein, percent whey proteins or percent noncasein nitrogen can have an effect on casein number. Casein number may vary according to Table 1.

Individual Cows or Breeds. There is considerable variation among cows within breeds and among breeds. Blake et al. (1980) reported casein numbers from milk of Holsteins ranging from 64 to 81% with an average of 75%. Casein numbers from milk of Jerseys ranged from 72 to 85% with an average of 78% (Figure 2). Cerbulis and Farrell (1975) published similar results in an earlier paper and found that differences among breeds decreased when true casein number was used in place of casein number.

Period of Lactation. Much of the differences among cows may be explained by variability of milk from cows in different lactation periods. Waite et al. (1956) studied the variation of casein number during a lactation period. Although casein increases during the laction period as milk yield decreases, casein number decreases slowly up to 200

Table 1. Factors affecting casein number.

Factor	References
Cows or breeds	(Blake et al., 1980; Cerbulis and Farrell, 1975)
Lactation	(Waite et al., 1956)
Age	(Waite et al., 1956)
Season	(Harding and Royal, 1974; Davies and Law, 1980; McDowell, 1972; Szijarto et al., 1973)
Location	(Szijarto et al., 1973)
Disease	(Rowland, 1938b; Haenlein et al., 1973; Weaver and Kroger, 1977; Anderson and Andrews, 1977)
Storage	(Adams et al., 1976; De Beukelar et al., 1977; Aylward et al., 1980)

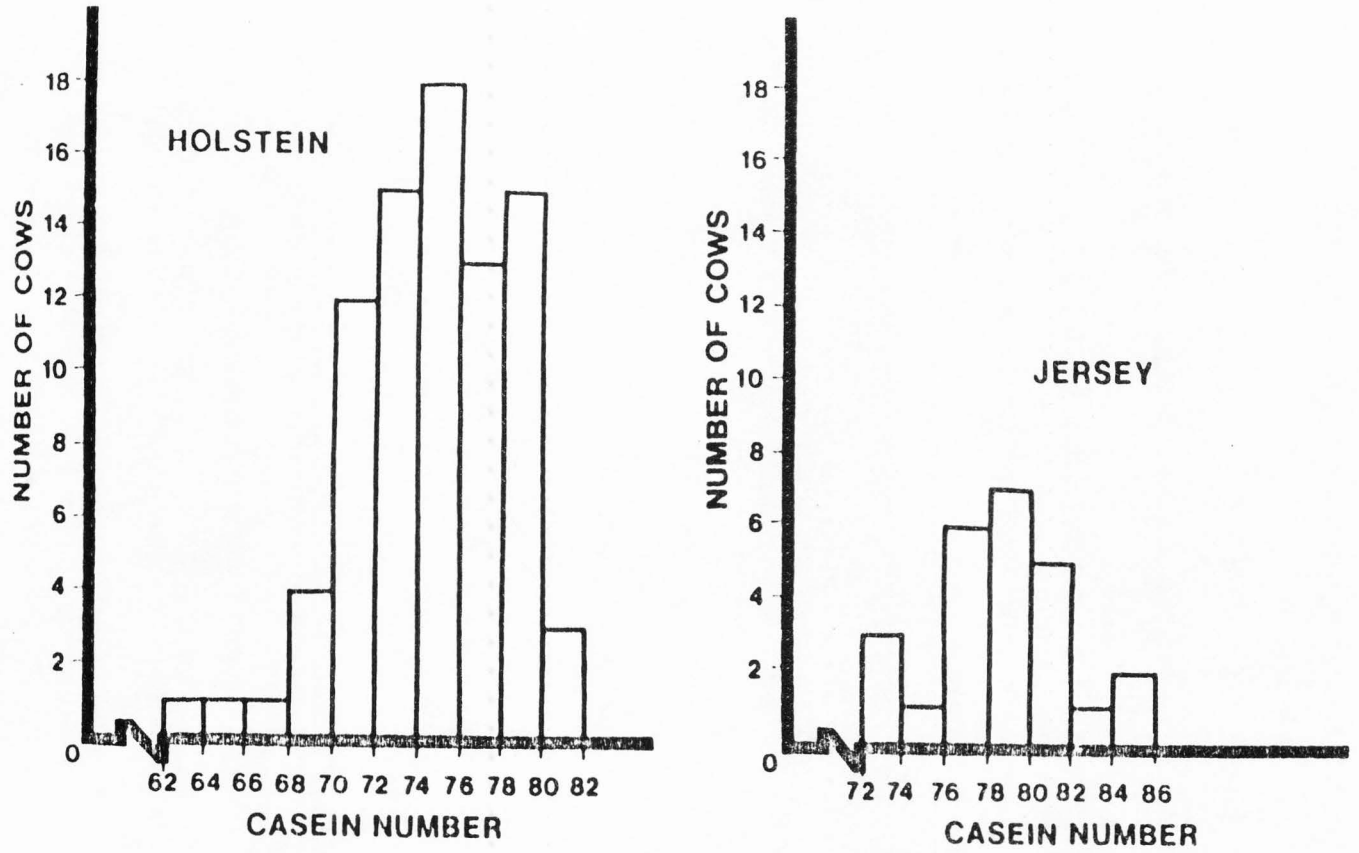


Figure 2. Histograms of casein number of milk from Holstein and Jersey breeds (Blake et al., 1980).

days and more rapidly thereafter (Figure 3).

This decrease in casein number may in part be due to the presence of proteinases naturally found in milk (Andrews, 1982). Proteinase activity of milk from cows in late lactation is double that of milk from cows in early lactation (Korycka-Dahl et al., 1983).

Age of the Cow. Age of the cow also can be a factor. Casein numbers were calculated from data (Waite et al. 1956) for cows of various lactations. Casein numbers of milk from cows between their first and fourth lactation averaged 80%. Casein numbers of milk from cows between their fifth and eighth lactations averaged 78%. Casein numbers of milk from cows in their ninth or greater lactation period averaged 77%.

Season. Percent casein can vary greatly with season (Davies and Law, 1980; Harding and Royal, 1974; McDowell, 1972). The most important seasonal influence is the abrupt change of diet occuring in the spring when cows go to pasture and when they return for the winter. Certain seasonal trends in casein number also may be detected. Seasonal casein number variability was reported by Szijarto et al. (1973) (Figure 4). He noted that casein number decreased in summer months and increased in winter months. Both NPN and WP as a percentage of total protein mirror casein number by increasing in the summer and decreasing in the winter. True casein number may not fluctuate as much since variability of NPN has been subtracted. The diet effect of season is probably greater than temperature effect but more work would need to be done to determine the exact causes responsible.

Location. Szijarto et al. (1973) reported variability in casein numbers from commingled milks of various dairy plants in Ontario. The

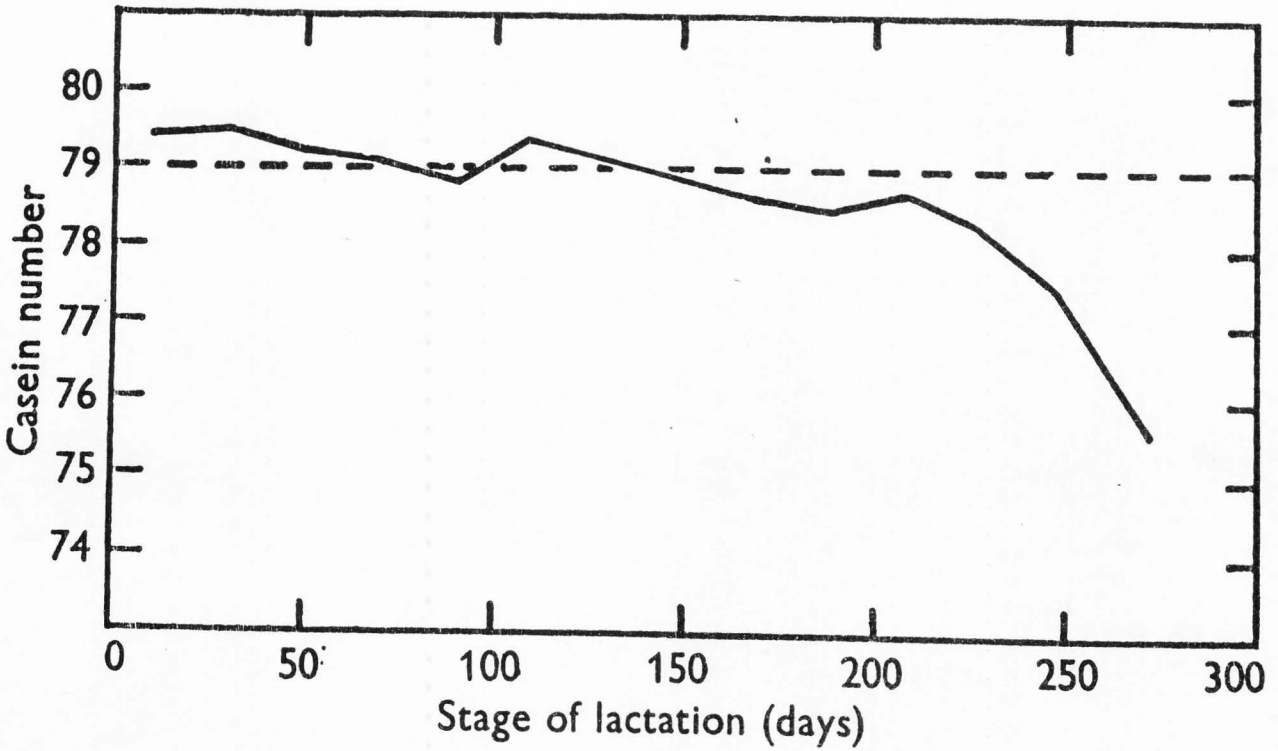


Figure 3. Variability of casein number with respect to period of lactation (solid line) and overall mean (broken line) (Waite et al., 1956).

