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Measurement of Proteins in Milk and Dairy Products

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MEASUREMENT OF PROTEINS IN MILK
AND DAIRY PRODUCTS

by

Marie K. Walsh

A thesis submitted in partial fulfillment

of the requirements for the degree

of

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in

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ABSTRACT

Measurement of Proteins in Milk

and Dairy Products

by

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The purpose of this study was to develop a short, easy procedure to measure five major proteins in milk and to detect concentrations of added protein to dairy products. Combinations of casein or whey protein with nonfat-dry milk were made with concentration ratios from 0:10 to 10:0. Similar mixtures of defatted goat milk with defatted cow milk were prepared. Samples were hydrolyzed in 6 N HCl at 145°C for 4 h and analyzed for amino acid composition. Multiple regression equations were derived to estimate the relative content of whey protein or casein added to nonfat-dry milk and goat milk added to cow milk employing amino acid profiles of whey protein, casein, nonfat-dry milk, goat milk and cow milk. Correlation coefficient values were all greater than .99. Measuring individual concentrations of milk proteins required separating casein and whey proteins by reverse phase high performance liquid chromatography on a C_3 column. α_S-, β-, and κ-casein were separated after dissociating casein micelles with mercaptoethanol and urea. A 40:60 to 0:100 gradient of .15 M sodium chloride/triethylamine (pH 2.5) and 40% acetonitrile was used. Whey proteins, α-lactalbumin and β-lactoglobulin were separated with a 95:5 to 0:100 gradient of .15 M sodium chloride (pH 2.4) and acetonitrile. Eluted proteins were collected from the column, analyzed for purity by electrophoresis, and

hydrolyzed in 6 N HCl at 145°C for 4 h. Purified proteins and mixtures of purified proteins were analyzed for amino acid composition. Estimates of individual protein concentrations in mixtures were made by solving simultaneous equations based on amino acid composition using a Tektronix 4052 computer.

(80 pages)

INTRODUCTION

Measuring milk protein concentrations and detecting added proteins to milk and dairy products is important for the dairy industry. Methods available for measuring milk proteins are lengthy and impractical. Determining quantities of casein and fat in milk are necessary for cheese yield formulas and milk payment methods used by cheese manufacturing companies. Because measurement of total milk protein is automated, casein is assumed to be a constant fraction of total milk protein (21). The amount of casein in milk is variable (8, 12) and can lead to incorrect cheese yield predictions if considered constant (60).

Procedures available for determining milk protein concentrations require precipitation of casein and separating proteins in both whey and precipitate by electrophoresis or column chromatography. Each protein fraction is measured individually. Casein can be determined by subtracting protein in the serum fraction from total protein. These methods are time consuming and subject to error.

Each milk protein has a specific amino acid composition. The purpose of this study is to develop a short procedure capable of measuring individual milk proteins. Amino acid compositions of milk proteins are determined and used to solve for each protein by simultaneous equations. Concentrations of α_S-, β-, and κ-casein summed give percent casein in milk. Addition of other protein sources to milk or dairy products can be detected by changes in total amino acid composition.

LITERATURE REVIEW

Milk Pricing

The basis for purchasing milk has changed over the years and is still being debated. Milk was first purchased by volume, leading to water addition, an easy form of adulteration. With the inventions of Gerber and Babcock milk fat tests, milk value rested solely on the amount of butterfat present in milk. After introduction and subsequent popularity of margarine, butterfat lost its ability to carry the milk value (31).

A milk payment method that received wide acceptance in the United States was devised by Froker and Hardin (34). Milk is given a base price per 100 pounds of milk testing 3.5% fat with a fat differential for variations in milk fat. Jacobson (45) demonstrated a general relationship between fat and solids-not-fat (SNF) in milk. A fat differential reflected an increase in fat and SNF as suggested by Jacobson. This pricing system discriminated against milk with high percentages of SNF, protein, and fat, and encouraged low production of low fat and low solids milk (31). This milk pricing system is reflected in current methods of management and selection of dairy cattle that increase milk yield per cow but decrease solids density (12).

Component pricing or pricing milk by the value of its major constituents became a popular idea. Fat and protein are the main milk components that reflect its economic value. Brog (13), Ladd and Dunn (50), and Zurborg (81) agreed that payment for milk should be based on protein and fat content. This eliminated problems of watering milk, unlike the Froker and Hardin payment plan. A problem with component pricing is establishing the price of each component when milk is used for different dairy products (31).

Purchasing milk by the Froker and Hardin system or component pricing works well for fluid milk but another method is needed for manufactured dairy products. Ernstrom (31) believes the milk price paid to producers should be based on the value of the product made from that milk. He calls this "end product pricing." If the end product is cheese, milk price

depends on its cheese yield ability. For cheese manufacturers, milk high in casein and fat and low in serum protein and lactose is desired (12). Cheese yield determination is based on the Van Slyke and Price Cheddar cheese formula (75):

$$Y = \frac{[.93F + (C - .1)] \, 1.09}{1-W}$$

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

This formula assumes 93% of milk fat is recovered in cheese, all the casein except .1% is recovered, and other milk solids recovered including salt are equivalent to 9% of the recovered fat and casein. Milk price is determined by Cheddar cheese yield if end product pricing is used.

Cheese Yield

Cheese yield depends on milk casein content, casein-to fat ratio (C/F), and mechanism of cheese production (7). Cheese yield as predicted by the Van Slyke and Price formula must be modified to accurately predict yield. As no easy, direct procedure for casein measurement exists, casein is given an average value of 78 percent of total protein (12, 21). Fat recovery in industry may be below the Van Slyke and Price predicted value of 93% (7). A working formula for predicting Cheddar cheese yield can be developed for each cheese company based on its individual fat and casein recovery (14, 31).

During cheese making, casein aggregates to form a network entrapping milk fat and some water. A direct linear relationship has been demonstrated between amount of fat and casein in milk and cheese yield (58). A cheese yield formula often predicts higher yield than

realized. One reason may be low casein content in milk (58). Casein is not a constant percent of milk protein and the C/F must be adjusted before cheese making. A low casein level in milk leads to more fat loss in whey and a low moisture cheese. Standardizing the C/F between 0.7 and 0.64 gives a quality cheese (31).

Accurately predicting cheese yield depends on the ability to calculate casein and fat present in milk. Blake et al. (12) believes a direct measurement of major protein fractions of milk is necessary to consistently predict cheese yield. Determining cheese yield from milk requires accurate analytical procedures for fat and casein measurement (7).

Dairy Adulteration

Whey protein (WP) and cow milk are less expensive than nonfat-dry milk (NDM) or goat milk. Adulteration of NDM with WP, or goat milk with cow milk is financially attractive but prohibited by the Code of Federal Regulations (24). Addition of WP to NDM may be detected by the turbidometric method of Harland and Ashworth (42) with modification by Leighton (51), or by the HPLC method of Olieman and Van de Bedem (59). These procedures have limitations in sensitivity and are unable to detect addition of acid or heat-denatured whey proteins to NDM. An electrophoretic method of Basch et al. (11) can detect greater than 1% added WP but the procedure is time consuming. Amino acid analysis procedure by Greenberg and Dower (37) can detect greater than 10% added WP to NDM. Detection of 2 to 5% added cow milk to goat milk can be done by HPLC (38), immunochemical or electrophoretic methods (2, 4, 9, 35, 62).

Factors Influencing
Protein Concentration

Proportion of casein to total protein in milk is influenced by three major factors (41, 76): genetic variations are found in species, breeds and individuals; physiological differences

include stage of lactation and age of cow; environmental factors include feed and climate. The estimated ratio of casein to serum protein is 80:20.

Casein number described by Rowland (67) is percent casein nitrogen divided by percent total nitrogen times one hundred. An average casein number for normal milk is 77 (67) but deviation occurs depending on season, breed, diet, disease, storage and stage of lactation.

Season. The casein proportion of total protein varies with season. Kinstedt et al. (48) found casein number in Vermont milk ranged from 74.9 in summer to 79.3 in winter. Szijarto et al. (70) showed pooled milk in Ontario (80% Holstein) had similar significant differences in amounts of casein, whey protein and nonprotein nitrogen among milks from different seasons. Szijarto et al. also found highest casein concentration in winter months, 77.19%, and the lowest in summer months, 73.07%. Change in percent casein was accompanied by an opposite trend in nonprotein nitrogen. Nonprotein nitrogen was highest in summer, 7.59% and lowest in winter, 4.93%. Bruhn and Franke (18), McDowell (53), and Nickerson (58) also reported seasonal variation similar to Szijarto et al in casein as percent of total protein.

Davies and Law (25) found concentrations of casein fractions varied with higher concentrations in winter months and lower in summer. Harding and Royal (41) measured seasonal changes in milk proteins and found relative proportions of casein, whey proteins and nonprotein nitrogen as a percent of total protein remained reasonably constant through the year.

Breeds. Bruhn and Franke (18) and Cerbulis and Farrell (21) demonstrated the statistical difference in protein concentrations by breed. Jersey milk had the highest protein content, 4.07%, followed by Brown Swiss, Guernsey, Ayrshire, Shorthorn, and Holstein with the lowest level, 3.07% (21). Casein number also varied among breeds (12, 21). Holstein and Shorthorn milk had a wider variation than other breeds with values ranging from 62 to 82. Jersey milk showed the least variability in casein number, 72 to 86 (12).

Diet. A diet high in grain and low in roughage (hay) increases protein content in milk. Energy deficiencies in diet result in milk with lower solids (32). Bartsch et al. (10) found cows fed roughage only produced less milk, SNF, protein and casein than cows fed roughage and grain. Increasing grain in diets increased casein, β-lactoglobulin and α-lactalbumin proportionally to total protein increase (10). Yousef et al. (79) also showed a diet with higher grain-to-roughage ratio increased total protein concentrations with proportional increases in α_s-casein and β-lactoglobulin fractions.

Disease. Mastitis is the most common disease in dairy cattle (32). This infection, characterized by greater than 500,000 somatic cells per milliliter of milk, causes compositional changes in milk (77). Rowland (67) first noted milk from cows with mastitis or subclinical mastitis had decreased casein and lactose levels and increased total protein, immunoglobulins and serum proteins compared to normal milk.

Feagan (32) and Haenlein et al. (39) noted specific changes of protein fractions in mastitic milk. Concentrations of α_{s1}-casein, β-casein, β-lactoglobulin, and α-lactalbumin decrease and κ-casein, immunoglobulin and serum albumin concentrations increase. Later, Anderson and Andrews (3) showed results similar to Feagan (32) and Haenlein et al. (39) except concentration of α-lactalbumin and β-lactoglobulin did not change in mastitic milk and β-casein concentration decreased more than α_{s1}-casein concentration. Variations in results may be caused by type of pathogen involved, stage of infection, or methods used to quantify protein fractions.

Storage. In the dairy processing industry, large volumes of milk are often stored at 0 to 5°C for as long as 72 h (23, 26). Raw milk is usually kept at or below 5°C during transport and before processing. Storage of raw milk at cold temperatures is selective for the growth of gram negative psychrotrophic microorganisms. Storage of raw milk at 5°C for 3 d can increase psychrotrophic counts from 4.3×10^4 to 1.3×10^6 colony forming units (23).

Gram negative psychrotrophs display proteolytic activity while producing little acid (6). DeBeukelar et al. (26) showed degradation of α_s-casein and β-casein from this proteolytic

activity with no decrease in whey protein concentrations. Adams et al. (1) reported slightly different results showing κ– and β-caseins are most susceptible to proteolytic activity of psychrotrophs and whey proteins are also attacked. Dzurec and Zall (29) noted heat treatment of raw milk before storage decreases psychrotrophic count and keeps casein concentration constant during normal storage.

Stage of Lactation. After parturition, first milk or colostrum contains more mineral salts, total protein, and immunoglobulins but less lactose than normal milk. During the first five weeks after lactation, lactose and protein decrease whereas fat content either remains the same or increases. Lactose, protein and pH increased over 10 mos of lactation and fat content gradually tapers off. Casein content increases during lactation as milk yield decreases (76).

Variations in Cheese Yield

Casein number can be used to evaluate suitability of milk for cheese manufacture (12). A high casein number in milk generally leads to an increase in cheese yield because there is enough casein present to entrap the fat. This may be obtained by feeding cattle a high grain/low roughage diet and using milk from winter months or Jersey milk. Jersey milk has a high casein number with little variability (60).

Poor cheese is made from stored raw milk or from milk with high psychrotroph count. Cheese from spoiled milk usually has high moisture, weak body and sour or stale flavor (22, 23). Cottage cheese yield decreases 2.5 to 3% for each day of low temperature storage because of a decrease in casein content from bacterial proteolytic activity (6).

Variations in Milk Coagulation

Rennet curd firmness and clotting time of milk vary depending on the amount and quality of casein in milk. Seasonal changes in milk proteins lead to variations in clotting time of milk used for processing (52). Mastitis and prolonged storage of raw milk cause an

increase in milk pH leading to increased clotting time. Curd from mastitic milk lacks firmness and such cheese has a weak body (16, 32). A delayed rennet coagulum also results from prolonged storage of milk at 5°C (23).

Amino Acid Analysis

Accuracy of amino acid analysis is limited by sample preparation and hydrolysis rather than analysis conditions. Amino acid hydrolysis is typically carried out in 6 N HCl at 110°C (54). Under these conditions tryptophan is completely destroyed. Threonine, serine, and tyrosine are partially destroyed, cystine and methionine are destroyed or oxidized while valine, isoleucine and leucine are incompletely hydrolyzed. The rate of amino acid decomposition during hydrolysis depends on concentration of acid, time and temperature of hydrolysis, specific proteins and presence of carbohydrates, aldehydes or metal impurities (65).

Moore and Stein (54) and Savoy et al. (68) reported 5 mg of protein to 1 ml of 6 N HCl is the optimum ratio in sample hydrolysis for high amino acid yields. Moore and Stein sealed hydrolysis tubes under vacuum while Savoy et al. (68) nitrogen flushed sample tubes. Kaiser et al. (46) added ultrasonic removal of dissolved air. Hydrolysis at 145 ± 2°C for 4 h ± 5 min (65) gives amino acid yields in good agreement with hydrolysis at 110± 1°C for 20 to 26 h (28, 54, 68). One hundred percent recovery of each amino acid is not possible with one sample preparation and hydrolysis technique; however, consistent values can be obtained by following one procedure.

Milk Proteins

Milk proteins can be divided into two main categories based on solubility at pH 4.6 and 20°C. About 80% of milk proteins precipitate at pH 4.6. This group of proteins is known as caseins. They are colloidal dispersions of protein-mineral complexes called micelles

(17). Proteins remaining in solution at pH 4.6 constitute 20% of milk protein. These whey or serum proteins may contain proteolytic derivatives of caseins (76).

