5-1972

Continuous Flow Analysis of Non-Casein Protein in Milk

Robert Steven White
Utah State University

CONTINUOUS FLOW ANALYSIS OF NON-CASEIN PROTEIN IN MILK

by

Robert Steven White

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

1972

ACKNOWLEDGMENTS

I would like to express my appreciation to those persons whose stimuli and counsel have contributed to the preparation of this thesis:

To Dr. G. H. Richardson for his extremely helpful suggestions and discussions during the course of this investigation; also for his patient and thorough review of the methodology and data contained in this thesis.

To Dr. C. A. Ernstrom, Dr. J. C. Batty, and Dr. R. C. Anderson for their consideration, interest, and optimism.

To. Dr. N. R. Gandhi for his support in obtaining part of the data used.

To Technicon Industrial Systems, Tarrytown, New York 10591 for their generosity in providing part of the equipment used in this study.

To my wife Connie for her patient understanding and expert typing of this thesis.

To my beloved parents, Robert Nelson and Frances Meidlinger White, for their interest and unending encouragement in my academic career.

Robert S. White

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Continuous Flow Analysis

of Non-casein Protein in Milk

by

Robert S. White, Master of Science

Utah State University, 1972

Major Professor: Dr. Gary H. Richardson
Department: Nutrition and Food Sciences

The Technicon AutoAnalyzer II was evaluated for automatic quantitation of non-casein protein (NCP) in milk. The Lowry method for colorimetric measurement of proteins was adapted to the automated method and found to be accurate in the determination of non-casein protein in milk. The automated Lowry method obeyed Beer's law. Casein content was calculated by difference between total protein determined by infrared milk analysis (IRMA) and non-casein protein. In the analysis of twenty-five replicate milk samples, the standard deviation was 0.32 and the coefficient of variability was 0.90. The casein content as a percentage of total protein in the milk of cows in the Utah State University dairy herd was found to vary from 41.0 to 81.3%. The automated or continuous flow method was affected by as little as 0.05% (w/w) mercuric chloride, a chemical preservative used in milk, whereas potassium dichromate and formaldehyde had insignificant effects upon the assay.

(78 pages)

INTRODUCTION

During the last half century, the dairy industry has developed into a highly organized business. The control of solids content in milk and accounting for the same has centered mainly around the fat portion of the milk. According to D. A. Biggs (unpublished studies): "Early recognition of the contribution of milk fat to quality and flavor, plus the discovery of a practical fat test, led to its acceptance as a controlling factor in the price structure." One of the consequences has been that many selective breeding programs have been designed to increase fat production. Testing for fat became widespread in the industry, and this created a belief that fat was the most important constituent of dairy products.

While fat was becoming a monitor of quality in dairy products, dietary trends were changing. Advice to decrease fat consumption and advertisement of the nutritional value of protein has diminished the importance of the fat content of milk and has shifted attention to milk as an inexpensive source of protein. Consequently, more recent selective breeding programs have been aimed at increasing protein production (Biggs, unpublished studies).

Horlamus (56) demonstrated that selective breeding can increase the protein content of milk in confirmation of claims of some Netherlands researchers. Wilcox, Gaunt, and Farthing (126) indicated that attempts to increase the protein content of milk by direct selection would increase the protein percentage but result in a

correlated loss in milk yield. Direct selection for milk yield alone would result in a correlated increase in protein yield that is 92% as efficient as direct selection for protein.

Protein levels in milk are especially important in the manufacture of cheese, the demand for which is growing in the United States. Higher protein content in the milk means greater yields. That the determination of protein levels in milk is essential in efforts to maximize cheese yields has been demonstrated by N. R. Gandhi and G. H. Richardson (unpublished studies). Using infrared milk analysis (IRMA) to quantitate total protein levels in mixed herd samples in various areas of the state of Utah, they found an average total protein content of 2.8% in one area of the state where a low cheese yield was obtained at the factory. If a substantial proportion of the total protein in milk was non-casein protein, it is understandable why yield problems existed. In similar studies total protein ranging from 2.4 to 4.4% was observed on milk samples from individual animals.

The work of Gandhi and Richardson was substantiated by Cziardo, Irvine, and Biggs (unpublished studies) who reported a variability of 2.9 to 3.4% total protein in the Ontario milk market. With these values representing bulk mixed herd milk, it is easy to realize that numerous individual animals varied much beyond these levels. These levels represented averages for the entire province; thus there was considerable variation within milk arriving at different plants in the province. Those herds found to be low in protein production caused cheese plants to experience low cheese yields.

Cziardo, Irvine, and Biggs (unpublished studied) also observed great variation in average casein values in mixed plant milk. Further evidence can be found supporting the variability of the casein content in milk. Jenness et al. (58), Brunner et al. (13), and Starkenburg (104) have all reported considerable variation in the quantity of casein in milk. If this kind of variability exists, then it certainly should be monitored, especially in milk used for cheese manufacture.

Yet, for all the importance put on the determination of protein in milk, such quantitation has been less than enthusiastically attempted on a large scale. This has been primarily due to the time and expense involved in the analysis of a large number of samples by classical techniques. These procedures all involve at least one time-consuming manual step. For example, the extensive study conducted by Wilcox, Gaunt, and Farthing (126) used Kjeldahl digestion, colorimetry employing orange -G and amido black dyes, formol titration, and steam distillation. They mentioned that other researchers were unable to measure the protein content of milk with the implication that existing procedures were either too time-consuming or inadequate or both.

A breakthrough in automating total protein analysis came with the development of IRMA. Using IRMA, DeMediros (26) spearheaded a large-scale analytical program for protein determination. His quantitative operation was enthusiastically supported by his California cooperative dairymen. In addition, the IRMA has helped to evaluate sire selection for high volume milk containing more

protein, aroused greater interest in a mastitic control protram, and provided a straight forward program of pricing related to both fat and solids-not-fat.

Since cheese consumption remains a very bright spot in the dairy industry, it is imperative that more be learned about the quantity and quality of the protein involved in cheese manufacture. Specifically, a study is needed to determine how casein levels vary with relationship to total protein content. There have not been adequate methods for measuring protein, especially casein, economically, rapidly, or accurately.