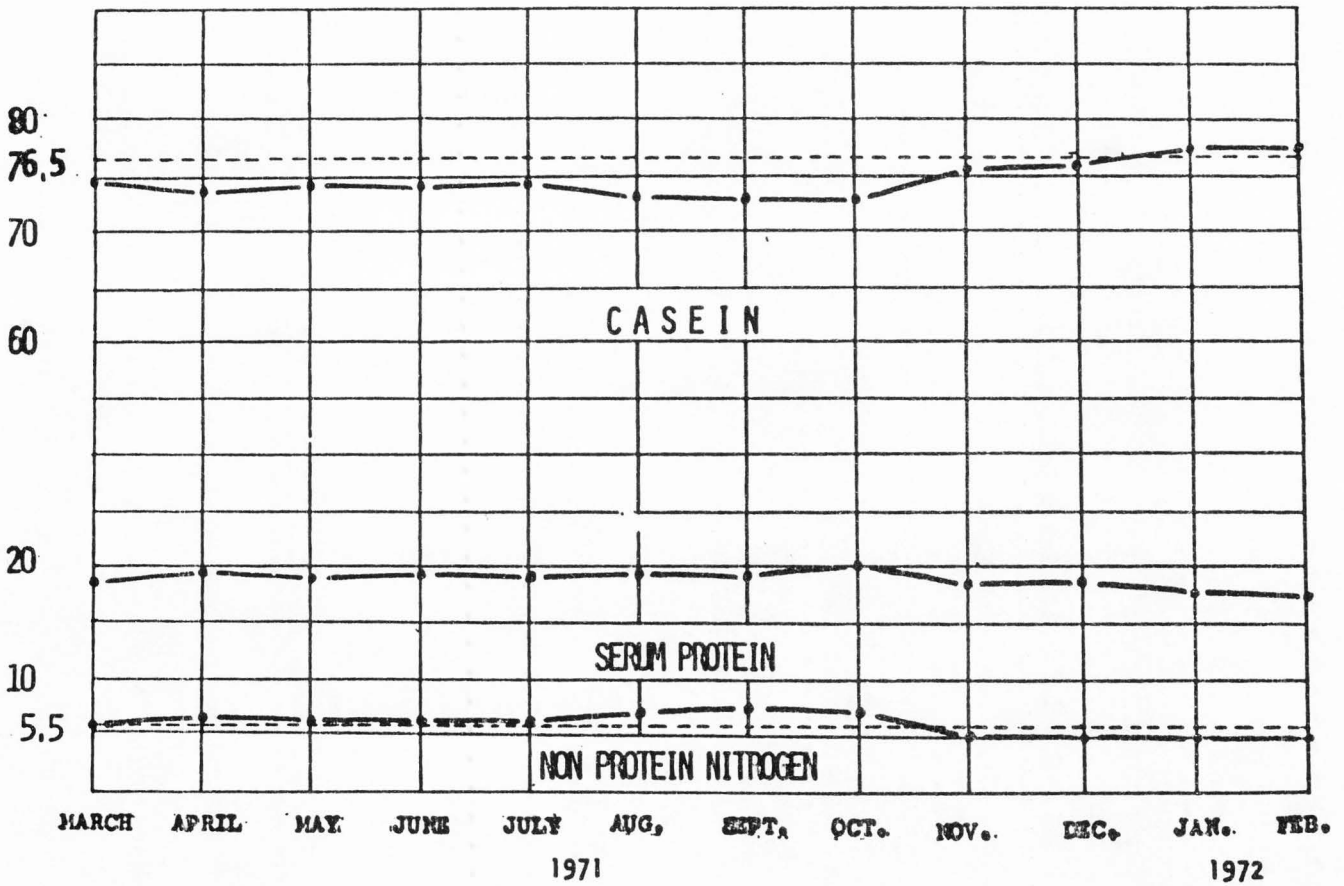


Figure 4. Monthly mean percentages of casein, serum protein and nonprotein nitrogen in milk from Ontario plants (Szijarto et al., 1973).

lowest average casein number was 72.06 with the highest being 76.40. The lowest average casein number coincided with the highest serum protein (20.57) and the highest NPN (7.38) percentages. Casein numbers for these milk plants ranged from 59 to 82 which gives an indication of the variability expected within a region for commingled milk.

Disease. Abnormal milk as in the case of mastitis affects casein number. Rowland (1938b) reported that milk from cows with clinical mastitis is low in solids-not-fat and is characterized by decreased casein and increased whey protein. He even suggested that casein number be used as an indicator of subclinical mastitis because of its high correlation with mastitis.

This effect on casein number has been supported by more current research. Weaver and Kroger (1977) demonstrated the relationship between somatic cell counts and total protein divided by noncasein protein, a number mathematically convertible to casein number (Figure 5). The values 3.5, 4.0, 4.5, and 5.0 correspond to casein numbers of 71, 75, 78 and 80 respectively. Haenlein et al. (1973), Weaver and Kroger (1977), and Anderson and Andrews (1977) have correlated somatic cell counts with casein and studied the effect on individual caseins. Haenlein et al. (1973) reported decreases in α-casein, α-casein, α-lactalbumin and β-lactoglobulin and increases in amounts of immunoglubulins, serum albumin and κ-casein. Anderson and Andrews (1977) demonstrated that β-casein decreased relatively more than α-casein. They postulated that there may be some degree of proteolysis occuring as well as a decrease in milk protein synthesis.

Storage. Prolonged storage of milk can affect percent casein and cheese yields (Aylward et al., 1980). The effect of storage of milk on

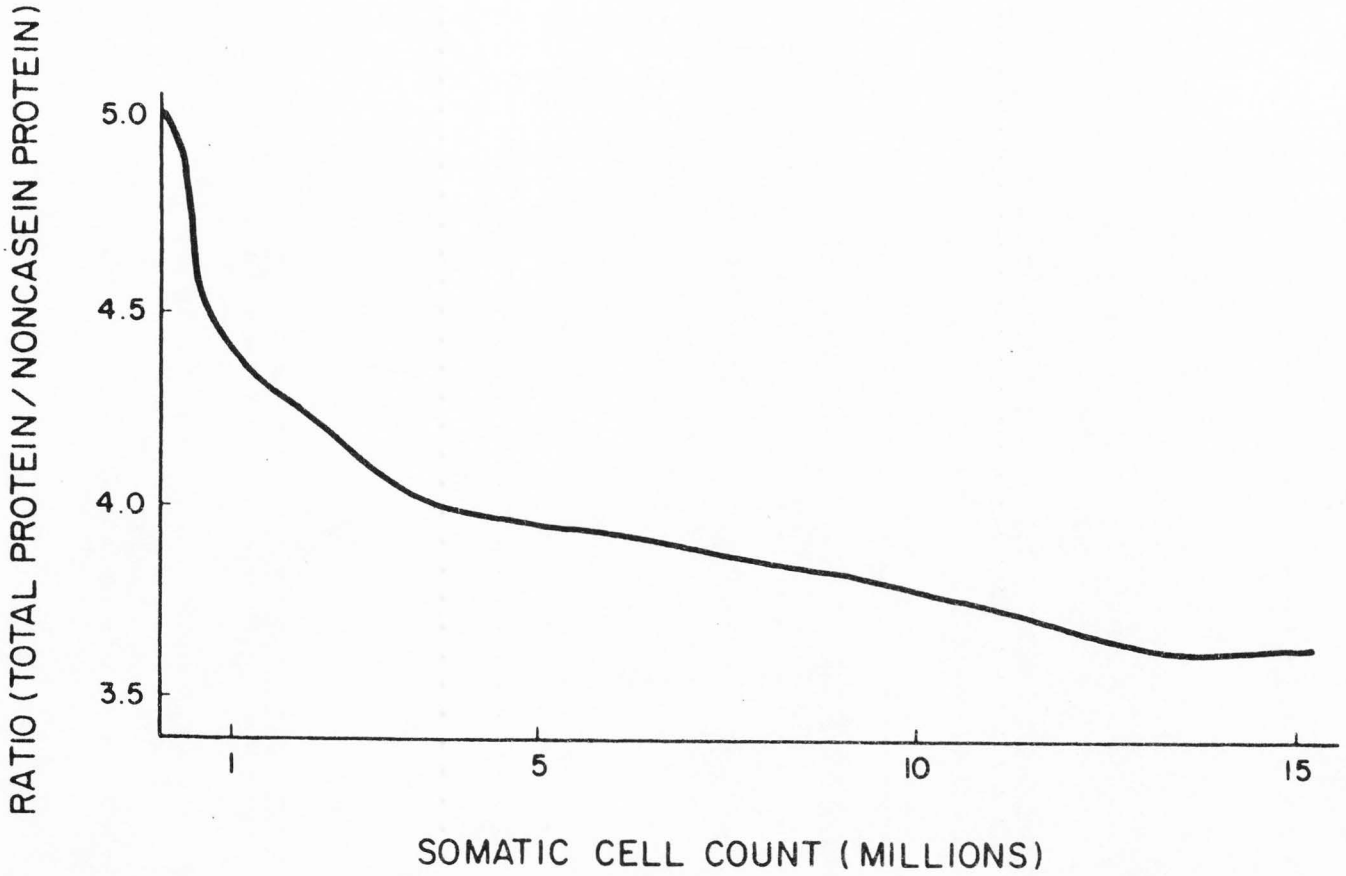


Figure 5. Relationship between somatic cell count and the ratio, total protein/noncasein protein (Weaver and Kroger, 1977).

casein and cheese yields is primarily due to proteolysis of casein by psychrotrophic bacteria (Adams et al., 1976). β-casein is decreased most during storage in the presence of psychrotrophs (De Beukelar et al., 1977).

Tests to Determine Casein

There is no test which is completely satisfactory for measuring casein. Those which are accurate are time consuming and those which are rapid and easy to perform lack the accuracy needed to make them valuable.

One problem encountered when testing for casein in milk is the presence of whey proteins. Casein must either be separated from whey proteins before measurement or measured in their presence by overcoming their interference. Most determinations are preceded by a separation step. This is normally accomplished by acid precipitation at pH 4.6 and 20 C (Whitney et al., 1976). A few tests require rennin to precipitate casein while fewer use ammonium sulfate. Casein may be quantified by protein measurement directly on casein precipitate or indirectly by measuring milk and whey proteins and calculating casein by difference.

Standard Casein Test. Various casein tests have been devised. The standard and generally preferred separation method and protein assay has been acid precipitation of milk and Kjeldahl nitrogen determination (Association of Official Analytical Chemists., 1980, Rowland, 1938a). In Kjeldahl nitrogen determinations, a sample is chemically digested in a way which converts protein nitrogen to ammonia. Nitrogen is then determined from the amount of ammonia distilled after neutralization with NaOH. Protein is obtained when percent nitrogen is multiplied by a

factor. The factor for casein was determined to be 6.38 but has since been questioned. After elucidating amino acid sequences of the various caseins, a more correct factor was found to be 6.52, higher than originally concieved and variable according to the ratios of individual casein molecules with respect to each other (Jennes, 1970). Therefore, "Percent casein" is more correctly reported as "percent casein nitrogen" since percent casein is only an estimate of casein.

While this method is used as the standard method with which to judge others, it is very time-consuming and involves the use of caustic chemicals. Other tests have been developed to replace these methods with the hope that determinations could be made easier, faster or more accurate (Table 2).

Formol Titration. Formol titration was developed for casein determinations by Walker (1914) and further studied by Gilmore and Price (1953), Pyne (1932 and 1933) and Skwarska et al. (1977). This test utilizes formaldehyde to modify amino groups on proteins, lowering their pKa's. The volume difference between the titration (NaOH) of milk with and without added formaldehyde is a value which when multiplied by a factor estimates percent casein. Since whey protein remains in the milk, this test is of value only if the ratio of casein to whey proteins does not deviate far from the average. The accuracy of this test has been shown to be somewhat poor compared to either Kjeldahl or dye binding methods (Skwarska et al., 1977).

Dye Binding. Dye binding is a protein test which has been widely used in analysis of milk for protein. Amido black is a common dye for this purpose (McGann et al., 1972; Renner and Oemeroglu, 1971; Wagner et al., 1973). Some of the other dyes are Acid Orange-12, Orange G, and

Table 2. Tests which determine percent casein on milk.

Method	References
Kjeldahl nitrogen	(Rowland, 1938a; Skwarska et al., 1977; Association of Official Analytical Chemists, 1980)
Formol titration	(Walker, 1914; Pyne, 1932 and 1933; Skwarska et al., 1977)
Dye binding	(Vanderzant and Tennison, 1961; Ashworth, 1965 and 1966; McGann et al., 1972; Wagner et al., 1973; Renner and Uemeroglu, 1971; Renner and Ando, 1974)
Infrared	(Goulden, 1967; Harding, 1973; Thomasow, 1976; Skwarska et al., 1977; Foss Electric, 1980)
Isoelectric interval	(Kirchmeier, 1968)
Refractometry	(Munchberg et al., 1969)

Buffalo Black (Ashworth, 1965 and 1966; Vanderzant and Tennison, 1961). The principle behind the test is that certain dyes bind to protein molecules, causing them to precipitate. The proteins are then removed with filters or centrifuged. Differences in colorimetric readings between standard dye solutions and solutions in which a portion of the dye has been bound to the precipitated protein are correlated to percent protein. Using dye binding as the protein detection method, casein is measured either by difference (McGann et al., 1972) or directly on redissolved casein (Wagner et al., 1973). Dye binding methods are of comparable accuracy to Kjeldahl (Skwarska et al., 1977). Dye binding offers some benefits in time and ease in testing but relies on calibration by Kjeldahl.