Casein micelles have little secondary or tertiary structure but have a complex quarternary structure. The caseins are nonglobular, phosphorylated proteins with high proline content and little or no cystine residues. α_{s1}-, α_{s2}-, β-, and κ-casein comprise 38, 10, 36, and 13% of the total casein in milk. γ-casein, a plasmin caused fraction of β-casein, comprises 3% of the total casein in milk (33).

Most serum proteins are globular and do not contain ester-bound phosphates. They are more heat sensitive and less calcium sensitive than caseins (17). α-lactalbumin and β-lactoglobulin comprise 21 and 54% of total serum proteins. They have a higher cystine content than caseins with little or no proline residues. Other serum proteins include serum albumin and immunoglobulins (33).

Milk proteins are products of co-dominant allelic autosomal genes (71). Gene products are not observed at random or with equal frequencies. Protein variances are determined by amino acid sequence, not electrophoresis, because post-translational modifications can change a protein's mobility in polyacrylamide or starch gels (30). Some protein variants have only one amino acid substitution but others have as many as nine. Many variants are not found in Western cattle but only in Indian cattle or in yak milk (30, 76).

α_{s1}-, α_{s2}-, and β-casein are calcium sensitive. Their ability to bind calcium depends on temperature, pH, and ionic strength of the medium and precipitate from solution. α_{s2}-casein, having 10 to 13 serine bound phosphates, is the most calcium sensitive and hydrophilic of the caseins (76). There are five variants of α_{s2}-casein, A, B, C, D, and E, each with two disulfide bonds (17).

α_{s1}-casein has five variants, A B, C, D, and E with B being the most common in Western cattle and C predominant in Indian cattle. There are eight or nine phosphates per protein depending on the variant (76).

β-casein has four or five phosphates and is less sensitive to precipitation by calcium. The six variants of β-casein are A^1, A^2, A^3, B, C, D, and A^2 is the most common (76). β-casein is the most hydrophobic of the caseins and easily dissociates from micelles at 4°C (17).

κ-casein, with A and B variants, is different from other caseins because it has only one phosphate group and is soluble over a wider range of calcium concentrations (76). One end of κ-casein is polar, charged, and contains sugars, while the other two-thirds is hydrophobic. This diversity is responsible for stabilizing casein micelles (76).

β-lactoglobulin exists naturally as a dimer and has seven variants A, B, C, D, E, F, and G. A and B variants are the most common in Western cattle while E is found only in yak milk (30, 76). The two disulfide bridges and one thiol group of β-lactoglobulin can form crosslinks with the two disulfides in κ-casein and α_{s2}-casein when heat treated (17).

Two genetic variants of α-lactalbumin exist, A and B. Only B is found in Western cattle (76). α-lactalbumin acts as a coenzyme in lactose synthesis (17).

Milk Protein Measurement

Measuring casein or whey proteins is difficult because most accurate procedures are lengthy and quick methods are often inaccurate (15). Time-consuming methods involve separation of casein from whey and measuring fractions separately for protein. Faster methods often involve total milk protein measurement with casein considered a constant fraction.

The standard Association of Official Analytical Chemists (AOAC) procedure for measuring casein and serum proteins is precipitation of casein at pH 4.6 from whole milk with acetic acid and sodium acetate at 40°C (44, 66). Percent casein nitrogen is determined by difference of total nitrogen content in milk and nitrogen content in the supernatant. Multiplying percent nitrogen by 6.34 or 6.38 estimates percent casein protein or whey protein (47).

Protein dye binding methods of Udy (73), Vanderzant and Tennison (74), and Ashworth (5) to measure total protein and casein in milk are faster than Rowland's method by Kjeldahl nitrogen determination. Dyes such as Orange G, Orange 12, Buffalo Black, and amido black bind to specific amino acids. Dyes bind to proteins quantitatively forming an insoluble complex that can be separated by centrifugation or filtration. Unbound dye is measured by optical density. A binding capacity is calculated, allowing a conversion factor for each protein to give percent protein.

Dye binding procedures should be performed using fresh milk. Casein content is determined by measuring non-casein protein separated from milk at pH 4.6. Casein is calculated by difference of dye binding of original milk and dye binding of whey proteins (56). A drawback to this procedure is that the same conversion factor is used for both caseins and serum proteins while their dye binding capacities are different (8). Dye binding methods became automated by instruments such as the Pro-Milk Tester, Pro-Milk Automatic and Pro-Milk MKII (40).

A popular method for measuring constituents in bulk milk samples is infrared spectrometry (36, 40, 72). Fat, protein, and lactose are measured at wavelengths of 5.73 μm, 6.46 μm, and 9.6 μm. Barbano and Dellavalle (8) improved Sjaunja and Schaar's (69) procedure of measuring casein by infrared spectrometry by using phosphoric acid in place of acetic acid to precipitate casein. Carboxyl groups of acetic acid absorb infrared radiation at the same wavelength as protein. Milk is first tested for total protein by infrared spectrometry with an infrared milk analyzer. Casein is precipitated by adjusting pH to 4.6, filtered twice and non casein protein filtrate tested for protein content by an infrared milk analyzer (8). The difference between total protein and non-casein protein filtrate estimates percent milk casein.

Other methods are available to measure casein or serum proteins. Immuno-electrophoresis (49) quantifies casein by using antibodies specific against various milk proteins. This procedure works well for heat-treated milk or milk products because it does

not measure heat-denatured whey proteins that acid precipitate with casein. For an immunological method to be accurate, specific antibodies against each milk protein variant must be made that do not cross react.

Refractometry can be used to measure casein (55). Casein is acid precipitated from whole milk and dissolved in a basic solution to be measured by refractive index. This procedure gives results in good agreement with AOAC methods.

Size exclusion column chromatography (20) can be used to separate casein micelles from non-casein protein. This method can easily be linked to infrared instruments for casein measurement at 6.46 μm wavelength.

Reverse Phase Protein Separation

Variation in hydrophobicity among peptides and proteins provides a means of separating mixtures of proteins by reverse phase high performance liquid chromatography (RP HPLC). Reverse phase (RP) supports are porous silicas with organosilane bonded phases such as ethyl-, octyl-, or octadecyl-silane. Solute retention in RP systems depends on hydrophobicity of proteins relative to the polarity of mobile and stationary phases (57). Polar mobile phases (buffers) favor solute retention while nonpolar solvents (acetonitrile, methanol, propanol) diminish it (63). Proteins can be made more hydrophobic, with increased retention time by ion pairing. A simple means of ion pairing is pH modification. Under acidic conditions protein carboxyl groups are protonated, decreasing solubility in the mobile phase and increasing retention time. Increasing retention time allows selective desorption of proteins by non-polar solvents (63).

Reverse phase separation of caseins and serum proteins has recently been investigated. Carles (19) separated caseins on a C_{18} column using a neutral phosphate buffer, propanol, and sodium dodecyl sulfate. Casein separation must first involve dissociation of casein micelles. This can be done by using reducing agents such as mercaptoethanol in sample preparation.

Diosady and Bergen (27) separated whey proteins on a C_8 reverse phase column. Pearce (61) showed improved results of whey protein separation on a C_6 column, using sodium chloride buffer (pH 2.1) and acetonitrile. Decreasing the polarity of the stationary phase showed improved separation of proteins but these columns did not become commercially available until recently.

Measuring Proteins

Each protein has unique characteristics which allow quantitative measurement in a mixture. Quantifying often involves separating proteins in a mixture and analyzing each protein fraction. Proteins differ in amino acid sequence, charge, size, three dimensional structure, amino acid composition and number of bound phosphate or sugar groups.

Ultrafiltration, dialysis, SDS electrophoresis and gel permeation separate proteins based on size. Ultracentrifugal analysis can resolve protein mixtures into their various components based on molecular size. Ion exchange chromatography and gel electrophoresis can be used to separate proteins based on charge. Immunochemical methods of diffusion and electrophoresis can quantitate proteins in a mixture. Procedures of measuring individual proteins in a mixture which require separation of each protein are time consuming and have many sources of error.

Measuring individual components of a mixture would be easy if individual components absorbed a different wavelength of light. Mixtures could then be scanned at different wavelengths to measure each component. Proteins do not absorb light at different wavelengths, but each protein has an amino acid composition that is unique to that protein. Amino acid compositions of each protein can be used to measure proportions of specific proteins or groups of proteins using regression analysis or simultaneous equations.

OBJECTIVES

The purpose of this study was to find an easy, fast procedure to estimate the concentration of the five major proteins in milk simultaneously with amino acid analysis. Because concentrations of milk protein variants vary, each protein must be separated from milk and analyzed individually for amino acid composition. Amino acid compositions of each milk protein were used to solve for concentrations of each protein in mixtures.

Relative amino acid compositions of groups of proteins such as whey, NDM, goat milk, and casein vary. Additions of one of these proteins to another was measured by changes in total amino acid composition.

MATERIALS AND METHODS

Preparation of Casein and Whey Proteins

Whole, fresh milk obtained from Utah State University Dairy Products Laboratory, Logan, Utah, was skimmed by centrifuging at $5,000 \times g$ for 30 min at 5°C. Skimmilk was acidified to pH 4.6 with concentrated HCl at 25°C. The mixture was stirred for 15 min before centrifuging at $3,000 \times g$ for 10 min at 2°C. Supernatant was decanted and saved while casein pellets were washed with distilled water. After washing and centrifuging the casein fraction twice, casein was suspended in distilled water and brought to pH 6.7 with 1 N NaOH. Both the original supernatant and neutralized casein were lyophilized for experiments. Kjeldahl nitrogen determination was done for each fraction.

Separation of Caseins

<u>Column Parameters.</u> Reverse phase chromatography was used to separate casein into its fractions. A Hamilton C_{18} (Hamilton Co., Reno, NV), 0.007 µm (70 Å) pore size column was rejected as the stationary phase was too hydrophobic to allow separation of α_s-casein and β-casein. A Beckman C_3 (Beckman Instruments Inc., Fullerton, CA), 0.03 µm (300 Å) pore size column improved separation of casein.

<u>Determining Mobile Phase</u>. The mobile phase consisted of a buffer and an organic solvent. Solvent A, .15 M NaCl/triethylamine (1 ml/L, pH 2.5), was acidified with nitric acid. A low pH buffer is common in reverse phase protein separation (63). Different mixtures of HPLC grade acetonitrile and water (solvent B) varying from 90 to 20% acetonitrile were used to find the best combination for protein separation. Other solvents such as methanol and propanol were also experimented with as the B solvent. Both solvent A and acetonitrile:water solvent B were filtered (0.2 µm) and degassed by placing in an ultrasonic cleaner under vacuum.

Casein Sample Preparation. Mercaptoethanol and urea were used to dissociate casein micelles. Effects of temperature and addition of either mercaptoethanol, urea or both on sample separation were tested. Lyophilized casein was dissolved in various ratios of sample buffer, acetonitrile, mercaptoethanol, and 5 M urea in sample buffer. Some samples were heat treated in a 50°C water bath for 15 min. All samples were filtered (0.2 µm) and separated by HPLC.

Separation of Whey Proteins

Column Parameters. Whey proteins were separated on a Beckman C_3 column. Mobile phase was as described by Pearce (61). Solvent A was degassed 0.15 M sodium chloride acidified to pH 2.4 with nitric acid. Solvent B was HPLC grade acetonitrile. Both solvents were filtered (0.2 µm). The column temperature was 30°C.

Sample Preparation and Gradient. Solvent A and 5 M urea in solvent A were tested to dissolve whey protein standards and samples. Gradient and flow rate were varied to separate α-lactalbumin and β-lactoglobulin.

High Performance Liquid Chromatography

A Beckman HPLC that included a Model 420 controller, Model 164 variable wavelength detector and a Model 427 integrator was used. Detector wavelength was set at 280 nm to measure protein peaks. Detector range was set at 0.1 and integrator attenuation was 16. A 20 µl sample loop was attached to the injector valve.

Collecting Protein Fractions

Casein and whey protein samples and protein standards were measured at 280 nm. Detector outlet tubing was connected to a fraction collector. The fraction collector was programmed to collect between 5 and 8 ml of solvent. Test tubes containing proteins were dried under nitrogen in a waterbath at 40°C.

Gel Electrophoresis

Electrophoresis of Casein. Polyacrylamide gel electrophoresis of casein fractions was as described by Yiadom-Farkye (78) using a vertical Hoefer Scientific SE600 (Hoefer Scientific Instruments, San Francisco, CA) apparatus. All buffers and gels were made with distilled water and filtered (0.2 μm). Stacking gel buffer was 0.05 M tris (hydroxymethyl) methylamine adjusted to pH 6.8 with concentrated HCl. Resolving gel buffer was 0.3 M tris (hydroxymethyl) methylamine adjusted to

pH 8.8 with concentrated HCl. Reservoir buffer, pH 8.3, consisted of 0.025 M tris (hydroxymethyl) methylamine and 0.019 M glycine.

Stacking gel contained 7 M urea and a commercial mixture of 5% acrylamide with N,N'-methylene-bis-acrylamide (Sigma Chemical Co., St. Louis, MO) in stacking gel buffer. Ammonium persulfate 1.75 ml (1.5% w/v) and 0.015 ml N,N,N,'N'-tetra-methylethylene-diamine (TEMED) were added to 50 ml stacking gel.

Resolving gel contained 7 M urea and 7% acrylamide and N,N'-methylene-bis-acrylamide in resolving gel buffer. Ammonium persulfate (1.0 ml) and TEMED (0.015 ml) were added to 50 ml resolving gel.

Test tubes containing dried casein fractions or commercial casein standards were dissolved in 0.45 ml sample buffer (8 M urea in stacking gel buffer). Mercaptoethanol (0.03ml) and 0.02 ml tracking dye (10% sucrose in 1% bromophenol blue) were added. Samples were placed in a 40°C water bath for 30 min before electrophoresis.

Electrophoresis of Whey Proteins. Procedures of Yiadom-Farkye (78) and Hillier (43) were modified to separate whey proteins by polyacrylamide electrophoresis. Reservoir and stacking gel buffers were the same as in casein electrophoresis. Resolving gel buffer was 0.3 M tris (hydroxymethyl) methylamine (pH 8.9).

Stacking gel was 5% acrylamide and N,N'-methylene-bis-acrylamide in stacking gel buffer. Resolving gel was 8% acrylamide and N,N'-methylene-bis-acrylamide and 7 M

urea in resolving gel buffer. Final concentrations of ammonium persulfate were 2% in stacking gel and 3.5% in resolving gel. TEMED (.03%) was used in both gels.

Whey protein fractions and whey protein standards were dissolved in 200 μl sample buffer (3 M urea in stacking gel buffer). Mercaptoethanol (0.4 ml) and tracking dye (0.01 ml) were added to samples before placing in a boiling water bath for 5 min.