The purpose of this work has been to develop a continuous and automated method for the quantitation of non-casein protein. The casein content of milk can then be calculated by difference between total protein determined for example by infrared milk analysis (IRMA) and non-casein protein. This approach was then applied to study the variation of the casein content in the milk of the Utah State University dairy herd.

REVIEW OF LITERATURE

Definition and characterization of casein

The classical definition of casein is that portion of skimmilk precipitated by acid at pH 4.6 (13). Casein is a protein that is typically insoluble at its isoelectric point, generally accepted to be pH 4.6 to 4.7 (97). Thompson et al. (105) extended the definition to include the parameter of temperature by declaring that whole casein is a heterogenous group of phosphoproteins precipitated from skimmilk at pH 4.6 and 20C. Moir (80) preferred to define casein as the material precipitated from cows milk at pH 4.6 by an acetic acid-sodium acetate (HOAc-NaOAc) buffer.

Casein was considered to be "one of the best examples of a pure, homogenous protein" long into the twentieth century, the theory being perpetuated mainly on the contentions of Hammersten as quoted by Warner (118). Although Mann (70) presented some evidence to the contrary, Warner (118) reported that the Hammersten theory was not disproved until 1925 when solubility studies showed that casein was definitely a mixture of proteins. Warner (118) characterized these conponents electrophoretically while more recent studies by Hipp et al. (53,54) centered around fractionation of casein by chemical means.

Jenness et al. (58) declared that the classical casein fraction obtained by the Rowland procedure involving isoelectric precipitation (99) was composed of at least three different proteins: α-, β-, and γ caseins. Jenness et al. (58) reported a variation in casein content

of skimmilk of 2.2-3.5 g/100 ml with a breakdown range for α of (1.4-2.3), β (0.5-1.0), and γ (0.06-0.22). They defined casein as "the protein fraction precipitated by acidifying raw skimmilk to pH 4.6 to 4.7" that "comprises about 80% of the proteins of milk and consists of a mixture of three principal components, α-, β-, and γ caseins." Brunner et al. (13) found that casein comprised 76-86% of the skimmilk protein and non-casein proteins 14-24%. Of the classical whole casein, fraction α made up 45-63%, β 19-28%, and γ 3-7%. These valued were based on Rowland nitrogen distribution data and electrophoretic pattern and protein yield studies (53,58,64,95,99,102).

Other studies have shown that additional fractions and subfractions exist (14,106,127).

Casein precipitation

The traditional and classical method of measuring casein requires preliminary precipitation of the protein. Precipitation of casein at its isoelectric point is most common. At the isoelectric point, the net charge on the casein micelle is zero, electrostatic interactions with the solvent in an aqueous medium approach a minimum, and a state of least solubility is attained.

Isoelectric precipitation is not the only method whereby casein may be isolated. Dumazert and Bozzi-Tichadou (30) used coagulation by rennin, salting out by K_2HPO_4-KH_2PO_4 solution, and precipitation by $CdSO_4$ to isolate the casein fraction of milk in addition to precipitation at the isoelectric point by an HOAc-NaOAc buffer. When casein was measured colorimetrically by the biuret reaction, the first three methods gave practically identical values; casein

samples precipitated isoelectrically with HOAc-NaOAc buffer gave slightly higher values. Jakubowski, Sienkiewicz, and Nowak (57) have also used coagulation by rennin to isolate casein. Cluskey, Thomas, and Coulter (20) attempted to precipitate casein from a simulated milk ultrafiltrate system using sodium carboxymethyl cellulose under controlled conditions of pH and ionic composition.

Anions have been reported to precipitate casein. Zittle (128) found that 0.005M concentrations of sulfate, pyrophosphate, and other divalent anions precipitated casein while similar concentrations of phosphate and citrate (pH 3.2) did not. The presence of sulfate in casein solutions broadens the isoelectric precipitation zone on the acidic side. Precipitation was also found to be markedly pH dependent: the lower the pH below isoelectric pH the greater the concentration of Na_2SO_4 required for precipitation (128). A method for preparing casein at pH 6.6-7.0, in an aqueous environment, and utilizing only cations naturally present in the casein micelle is described by von Hippel and Waugh (116).

Use of acids as precipitants is common. Hydrochloric (17,29,31, 53,54,55,73,103) and acetic (32,64,66,95,97) acids are frequently used. Concentrations of HCl between 0.1 N and 1.0 N are most usual. The most refined procedure is that of Warner (69,118). Warner (118) separated α- and β- casein fractions in skimmilk using differential precipitation with HCl.

Trichloroacetic (final concentration 12%) and phosphotungstic and phosphomolybdic acids have been employed also as precipitating agents (1,43).

Precipitation with either hydrochloric or acetic acid is based on the addition of sufficient acid to lower the pH to the isoelectric point of casein. The isoelectric point of casein is most commonly placed at 4.6 although pHs in the range 4.5-4.7 are not unusual in the literature (1,3,13,16,17,29,31,53,54,55,58,73,80,95,103,105). Optimum precipitation of casein has been supposedly observed at pH values not within this range, the extremes being pH 4.20 and pH 4.89 (79).

Moir (79) attributed the beginnings of the idea of isoelectric precipitation to Waterman (119,120). "Long before" isoelectric precipitation acquired theoretical significance from the colliodal chemists, and a mathematical definition in terms of electrolytic dissociation was developed, the purification [isolation] of casein depended upon its relative insolubility under certain specified conditions (21). Although Waterman experienced difficulty with his isoelectric separation at first, he was able to overcome filtration and turbidity problems (121,122,123). Isoelectric precipitation has become the most effective method to quantitatively precipitate casein, and the work of Waterman has developed into two widely recognized methods of the Association of Official Agricultural Chemists (16.042-16.043, eleventh edition, 1970).

Owing to varying ionic composition and pH of milk samples, the use of acetate buffers as precipitants has grown (16,19,30,64,66,88, 97). According to Lebermann (66), the buffering of cow's milk is so great that it must be properly considered in precipitation of the casein at its isoelectric point. Acetate buffers of several compositions

and buffer capacities have been suggested, all with the purpose of precipitating the casein as close to pH 4.6-4.7 as possible.