Infrared Analysis. A relatively new method for determining protein in milk is infrared (IR) spectroscopy (Goulden, 1967; Harding, 1973). Foss Electric (1980) describes a method of casein analysis using their infrared instrument, however the method must be modified if accurate casein percentages are to be obtained (Okigbo, 1982). IR analysis takes advantage of the absorption of peptide bonds at 6500 nm wavelength (Goulden, 1967). Comparisons between dye binding and infrared spectroscopy with Kjeldahl as the reference method shows little difference in repeatability or accuracy (Grappin et al., 1980).

Miscellaneous Methods. Several other casein tests belong in the miscellaneous category. These are procedures which have been developed but have not been used to any great extent.

The isoelectric point of casein is about pH 4.6 (Gordon and Kalan, 1974). A method based upon titration of casein in the isoelectric interval from pH 4.9 - 4.6 has been described (Kirchmeier, 1968). The

amount of acid consumed during passage through this interval is indicative of casein content. Errors occur in this method due to interference from other proteins and dissolved salts. Also, individual caseins are not equal with respect to isoelectric points (Gordon and Kalan, 1974).

Refractometry (Munchberg et al., 1969) has been used to determine casein in milk. This analysis employs the refractive index of casein which has been precipitated with acid and redissolved in a basic solution.

Sources of Experimental Error. There are many sources of experimental error within these casein determinations. Many tests are performed on the difference between some physical or chemical property of milk and its whey. This is usually done because a measurement cannot be taken effectively on the precipitated casein. Difference measurements have double the experimental error since two measurements are made for each casein determination. Whenever casein is precipitated and redissolved there is a possible error from dilution. Such is the case with the refractometric method. Dye-binding and infrared techniques can be used for determining casein in milk by difference. Automated methods are sought to increase the desirability of determining casein content of industrial milk.

Casein Micelles

Casein micelles are spherical (Bloomfield, 1979), highly hydrated and spongelike (Bloomfield and Mead, 1975) protein particles suspended in milk. Bloomfield and Morr (1973) reported an average particle diameter of 80 nm. While 80% of the particles range in size from 50 to

100 nm, 95% range from 40 to 220 nm (Bloomfield and Morr, 1973). Laser light scattering detects an even broader molecular weight distribution (Holt et al., 1973). Although 80% of casein micelles are less than 20 nm in diameter, their volume comprises only 3% of total micellar volume (Schmidt et al., 1973). Casein micelle structure has recently been reviewed by Schmidt (1982).

Casein micelles may be easily separated by size from other milk proteins. A small micelle of 250,000 daltons is relatively large compared to whey proteins which range from 15,000 to 70,000 daltons (Gordon and Kalan, 1974). Immunoglobulins are larger, 150,000 to 300,000 daltons, but exist in very low concentrations (about 4% of total protein) in milk (Gordon and Kalan, 1974).

Calcium is important in the structural stability of the casein micelle (Bingham et al., 1972). As calcium ion (Ca^{2+}) activity is reduced below a certain level, casein micelle framework begins to come apart and micelles dissociate. Small additions of Ca^{2+} cause a transfer of soluble casein to micelles without changing the radii while addition of more Ca^{2+} causes larger micelles to form (Bloomfield and Morr, 1973). It would seem reasonable then to suppose that addition of Ca to milk would help to maintain micelle structure.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) is a technique of liquid chromatography which separates molecules in solution according to their size (Yau et al., 1979). A SEC column is packed with small, rigid-structured porous particles. A sample is introduced into the column and carried through by the solvent (mobile phase) or eluent. The

process is called elution.

Sorting by size occurs by repeated exchange of molecules between the bulk solvent of the mobile phase and the stagnant liquid within the pores of the packing (Yau et al., 1979). Small molecules elute more slowly as they spend more time in the pores than large molecules. If the molecules are large enough, they spend no time in the pores and elute in the void volume (volume of the mobile phase). The criterion for selecting pore size is the range of size separation desired.

Chromatography is a relatively recent development. Ettre (1971) described how David Talbot Day in 1897 separated crude oil fractions through pulverized fuller's earth. Day did not accurately relate what occured so the founding of chromatography is generally credited to Michael S. Tswett. In 1903 to 1906 Tswett described the phenomenon of chromatography and used it to separate vegetable pigments in petroleum ether on calcium carbonate. More recently, cross-linked polydextran gels were found useful in chromatographic separation (Porath and Flodin, 1959). Cross-linked polystyrene gels were then developed (Moore, 1964) having the capability of higher pressures and flow rates. Finally, completely rigid inorganic-based porous packing material was introduced. These are described by Unger et al. (1974) and Kirkland (1976). More complete description of SEC and other liquid chromatographic techniques are available (Fischer, 1980; Snyder and Kirkland, 1974; Yau et al., 1979).

In milk, filtration chromatography has been utilized in the study of the physical and chemical properties and composition of casein micelles (Eckstrand et al., 1981; Heth and Swaisgood, 1982; McGann et al., 1979 and 1980; Ono et al., 1983; Yaguchi and Rose, 1971) but it has

not before been used as a method of separation for determining percent casein.

MATERIALS AND METHODS

Development of Separation Method

Column Parameters. Glycophase coated controlled pore glass (CPG) supports from Pierce Chemical Company were chosen as packing material because of their ability to endure faster flow rates and higher pressures than gels. The glycophase coating is a covalent bonding of glycerol molecules onto the glass surface of the packing to retard protein adsorption. Pore diameter and particle size were selected to effect separation of casein micelles from whey proteins. Two pore diameters (4600 and 2000 nm) were evaluated. In preliminary glass columns, CPG/200 performed better than CPG/460. The distance between the two peaks were visibly farther apart when CPG/200 was used, hence, better separation was achieved.

In preliminary separations, columns were made of glass and solutions were pumped using a Cole-Palmer Masterflex peristaltic pump. Irregular flow rates and pressure fluctuations in the column necessitated the use of high performance liquid chromatography (HPLC) machinery. The pump and sample injector were from a Perkin-Elmer series 2 chromatography system. While separation occured with column length as low as 15 cm, a length of 100 cm produced two very well defined peak, and was used in later experiments. Inside diameter of the column is not a critical parameter in the separation of casein micelles from whey proteins but was selected at .4 cm so that small sample sizes (10 - 150 µL) could be used.

Protein Measurement. A spectrophotomer was used to monitor the protein at 280 nm as it was eluted and to evaluate the parameters of

plots obtained from the elution. Infrared analysis would be the first choice for measurement of protein but absorbance at 280 nm was suitable for demonstrating the separation method.

Laboratory Methods

Equipment. Figure 6 illustrates the operations involved in milk protein separation and data collection. A solution of $CaCl_2$ (40 mM) was pumped at a steady rate (1 to 6 mL per min). The eluent passed through the sample injector, then through the column and finally through the flow cell of a Beckman DU-8B spectrophotometer. The spectrophotometer begans monitoring as the sample was injected. Every 4 sec an absorbance value was sent from the spectrophotometer to a Tektronix 4052 computer which stored these values on tape and produced an elution plot to be evaluated later. Computer interfacing and the program written especially for the Tektronix 4052 can be found in Appendix A.

Elution Data. In a typical elution plot of milk there are two peaks (Figure 7). For each peak the computer calculated the following: The volume at which the peak eluted, the absorbance reading at this point and the area under the curve according to Simpson's approximation. Area of the first peak was calculated from base line to a point midway between the two peaks. Area of the second peak was calculated from the same midpoint to a point after the second peak at which a baseline occured once again. These six parameters were used later in statistical analyses. Elution data were stored on computer tape.

Column Preparation. The column was prepared by attaching a funnel to the top end of the column and capping the bottom. Approximately 100

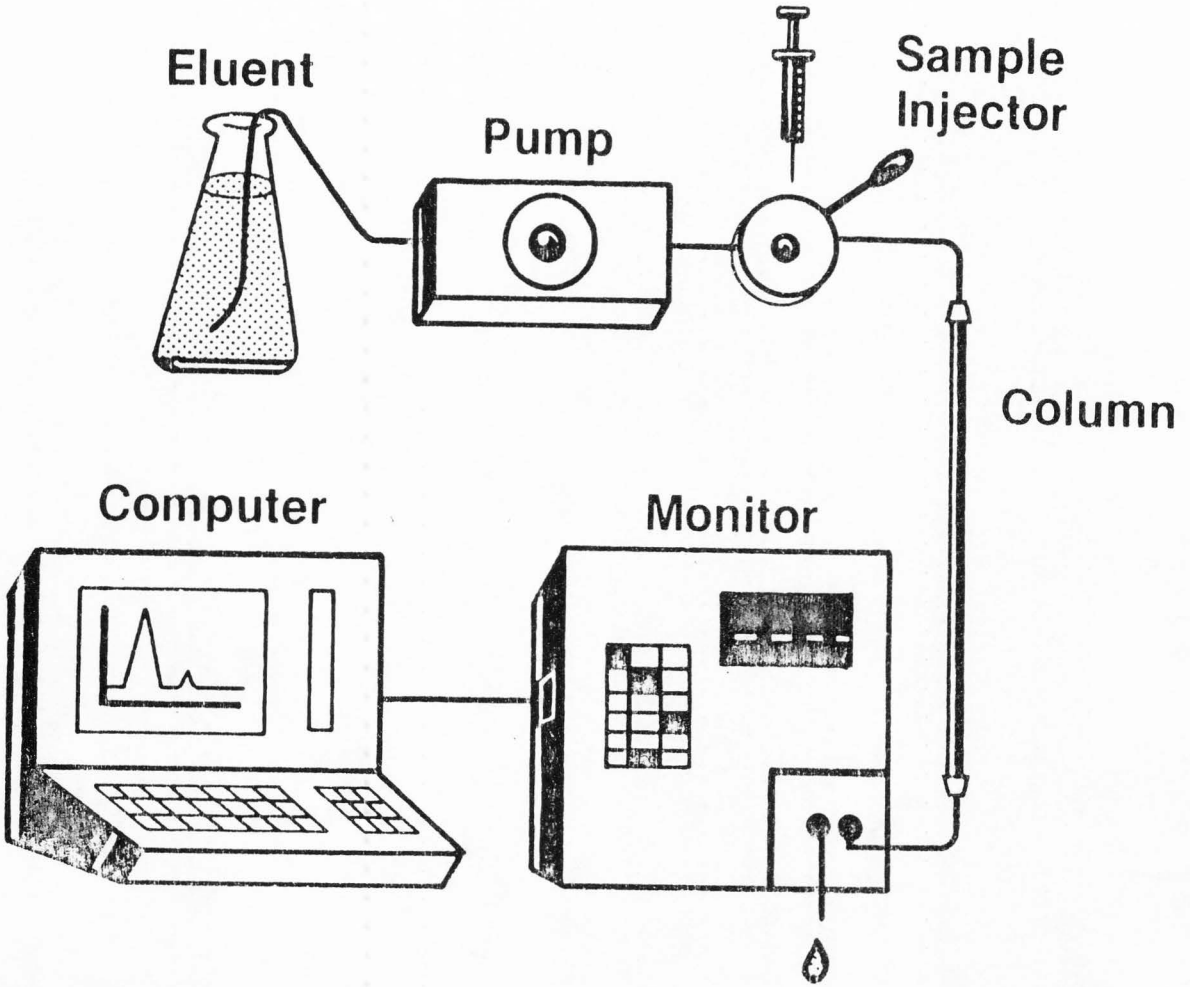


Figure 6. Equipment and flow diagram for separating casein micelles from whey protein and generating elution plots.