Gel Running. Gels were pre-run for 30 min at 0.2 kV. A current of 2 mA was applied until samples reached the gel interface. For the remainder of the 3 h running period a constant voltage of 0.2 kV was applied.

Staining and Destaining. Gels were stained for 30 min in a solution of Coomassie Blue: water: methanol: glacial acetic acid (.001: 65: 25:10). Destaining was in a solution of glacial acetic acid: methanol: water (7.5: 25: 67.5).

Protein Fraction Hydrolysis Preparation

Dried protein fractions were transferred from HPLC collection test tubes to hydrolysis vials rinsing three times with distilled water. Distilled water was dried from vials under nitrogen in a 40°C water bath. Some protein fractions were combined in one vial before drying.

Detection of Added Protein
to Dairy Products

Casein, Whey and Nonfat-dry Milk. Total protein determination of casein, whey and NDM was by Kjeldahl nitrogen multiplied by 6.38. Total protein was used to calculate hydrolysis sample weights. Eleven samples were weighed in duplicate or triplicate ranging from 0 to 100% casein added to NDM and 0 to 100% whey protein added to NDM. Each sample contained about 25 mg of protein.

Goat and Cow Milk. Whole goat milk was from a Logan goat farm and whole cow milk was from Utah State University Dairy Products Laboratory. Both milks were centrifuged at 5,000 × g for 30 min at 2°C to separate fat. The top fat layer was removed and Kjeldahl

nitrogen determination of the skimmilks was performed. Eleven samples were weighed in duplicate ranging from 0 to 100% goat milk in cow milk. Each sample contained about 25 mg of protein.

Amino Acid Analysis

Sample Hydrolysis. Hydrochloric acid (6 N) was added to sample vials for a final concentration of 5 mg of protein per milliter of acid. Hydrolysis vials were attached by tubing to a nitrogen tank and a vacuum. A valve attached to the tubing allowed selection of either nitrogen or vacuum. Vials were placed in an ultrasonic cleaner and flushed with nitrogen, alternating with vacuum. After 4 min, vials were sealed under vacuum. Samples were hydrolyzed in a heating block for 4 h at $145 \pm 2°C$.

After hydrolysis, samples were allowed to cool. Ten microliters from each sample were filtered (0.2 μm) and dried under nitrogen. Dried samples were stored in a freezer.

Amino Acid Analysis. Samples were analyzed for amino acids using a Beckman 6300 Amino Acid Analyzer. Hydrolysates were dissolved in 250 μl sample dilution buffer (Beckman) and loaded into a sample coil. As many as 15 samples were run at one time with data given in peak area. Areas of amino acid standards were used to calculate quantity of each amino acid in samples.

Statistical Procedures

Forward stepwise regression on SAS (SAS Institute Inc., Cary, NC) was used to select the best amino acids to predict added protein to NDM or cow milk.

Computer programs written for a Tektronix 4052 computer were used to solve for each major milk protein. The programs stored amino acid compositions of protein standards, changed matrix dimensions and solved for α_S-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin concentrations using the Gauss-Jordan procedure of solving simultaneous equations. The programs are presented in Appendix A.

RESULTS AND DISCUSSION

HPLC Separation of Caseins

Urea and mercaptoethanol were used for dissociation of casein micelles. Casein was dissolved in 7% 5 M urea in solvent A, 10% mercaptoethanol, and 10% acetonitrile. Casein did not separate or dissolve as well with other combinations of urea, mercaptoethanol, buffer or acetonitrile. Heating samples before separation did not improve separation.

The organic solvent B was a mixture of HPLC grade acetonitrile and water. Concentrated acetonitrile elutes caseins at the same time. Less than 20% acetonitrile, or other solvents such as methanol or propanol, was not strong enough to elute proteins from the column. A concentration of 40% acetonitrile eluted κ-, α_S-, and β-casein. Higher concentrations of acetonitrile decreased β-casein retention time and lower concentrations increased peak width. A gradient, beginning with 60% B, was used to separate caseins (Table 1).

A chromatograph of casein standards is shown in Figure 1. Major peaks numbered 1, 2, and 3 correspond to κ-, α_S-, and β-casein. α_S-casein is both α_{S1}-casein and α_{S2}-casein.

A chromatograph of unknown casein is shown in Figure 2. Retention times of major peaks are similar to κ-, α_S-, and β-casein standards in Figure 1. Quantities of each unknown casein peak were calculated based on standard peak areas.

HPLC Separation of Whey Proteins

Whey proteins were separated using solvent A, 0.15 M sodium chloride (pH 2.4), and solvent B, acetonitrile. Whey protein standards and samples did not dissolve in solvent A but did dissolve in 5 M urea in solvent A. Table 2 is the gradient separating whey proteins beginning with 5% B.

Table 1. HPLC gradient separating κ-, α_S-, and β-casein.

Time (min)	Flow Rate (ml/min)	B Solvent (%)	Duration (min at %B Solvent)
0	1	60	1
1	1	80	7
17	1	87	0.5
24	1.5	80	0.2
25.2	1.5	80	1
30	1.5	100	2
39	1	60	3

Figure 1. Chromatograph of commercial κ-, α_S-, and β-casein standards. Major peaks numbered 1, 2, and 3 correspond to κ-, α_S-, and β-casein.

Absorbance(280 nm)

12.11   24.72   34.59
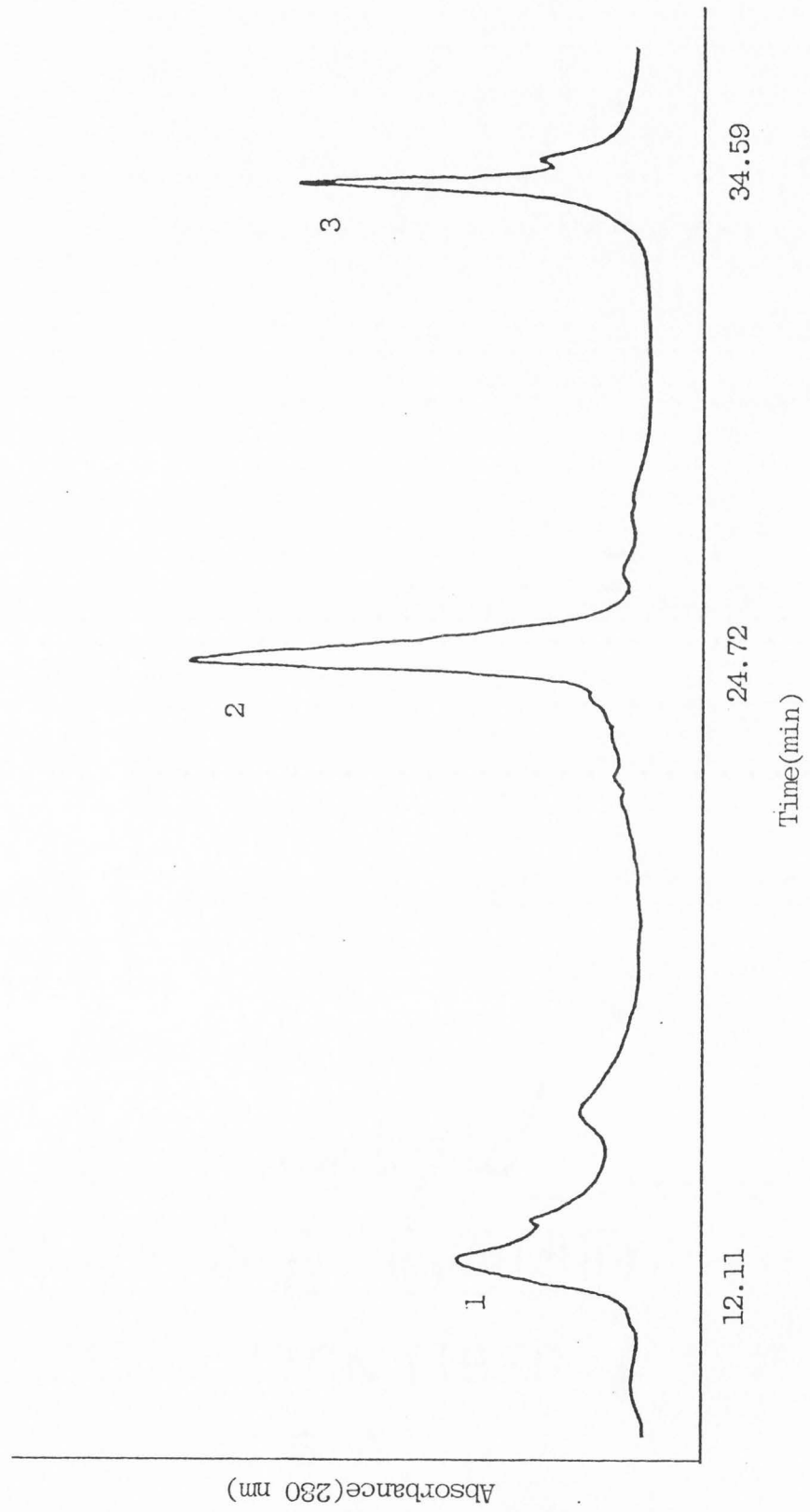
Time(min)

Figure 2. Chromatograph of unknown casein sample. Major peaks numbered 1, 2, and 3 correspond to κ-, α_S-, and β-casein.
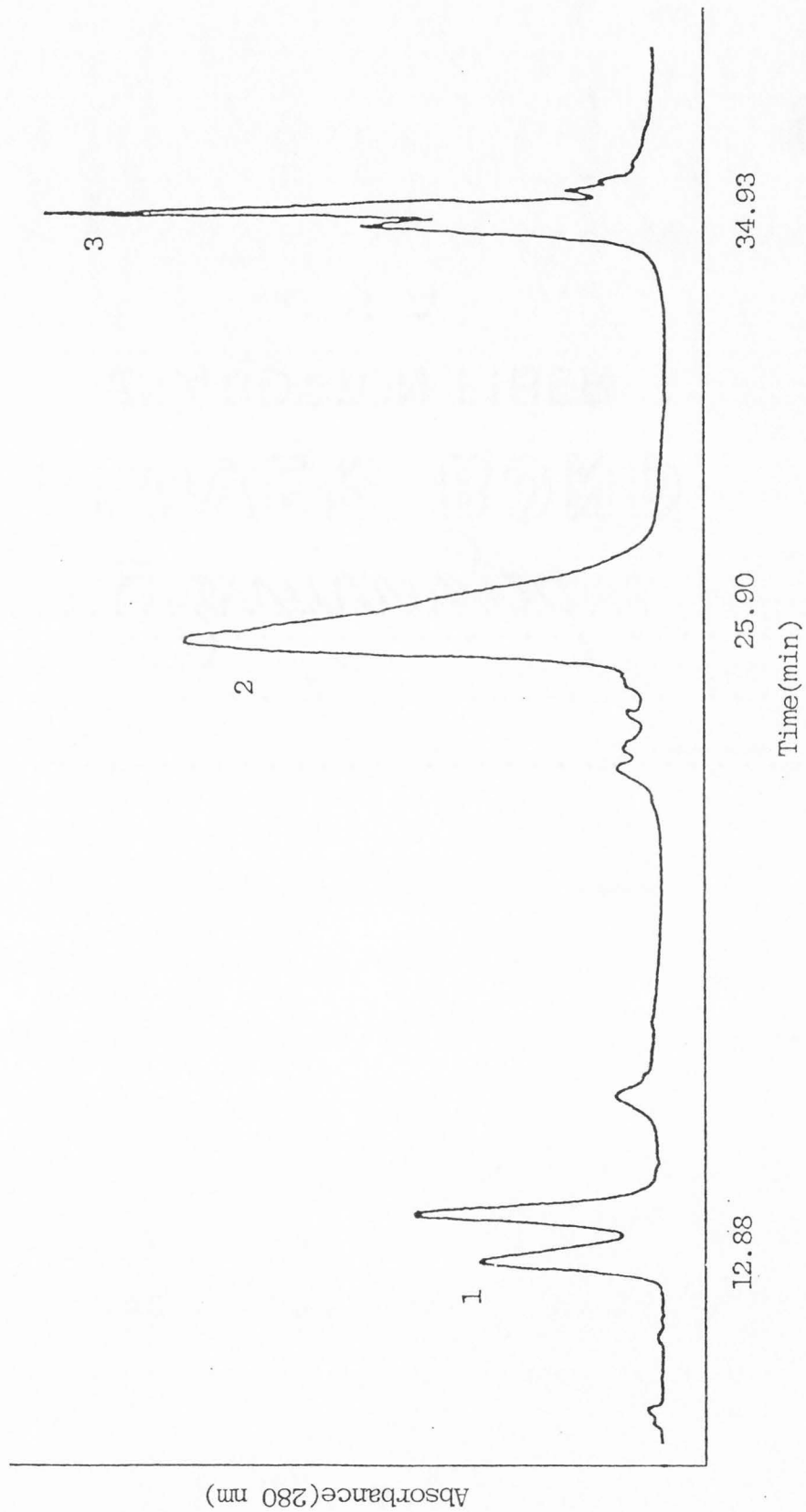
Table 2. HPLC gradient separating α-lactalbumin and β-lactoglobulin.

Time (min)	Flow Rate (ml/min)	B Solvent (%)	Duration (min at %B Solvent)
0	1	5	1
1	1	35	6
8	1	36.2	5
11.2	1.75	36.2	0.3
12.8	1.75	33	1
19	1.75	100	2
25	1	35	5

Figure 3 is a chromatograph of separated whey protein standards. Peak 1 is α-lactalbumin and peaks 2 and 3 are β-lactoglobulin. Figure 4 is a chromatograph of unknown whey proteins. Retention times of whey protein standards are similar to retention times of whey protein samples. Quantities of each unknown whey protein peak were calculated based on standard peak areas.

Electrophoresis of Casein and Whey Proteins

Figure 5 is an electrophoresis gel of purchased casein standards and casein peaks collected as they eluted from the column. The first three bands are eluted β-casein, α_S-casein and κ-casein.

Lane 4 is casein before HPLC separation. Lanes 5, 6, and 7 are commercial standards of κ-, α_S-, and β-casein. The α_S-casein commercial standard contains some β-casein but the collected caseins are pure fractions.

Figure 6 is an electrophoresis gel of commercial α-lactalbumin and β-lactoglobulin and eluted whey protein fractions. Lanes 1 and 2 are α-lactalbumin and β-lactoglobulin commercial standards. Lane 3 is whole whey protein before HPLC separation. Lanes 4, 5 and 6 and lanes 7, 8, and 9 are whey protein fractions of α-lactalbumin and β-lactoglobulin peaks.

These gels show protein peaks collected from the column are pure fractions of milk proteins.