By precipitating casein from skimmilk with a high capacity buffer, a filtrate with a pH very near the isoelectric point may be obtained which has a variation of only 0.04 pH unit (120). Increasing the buffer capacity decreases the pH of maximum precipitation because the pH of minimum solubility varies with the salt concentration of the casein solution (97).

Petersen (88), continuing the work of Waterman, studied the pH at which maximum precipitation of casein occurred by preparing a series of acetate buffers made from N HOAc and N NaOH. Very slightly higher casein values were obtained from buffers prepared from 250 ml HOAc and 75 and 100 ml of NaOH than those containing either more or less NaOH. The milk buffer mixtures in these two cases were reported to have had pH values of 4.321 and 4.484 respectively.

Moir (79) and Rowland (97) also experimented with the use of buffer mixtures to effect isoelectric precipitation. Using a quinhydrone electrode, Moir (79) found that a mixture of 90 ml of N HOAc and 35 ml of N NaOH gave a pH of 4.42. This buffer was essentially the same as the one that Petersen (88) used later, the two differing only in buffer capacity. Moir's buffer effectively precipitated milks of widely varying composition within the comparatively narrow range of pH 4.59-4.74.

Rowland (97) showed that maximum precipitation or casein occurred when 10% HOAc and N NaOH were used in the ratios of 3:2 of 1:1 and the pH of the filtrates obtained were 4.73 and 4.66 respectively.

Rowland persisted in the one-at-a-time addition of acetic acid and sodium acetate to milk samples, claiming that "the addition to the milk of acetic acid and sodium acetate at the same time, in the form of a buffer giving a suitable final pH for the mixture, always results, as Moir showed, in incomplete casein precipitation (97). In so doing, they seemed to have ignored the finding of Petersen (88).

However, buffers having an average pH of 4.680 to 4.177 resulted in average casein nitrogen readings which ranged from 0.374 to 0.731 (88). Work by Moir (80) has substantiated this narrow range of readings resulting from a relatively larger range of pH values. Moir precipitated casein using an acetic acid-sodium acetate buffer at pH 4.6 (4.65) and 10% acetic acid at pH 4.2 (4.23). Digestion of the two samples of casein by NaOH and oxidation of both by NaOBr under comparable conditions gave similar rates for each reaction respectively. Nitrogen content of the casein precipitated by acetic acid alone at pH 4.23 was 0.405 g N/100 ml milk while the nitrogen content of the casein precipitated at pH 4.65 by the acetic acid-acetate buffer was 0.410 g N/100 ml milk. Lebermann (66) found that optimum precipitation occurred when the HOAc-NaOAc buffer precipitant had a pH of 3.74.

One of the problems associated with isoelectric precipitation of casein is the possibility that a pH slightly above that at the isoelectric point will result in incomplete precipitation while a pH slightly below that at the isoelectric point will cause dissolution of some of the precipitate. Mascre and Bouchara (74) have discovered that addition of up to 20% formol to the milk required more HOAc for precipitation of the casein but less nitrogen was left in solution

after precipitation and there was less dissolution of the precipitated casein upon addition of acid.

Methods of measuring casein

The estimation of casein based on fat content (2) has serious limitations (although accuracy is claimed to be equal to that of chemical analyses (2)) as does the calculation of casein as a fixed percentage of total protein (65). Flourimetric analysis of casein solutions has been proposed by Timita (107) using the dye sodium 1-phenyl-naphthylamine-8-sulfonate and measuring fluouresence at an excitation frequency of 365 nm.

Fleury and Eberhard (32) have suggested a gravimetric analytic approach after acid precipitation with HOAc. This method is said to be in close accord with standard methods.

Hart (50,51), Van Slyke and Bosworth (111,112), and Pfyl and Turner (90) all have advocated a volumetric analysis. Hart (50) pointed out the need of a simple test for casein corresponding to the Babcock test for fat. His procedure calls for precipitation of the casein by dilute HOAc with the fat being dissolved in $CHCl_3$. The casein is collected by centrifuging into a pellet and read directly in a graduated tube.

Estimation of casein by ferric alum as the precipitant was suggested by Arny and Schaefer (5). An excess of ferric alum of known titer is employed, followed by estimation of the unused iron in the filtrate, and calculation of the amount of iron used by the casein in the precipitation.

Another method (92) involves determination of casein by measuring the heat of coagulation at pH 4.6. A 100 ml sample of milk diluted 1:1 with H_2O was coagulated with 10 ml of HOAc-NaOAc buffer in a specially constructed 30C thermostat. The maximum rise in temperature is said to occur in five minutes. Using thermal data, casein content could be determined.

Assays for casein based on nephelometric measurements have been developed by Kober (62) and Deniges (27). The former method involves dilution of the milk sample, extraction, centrifugation, and finally resuspension of the casein for comparision with a standard.

The first apparent attempt to determine casein content by refractometry was made by Robertson (94) in 1909. Improvements were made by Brereton and Sharp (11), who used a dipping refractometer, to achieve an accuracy of one part in seventy. Schober and coworkers (100) further simplified the Brereton and Sharp method using an immersion refractometer to obtain results in close agreement with those of the Kjeldahl method. Other refractometric assays and studies have been carried out by Joost (60) and Hansson (46), the latter having presented an equation for determining casein using two refractometric readings and the fat content. The Hansson method has been demonstrated to be very accurate when compared with the Kjeldahl (91). Muenchberg, Leskova, and Svastics state that statistical analysis showed no significant differences between the two methods (83).

The Kjeldahl procedure to determine nitrogen content (and thus protein using the factor 6.38) is perhaps the method found most often in the literature. The Kjeldahl procedure consists of an oxidation

of all carbonaceous material in the sample by H_2SO_4 during which the nitrogen is converted to the form $(NH_4)_2SO_4$. Upon neutralization of the H_2SO_4, NH_3 is liberated into a known quantity of boric acid and the amount of nitrogen present is calculated by means of a back titration (3).

Automation of the Kjeldahl procedure to determine total nitrogen in milk was accomplished by Brisson (12) using the Technicon AutoAnalyzer System (Technicon Industrial Systems, Tarrytown, New York 10591). Twenty samples per hour were analyzed with an accuracy of 1% based on the standard deviation of the differences between the standard Kjeldahl and the AutoAnalyzer methods. Another continuous flow assay for total nitrogen was developed by Joe, Sakai, and Moffitt (59).