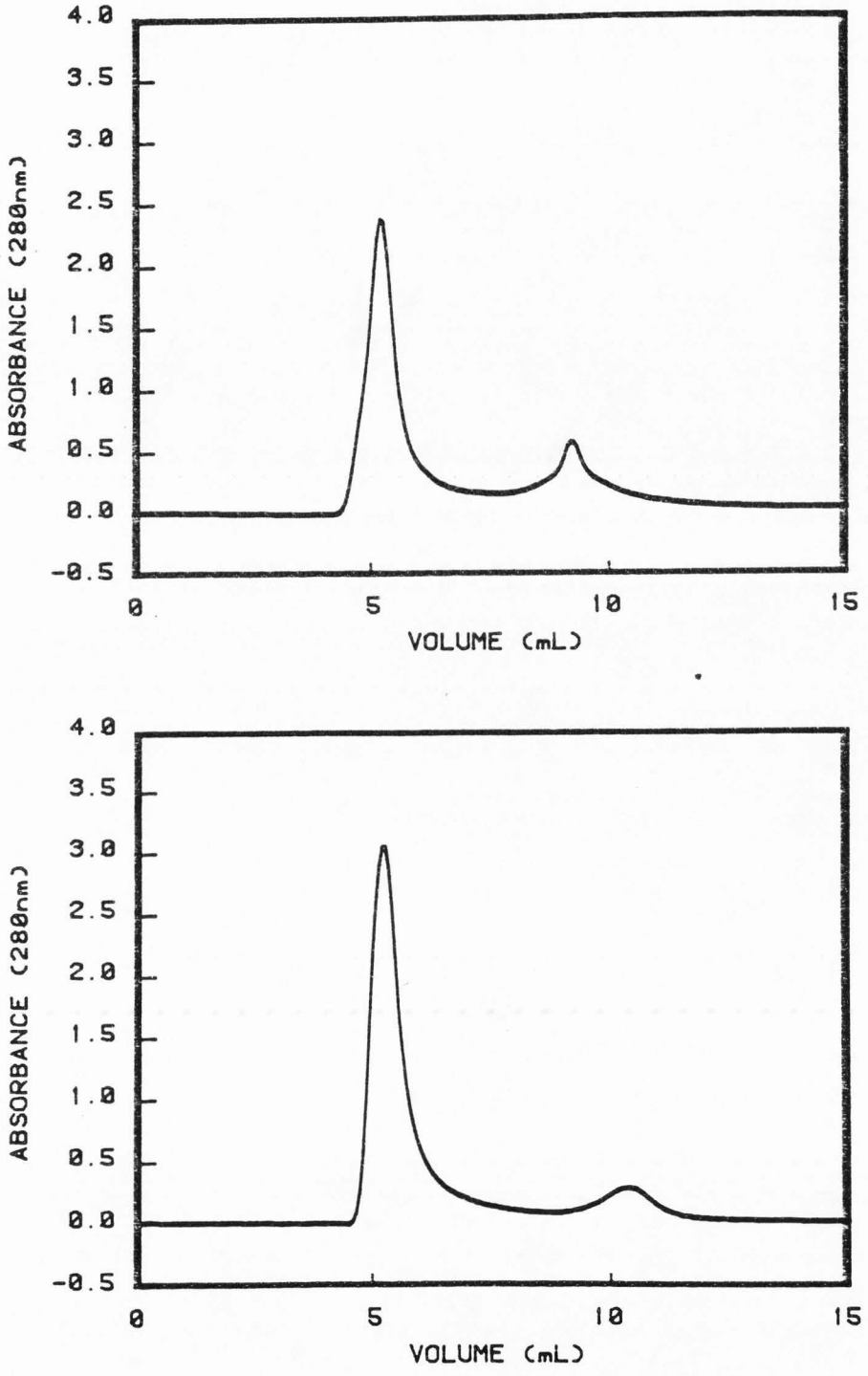


Figure 7. Characteristic protein elution plots of non-treated (top) and calcium treated (bottom) milk samples.

to 200 mg of Glycophase coated CPG glass supports were measured at a time into the column. The column was held vertical and tapped gently on a hard surface 20 to 30 times. A pencil was used to tap the sides of the column which was rotated slowly at a slight angle. This procedure was repeated until the column was completely filled. Degassed, deionized water was pumped into the bottom of the vertical column at a slow flow rate (.1 mL per min) until air bubbles no longer appeared in the effluent. The column thereafter was maintained in a horizontal position for sample elution.

Milk Sample Treatment. Milk samples containing $K_2Cr_2O_7$ preservative were obtained from the Utah Dairy Herd Improvement Association (DHIA) Laboratory located in the Nutrition and Food Sciences building of Utah State University, Logan. Each sample was filtered through Whatman no. 1 filter paper and run twice at 40 C through a small sample homogenizer (Fischer Scientific Company). To each 50 mL sample, .1 mL of 5 M $CaCl_2$ was added and allowed to equilibrate for 24 h at 4 C. Before injection, samples were brought to 40 C for at least 2 h. Samples (30 μL) were injected into sample injector for elution through the column. The 40 mM $CaCl_2$ solution aided in maintaining casein in micellar form.

Standard Casein Test. The reference method for determining casein in milk consisted of separation of casein by acid precipitation and Kjeldahl nitrogen determination (Rowland, 1938a; Association of Official Analytical Chemists, 1980). These procedures were modified in the following ways: Sample size and solutions of the separation step (section 16.047) were reduced 1/20th so that micro-Kjeldahl procedures (sections 47.021, 47.022, 47.023) could be followed. Semi-micro

Kjeldahl flasks (100mL) were used in place of micro Kjeldahl (30 mL). A .4 mL solution of 10% murcuric sulfate was used as the catalyst. The catalyst was prepared by diluting 12 mL H_2SO_4 and 10 gm murcuric oxide to 100 mL with distilled water. An extra .5 mL H_2SO_4 (2.5 mL total) was added to each reaction flask. Samples were digested until clear and then cooled. Three milliliters of water were added to the samples and heated for another 45 min. Flasks (125 mL) containing 5 mL of saturated boric acid solution and 3 drops of methyl red/bromecresol green indicator were prepared to recieve the ammonia distillate. The solutions were titrated with 0.0258 M HCl to a pinkish-grey endpoint. Percent nitrogen was calculated from the molarity of acid, the sample weights, the volume of acid and the molecular weight of nitrogen. Percent protein was obtained by multiplying percent nitrogen minus the reagent blank by a factor of 6.52.

Polyacrylamide Gel Electrophoresis. Effluent containing casein and whey protein peaks was collected from the column. The samples were dried in a vacuum oven at 50 C. A mercaptoethanol solution (100 µL of 1:9 dilution) was added directly to the samples and refrigerated for 2 h. Policks modified buffer (50 µL) was added to each sample. No dye marker was added to the samples but separate gel slits on both sides of the samples were used for the dye. Dye marker, acrylamide solution, preserving solution and staining solution were prepared according to LKB Application Note 306 (Fehrnstrom and Moberg, 1977). Buffer solution and gel solutions were made according to Kiddy (1974). The gel was prepared (LKB, Application Note 306) at least 12 h before use and applied to the electrophoresis unit (LKB multiphor model 2117). Samples (10 µL) were syringed into gel slits and 20 mA of current was applied with the power

supply (LKB model 2103) for 15 min, then 200 V for about 4 h.

Experimental Design

Elution Volumes of Casein Micelles and Whey Proteins. A solution of casein was obtained by acid precipitation of caseins from milk. The precipitate was redissolved in simulated milk ultra-filtrate (SMUF) (Jennes and Koops, 1962). The casein solution was precipitated with 1 N HCl and the resulting precipitate was dissolved in SMUF. It is recognized that this solution of casein aggregates is not the same as native micellar casein but is of sufficient character as to give and indication of a casein micelle elution pattern. Also a solution of α-lactoglobulin and β-lactalbumin was injected into the column to determine their elution rates.

Determining Best Sample Treatment. Ali et al. (1980a, 1980b and 1980c) described the effect of calcium phosphate, pH, temperature and mastitis on soluble casein in solution. It was decided to test the effects of pH, temperature and addition of calcium on the elution pattern. A factorial design experiment was performed on a single milk sample to determine the effect of various treatments. Commingled milk was obtained from Utah State University Dairy Products Laboratory in Logan, Utah. Treatments were run in duplicate through the column. Temperature treatments were: milk injected cold at 4 C, milk brought to 40 C at least 2 h before injection, and milk heated to 72 C for 1 min and then cooled to 40 C. PH treatments were: milk at pH 6.58 (no treatment), pH 6.7 (100 µL 1 N NaOH per 50 mL), and pH 6.4 (50 µL 1 N NaOH per 50 mL). Half of the milk samples were treated with calcium (100 µL $CaCl_2$ per 50 mL milk). When calcium was added to milk, a

solution of 40 mM $CaCl_2$ was eluted through the column and when no calcium was added to the milk, distilled water was the eluent. Samples were randomly selected to be introduced to the column except that calcium treated samples were not run at the same time as non-treated samples since it was not possible to continually change eluents. Calcium treatment was crossed with pH which was crossed with temperature for this factorial design. Thirty six elution plots were obtained and parameters calculated therefrom. The parameters were evaluated using analysis of variance from SAS statistical package (SAS Institute, 1982) to determine each treatment effect.

Estimating Casein Percentage of Milk. Percent casein was estimated from the parameters of three elution curves on eight samples from individual cows. For each sample, fat and protein percentages were obtained from a Multispec infrared instrument (Berwind Instruments Inc.). Casein percentage was obtained by acid precipitation and Kjeldahl nitrogen determination. Averages of one two or three samples of the same milk were used in the casein determination. For the parameters of elution curves, three samples were averaged. The parameters obtained from the plots as well as fat and protein percentages were correlated with percent casein. Analysis of covariance and stepwise regression were performed and a regression equation to estimate casein was produced.

RESULTS AND DISCUSSION

Separation of Casein Micelles from Whey Protein

When artificial casein aggregates passed through the column there was a peak at 5.1 mL and a very slight rise in absorbance near 10 mL (Figure 8, bottom). The larger peak contained 97% of the total area under the elution curve. It consisted of casein micelles while the small bulge probably contained either whey proteins not separated from the casein with the precipitation step or non-micellar caseins. The amount of protein contained in this part is probably insignificant for this separation method.

A solution of two whey proteins (α-lacalbumin and β-lactoglobulin) was also eluted from the column (figure 8, top). These proteins both eluted at 10.2 mL. The two peaks of an elution of milk are in the same place as the casein aggregate and whey protein peaks in figure 8 (compare with figure 7).