Amino Acid Analysis of Protein Fractions

The amino acid analysis of each milk protein fraction is presented in Appendix B. Figure 7 graphically shows differences in amino acid composition among aspartic acid, proline and leucine in milk proteins. Whey proteins, α-lactalbumin and β-lactoglobulin, have higher

Figure 3. Chromatograph of commercial α-lactalbumin and β-lactoglobulin. Peak 1 is α-lactalbumin and peaks 2 and 3 are β-lactoglobulin.
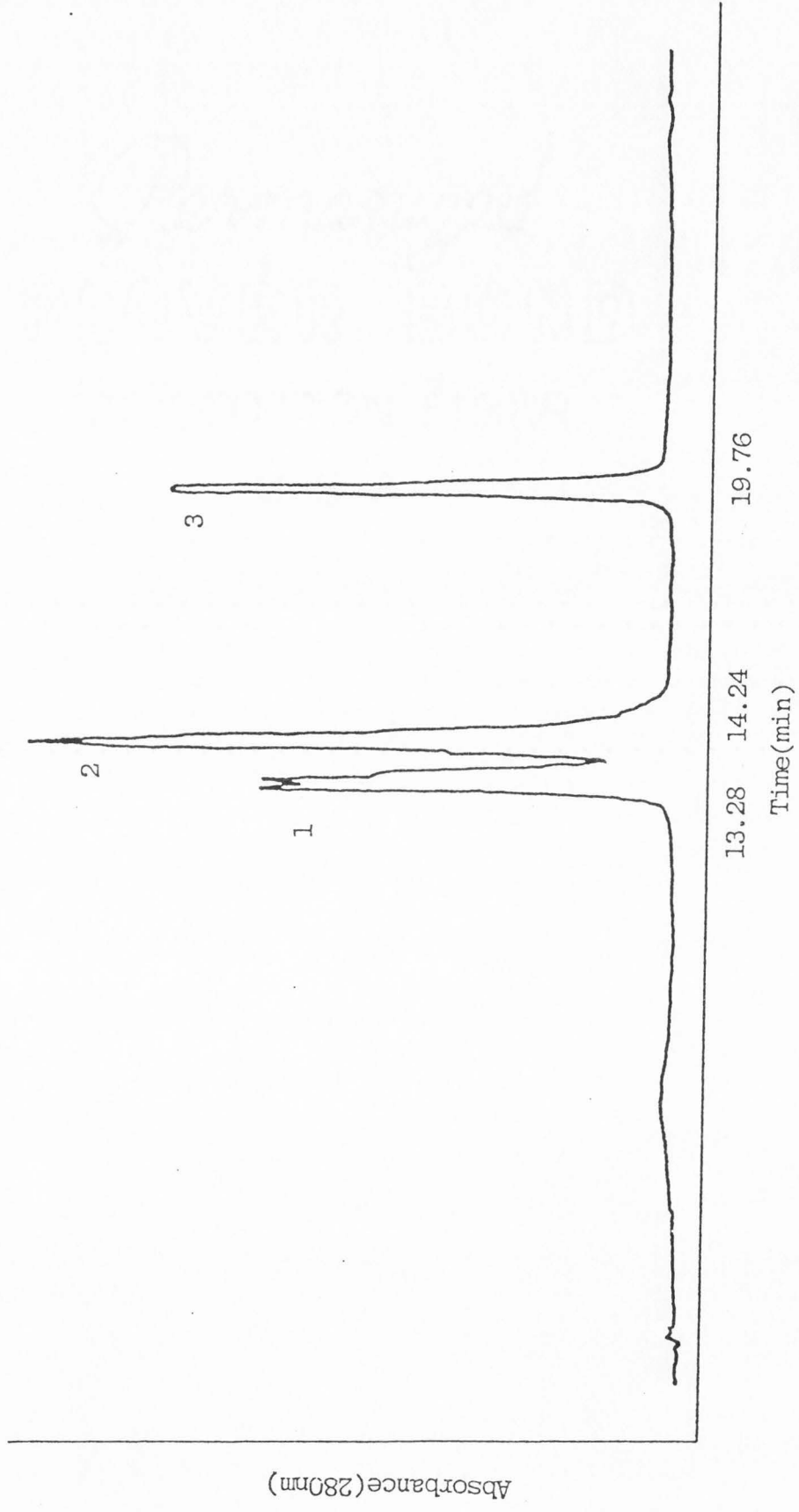
Figure 4. Chromatograph of unknown whey protein sample. Peak 1 is α-lactalbumin and peaks 2 and 3 are β-lactoglobulin.
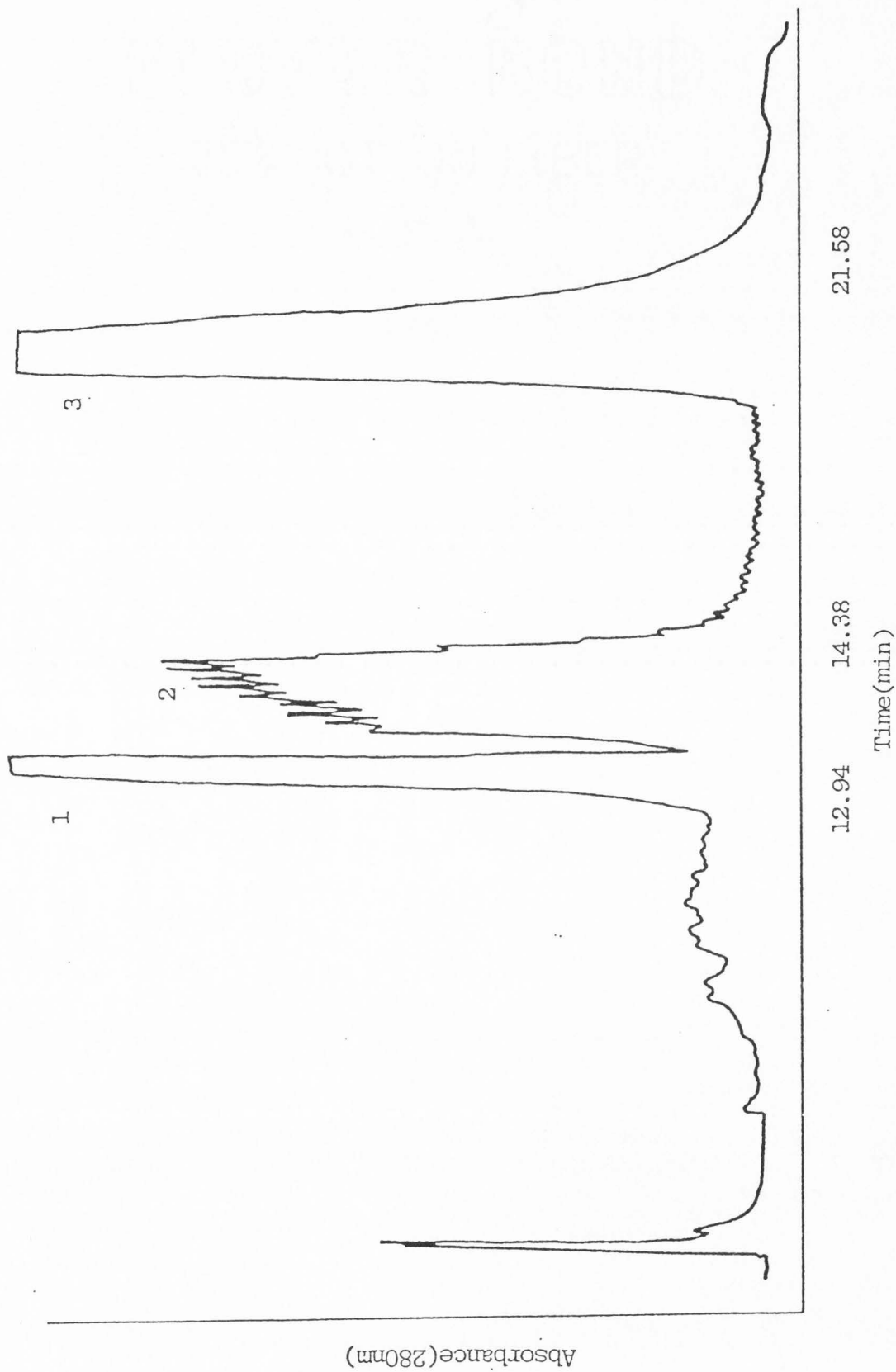
Figure 5. Electrophoresis gel of casein standards and unknown casein fractions. Lanes 1, 2, and 3 are commercial casein standards. Lane 4 is whole casein before HPLC separation. Lanes 5, 6, and 7 are eluted casein fractions.
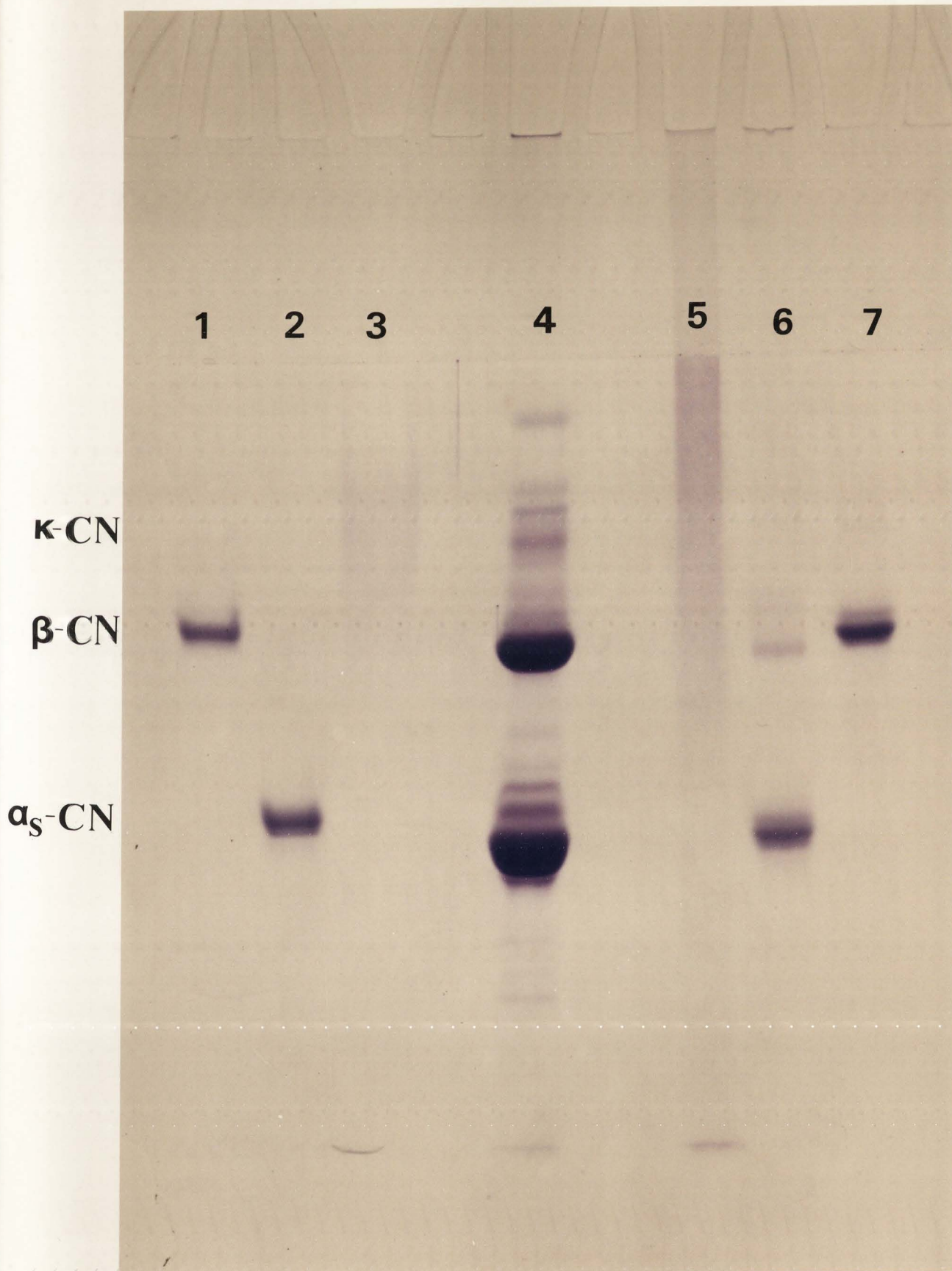
Figure 6. Electrophoresis gel of whey protein standards and unknown whey protein fractions. Lanes 1 and 2 are commercial α-lactalbumin and β-lactoglobulin standards. Lane 3 is whole whey protein.before HPLC separation. Lanes 4, 5, and 6 are eluted whey protein fractions. Lanes 7, 8, and 9 are duplicates of lanes 4, 5, and 6.