Experiments have shown that a semimicro Kjeldahl procedure was as accurate as the macro methods for the determination of nitrogen in milk samples (76,77,101). Accuracy of various analytical methods are usually determined on the basis of their agreement with the Kjeldahl method (28).

Chibnall and coworkers (18) claimed that Kjeldahl estimations of nitrogen in proteins and protein hydrolysates gave low results, especially if micro-methods were used. One possible cause was that the digestion period has been too short. They claimed that with proteins and protein hydrolysates it was necessary to continue the heating for eight hours or more after the digest has cleared. A variation of the Kjeldahl procedure in which the NH_3 liberated is due largely to the glutamine and asparagine content of milk proteins

is the Kofranyi method (63,85). Although claimed to be ten times as fast as the Kjeldahl, the closely controlled standardized conditions required were a disadvantage.

A method (114) using perhydrol enabled determination of nitrogen and phosphorus in the same milk sample. Advantages compared to the Kjeldahl method were reported to be: oxidation of organic substances accelerated three to four times; elimination of the catalysts; elimination of foaming and splashing during combustion. Somewhat higher results were obtained on protein and casein by this method compared to the Kjeldahl due to lower losses of organic substances during combustion.

Titrimetric assays of casein have been attempted. A method using NaOH as the titrant and phenolphthalein as the indicator has been used on occasion (44,45,75,87,113). Moir (79) stated that the method of Van Slyke (113) which is typical of those just mentioned was not as accurate as those procedures based on the nitrogen content of the precipitate. Moir also claimed that Hart's centrifugal method (50), Robertson's refractometric method (94), Arny and Schaeffer's ferric alum titration method (5), and Harris' potentiometric titration method (49) were not as accurate as the Kjeldahl.

Several workers have improved on the above general titrimetric method by substituting the formol titration (8,71,83). Pyne (93) found that disturbances in the titration caused by side reactions involving colloidal phosphates could be remedied by treating milk with an oxalate prior to formaldehyde titration. However, the formol titrimetric method had an accuracy of only 3% (86). Furthermore, this

method assumed a standard percentage of casein in the milk which has been shown to be incorrect (13,58). The so-called Walker method (117), a variation of the formol titration, has been developed and improved upon (72,99).

Recently developed assay procedures include dye-binding techniques. The dye-binding capacity of a protein is defined as the milligrams of dye bound per milligram of protein (nitrogen X 6.38). This dye-binding constant assumes a stoichoimetric reaction between dye and protein (6). Udy (110) developed an assay based on the capacity of proteins to bind certain dyes. Treece, Gilmore, and Fechheimer (108) found this method to agree closely with the Kjeldahl method. However, the dye-binding capacity of casein is different than that of the serum proteins (D. A. Biggs, personal correspondence). Thus, the dye-binding test for individual cows, especially those which may be abnormal, may incorrectly quantitate the casein content of milk and may be misleading in predicting cheese yields. Ashworth (7) determined the casein content of milk by dye-binding by measuring total protein and protein content of the filtrate after isoelectric precipitation of the casein. Zhebrovskii and Pavlyuchenko (127) developed a method based on the adsorption of dye by the milk proteins, followed by fractional precipitation and colorimetric analysis by difference.

Colorimetric assays are also numerous. Buruiana (14,15,16) has employed the xanthoproteic reaction to determine casein levels. In this reaction, nitration of the protein solution precipitates the protein which on heating produces a lemon yellow color. When this

acidic mixture is neutralized with base, a burnt orange color results. Routine colorimetric determination is made against a $Cr_2O_7^=$ or MnO_4^- standard (3,12).

Others have devised colorimetric techniques for the quantitation of casein (37), including the perhydrol assay of Vereshchagina (114) and the colorimetric tyrosine index method of Goiffon (41).

The two most common colorimetric procedures for determining protein are the biuret method and that of Lowry et al. (68). The biuret test involves the addition of a strong alkali to a protein solution followed by a few drops of a dilute $CuSO_4$ solution, which yields a violet color. The color is allowed to develop for 30 minutes at room temperature and then the color intensity is read at 540 nm (30,32,33). Using the procedure of preparing the biuret reagent according to Weichselbaum (124), an analysis for protein has been automated using the Technicon AutoAnalyzer II. Such an apparatus was used at Rutgers University to monitor bacterial growth (4).

In the Lowry method, the protein solution is mixed well with a copper reagent (a 50:1 mixture composed of a 2% solution of Na_2CO_3 in 0.10N NaOH and a 0.5% solution of $CuSO_4 \cdot 5H_2O$ in 1% sodium or potassium tartrate respectively) and allowed to stand for ten minutes. Folin-Ciocalteau phenol reagent diluted with water to N acid is added to the protein-copper mixture. A blue-violet color is developed as the copper treated protein mixture reduces the Folin reagent. After 30 minutes or longer at room temperature, the color is read at 750 nm or 500 nm depending on the concentration of the protein solution.

While casein is measured directly in a majority of the methods described, indirect calculation of casein is widely accepted. Both the Rowland method (98) and AOAC method 16.043 (eleventh edition, 1970) determine casein nitrogen as the difference between the total nitrogen and the non-casein nitrogen determined on the filtrate from the precipitation of casein. According to Ashworth (7), these two methods are the most widely used in the quantitation of casein. Goiffon (41) and Larson and Rolleri (64) also quantitated casein as the difference between total nitrogen and non-casein nitrogen.

Moir (79) objected to the quantitation of casein by indirect means. Such a procedure neglected the volume of fat, casein, and other solids-not-fat, therefore giving lower values for casein than direct determinations on the precipitate itself. According to Cerbulis and Zittle (17), acid precipitated casein contains 4.5-7.0% lipids on a dry weight basis. Rowland (98) argued, however, that correction factors for these volumes introduced an extremely small error into the non-casein nitrogen figure and a negligible error into the final casein nitrogen figure (when the quantitation was done by difference).

Distribution of protein nitrogen in milk

Rowland (97) claimed that casein constituted over 80% of the total protein of normal milk. In individual samples tested by Rowland (99), casein nitrogen averaged 78.7% of the total nitrogen with a range of 77.3 to 80.7%. In bulk samples, the average was 78.3% with a range of 77.7 to 78.5%.