Electrophoresis of Protein Peaks

During the factorial experiment in which treatment of samples were evaluated, casein and whey protein peaks were collected for temperature and calcium treatments. Temperature had little effect on samples at the same calcium level. Electrophoresis of proteins in the first peak of without added calcium had dark casein bands and no whey protein bands. Electrophoresis of the first peak to which calcium had been added demonstrated the same casein bands with no apparent whey protein bands. Electrophoresis of proteins in the whey protein peak to which calcium

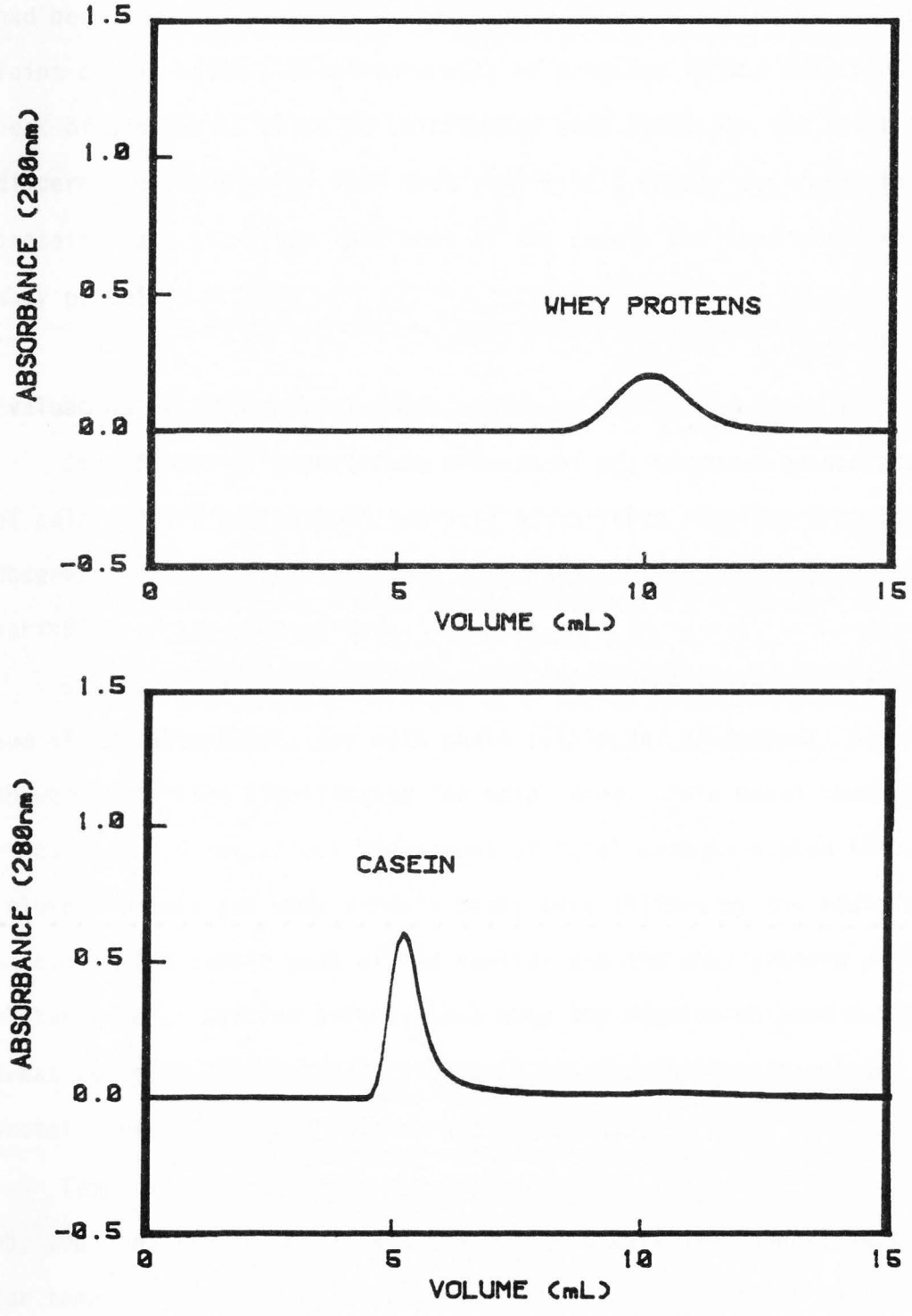


Figure 8. Protein elution curves of α-lactalbumin and β-lactoglobulin (top), and artificial casein aggregates (bottom).

had been added displayed clearly visible whey protein bands and very faint casein bands. Electrophoresis of proteins in the whey protein peak of samples to which no calcium had been added was too faint to discern the bands. The fact that very little casein was found in whey protein peaks signifies that most of the casein was separated from the whey proteins.

Evaluation of Sample Treatments

In a factorial experiment, effects of pH, temperature and addition of calcium were evaluated. Analysis of variance obtained from 36 observations is listed in Tables 2 through 12 in Appendix B for several variables of the elution data.

Calcium treatment was significant for all variables except for the sum of the absorbances for both peaks (Table 9a. of Appendix B), and showed borderline significance for total area. This means that addition of calcium did not affect the amount of total protein eluted through the column. Casein and whey protein peaks were shifted by the addition of calcium. The casein peak eluted earlier and the whey protein peak eluted later. Calcium affected not only the elution volumes but peak areas as well. Casein peaks increased in area and absorbance and whey protein peaks decreased in area and absorbance.

Temperature treatments were significant. Tables 4b, 5b, 6b, 7b, 9b, 10b, and 11b in Appendix B are Duncan's multiple comparison tests for temperature treatment for the accompanying analysis of variance tables. These show that in most cases the temperature of 40 C was better than the others. The cold (4 C) treatment was usually the worst and the high temperature (72 C for 1 min, then 40 C) treatment produced

in-between values and in many cases were not significantly different from the 40 C treatment. Figure 9 shows the relationship between percentage of casein peak to total area with respect to calcium and temperature treatments. The temperature effect was reduced when calcium was added. Calcium by itself did a very good job of eliminating soluble casein so that temperature did not affect it as much as when calcium had not been added. The shaded bars representing samples treated with calcium ranged from 78% to 80% of total peak area. This is approximately the average casein number of normal milk. The plain bars representing milk without calcium were below the average casein number. This showed that not all of the casein was included in the first peak.

PH did not have a significant effect in any of the analysis of variance tables. This may be partly due to the small pH range studied.

Estimating Casein Percentage of Milk

Casein in milk may be estimated from the parameters obtained from the protein elution data. This serves to demonstrate the usefulness of the method for separating casein, and that testing for casein percentages of milk is practical. Eight samples of milk from individual cows ranging in fat from 2.1 to 3.85% and in protein from 2.19 to 4.42% were prepared and run through the column. Elution plots were obtained and parameters of each plot were calculated. Correlation coefficients of these variables demonstrated that casein was contained in the first peak. The correlation coefficient (r) of casein with respect to absorbance of the first peak was .77 and with respect to the area under the first peak was .65. The second peak parameters correlate poorly with casein. The correlation coefficients between casein, and

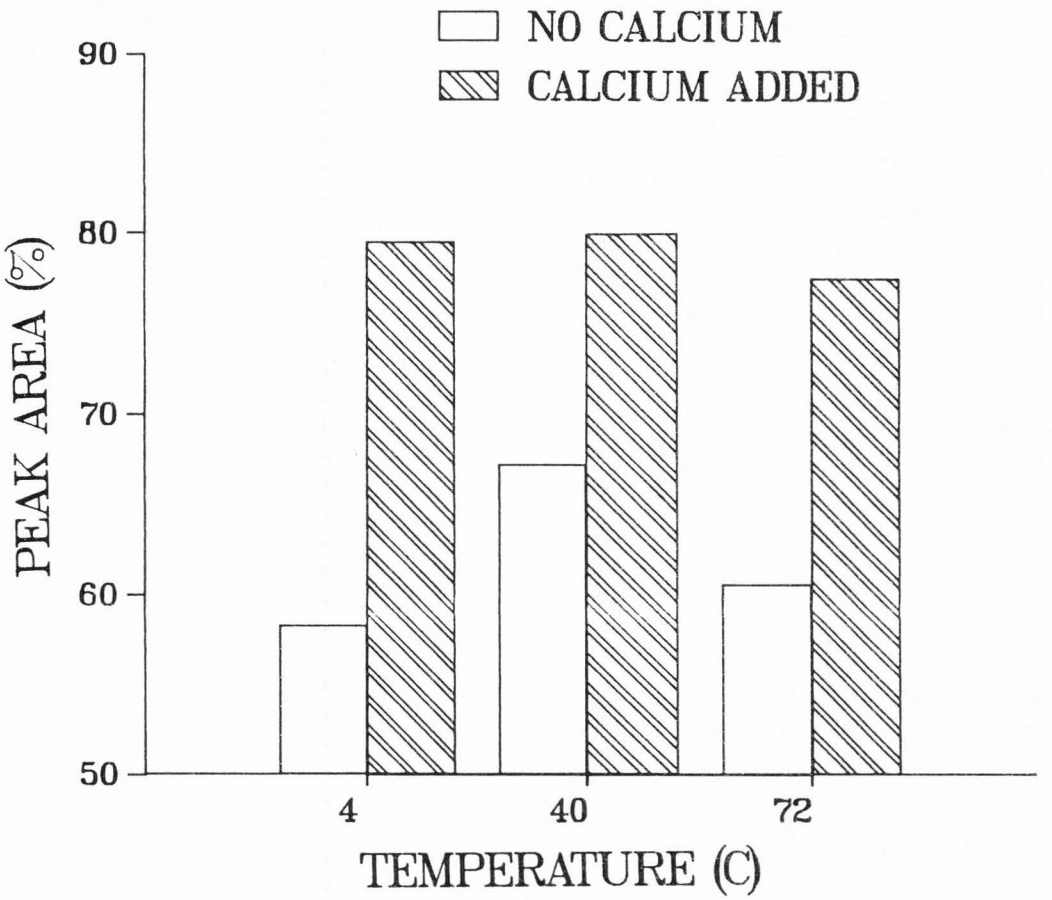


Figure 9. Temperature and calcium treatment effects on percent area of first (casein) peak.

absorbance and area of the second peak were .03 and .25. Analysis of covariance also demonstrated the good relationship between peak area and absorbance of the first peak with percent casein (Table 11 in Appendix B).

Stepwise regression was performed to estimate casein percentages. The area and absorbance of the first peak were the only variables which could estimate casein. When these were used in the regression, an equation which predicts casein resulted in an r of .92. An estimate of casein was calculated for each sample using absorbance and area from each sample elution. A regression equation was calculated between the estimate of casein using the column and the reference method (Figure 10). The r of this equation (Appendix B, Table 12) was also .92. Hence a linear relationship was found between percent casein, and the height and area of the first peak.

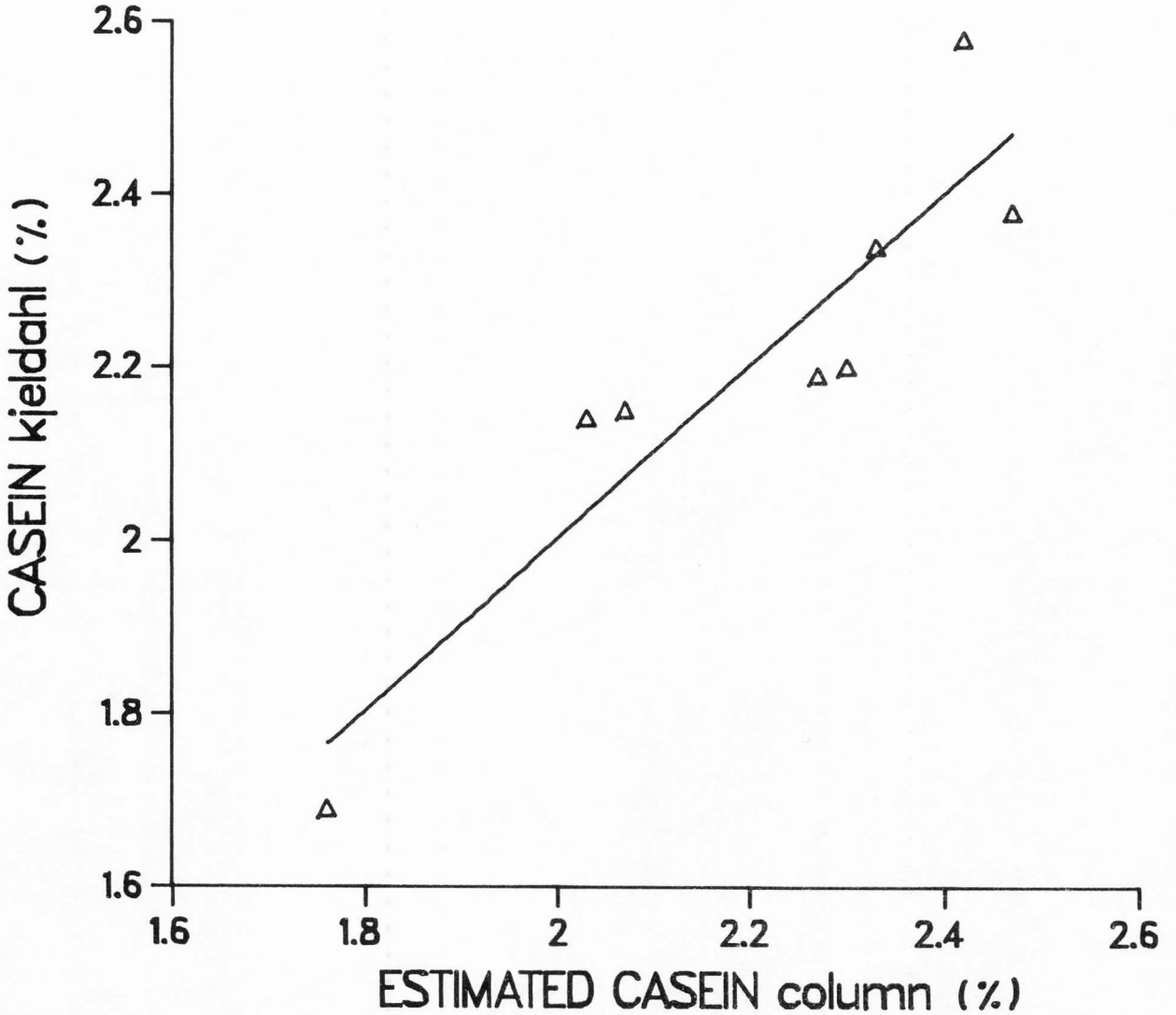


Figure 10. Estimated percent casein of milk (column) versus actual casein (acid precipitation and Kjeldahl).