β-LG
α-LB
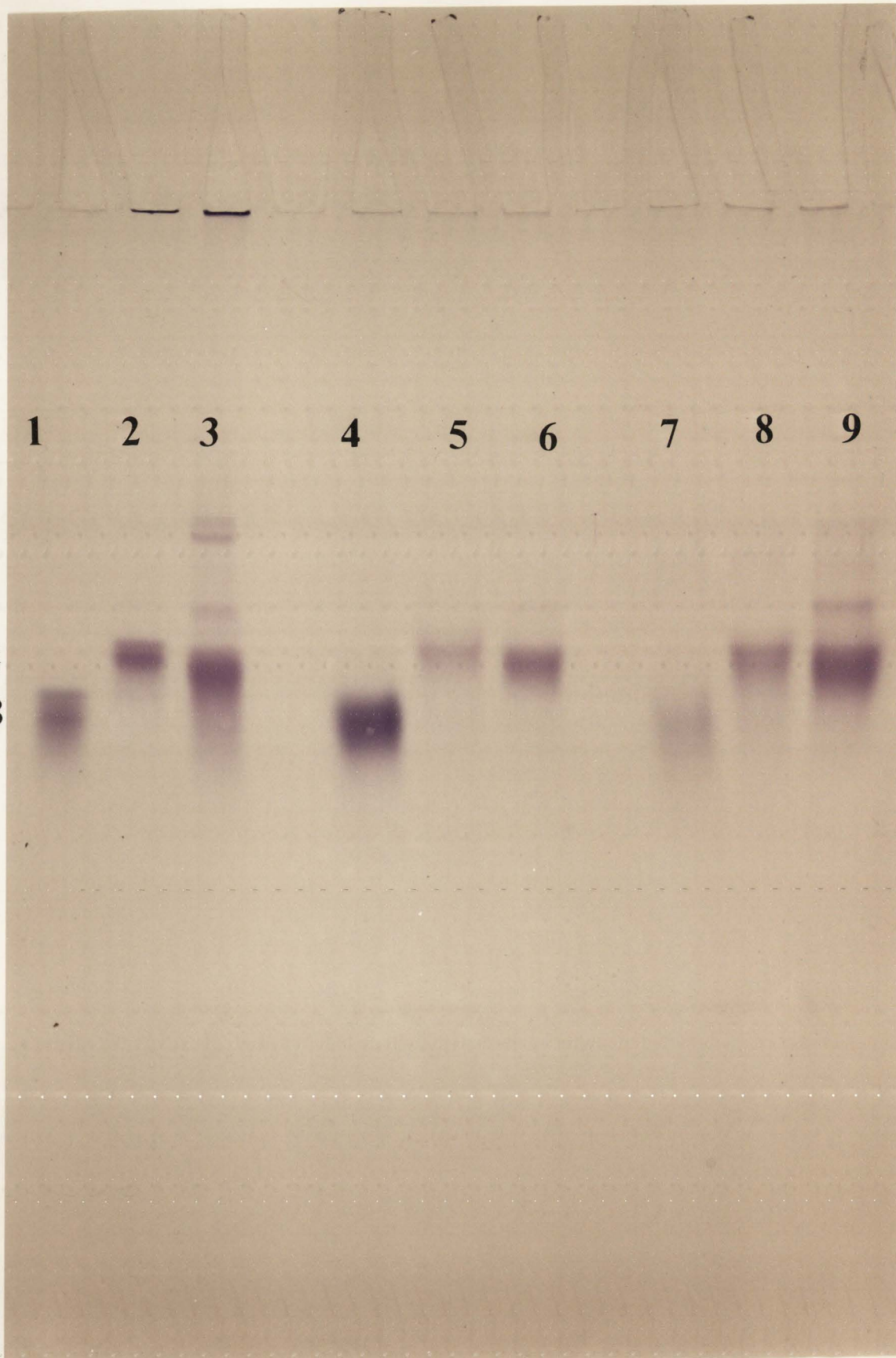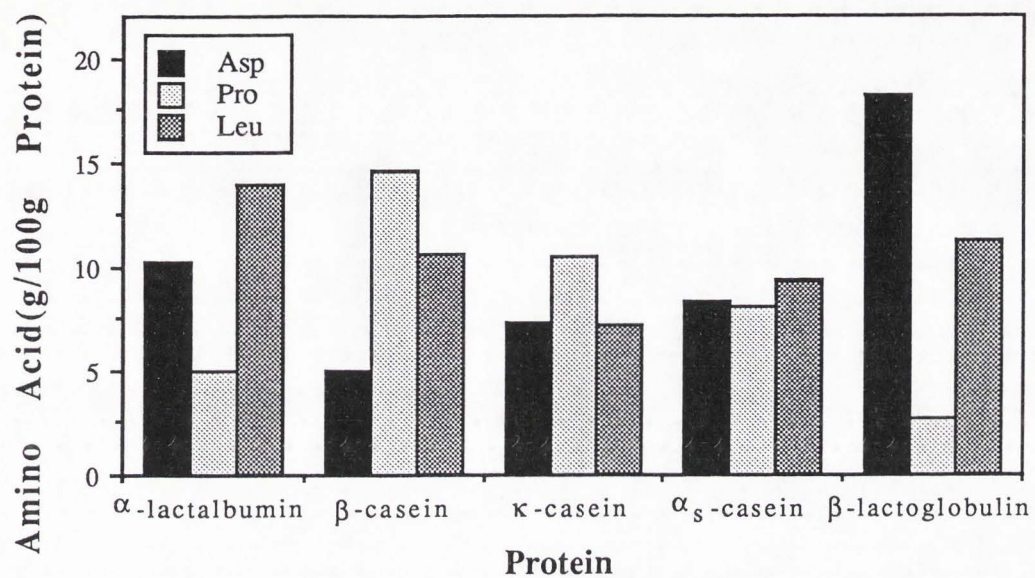
Figure 7. Relative amino acid quantities in milk proteins. The variability in aspartic acid, proline and leucine among milk proteins is shown.

concentrations of aspartic acid and leucine while caseins have higher concentrations of proline.

The amino acid values of Appendix B were entered into the computer programs (Appendix A). Amino acid compositions of mixtures of these proteins were also entered into the program and concentration of each protein in mixtures was determined by solving simultaneous equations.

Figures 8 through 12 are different mixtures with varying ratios of milk proteins. Estimated values were computed by solving for each protein based on amino acid analysis. Actual values are quantities of each protein in mixtures based on HPLC calculations comparing peak areas of unknown versus protein standards. The percent difference between estimated and predicted values is listed in Appendix C. The maximum percent difference is less than 20. Unfortunately each method of calculating concentration of milk proteins in mixtures involves error. Such error is the result of amino acid analysis or HPLC calculations.

Detection of Added Casein to Nonfat-dry Milk

Our NDM samples contained 33.56% protein and our casein contained 79.8% protein. Figure 13 shows eight graphs of added casein to NDM versus selected amino acids. No added casein is 100 percent NDM. Boxed symbols on each graph represent results of amino acid analysis of a specific mixture of casein and NDM. Each amino acid in Figure 13 shows a change in amino acid composition with varying casein ratios. Stepwise regression was used to select amino acids to predict the concentration of casein added to NDM.

Tyrosine and aspartic acid were selected by stepwise regression to form the multiple regression equation in Table 3. Changes in quantities of tyrosine and aspartic acid with an increase in casein concentration are small and opposite, shown in Figure 14. Because NDM
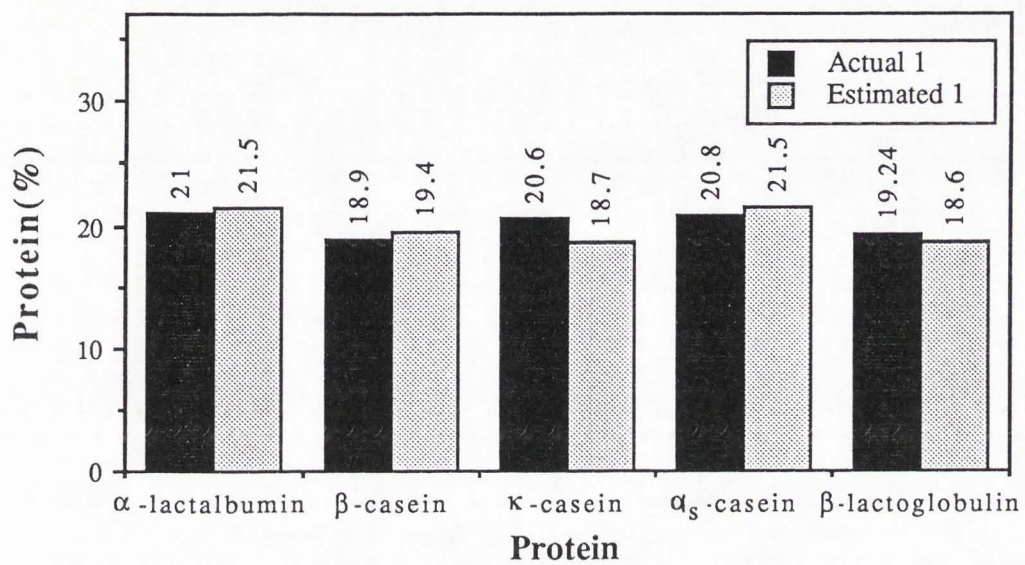
Figure 8. Milk protein estimates: 1. Concentrations of milk proteins in a mixture based on amino acid analysis estimates and HPLC actual values.
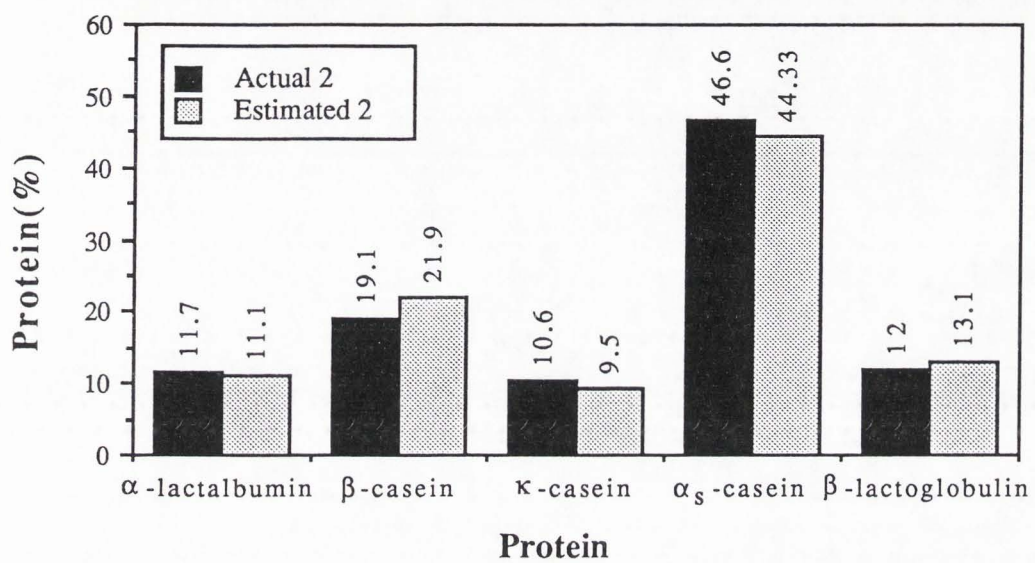
Figure 9. Milk protein estimates: 2. Concentrations of milk proteins in a mixture based on amino acid analysis estimates and HPLC actual values.
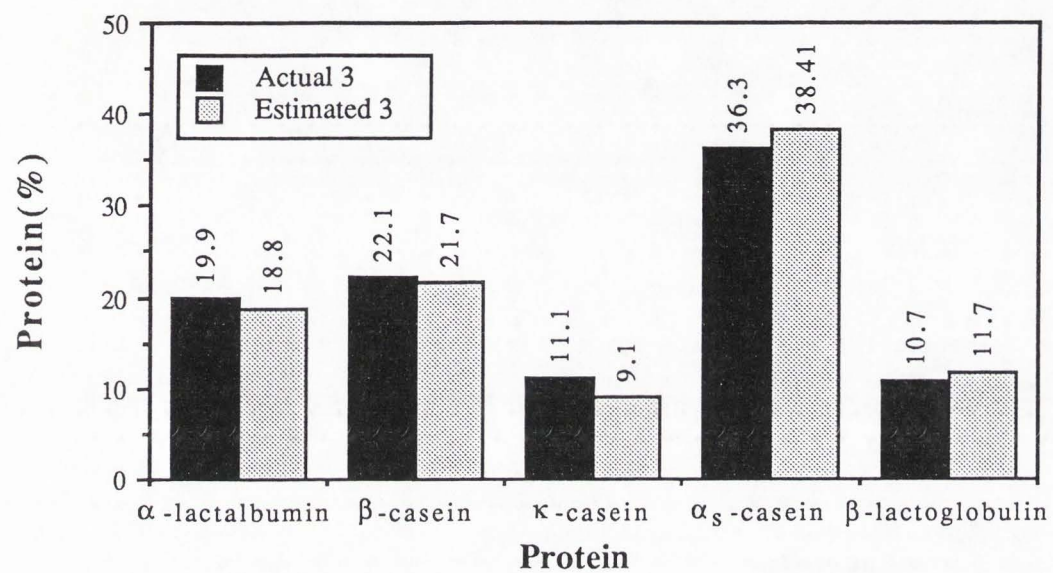
Figure 10. Milk protein estimates: 3. Concentrations of milk proteins in a mixture based on amino acid analysis estimates and HPLC actual values.
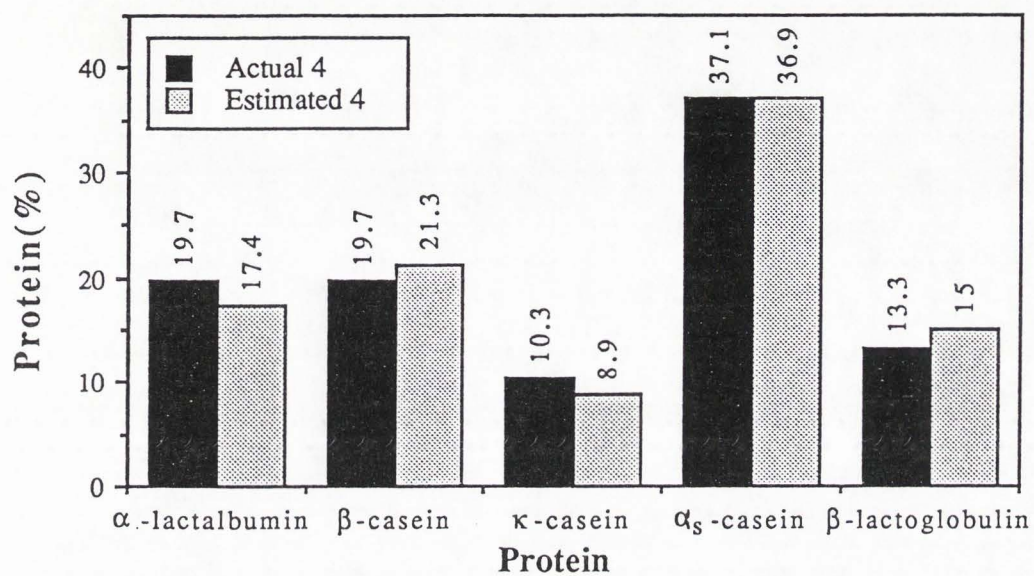
Figure 11. Milk protein estimates: 4. Concentrations of milk proteins in a mixture based on amino acid analysis estimates and HPLC actual values.
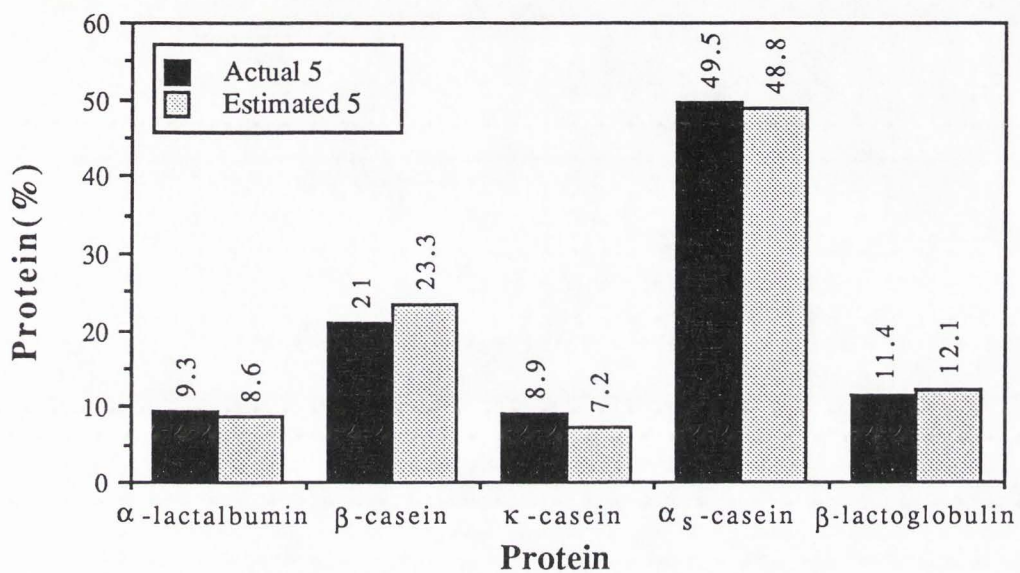
Figure 12. Milk protein estimates: 5. Concentrations of milk proteins in a mixture based on amino acid analysis estimates and HPLC actual values.