Davies (24) found casein nitrogen to be 76.7% of the total nitrogen. Golding (42) obtained an average value of 76.5% for casein nitrogen while Shahani and Sommer (102) arrived at values of 76.6% and 78.9% on studies of individual and bulk samples respectively.

Three mastitic cows were studied by Rowland (99). Cow X suffered from acute mastitis. Cows Y and Z were clinical cases and had only slightly inflamed quarters but bacteriological examination of their milk revealed large numbers of S. agalactiae. Their milk was of normal appearance. In cow X, casein nitrogen represented only 51.7% of the total nitrogen. Cows Y and Z had values of 66.2 and 66.8% respectively.

From this study Rowland concluded:

> "Milk samples of normal appearance, produced by apparently healthy cows and having persistently low content of solids-not-fat, are of two kinds: (a)samples from cows with occult subclinical mastitis, i.e. pathological samples; and (b)samples from uninfected cows, the cause of the low solids-not-fat being physiological. These kinds differ in chemical composition. The pathological samples have the low casein and high soluble protein content of mastitis milk, on account of the presence of an isotonic diluent containing only soluble proteins. The physiological cases have a normal protein distribution."

Cziardo, Irvine, and Biggs (unpublished studies) observed average casein values in mixed plant milk to vary from 59 to 82% of the total protein. Instead of finding 78-79% casein in the total protein as ordinarily reported, they found that less than 76% casein was obtained 42 weeks out of the year. The average in the province of Ontario was 73-74% during the flush period. The only time 76% was reached was during January and February. These workers also reported that the

serum protein remained very stable in the Ontario market near 19.5% whereas non-protein nitrogen varied from 3 to 13% with an average of 6.2%. This is higher than the 5.0% considered by Rowland (98) to be a standard non-protein nitrogen value.

Jenness et al. (58) have reported a variation of casein content (g casein/100 ml of skimmilk) of 2.2-3.5g. Brunner et al. (13) stated that casein composed 76-85% of skimmilk protein. Starkenburg (104) indicated that milk of registered Holsteins with an average fat content of 3.6% varied in solids-not-fat from 8.0 to 9.0%.

A comparison of casein content of milk of different breeds of cows is summarized in Table 1.

Table 1. Comparison of casein content of milk of different breeds of cows

	g casein/100 ml skimmilk					
Study	Ayrshire	Brown Swiss	Holstein (Friesian)	Guernsey	Jersey	Shorthorn
(24)	--	--	--	--	--	2.53
(95)	2.64	2.78	2.38	2.88	2.72	--
(99)	--	--	2.36 2.60	2.64 2.95 3.38 3.17	--	2.72 2.58 2.86 3.15
(102)	--	2.81	2.42 2.54	2.38 2.63 2.43	--	--

Automation of protein and nitrogen assays

Automated determination or continuous flow analysis of protein and nitrogen has grown rapidly with the advent of the Technicon AutoAnalyzer system. This system consists of a series of modules each of which performs a specific function. These modules may be connected in a manner suitable to effect nearly any wet chemistry. The AutoAnalyzer is unique in that each sample passing through the system experiences identical reaction conditions of time, temperature, reagent, volume, etc. This kind of reproducibility cannot be achieved manually even by the most conscientious technician using the best techniques.

Using this apparatus, total nitrogen and/or protein assays have been developed for fertilizers and feeds (39,82), forages and grain (40), foodstuffs (67), and brewing materials (38). Of greater interest, however, are procedures designed to quantitate total nitrogen and/or protein in milk.

Adaptation of the Kjeldahl method for measuring total protein has been described by Brisson (12). This procedure features continuous digestion. Quantitation of the protein is obtained by reaction of the digested nitrogenous material with an alkaline phenol reagent followed by colorimetric determination at 630 nm. The accuracy of this method was 1% when compared with standard Kjeldahl determinations.

The method of Conetta, Stookey, and Zehnder (23) for determination of protein in milk is based on the dye-binding capacity of the amino groups of the arginine, lysine, and histidine residues. Protein concentration was determined colorimetrically at 420 nm using the dye Orange -G because of superior wash characteristics. The drawback

of this method is that individual protein components cannot be quantitated due to their different dye-binding characteristics. Therefore, measurement of casein, critical to predicting cheese yield, cannot be effected.

EXPERIMENTAL METHODS

Description of Technicon AutoAnalyzer II instrumentation

Non-casein protein was determined using the Technicon Auto-Analyzer II system (Technicon Industrial Systems, Tarrytown, New York 10591).

The AutoAnalyzer II uses continuous flow analysis. The samples are sequentially aspirated from a sample tray into a continuous flowing analytical stream. A wash solution is introduced between each sample for sample separation. Air bubbles are injected at two second intervals to separate sample segments, maintain sample integrity, create a turbulent flow which mixes the sample, and clean the tubing. Besides being continuous and automatic, the AutoAnalyzer presents each sample with identical reaction parameters of temperature, time, volume of reagents, mixing, etc.

The system is composed of separate modules, each of which perform a separate function and are interconnected by tubing and electrical cables. These modules can be adapted to run a wide variety of wet chemical analyses as is evident from the literature. The use of the AutoAnalyzer has become widespread since its introduction in 1957; there are more than 20,000 in operation in laboratories worldwide.

In the quantitation of non-casein protein, the following Auto-Analyzer modules were employed: 1) sampler, 2) proportioning pump, 3) continuous filter, 4) water bath manifold, 5) colorimeter, and 6) recorder. In addition to the regular Technicon colorimeter and

recorder, a Beckman spectrophotometer model DBG and a Heath pH recorder were used together as an alternate readout system.

Filtration

A. Prior to the arrival of the Technicon continuous filter, a temporary filter system was devised to facilitate development of the rest of the analytical system.

A section of pump tube (0.056 inches, ID) was inserted snugly into a cylindrical glass casing to give it rigidity. A piece of rubber tubing encircled the glass so that the whole structure could be better held in a clamp. Two of these structures were made, aligned, and held in place such that a piece of filter paper (Whatman No. 41) could be placed between them. Using trial and error, the proper distance for effective filtration was determined. The filter paper was manually rotated to prevent casein precipitate from plugging the tube.