CONCLUSIONS

1. Size exclusion chromatography can be used to separate casein micelles from whey proteins. Glycophase coated porous glass beads with particle size and pore diameter of 37-74 µm and 2000 nm respectively, performed this separation in a 100 by .4 cm column.

2. Best separation of casein from whey protein in milk was achieved by addition of 100 µL of 5 M $CaCl_2$ per 50 mL sample 24 h before testing and heating to 40 C for at least 2 h before testing.

3. Casein can be estimated from the absorbance and area of the first peak with a fair degree of accuracy (r=.92) when monitoring at a wavelength of 280 nm.

4. Separating casein micelles from whey proteins without coagulation is advantageous for further testing of the casein fraction. This fraction may be assayed for percent protein using a variety of methods not used before. Infrared analysis of protein content in milk is highly automated and may be used to measure protein in the casein peak after some modifications.

REFERENCES

Abu-Tarboush, H. M. 1982. Prediction of Mozzarella cheese yield from milk composition. M. S. Thesis, Utah State University, Logan, Utah.

Adams, D. M., J. T. Barach, and M. L. Speck. 1976. Effect of psychrotrophic bacteria from raw milk on milk proteins and stability of milk proteins to ultrahigh temperature treatment. J. Dairy Sci. 59:823.

Ali, E. A., A. T. Andrews, and G. C. Cheeseman. 1980a. Influence of storage of milk on casein distribution between the micellar and soluble phases and its relationship to cheese making parameters. J. Dairy Res. 47:371.

Ali, E. A., A. T. Andrews, and G. C. Cheeseman. 1980b. Factors influencing casein distribution in cold stored milk and their effects on cheese making parameters. J. Dairy Res. 47:383.

Ali, E. A., A. T. Andrews, and G. C. Cheeseman. 1980c. Influence of elevated somatic cell count on casein distribution. J. Dairy Res. 47:393.

Anderson, M., and A. T. Andrews. 1977. Progressive changes in individual milk proteins associated with high somatic cell counts. J. Dairy Res. 44:223.

Andrews, A. T. 1982. Proteinases in normal bovine milk and their action on caseins. J. Dairy Res. 50:45.

Ashworth, U. S. 1965. Rapid method for the determination of casein in milk by the dye binding method and for the detection of mastitis. J. Dairy Sci. 48:537.

Ashworth, U. S. 1966. Determination of protein in dairy products by dye-binding. J. Dairy Sci. 49:133.

Association of Official Analytical Chemists. 1980. Official and Tentative Methods of Analysis. 13th ed. A.O.A.C., Washington, D.C.

Aylward, E. B., J. O'Leary, and B. E. Langlois. 1980. Effect of milk storage on cottage cheese yield. J. Dairy Sci. 63:1819.

Bingham, E. W., H. M. Farrell Jr., and R. J. Carroll. 1972. Properties of dephosphorylated α_{s1}-casein. Biochemistry 11:2450.

Blake, R. W., I. B. Nmai, and R. L. Richter. 1980. Relationship between distribution of major milk proteins and milk yield. J. Dairy Sci. 63:141.

Bloomfield, V. A., and C. V. Morr. 1973. Structure of casein micelles: physical methods. Neth. Milk Dairy J. 27:103.

Bloomfield, V. A., and R. J. Mead Jr. 1975. Structure and stability of casein micelles. J. Dairy Sci. 50:592.

Bloomfield, V. A. 1979. Association of proteins. J. Dairy Res. 46:241.

Brog, R. A. 1971a. Economic incentives for cheese processors using a protein-fat milk pricing model. J. Dairy Sci. 54:1006.

Brog, R. A. 1971b. Proposed economic formula (model) for deriving the value of cheese milk. J. Dairy Sci. 54:1135.

Cerbulis, J., and H. M. Farrell, Jr. 1975. Composition of milks of dairy cattle. I. Protein, lactose and fat contents and distribution of protein fraction. J. Dairy Sci. 58:817.

Chapman, R. H. 1974. The effect the chemical quality of milk has on cheese quality. Dairy Ind. 39:329.

Davies, D. T., and A. J. R. Law. 1980. The content and composition of protein in creamery milks in south-west Scotland. J. Dairy Res. 47:83.

Davis, J. G. 1965. Cheese. Vol. I. American Elsevier Publ. Co., Inc., New York, NY.

De Beukelar, N. J., M. A. Cousin, R. L. Bradley Jr, and E. H. Marth. 1977. Modification of milk proteins by psychrotrophic bacteria. J. Dairy Sci. 60:857.

Eckstrand, B., M. Larsson-Raznikiewicz, E. Brannang, and C. Swensson. 1981. Size distribution of casein micelles related to coagulation properties: A comparison between different breeds of cattle. Swedish J. Agric. Res. 11:57.

Ernstrom, C. A. 1980. A workable pricing system based on cheese yield. Utah State University 4rth Biennial Cheese Industry Conference, Logan.

Ettre, L. S. 1971. The development of chromatography. Anal. Chem. 43:20A.

Fehrnstrom, H., and V. Moberg. 1977. SDS and conventional polyacrylamide gel electrophoresis with LKB 2117 multiphor. Application note 306. LKB - Producter AB, Bromma, Sweden.

Fischer, L. 1980. Laboratory Techniques in Biochemistry and Molecular Biology: Gel filtration chromatography. 2nd ed. Elsevier/North-Holland Biomedical Press, Amsterdam, The Netherlands.

Foss Electric A/S N. 1980. Milko Scan 104. Applications Guide. N. Foss and Co. 66 Hellerupvej - DK 2900 Hellerup - Denmark.

Gilmore, T. E., and W. V. Price. 1953. Butt. Cheese Milk Prod. J. 44:28 (Dairy Sci. Abstr. 15:740d).

Gordon, W. G., and E. B. Kalan. 1974. Proteins of milk. Pages 87-124 In B. H. Webb, A. H. Johnson, and J. A. Alford eds. Fundamentals of Dairy Chemistry 2nd ed. The AVI Publishing Co., Inc., Westport, CT.

Goulden, J. D. S. 1967. Quantitative analysis of milk and other emulsions by infra-red absorption. Nature 191:905.

Grappin, R., V. S. Packard, and R. E. Ginn. 1980. Repeatability and accuracy of dye-binding and infra-red methods for analyzing protein and other milk components. J. Food Prot. 43:374.

Haenlein, G. F. W., L. H. Schultz, and J.P. Zikakis. 1973. Composition of proteins in milk with varying leukocyte contents. J. Dairy Sci. 56:1017.

Harding, F. 1973. Instrumental techniques for the determination of the compositional quality of milk and milk products. Dairy Ind. 38:311.

Harding, F., and L. Royal. 1974. Variations in the nitrogen-containing fractions of bulked milk in England and Wales during the period 1947 to 1970. Dairy Ind. 39:372.

Heth, A. A., and H. E. Swaisgood. 1982. Examination of casein micelle structure by a method for reversible covalent immobilization. J. Dairy Sci. 65:2047.

Holt, C., D. G. Dalgleish, and T. G. Parker. 1973. Particle size distribution in skim milk. Biochim. Biophys. Acta 328:428.

Jennes, R., and J. Koops. 1962. Preparation of a salt solution which simulates milk ultra filtrate. Neth. Milk and Dairy J. 16:153.

Jennes, R. 1970. Protein composition of milk. In H. A. McKenzie, Milk Proteins Vol. 1 Academic Press, New York, NY.

Kiddy, C. A. 1974. Gel electrophoresis in vertical polyacrylamide beds. Pages 14-15 In H. E. Swaisgood, ed. Methods of Gel Electrophoresis of Milk Proteins. Department of Food Science, North Carolina State University, Raliegh.

Kirchmeier, O. 1968. A method of rapid and quantitative determination of the casein in milk. Milchwissenschaft 23:403.

Kirkland, J. J. 1976. Porous silica microspheres for high-performance size exclusion chromatography. J. Chromatogr. 125:231.

Korycka-Dahl, M., B. R. Dumas, N. Chene, and J. Martal. 1983. Plasmin activity in milk. J. Dairy Sci. 66:704.

Kosikowski, F. 1968. Cheese and Fermented Milk Foods. 2nd ed. F. V. Kosikowski and Associates, Brooktondale, NY.

Ladd, G. W., and J. R. Dunn. 1979. Estimating values of milk components to a dairy manufacturer. J. Dairy Sci. 62:1705.

Larson, B. L., G. D. Rolleri, and K. A. Kendall. 1956. Protein production in the bovine. Comparison of daily protein, fat, and milk production during the entire laction period. J. Dairy Sci. 39:204.

Majeed, G. H. 1982. The relationship between milk composition and Swiss cheese yield. M. S. Thesis. Utah State University, Logan.

McDowell, A. K. R. 1972. Seasonal variations in the total nitrogen, non-protein nitrogen and urea nitrogen contents of Fresian and Jersey milk. J. Dairy Res. 39:27.

McGann, T. C. A., A. Matthiassen, and J. A. O'Connell. 1972. Applications of the pro-milk II. III. Rapid estimation of casein in milk protein and whey. Lab. Pract. 21:628.

McGann, T. C. A., R. D. Kearney, and W. J. Donnelly. 1979. Developments in column chromatography for the separation and characterization of casein micelles. J. Dairy Res. 46:307.

McGann, T. C. A., W. J. Donnelly, R. D. Kearney, and W. Buchheim. 1980. Composition and size distribution of bovine casein micelles. Biochim Biophys. Acta 630:261.

Moore, C. A. 1983. Comparison of several forms of equations for predicting Cheddar cheese yield from milk composition. M. S. Thesis. Utah State University, Logan.

Moore, J. C. 1964. Gel permeation chromatography. I. A new method for molecular weight distribution of high polymers. J. Polym. Sci. Part A 2:835.

Munchberg, F., R. Leskova, and D. Svastics. 1969. On the refractometric determination of casein and total protein of milk. Milchwissenschaft 24:65-67.

Okigbo, L. M. 1982. Variation in the coagulation properties of milk from individual cows. M. S. Thesis. Utah State University, Logan.

Ono, T., S. Odagiri, and T. Takagi. 1983. Separation of submicelles from micellar casein by high performance liquid chromatography on a TSK-Gel G4000SW column. J. Dairy Res. 50:37.

Porath, J., and P. Flodin. 1959. Gel filtration: a method for desalting and group separation. Nature 183:1657.