Figure 13. Relative amino acid quantities in casein/nonfat-dry milk mixtures. Graphs show the change in amino acid value with varying casein concentration.

Table 3. Regression analysis of casein added to nonfat-dry milk.

Source	df	MS	F value	Pr > F
Model	3	25878.07	2711.56	0.0001
Tyr	1	60579.01	6347.60	0.0001
Asp	1	16823.19	1762.77	0.0001
Tyr*Asp	1	232.01	24.31	0.0001
Error	19	9.54		
Uncorrected Total	22			

Casein(%) = 164.00 Tyr + 1.86 Asp - 22.05 Try*Asp $R^2 = 0.998$
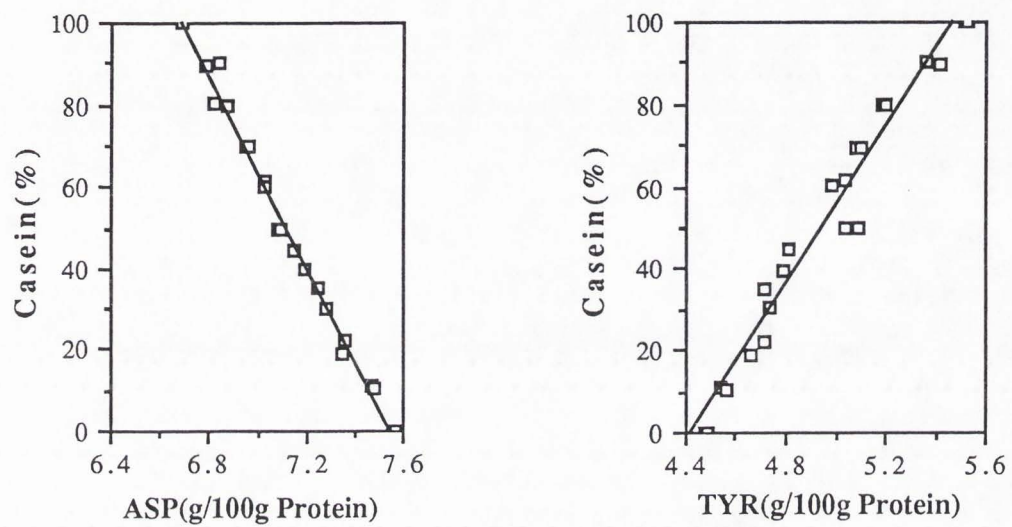
Figure 14. Quantities of tyrosine and aspartic acid in casein/nonfat-dry milk mixtures. Graphs show the change in value of tyrosine and aspartic acid with varying casein concentration.

is about 80% casein, amino acid analysis of mixtures detects this slight change in casein concentration or the loss of whey proteins.

The multiple regression equation uses values of tyrosine, aspartic acid and their interaction to predict concentrations of casein added to NDM. The R^2 for this equation is 0.998. This equation was used on known mixtures of casein and NDM to find the predicted values (Figure 15). Other amino acids are not needed in the equation to predict concentrations of casein in mixtures. Figure 16 shows that addition of other amino acids to the multiple regression equation does not improve the accuracy. After adding tyrosine and aspartic acid to the equation the correlation coefficient is 0.998 which does not increase significantly if more amino acids are added.

Detection of Added Whey Powder to Nonfat-dry Milk

Our whey powder samples contained 76.2% protein. Figure 17 shows eight graphs of WP added to NDM versus selected amino acids. No added casein is 100% NDM. Boxed symbols on each graph are the result of an amino acid analysis of a mixture of WP and NDM. Each amino acid shows a change in composition with varying ratios of WP. Stepwise regression was used to select amino acids to predict the concentration of WP added to NDM.

Proline and threonine (Figure 18) were selected by stepwise regression to form the multiple regression equation in Table 4. Proline and threonine have opposite slopes and show high variability in amino acid quantities between 0 and 100% added WP. NDM is about 20% WP. The scale of their axes shows the change in amino acid quantity and is larger for proline and threonine than tyrosine and aspartic acid in casein/NDM study.

The multiple regression equation was used to predict the concentration of WP added to NDM with known mixtures (Figure 19). Figure 20 shows that the addition of more than
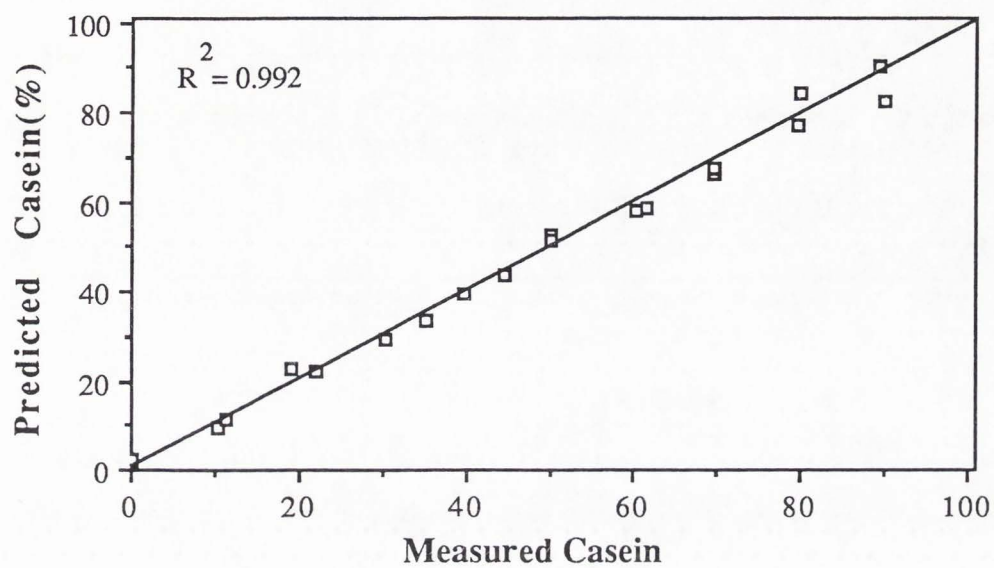
Figure 15. Multiple regression equation using tyrosine, aspartic acid and their interaction to predict concentration of casein added to nonfat-dry milk.

Figure 16. Number of amino acids required in multiple regression equation predicting concentration of casein added to nonfat-dry milk.
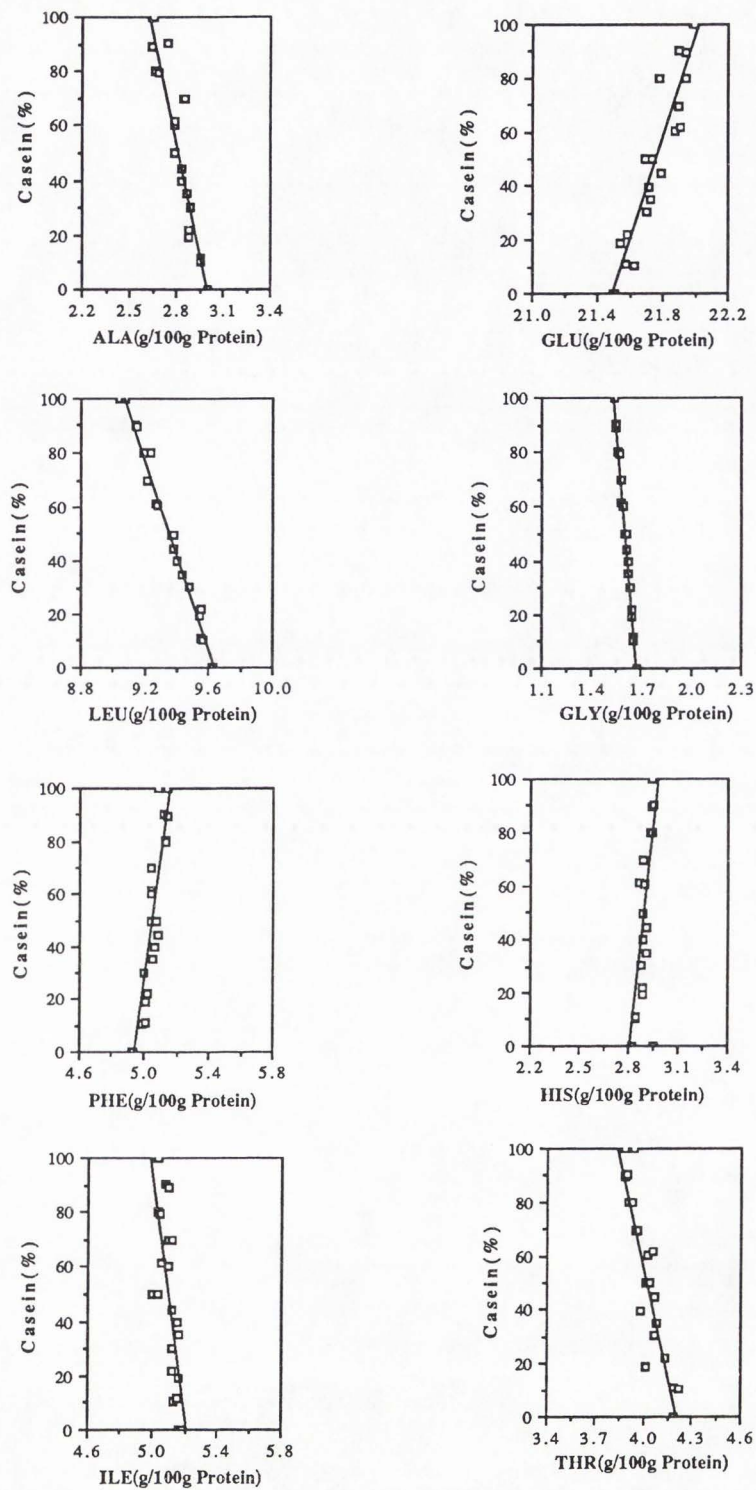
Figure 17. Relative amino acid quantities in whey powder/nonfat-dry milk mixtures.

Graphs show the change in amino acid value with varying whey powder concentration.
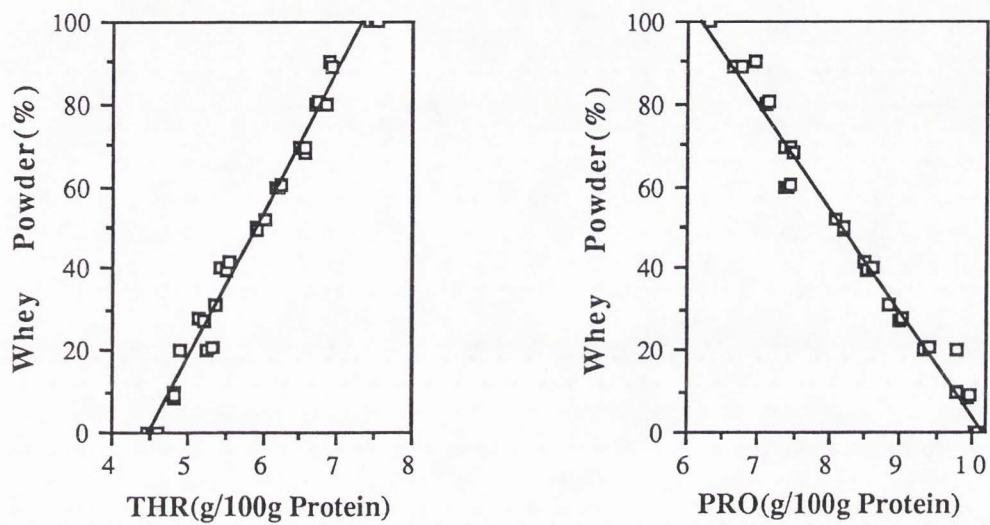
Figure 18. Quantities of proline and threonine in whey powder/nonfat-dry milk mixtures. Graphs show the change in value of proline and threonine with varying whey powder concentration.

Table 4. Regression analysis of whey powder added to nonfat-dry milk.

Source	df	MS	F value	Pr > F
Model	2	57372.12	5666.92	0.0001
Thr	1	97096.91	9590.72	0.0001
Pro	1	17647.33	1743.11	0.0001
Error	31	10.12		
Uncorrected Total	33			

Whey Powder(%) = 22.29 Thr - 9.28 Pro $\quad$ R^2 = 0.997
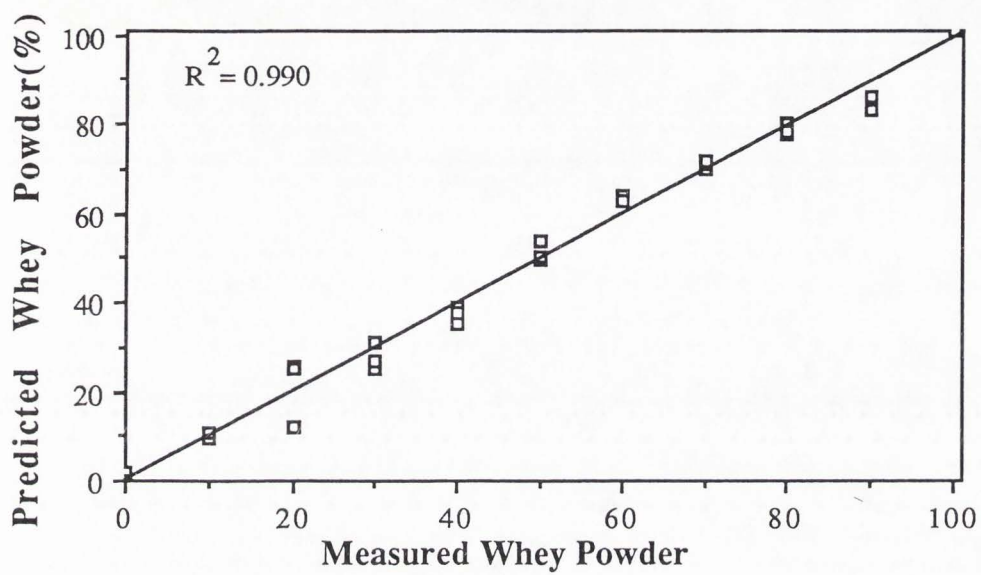
Figure 19. Multiple regression equation using threonine and proline to predict
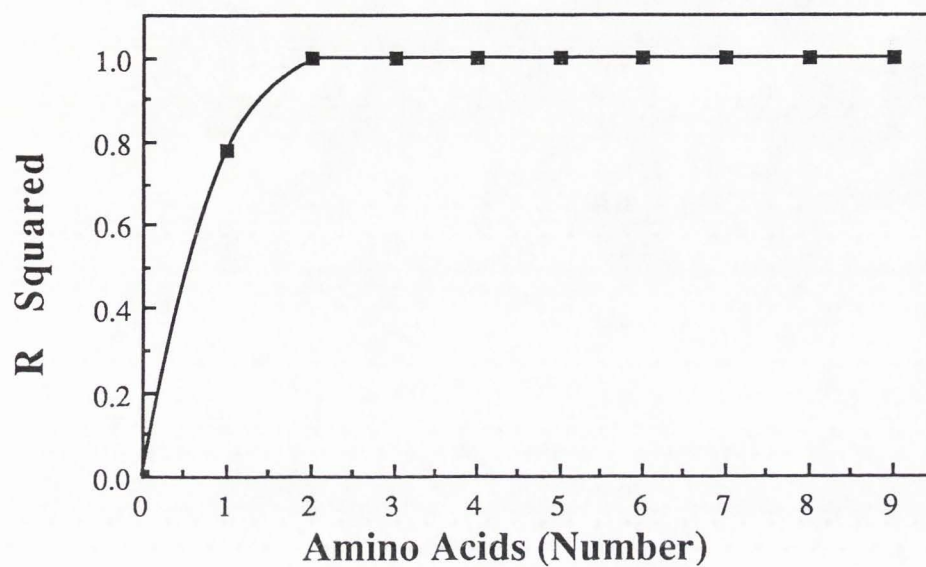concentration of whey powder added to nonfat-dry milk.