This temporary filter was used in the evaluation of all the methods of quantitation of non-casein protein. The temporary filter adequately removed the casein precipitate and gave a clear filtrate. However, the data from evaluation of the quantitative procedures were irreproducible.

One of two reasons for these poor results was traced to the irregular air bubble pattern in the filtrate line which did not promote sample integrity. The other was the redissolution of casein on the filter paper as the NH_4OH wash passed through.

B. The arrival of the continuous filter brought a new dimension to the project. It was found that precipitation of casein could be more easily effected within the filter block than in a mixing coil.

Preliminary methods evaluated

Each of the following methods were adapted to continuous flow analysis on the Technicon AutoAnalyzer before arrival of the continuous filter so as to reflect the steps of ordinary chemical analysis.

A. Precipitation of casein by ammonium sulfate (22) ($(NH_4)_2SO_4$) was attempted because it is a common means of protein precipitation. This procedure decreased the sticky character of the casein precipitate but precipitate residue still accumulated in the mixing coils. The residue was reduced by implementing an ammonium hydroxide (NH_4OH) wash. Non-casein protein was measured at 280 nm using the Beckman-Heath readout system (125).

B. To eliminate precipitate residue in coils, casein precipitation was attempted using acetic acid (HOAc). Wich 0.5M HOAc as the precipitant and 1.0M NH_4OH as the wash solution, good precipitation and wash characteristics were obtained at 20 samples per hour (sph) and a 1:1 sample to wash ratio. Precipitate residue was kept to a minimum by having the milk sample enter the mixing coil through a platinum nipple rather than a DO fitting. The only residue following six hours of continuous operation was a slightly visible ring inside the mixing coil.

C. Due to the pH variability of milk samples, a buffer was chosen to isoelectrically precipitate the casein. An acetic acid-sodium acetate buffer (page 25) was prepared according to AOAC method 16.042

(eleventh edition, 1970). The precipitation pH was monitored by running a tube down the side of a combination pH electrode. The tube through which flowed the fully mixed precipitation reaction system was held in place with tape so that the casein precipitate and the accompanying aqueous phase emptied onto the electrode just above the wick. The casein precipitate was washed off the electrode with distilled water whenever there was sufficient particulate casein to interfere with the measurement of the pH of the aqueous phase. A series of experiments demonstrated that if the buffer system were adjusted to pH 4.48-4.50 with HOAc, it precipitated casein at and gave a filtrate pH of 4.60.

This concurred with the findings of Petersen (88). The above conditions were chosen over those of Lebermann (66) who used an acetate buffer precipitant of pH 3.74 which gave a pH below 4.6.

A wash of 2M NH_4OH proved satisfactory without adversely increasing the filtrate pH.

While this method adequately precipitated the casein, quantitation of non-casein protein at 280 nm with the Beckman-Heath readout system proved unsuccessful because of a wildly erratic readout pattern.

D. Assuming that measurement at 280 nm was difficult due to a possible turbidity problem, an attempt was made to quantitate non-casein protein colorimetrically using the biuret method. The procedures of Technicon AutoAnalyzer Methodology N-14b I/II: Total Protein and of Weichselbaum (124) were adapted. Casein precipitation was effected by a buffer composed of 250 ml of N HOAc and 125 ml of

N NaOH per liter of water and adjusted to pH 4.48-4.50 with HOAc. The wash solution was 2M NH_4OH. Meaningless data were obtained using the Beckman-Heath readout system at 540 nm. Switching to the Technicon colorimeter (600 nm) and recorder modules for a detection system, it was observed that the biuret method was insensitive to the levels of non-casein protein even when the milk sample remained undiluted.

E. The colorimetric method for protein measurement developed by Lowry, Rosebrough, Farr, and Randall (68) was considered to increase the sensitivity. Lowry's procedure had the advantage of being 10 to 20 times more sensitive than measurement of the ultra-violet absorption at 280 nm, 100 times more sensitive than the biuret reaction, and much less subject to disturbance by turbidity than measurement at 280 nm (68). The same HOAc-NaOAc buffer of pH 4.48-4.50 and the 2M NH_4OH wash solution were used. Using the Technicon colorimeter and recorder modules, a readout pattern was established which became more defined and regular when the temporary filter was replaced by the continuous filter. Non-casein protein was determined colorimetrically at 600 nm. Manually, the color developed is measured at 750 or 550 nm.

It became apparent after evaluation of the foregoing methods that the temporary filter had introduced instability into the system and thus created meaningless readout data. Shorted cables were found in the Beckman-Heath detection system which compounded that problem.

Standardization of the AutoAnalyzer II system

In order to establish identical reaction conditions within the AutoAnalyzer system, on a daily basis, a standard must be used. This standardization procedure would compensate for the slight deterioration of the Folin phenol reagent (89).

The standard had to be aqueous because organic standards such as phenol would corrode the clear standard tubing on the AutoAnalyzer. Tryptophan is undesirable because of rapid deterioration over the course of a few weeks (35). Solutions of tyrosine on the other hand are very stable. L-tyrosine can withstand 5N H_2SO_4 of 5N NaOH for up to 30 hours at 100C (25).

A standard tyrosine solution was prepared by dissolving 1.000 g of L-tyrosine in one liter of deionized water which contained 0.122 g NaOH to increase the solubility of the L-tyrosine. The standard was stored at 4C. If allowed to stand at room temperature, the tyrosine solution discolored slowly.

The Technicon readout system was calibrated to give a readout value of 30.0 (% full scale of Technicon-Bristol recorder) for the standard tyrosine solution. The recorder was equipped with an internal log circuit which provided direct linear concentration versus reading relationships.

The following procedure quoted in part and otherwise adapted from the Operation Manual for the Technicon AutoAnalyzer II System, Technicon Publication No. TA0-0170-00, September,1970 entitled "Detailed Operation Procedures--Reference Sample (RS): One Channel Operation--Direct," was used to quantitate non-casein protein in milk samples.