Pyne, G. T. 1932. The determination of milk-proteins by formaldehyde titration. Biochem. J. 26:1006.

Pyne, G. T. 1933. Note on the formaldehyde titration of milk protein and its application with its estimation of caseinogen. Biochem. J. 27:58.

Renner, E., S. Oemeroglu. 1971. Determination of casein in milk with devices based on the amido-black method. Deutsche Molkerei-Zeitung 92:943 (Dairy Sci. Abstr. 33:732).

Renner, E., and S. Ando. 1974. Methods for the determination of protein conponents in milk. V. Casein and whey protein content with amido black methods. Z. Lebensm. Unters.-Forsch. 155:23 (Dairy Sci. Abstr. 36:612).

Richter, R. 1980. Estimating and calculating cottage cheese yields. Am. Dairy Rev. 42(1):18A.

Rowland, S. J. 1938a. The determination of the nitrogen distribution in milk. J. Dairy Res. 9:42.

Rowland, S. J. 1938b. The protein distribution in normal and abnormal milk. J. Dairy Res. 9:47.

SAS Institute. 1982. SAS Users Guide. SAS Institute Inc., Raleigh, NC.

Schmidt, D. G., P. Walstra, and W. Buchheim. 1973. The size distribution of casein micelles in cows milk. Neth. Milk Dairy J. 27:128.

Schmidt, D. G. 1982. Proteins: Association of casein and casein micelle structure. in Developments in Dairy Chemistry. Applied Science Publishers LTD, Essex, England.

Skwarska, S., H. Garszczynska, and K. Laskowski. 1977. Evaluation of methods for determination of casein in milk. Roczniki Instytutu Przemyslu Mleczarskiego 19:99 (Food Sci. Technol. Abstr. 78:308).

Snyder, L. R., and J. J. Kirkland. 1974. Introduction to Modern Liquid Chromatography. Wiley, New York.

Szijarto, L., D. A. Biggs, and D. M. Irvine. 1973. Variability of casein, serum protein and nonprotein nitrogen in plant milk supplies in Ontario. J. Dairy Sci. 56:45.

Taylor, W. J., and H. H. Van Horn. 1962. Genetic and environmental influences on protein and solids not fat. Iowa State University, Cooperative Extension Service, Ames.

Thomasow, J. 1976. Simultaneous determination of milk fat and protein content by measurement of IR absorption with the Milko-scan instrument. Milchwissenschaft 31:149.

Unger, K., R. Kern, M. C. Ninou, and K. F. Krebs. 1974. High performance gel permeation chromatography with a new type of silica packing material. J. Chromatogr. 99:435.

Vanderzant, C., and W. R. Tennison. 1961. Estimation of the protein content of milk by dye binding with buffalo black. Food Technol. 15:63.

Van Slyke, L. L., and W. V. Price. 1952. Cheese. Orange Judd Publ. Co., New York, NY.

Wagner, A., I. Merenyi, and M. D. Kovacs. 1973. Rapid determination of casein in fresh farm milk with particular respect to pathological milks. Elelmiszervizsgalati Koezlemenyek 19:133 (Food Sci. Technol. Abstr. 74:237).

Waite, R., J. C. D. White, and A. Robertson. 1956. Variations in the chemical composition of milk with particular reference to solids-not-fat 1. The effect of stage of lactation, season on year and age of cow. J. Dairy Res. 23:65.

Walker, W. O. 1914. A rapid method for determination of casein in milk. J. Ind. Eng. Chem. 6:131.

Weaver, J. C., and M. Kroger. 1977. Protein, casein and noncasein protein percentages in milk with high somatic cell count. J. Dairy Sci. 60:878.

Webb, B. H., and E. O. Whittier. 1970. By-Products from Milk. AVI Publishing Company, Inc., Westport, CN.

Whitney, R. McL., chairman, J. R. Brunner, K. E. Ebner, H. M. Farrell Jr., R. V. Josephson, C. V. Morr, and H. E. Swaisgood. 1976. Nomenclature of the proteins of cow's milk: Fourth revision. J. Dairy Sci. 59:795.

Yaguchi, M., and D. Rose. 1971. Chromatographic separation of milk proteins: a review. J. Dairy Sci. 54:1725.

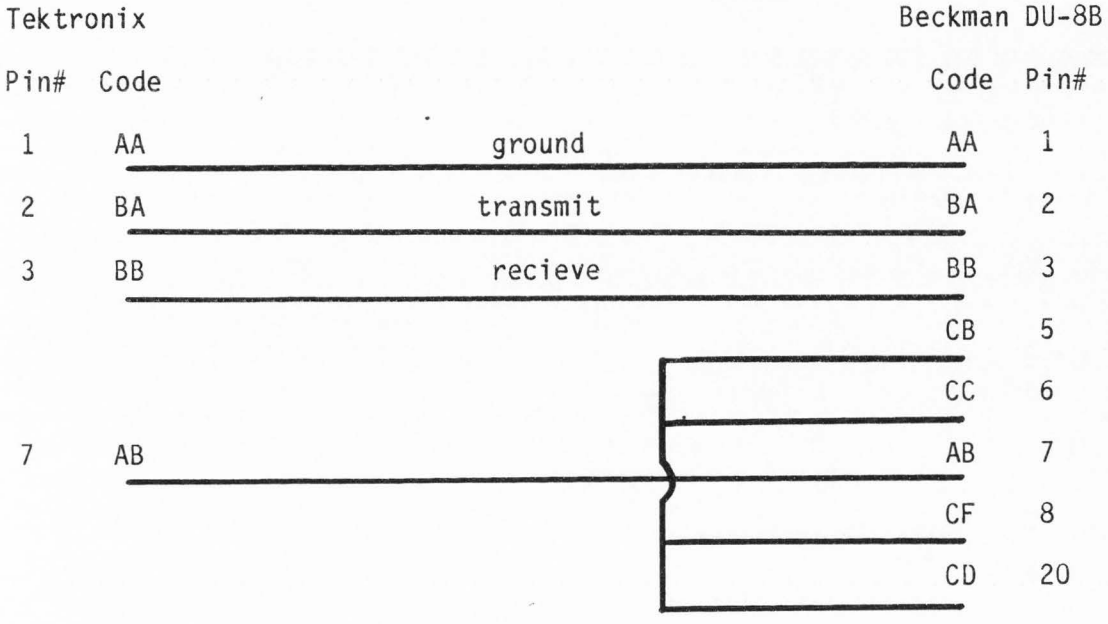
Yau, W. W., J. J. Kirkland, and D. D. Bly. 1979. Modern Size Exclusion Liquid Chromatography: Practice of Gel Permeation and Gel Filtration Chromatography. John Wiley & Sons, Inc., New York.

Zurborg, Z. E. 1978. Practical experiences with payment for milk for protein and fat. J. Dairy Sci. 61:801.

APPENDICES

Appendix A: Computer
Interfacing and Program

Data was transmitted from the Beckman DU-8B spectrophotometer to the Tektronix 4052 computer. Interfacing was accomplished via RS232 plugs of both computer and spectrophotometer. The cable was modified on the spectrophotometer end as follows:



Baud rate was set at 1200, parity was odd and the spectrophotometer was set on protocol output mode. The following computer program facilitates data input from the spectrophotometer. Line number 1025 sets the baude rate and parity and line number 2150 inputs data from the spectrophotometer. The program also stores data on tape, calculates areas, absorbances and volumes of peaks and plots elution curves.

```
4 PAGE
5 GOSUB 1000
6 END
8 PAGE
9 GOSUB 2000
10 END
12 PAGE
13 GOSUB 5000
14 END
16 PAGE
17 W=32
18 GOSUB 4000
19 END
20 PAGE
21 W=1
22 GOSUB 4000
23 END
24 PAGE
25 GOSUB 6000
26 END
28 PAGE
29 Q9=32
30 GOSUB 6000
31 END
32 PAGE
33 Q9=41
34 GOSUB 6000
35 END
1000 REM ** INITIALIZE **
1010 INIT
1020 W=32
1025 CALL "RATE",1200,0,2
1030 G$=""
1040 G=0
1050 DIM Y(400)
1499 RETURN
2000 REM ** ENTER PARAMETERS AND RUN COLUMN **
2005 GOSUB 1000
2010 PRINT "JJJJJDATE..... ";
2020 INPUT D$
2030 PRINT "JFILE NUMBER..... ";
2040 INPUT F
2045 IF F=1 THEN 8
2050 PRINT "JSAMPLE NUMBER..... ";
2060 INPUT S$
2070 PRINT "JSAMPLE SIZE..... ";
2080 INPUT S
2090 PRINT "JFLOW RATE..... ";
2100 INPUT R1
2110 PRINT "JREADINGS PER MIN... ";
2120 INPUT R2
2122 PRINT "JRUN TIME..... ";
2125 INPUT T
2130 FIND F
```

```
2132 GOSUB 3000
2135 MOVE @32:0,0
2140 FOR I=1 TO T*R2
2150 INPUT @40:X1,X2,Y(I)
2155 DRAW @32:I/R2*R1,Y(I)
2160 NEXT I
2170 WRITE @33:D$
2180 WRITE @33:F
2190 WRITE @33:S$
2200 WRITE @33:S
2210 WRITE @33:R1
2220 WRITE @33:R2
2230 WRITE @33:T
2240 FOR I=1 TO T*R2
2250 WRITE @33:Y(I)
2260 NEXT I
2270 PRINT "GGGGGGGGGGGGGGGGGGGGGG"
2499 RETURN
3000 REM ** PLOT **
3010 IF W=1 THEN 3040
3020 VIEWPORT 70,128,15,95
3030 GO TO 3050
3040 VIEWPORT 40,120,20,80
3050 WINDOW 0,5*INT((T*R1+4.95)/5),-0.5,4*H
3060 AXIS @W:5,0.5,0,-0.5
3070 MOVE @W:0,-0.5
3075 Z=0
3080 FOR I=1 TO 5
3090 DRAW @W:0+Z*4.35,-0.5+Z*H
3100 DRAW @W:5*INT((T*R1+4.95)/5)-Z*4.35,-0.5+Z*H
3110 DRAW @W:5*INT((T*R1+4.95)/5)-Z*4.35,(4-Z)*H
3120 DRAW @W:0+Z*4.35,(4-Z)*H
3125 Z=Z+0.007
3130 NEXT I
3135 GOSUB 3600
3140 A1=0.6
3150 FOR J=0 TO 5*INT((T*R1+4.95)/5) STEP 5
3160 IF J<10 THEN 3180
3170 A1=0.3
3180 MOVE @W:J-1+A1,-0.5-0.3*H
3200 PRINT "H";
3210 PRINT @W:J;
3220 NEXT J
3230 FOR J=-0.5 TO 4*H STEP 0.5
3240 MOVE @W:-1.7,J-0.01
3250 IF W<>32 THEN 3270
3260 PRINT @32:"HH";
3270 PRINT @W: USING "2D.1D":J
3280 NEXT J
3420 MOVE @W:7.5,-0.5-0.6*H
3430 PRINT @W:"HHHHHVOLUME (mL)"
3440 IF W=32 THEN 3530
3450 MOVE @W:-2.3,1.75*H
3460 PRINT @W,25:90
```

```
3470 PRINT @W:"HHHHHHHHHABSORBANCE (280nm)";
3480 MOVE @W:-5.5,-0.11
3500 PRINT @W,25:0
3530 RETURN
3600 MOVE @W:5,-0.5
3610 RDRAW @W:0,0.15*H
3620 MOVE @W:10,-0.5
3630 RDRAW @W:0,0.15*H
3640 FOR I=0.5 TO 4*H STEP 0.5
3650 MOVE @W:0,I
3660 RDRAW @W:0.4,0
3670 NEXT I
3680 RETURN
4000 REM ** PLOT DATA **
4010 GOSUB 3000
4015 Z=0
4016 FOR R=1 TO 3
4020 MOVE @W:0,0+Z
4030 FOR I=1 TO T*R2
4040 DRAW @W:I/R2*R1,Y(I)+Z
4050 NEXT I
4055 Z=Z+0.01*H
4056 NEXT R
4060 RETURN
5000 REM ** LOAD DATA FROM TAPE **
5005 GOSUB 1000
5010 PRINT "JJWHICH FILE ON TAPE? ";
5020 INPUT F1
5030 FIND F1
5040 READ @33:D$
5050 READ @33:F
5060 READ @33:S$
5070 READ @33:S
5080 READ @33:R1
5090 READ @33:R2
5100 READ @33:T
5110 FOR I=1 TO T*R2
5120 READ @33:Y(I)
5130 NEXT I
5140 RETURN
6000 REM ** CALCULATE AND PRINT PARAMETERS **
6010 PRINT "JJJSTARTING FILE NUMBER... ";
6020 INPUT B9
6030 PRINT "JENDING FILE NUMBER..... ";
6040 INPUT E9
6050 FOR L=B9 TO E9
6060 F1=L
6070 GOSUB 5030
6080 A=INT(4*R2/R1)
6090 E=INT(13*R2/R1)
6095 IF (E-A)/2<>INT((E-A)/2) THEN 6100
6097 E=E+1
6100 M=0
6110 FOR I=1 TO INT(7.5*R2/R1)
```

```
6120 IF Y(I)<M THEN 6150
6130 M=Y(I)
6140 B=I
6150 NEXT I
6160 M=0
6170 FOR I=INT(7.5*R2/R1) TO R2*T
6180 IF Y(I)<M THEN 6210
6190 M=Y(I)
6200 D=I
6210 NEXT I
6220 C=B+0.5*(D-B)
6230 IF (C-A)/2<>INT((C-A)/2) THEN 6250
6240 C=C+1
6250 A1=0
6260 FOR I=A TO C
6270 A1=A1+R1/R2*(Y(I+1)+Y(I))
6280 NEXT I
6290 A1=A1*0.5
6300 A2=0
6310 FOR I=C TO E
6320 A2=A2+R1/R2*(Y(I+1)+Y(I))
6330 NEXT I
6340 A2=A2*0.5
6350 S=0
6360 S=Y(A)+Y(C)
6370 FOR I=A+1 TO C-1
6380 S=S+2*Y(I)
6390 NEXT I
6400 FOR I=A+2 TO C-2 STEP 2
6410 S=S+2*Y(I)
6420 NEXT I
6430 A3=S/(R2/R1*3)
6440 S=0
6450 S=Y(C)+Y(E)
6460 FOR I=C+1 TO E-1
6470 S=S+2*Y(I)
6480 NEXT I
6490 FOR I=C+2 TO E-2 STEP 2
6500 S=S+2*Y(I)
6510 NEXT I
6520 A4=S/(R2/R1*3)
6530 PRINT @Q9:"JJSAMPLE NO. ";S$;"    FILE NO. ";F
6550 PRINT @Q9:"J     VOLUME  ABSORBANCE   AREA"
6560 PRINT @Q9: USING 6610:A/R2*R1,Y(A)
6570 PRINT @Q9: USING 6620:B/R2*R1,Y(B),A1,A3
6580 PRINT @Q9: USING 6610:C/R2*R1,Y(C)
6590 PRINT @Q9: USING 6620:D/R2*R1,Y(D),A2,A4
6600 PRINT @Q9: USING 6610:E/R2*R1,Y(E)
6610 IMAGE 3X,2D.2D,5X,2D.2D
6620 IMAGE 3X,2D.2D,5X,2D.2D,5X,2D.3D,5X,2D.3D
6630 NEXT L
6640 RETURN
```