Figure 20. Number of amino acids required in multiple regression equation predicting concentration of whey powder added to nonfat-dry milk.

two amino acids to the multiple regression equation does not increase the correlation coefficient significantly.

Detection of Added
Goat Milk to Cow Milk

Our goat milk sample contained 4.19% protein and cow milk contained 2.90% protein. Figure 21 shows eight graphs of goat milk added to cow milk versus various amino acids. No added goat milk is 100% cow milk. Each amino acid analysis, represented by a box symbol on the graphs, is a mixture of goat and cow milk. Each of the amino acids shows a change in composition with varying ratios of goat milk. Valine and isoleucine (Figure 22), with opposite slopes are used to predict the concentration of goat milk added to cow milk. The statistical analysis and multiple regression equation are in Table 5.

Goat milk and cow milk have the same casein (2.6%) and whey protein (0.6%) concentration but vary in concentration of some individual proteins. Cow and goat's α-lactalbumin and β-lactoglobulin are similar antigenically (76). The concentration of κ-casein is the same in both species, about 13% of casein (80). The principal milk protein, α_{s1}-casein, differs between the species. α_{s1}-casein is predominant in cow milk, 38% of casein, but almost absent from goat milk, less than 10% of casein (64, 80). The major component of goat casein is β-casein, 53% of casein (64). Amino acid analysis of mixtures of goat and cow milk detects these small changes in protein composition between species.

The multiple regression equation using valine and isoleucine to predict the concentration of goat milk in cow milk has a correlation coefficient of 0.99 (Table 5). This equation was used on known mixtures of cow and goat milk to find predicted values (Figure 23). Figure 24 shows only these two amino acids are needed to predict accurately the concentration of goat milk added to cow milk.
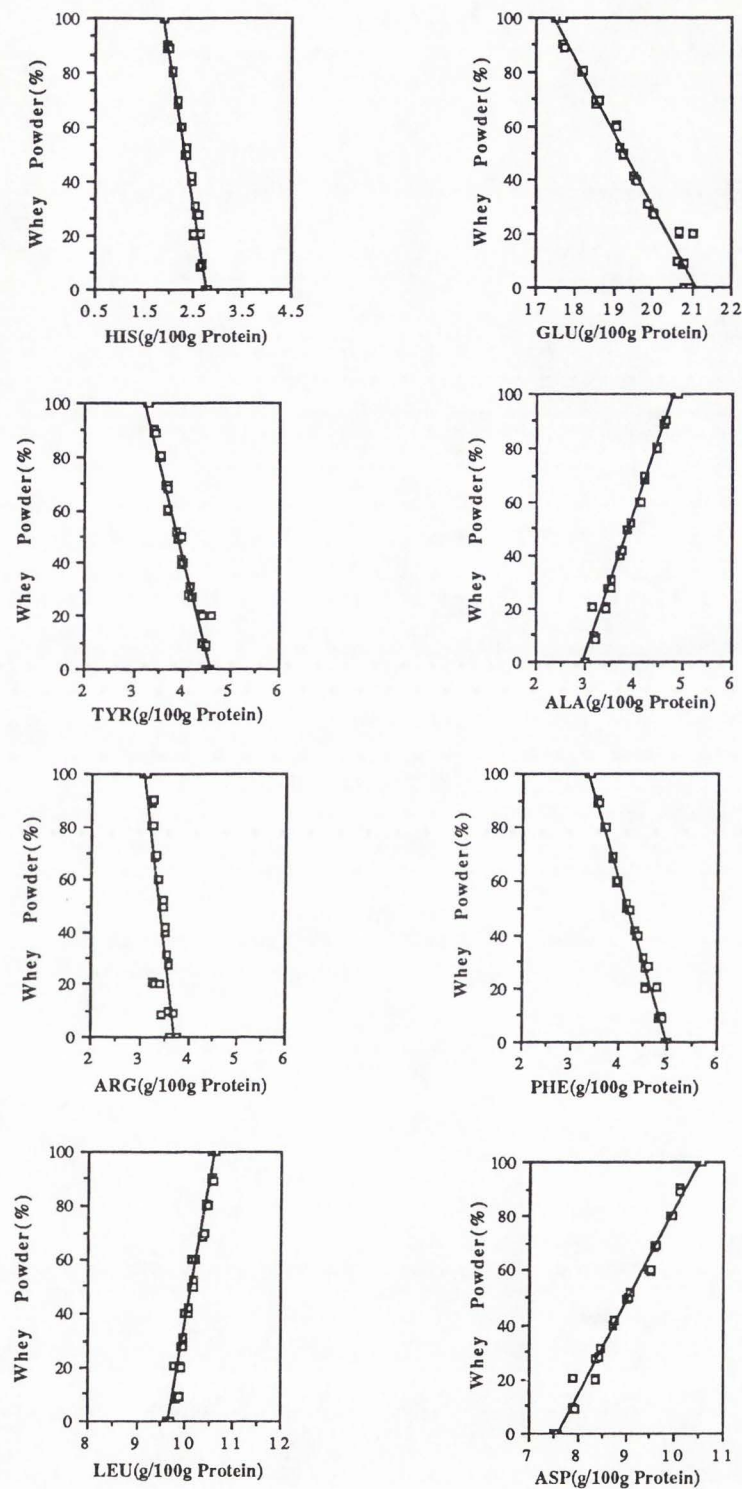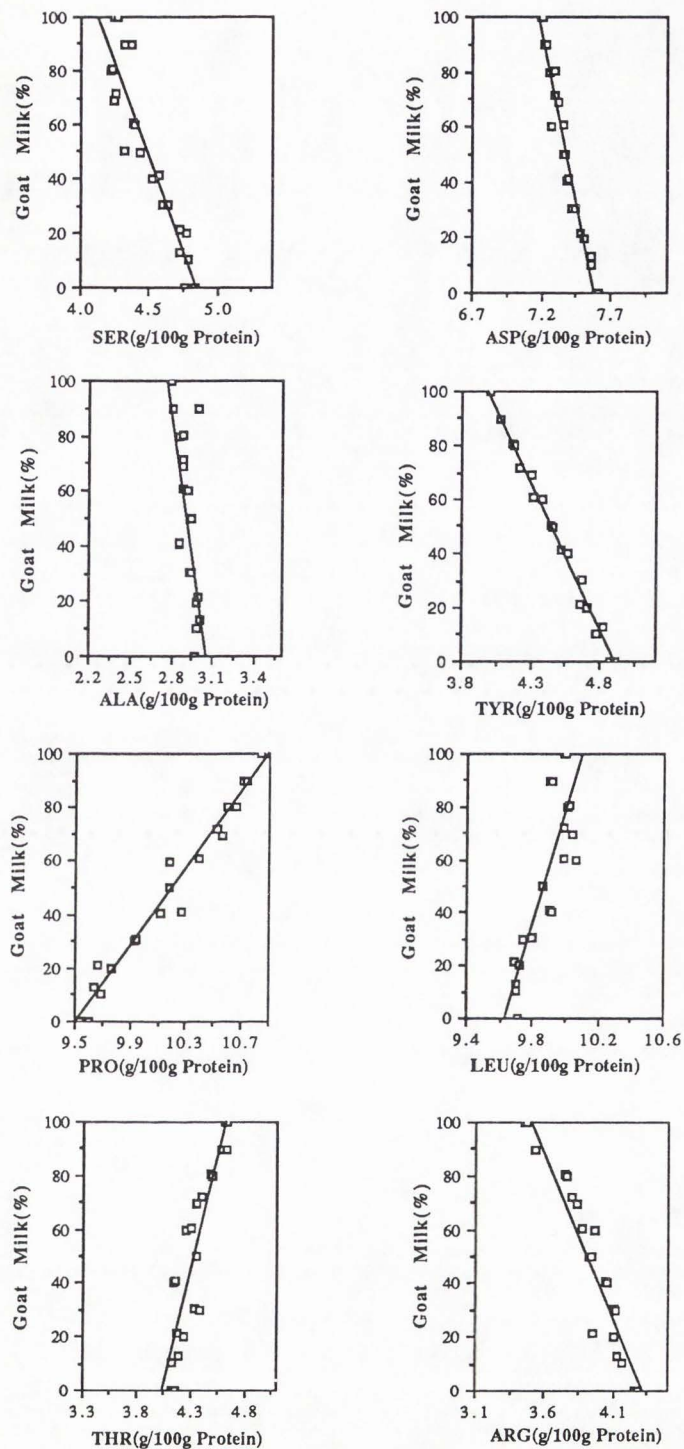
Figure 21. Relative amino acid quantities in goat milk/cow milk mixtures. Graphs show the change in amino acid value with varying goat milk concentrations.
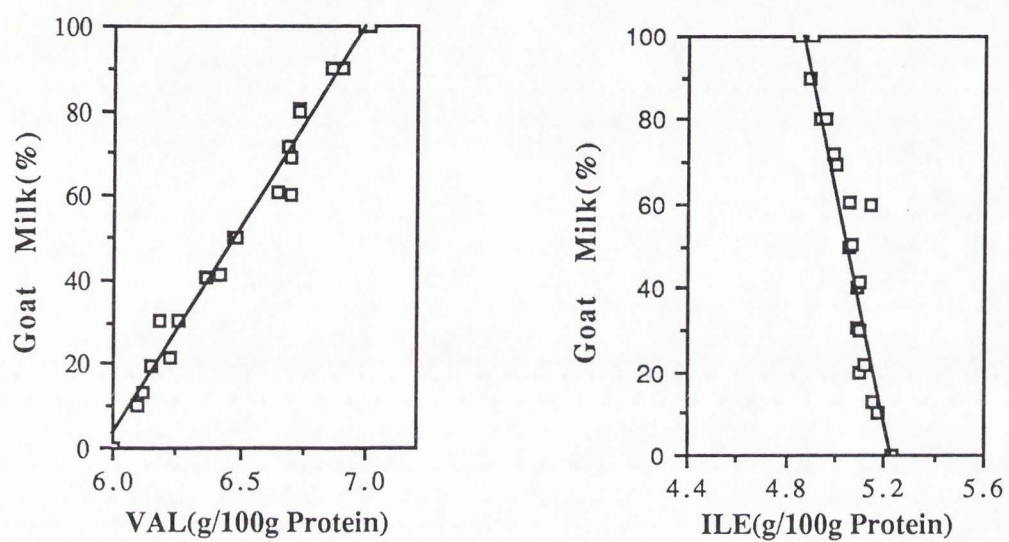
Figure 22. Quantities of valine and isoleucine in goat milk/cow milk mixtures. Graphs

show the change in value of valine and isoleucine with varying goat milk concentrations.

Table 5. Regression analysis of goat milk added to cow milk.

Source	df	MS	F value	Pr > F
Model	2	38668.39	5905.24	0.0001
Val	1	59046.44	9017.27	0.0001
Ile	1	18290.34	2793.21	0.0001
Error	20	6.55		
Uncorrected Total	22			