I. Start-up procedure and continuous analysis

1. Assure that filter is in place in the Colorimeter.
2. Turn on all Modules (except Sampler and chart drive of Recorder). Sample probe should be placed in water wash.
3. Plug in heating bath. Install platen and start Proportioning Pump.
4. Allow system to warm up for 15 minutes with reagents flowing. Check bubble pattern of flow and examine for leaks. Check temperature of heating bath.
5. Turn on chart drive switch of the Recorder.
6. Place MODE switch of the Colorimeter in position DIRECT.
7. Press the REC ZERO button of the Colorimeter, hold until zero adjust is complete, and then release.
8. Rotate the REC ZERO ADJ (screwdriver adjust) on the Colorimeter control panel until Recorder pen indicates zero on the chart paper.
9. Rotate the DISPLAY switch of the Colorimeter panel to position TEST.
10. Adjust STD CAL dial of the Colorimeter to obtain full scale deflection on the Recorder. (Reads 100.)
11. Rotate DISPLAY switch on the Colorimeter control panel to position SAMPLE. Adjust mechanical aperture for sample channel to obtain maximum energy. (Do not exceed 95.)
12. Rotate DISPLAY switch of the Colorimeter control panel to position REF. Adjust mechanical aperture of the Reference Channel to obtain a reading equal to that obtained with the sample adjustment on the previous step.

13. Rotate the DISPLAY switch of the Colorimeter control panel to position OPERATE.
14. Adjust BASELINE control of the Colorimeter to obtain zero on the Recorder.
15. Fill the Sampler Tray as follows: In positions 1 and 2, place 10 ml of standard tyrosine solution for the purpose of standardization. Fill the remainder of the tray with samples to be tested with a tyrosine standard in every tenth position.
16. Start Sampler. (Reset Sampler if it does not start.)
17. When the standard tyrosine solution reaches steady state, adjust the STD CAL dial on the Colorimeter control panel until the Recorder indicates the assay value of the standard tyrosine solution (defined to be 30.0).
18. Continue run of sample tray.

II. Shut-down procedure

1. After the last sample has been analyzed, all pump tubes should be allowed to draw deionized water for five minutes.
2. Turn off Sampler, Colorimeter, and chart drive on Recorder.
3. Unplug heating bath module.
4. Remove platen from proportioning pump when no liquid remains in entire system.
5. Thoroughly clean out wash solution reservoir to prevent accumulation of slime.

Reference method

The Technicon AutoAnalyzer II system was calibrated to the Kjeldahl method. Non-casein protein content of Utah State University 2% milk was assayed on the AutoAnalyzer and by Kjeldahl digestion using the procedure of Joslyn (61) with the Hiller, Plazin, and Van Slyke (52) modification.

Manually, the non-casein protein filtrate was obtained by using AOAC method 16.041 (eleventh edition, 1970). Replicate 10 g samples of milk were placed in a beaker with 90 ml H_2O at 40-42C and mixed well. Immediately 1.5 ml of 10% HOAc were added; the reaction system was stirred well and allowed to stand for three to five minutes. The solution was filtered using S&S filter paper #596 blue ribbon. If the non-casein protein filtrate was not clear or nearly so, it was refiltered.

RESULTS AND DISCUSSIONS

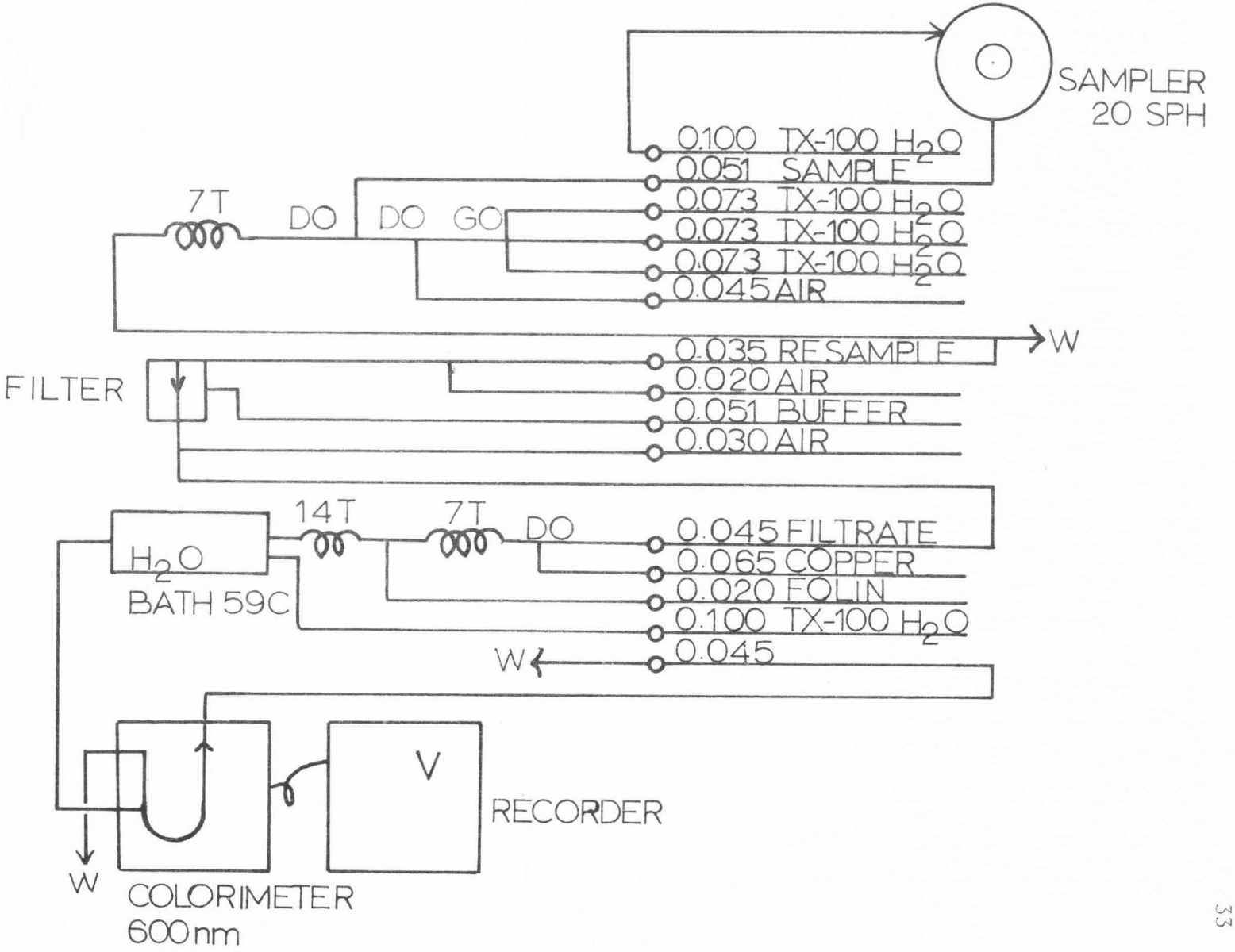
Automating the Lowry method for non-casein protein

The Lowry method (68) was chosen to quantitate non-casein protein because of its greater sensitivity. Protein levels as low as 0.2 µg/0.05 ml can be quantitated with "reasonable precision."