Appendix B: Statistical Tables

The following tables are the results of the factorial experiment for various sample treatments. Each table represents a different variable calculated from the elution plots. Treatment of pH was not significant and there were only two levels of calcium treatment. For this reason, Duncan's multiple mean comparison was performed only for those analyses which reported significance in temperature treatment. For Duncan's multiple mean comparison, temperature number 1 is 4 C, temperature number 2 is 40 C, and temperature number 3 is 72 C for 1 min and then 40 C.

Table 3. Analysis of variance: Elution volume of casein peak.

Source	DF	MS	F	Alpha
Calcium (CA)	1	.034	10.92	.0039 **
Temperature (T)	2	.005	1.63	.2234
pH	2	.005	1.50	.2495
CA X T	2	.006	2.09	.1532
CA X pH	2	.002	.52	.6058
T X pH	4	.002	.81	.5371
CA X T X pH	4	.003	.92	.4738
Error	18	.003		
Total	35		R2 = .62	

** alpha level less than .01

Table 4a. Analysis of variance: Elution volume of whey protein peak.

Source	DF	MS	F	Alpha
Calcium (CA)	1	11.357	2363.29	.0001 ***
Temperature (T)	2	.028	5.89	.0108 *
pH	2	.006	1.32	.2910
CA X T	2	.028	5.89	.0108 *
CA X pH	2	.006	1.32	.2910
T X pH	4	.004	.81	.5366
CA X T X pH	4	.004	.81	.5366
Error	18	.004		
Total	35		R2 = .99	

* alpha level less than .05

*** alpha level less than .001

Table 4b. Duncan's multiple mean comparison for temperature treatment.

Duncan grouping	Mean	Treatment
A	9.82	2
B	9.76	3
B	9.73	1

Table 5a. Analysis of variance: Absorbance of casein peak.

Source	DF	MS	F	Alpha
Calcium (CA)	1	2.14	17.32	.0006 ***
Temperature (T)	2	.67	5.39	.0146 *
pH	2	.39	3.12	.0687
CA X T	2	.22	1.80	.1945
CA X pH	2	.22	1.81	.1919
T X pH	4	.15	1.18	.3510
CA X T X pH	4	.61	4.90	.0075 **
Error	18	2.22		
Total	35		R2 = .79	

* alpha level less than .05

** alpha level less than .01

*** alpha level less than .001

Table 5b. Duncan's multiple mean comparison for temperature treatment.

Duncan grouping	Mean	Treatment
A	2.59	3
A	2.56	2
B	2.17	1

Table 6a. Analysis of variance: Absorbance of whey protein peak.

Source	DF	MS	F	Alpha
Calcium (CA)	1	1.210	501.63	.0001 ***
Temperature (T)	2	.011	4.71	.0227 *
pH	2	.008	3.35	.0580
CA X T	2	.011	4.52	.0257 *
CA X pH	2	.008	3.19	.0652
T X pH	4	.002	.77	.5581
CA X T X pH	4	.003	1.09	.3891
Error	18	.002		
Total	35		R2 = .97	

* alpha level less than .05

*** alpha level less than .001

Table 6b. Duncan's multiple mean comparison for temperature treatment.

Duncan grouping	Mean	Treatment
A	.492	3
A	.490	1
B	.438	2

Table 7a. Analysis of variance: Area of casein peak.

Source	DF	MS	F	Alpha
Calcium (CA)	1	1.011	11.95	.0028 **
Temperature (T)	2	.795	9.41	.0016 **
pH	2	.197	2.33	.1260
CA X T	2	.669	7.90	.0034 **
CA X pH	2	.342	4.04	.0355 *
T X pH	4	.209	2.48	.0811
CA X T X pH	4	.317	3.74	.0219 *
Error	18	.085		
Total	35		R2 = .82	

* alpha level less than .05

** alpha level less than .01

Table 7b. Duncan's multiple mean comparison for temperature treatment.

Duncan grouping	Mean	Treatment
A	2.39	3
B	2.13	2
C	1.88	1

Table 8. Analysis of variance: Area of whey protein peak.

Source	DF	MS	F	Alpha
Calcium (CA)	1	3.777	55.50	.0001 ***
Temperature (T)	2	.215	3.15	.0670
pH	2	.066	.97	.3994
CA X T	2	.265	3.89	.0394 *
CA X pH	2	.078	1.14	.3408
T X pH	4	.039	.58	.6823
CA X T X pH	4	.035	.52	.7225
Error	18	.068		
Total	35		R2 = .81	

* = alpha level less than .05

*** = alpha level less than .001

Table 9a. Analysis of variance: Sum of casein and whey protein peak absorbances.

Source	DF	MS	F	Alpha
Calcium (CA)	1	1.390	1.10	.3085
Temperature (T)	2	.607	5.10	.0176 *
pH	2	.385	3.24	.0629
CA X T	2	.255	2.15	.1458
CA X pH	2	.220	1.85	.1860
T X pH	4	.120	1.01	.4282
CA X T X pH	4	.651	5.47	.0046 **
Error	18	.119		
Total	35		R2 = .74	

* alpha level less than .05

** alpha level less than .01

Table 9b. Duncan's multiple mean comparison for temperature treatment.

Duncan grouping	Mean	Treatment
A	3.08	3
A	3.00	2
B	2.66	1

Table 10a. Analysis of variance: Total area.

Source	DF	MS	F	Alpha
Calcium (CA)	1	.880	5.51	.0305 *
Temperature (T)	2	1.250	7.83	.0036 **
pH	2	.170	1.07	.3646
CA X T	2	1.554	9.73	.0014 **
CA X pH	2	.594	3.72	.0444 *
T X pH	4	.334	2.09	.1238
CA X T X pH	4	.253	1.58	.2213
Error	18	.160		
Total	35		R2 = .78	

* alpha level less than .05

** alpha level less than .01

Table 10b. Duncan's multiple mean comparison for temperature treatment.

Duncan grouping	Mean	Treatment
A	3.43	3
B	2.91	2
B	2.84	1

Table 11a. Analysis of variance: Percentage of casein peak absorbance with respect to sum of casein and whey protein peak absorbances.

Source	DF	MS	F	Alpha
Calcium (CA)	1	.157	215.92	.0001 ***
Temperature (T)	2	.004	5.46	.0140 *
pH	2	.001	1.83	.1884
CA X T	2	.002	2.85	.0840
CA X pH	2	.001	1.31	.2956
T X pH	4	.001	.59	.6729
CA X T X pH	4	.001	1.47	.2533
Error	18	.001		
Total	35		R2 = .93	

* alpha level less than .05

*** alpha level less than .001

Table 11b. Duncan's multiple mean comparison for temperature treatment.

Duncan grouping	Mean	Treatment
A	.852	2
BA	.832	3
B	.815	1

Table 12. Analysis of variance: Percentage of casein peak area with respect to total area.

Source	DF	MS	F	Alpha
Calcium (CA)	1	.258	67.77	.0001 ***
Temperature (T)	2	.008	2.20	.1390
pH	2	.005	1.38	.2764
CA X T	2	.005	1.44	.2627
CA X pH	2	.002	.51	.6103
T X pH	4	.001	.33	.8530
CA X T X pH	4	.005	1.38	.2791
Error	18	.004		
Total	35		R2 = .83	

*** alpha level less than .001

Table 13. Analysis of covariance: Casein estimate.

Source	df	MS	F	alpha
Casein Peak Absorbance	1	.126	38.90	.0248 *
Casein Peak Area	1	.106	32.70	.0292 *
Whey Protein Peak Abs.	1	.016	4.92	.1569
Whey Protein Peak Area	1	.022	6.83	.1205
Whey Protein Peak Volume	1	.030	9.14	.0942
Error	2	.003		
Total	7		R2 = .99	

* alpha level less than .05

Table 14. Regression analysis: Casein estimate.

Source	DF	MS	F	Alpha
Regression	2	.194	13.25	.010 *
Error	5	.015		
Total	7	R2 = .84		

** alpha level less than .01

Regression Equation

C = .8977 + 1.495*(A) - 1.181*(B)

Where:

C = Estimate of percent casein

A = Absorbance of first peak

B = Area under the first peak