Goat Milk(%) = 71.96 Val - 82.42 Ile R^2 = 0.998
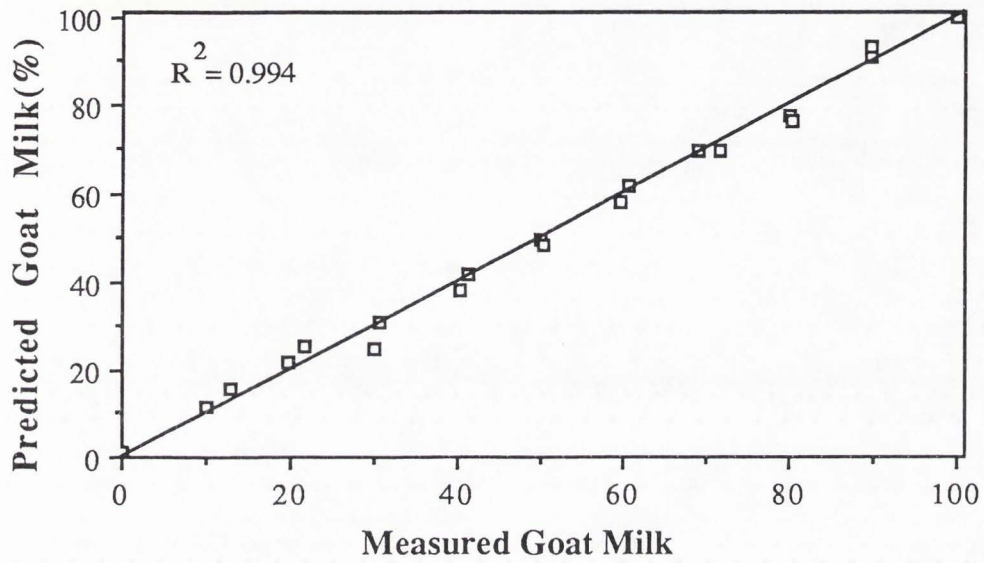
Figure 23. Multiple regression equation using valine and isoleucine to predict concentration of goat milk added to cow milk.
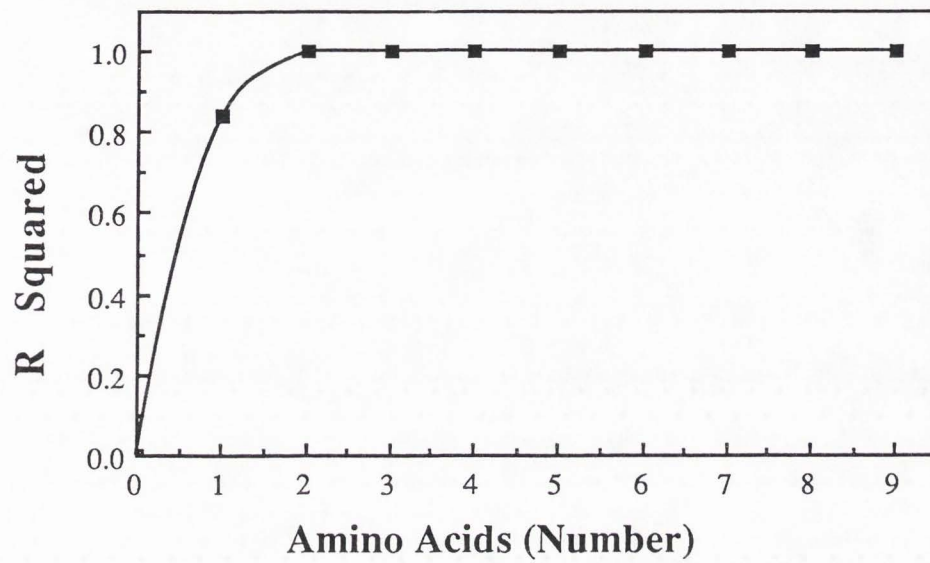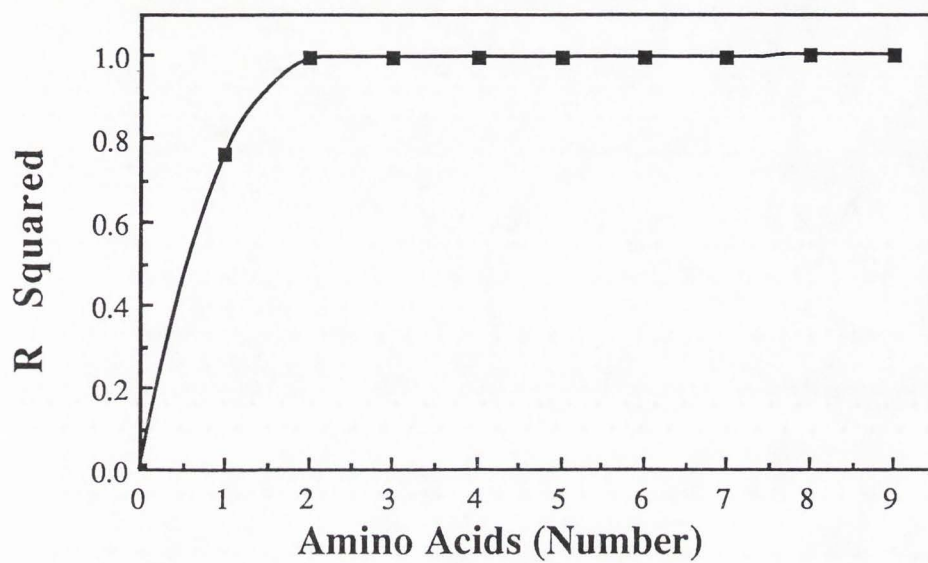
Figure 24. Number of amino acids required in multiple regression equation predicting concentration of goat milk added to cow milk.

CONCLUSIONS

1. Milk proteins κ-casein, α_S-casein, β-casein, β-lactoglobulin and α-lactalbumin are separated and quantified by reverse phase high performance liquid chromatography.

2. Concentrations of milk proteins in mixtures are simultaneously estimated by the information contained in a single amino analysis of the sample.

3. Relative amounts of proteins in mixtures are determined by regression equations based on amino acid differences among groups of proteins.

4. Addition of whey protein or casein to nonfat-dry milk or goat milk to cow milk are detected by amino acid analysis.

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APPENDICES

Appendix A: Computer Programs

Computer program 1. The following computer program was written for a Tektronix 4052 computer. The program stores the individual milk protein matrix in a file and transforms the (14,5) matrix to (5,5) and the (14,1) matrix to (5,1).

```
4       REM Enter Amino Acids for Each Protein

5       LIST

6       END

8       REM Enter Amino Acids for a Mixture (c)

9       GOSUB 3000

10      GO TO 24

12      REM Read Standards from File 5

13      GOSUB 2500

19      END

20      GO TO 100

24      REM Transpose Protein (a) Matrix = a1

25      GOSUB 4000

28      REM Multiply a1 and Mixture (c) = P

29      GOSUB 5000

32      REM Multiply a1 * a = c

33      GOSUB 6000

36      REM Gauss-Jordan on zx=p

37      GOSUB 7000

40      REM Print Answer

99      END

100     INIT

150     DIM A(14,5), C(14), A1(5,14), P(5,1), Z(5,5), X(5,1), Z1(5,5)
```

```
160    PAGE

170    PRINT "Use Buttons to Continue G"

180    END

2000   REM Enter Amino Acid Values for Proteins

2010   FOR I=1 to 5

2020   PAGE

2030   PRINT

2040   PRINT "Enter Amino Acids for Protein";I

2050   FOR J=1 TO 14

2055   RESTORE

2060   READ A$

2070   PRINT A$;"    ";

2080   INPUT A(J,I)

2090   NEXT J

2110   PAGE

2120   NEXT I

2150   DATA "ASX", "GLX", "SER", "GLY", "HIS", "ARG", "THR", "ALA"

2160   DATA "PRO", "TYR", "VAL", "ILE", "LEU", "PHE"

2170   RETURN

2500   FIND 5

2520   FOR I=1 TO 5

2550   FOR J=1 TO 14

2570   INPUT @33:A(J,I)

2590   NEXT J

2600   NEXT I

2610   RETURN

3000   REM Enter Amino Acids for c Matrix
```

```
3010    PAGE

3020    PRINT "Enter Amino Acid Values for Mixture ";

3040    FOR J=1 TO 14

3050    READ A$

3080    PRINT A$;"    ";

3090    INPUT C(J)

3100    NEXT J

3130    RETURN

4000    REM Transpose a Matrix

4020    A1=TRN(A)

4040    RETURN

5000    REM at*c

5020    P=A1 MPY C

5050    RETURN

6000    REM a1*a

6020    Z=A1 MPY A

6050    RETURN

7000    REM Gauss-Jordan for zx=p

7020    Z1=INV(Z)

7040    Z=Z1 MPY P

7050    PRINT @41:X(1,1);"    ";

7060    PRINT @41:X(2,1);"    ";

7070    PRINT @41:X(3,1);"    ";

7080    PRINT @41:X(4,1);"    ";

7090    PRINT @41:X(5,1);"    ";

7100    PRINT @41:Z

7110    PRINT @41:P
```

Computer program 2. The following computer program was written for a Textronix 4052 computer. The program uses the matrices from program 1 and calculates the concentrations of α_S-, β-, κ-casein, α-lactalbumin, and β-lactoglobulin in mixtures.

```
10      INIT

20      DIM C(20), W(20), A(20,20), E(20,20), X(20), X1(20,20), R(20), B(20)

30      PRINT

40      PRINT "TITLE? ";

50      INPUT H$

60      PRINT

70      PRINT "How Many Proteins in Mixture? ";

80      INPUT N

90      PRINT

100     PRINT

110     FOR I=1 TO N

120     PRINT "Characteristic Amino Acid of Protein # ";I;" ?";

130     INPUT W(I)

140     NEXT I

150     PRINT

160     FOR I=1 TO N

170     FOR J=1 TO N

180     PRINT "gm ";W(J);"/100 gm of Protein #";I;" ?";

190     INPUT A(I,J)

200     NEXT J

320     NEXT I

330     FOR I=1 TO N

340     FOR J=1 TO N
```

```
350    E(I,J)=A(I,J)

360    NEXT J

370    NEXT I

380    FOR J=1 TO N

390    PRINT "gm ";W(J);"/100 gm of  Mixture ?";

400    INPUT X(J)

410    NEXT J

420    PRINT

430    FOR I=1 TO N

440    X1(I,N+1)=X(I)

450    FOR J=1 TO N

460    X1(I,J)=E(J,I)

470    NEXT J

480    NEXT I

490    M=N

500    GOSUB 830

510    PRINT @41:"L"

520    PRINT @37,26:1

530    PRINT @41:"         ";H$

540    PRINT @41:

580    FOR I=1 TO N

590    PRINT @41:"PROTEIN  #";I:

600    PRINT @41:

610    PRINT @41:"AMINO ACID            gm/100gm Protein"

620    FOR J=1 TO N

630    PRINT @41:W(J),A(I,J)

640    NEXT J
```

```
650    PRINT @41:

660    NEXT I

670    PRINT @41:" --------------------------------------"

673    X1(I,N+1)=0

680    PRINT @41:

690    PRINT @41:"Mixture "

700    PRINT @41:

710    PRINT @41:" Amino Acid              gm/100g gm Protein         "

720    FOR J=1 TO N

730    PRINT @41:W(J),X(J)

740    NEXT J

750    PRINT @41:

760    FOR I=1 TO N

762    IF X1(I,N+1)>1.0E-3 THEN 770

763    X1(I,N+1)=0

770    PRINT @41:"Protein #";I:

780    PRINT @41:"   Concentration=    ";X1(I,N+1);" gm/100gm mixture"

790    PRINT @41:

800    NEXT I

820    END

830    M=N+1

840    FOR I=1 TO M

850    R(1)=I

860    B(1)=I

870    NEXT I

880    M1=N-1

890    FOR I=1 TO M1
```

```
900    IF ABS(X1(I,I))-1.0E-3>0 THEN 1160

910    FOR J2=1 TO N

920    FOR I3=1 TO N

930    IF ABS(X1(I3,J2))-1.0E-4>0 THEN 980

940    NEXT I3

950    NEXT J2

960    PRINT "***** No Solution *****"

970    GO TO 1520

980    IF J2-1<0 THEN 1070

990    B(M)=B(I)

1000   B(I)=B(J2)

1010   B(J2)=B(M)

1020   FOR J3=1 TO N

1030   X1(J3,M+1)=X1(J3,I)

1040   X1(J3,I)=X1(J3,J2)

1050   X1(J3,J2)=X1(J3,M+1)

1060   NEXT J3

1070   IF I3-I<0 THEN 1160

1080   FOR I4=1 TO M

1090   X1(N+1,I4)=X1(N+1,I4)

1100   X1(I,I4)=X1(I3,I4)

1110   X1(I3,I4)=X1(N+1,I4)

1120   NEXT I4

1130   R(M)=R(I)

1140   R(I)=R(I3)

1150   R(I3)=R(M)

1160   A9=X1(I,I)
```

```
1170   L1=I+1

1180   FIR J=L1 TO N

1190   B9=X1(J,I)

1200   IF ABS(B9)-1.0E-3<0 THEN 1240

1210   FOR K=1 TO M

1220   X1(J,K)=X1(J,K)-B9*X1(I,K)/A9

1230   NEXT K

1240   NEXT J

1250   NEXT I

1260   IF ABS(X1(N,N))-1.0E-4>0 THEN 1290

1270   PRINT  "***** NO SOLUTION *****"

1280   GO TO 1520

1290   X1(N,M)=X1(N,M)/X1(N,N)

1300   L2=M

1310   FOR J=1 TO M1

1320   L2=L2-L1

1330   L1-L2-1

1340   S9=0

1350   FOR I=L2 TO N

1360   S9=S9+X1(LI,I)*X1(I,M)

1370   NEXT I

1380   X1(L1,,M)=(X1(L1,M)-S9)/X1(L1,L1)

1390   NEXT J

1400   FOR L2=1 TO N

1410   IF B(L2)-L2=0 THEN 1520

1420   FOR J=1 TO N

1430   IF B(J)-L2<>0 THEN 1520
```

```
1440   X1(M+1,M)=X1(L2,M)

1450   X1(L2,M)=X1(M+1,M)

1460   X1(J,M)+X1(M+1,M)

1470   B(M)=B(L2)

1480   B(L2)=B(J)

1490   B(J)=B(M)

1500   NEXT J

1510   NEXT L2

1520   RETURN
```

Appendix B: Amino Acid
Analysis of Milk Protein Fractions

			Protein		
Amino Acid	α_S-casein	β-casein	κ-casein	β-lactoglobulin	α–lactalbumin
		(g Amino Acid/100g Protein)			
Aspartic acid	8.281	5.033	7.271	10.202	18.104
Glutamic acid	22.783	21.649	20.253	18.227	13.056
Serine	4.935	4.256	4.349	2.867	3.636
Glycine	2.464	1.248	1.133	1.174	2.617
Histidine	3.369	3.248	2.766	1.671	3.118
Arginine	4.393	3.096	4.432	2.981	1.631
Threonine	2.598	3.832	5.293	4.388	5.170
Alanine	2.854	1.723	4.101	5.435	1.719
Proline	8.036	14.545	10.523	4.938	2.717
Tyrosine	6.544	3.188	5.982	3.824	5.059
Valine	4.739	7.649	5.920	5.187	4.411
Isoleucine	5.185	4.639	6.470	5.695	6.530
Leucine	9.298	10.662	7.183	13.939	11.256
Phenylalanine	5.547	5.721	4.335	3.449	4.526

Appendix C: Percent Differences Between
Actual and Estimated Milk Protein
Concentrations

	Actual	Estimate	%Difference
Mixture 1			
α_S-casein	46.60	44.33	4.87
β-casein	19.10	21.90	-14.66
κ-casein	10.60	9.50	10.37
β-lactoglobulin	12.00	13.10	-9.17
α-lactalbumin	11.70	11.10	5.13
Mixture 2			
α_S-casein	36.30	38.41	-5.81
β-casein	22.10	21.70	1.81
κ-casein	11.10	9.10	18.02
β-lactoglobulin	10.70	11.70	-9.35
α-lactalbumin	19.90	18.80	5.53
Mixture 3			
α_S-casein	20.80	21.50	-3.36
β-casein	18.90	19.40	-2.65
κ-casein	20.60	18.70	9.22
β-lactoglobulin	19.24	18.60	3.33
α-lactalbumin	21.00	21.50	-2.38
Mixture 4			
α_S-casein	31.10	36.90	0.54
β-casein	19.70	21.30	-8.12
κ-casein	10.30	8.90	13.59
β-lactoglobulin	13.30	15.00	-12.78
α-lactalbumin	19.70	17.40	11.67
Mixture 5			
α_S-casein	49.50	48.80	1.41
β-casein	21.00	23.30	-10.95
κ-casein	8.90	7.20	19.10
β-lactoglobulin	11.40	12.10	-6.14
α-lactalbumin	9.30	8.60	7.53