Reagents were prepared according to Lowry et al. (68) with the exception of reagent D. Reagent A, a buffer, consisted of a 2% solution of Na_2CO_3 in 0.10 N NaOH. Reagent B was a 0.5% solution of $CuSO_4 \cdot 5H_2O$ in 1% sodium tartrate. A pellet of NaOH was added per liter to aid solution. Deionized water was used in making reagents A and B to avoid insoluble $Ca(OH)_2$ and $Mg(OH)_2$ precipitates which were formed when distilled water was used. Reagent C was prepared by mixing reagent A with reagent B in a 50:1 ratio. Reagent C was prepared fresh each day. Reagent D was Folin-Ciocalteau phenol reagent (Harleco, Philadelphia) diluted 1:1 with deionized water. The phenol reagent was refrigerated to retard the slight loss of activity (89).

The Lowry method was automated for non-casein protein analysis using the flow diagram shown in Figure 1. The milk sample was diluted 1:6 and then precipitated by a HOAc-NaOAc buffer prepared according to AOAC method 16.042 (eleventh edition, 1970) and adjusted to pH 4.50 (page 25). The continuous filter at a speed setting of 3, equivalent to one and one-half inches per minute, adequately collected the precipitate. Sample integrity and wash characteristics were found

Figure 1. Continuous flow diagram for automating the Lowry method


SAMPLER
20 SPH
0.100 TX-100 H_2O
0.051 SAMPLE
0.073 TX-100 H_2O
0.073 TX-100 H_2O
0.073 TX-100 H_2O
0.045 AIR
7T
DO
DO
GO
W
0.035 RESAMPLE
0.020 AIR
0.051 BUFFER
0.030 AIR
FILTER
14T
7T
DO
H_2O
BATH 59C
0.045 FILTRATE
0.065 COPPER
0.020 FOLIN
0.100 TX-100 H_2O
0.045
W
V
RECORDER
W
COLORIMETER
600 nm

to be improved if an air bubble was inserted just after uptake of the non-casein protein filtrate. The non-casein protein filtrate was first mixed with the copper solution (reagent C) and then with the Folin-Ciocalteau phenol reagent. After thorough mixing in 14 turns of coiled glass tubing, the reaction system was passed through a 59C waterbath for 3 minutes. The blue-violet color was read at 600 nm in the colorimeter and recorded continuously on the recorder module.

Precipitation was effected inside the filter block rather than a mixing coil, the advantage being that casein precipitate did not accumulate in the block as it did in a mixing coil. One disadvantage was that with time the sticky precipitate adhered to the continuously spinning mixing paddle located inside the filter block and restricted passage of sample and buffer precipitant through the block. To prevent this overflow and consequent high readings, the block was removed every 30 to 45 minutes and cleaned out. At the same time, the mixing paddle was wiped off with a Kimwipe. This cleaning procedure was completed within ten seconds. If done while the wash phase was passing through the block, the cleaning had no effect on the readout value.

With the adhesion of casein precipitate to the mixing paddle, NH_4OH wash solutions proved inadequate. A $2\underline{M}$ NH_4OH wash solution could not remove the adhering precipitate completely and what casein it did remove interfered with the readout pattern. It was found that a wash solution of 0.1 ml of a 1:1 (v/v) mixture of Triton X-100 with absolute methanol per liter of deionized water was adequate.

The system was operated at 20 sph with a 1:1 sample to wash ratio.

In all studies, the standard tyrosine solution was calibrated to read out at a value of 30.0 (% full-scale).

Reproducibility of the automated Lowry method

Two studies demonstrated that the automated Lowry method gave very reporducible data. In the first study, eighty replicate tyrosine samples were assayed having a mean readout value of 57.7% full scale. The range was 56.5-61.0% full scale with a coefficient of variability of 1.38. The standard deviation was 0.80.

The second repeatability study consisted of 25 replicate samples of USU skim fortified 2% milk. The mean readout value was 37.5% full scale with a range of 37.0-38.5% full scale. The coefficient of variability was 0.90 and the standard deviation was 0.32.

Linearity of the automated Lowry method

Folin and Ciocalteau (35) gave data which showed Beer's law was followed explicitly when 0.25-4.0 mg tyrosine/100 ml solution were quantitated colorimetrically with the Folin phenol reagent alone. Lowry et al. (68) also found the procedure to obey Beer's law even down to the range of 0.1 to 1.7 µg protein/0.05 ml protein solution.

Two experiments were conducted to determine if automated analysis of non-casein protein using Lowry's method also followed Beer's law. In the first USU 2% milk was mixed with instant non-fat dry milk and diluted with water to give ten solutions of varying protein concentrations. These protein solutions were then quantitated as follows:

First, the casein was precipitated according to AOAC method 16.041 (eleventh edition, 1970). The casein precipitate was collected on S&S filter paper #596 blue ribbon, and the non-casein protein content of the filtrate was quantitated by Kjeldahl nitrogen assay. Secondly, the series of milk solutions were assayed on the Auto-Analyzer II system. A plot of % full scale versus % NCP shown in Figure 2 represents ten sets of duplicate determinations. The data showed a linear correlation coefficient of 0.9894.

Figure 2 shows that the automated Lowry method is linear up to non-casein protein levels of 1.40% which is about twice that to be found in normal milk according to Shahani and Sommer (102).

Secondly, a tyrosine solution was prepared, the concentration of which was exactly three times that of the tyrosine reference solution used for standardization of the AutoAnalyzer. The following dilutions were prepared and analyzed in triplicate on the AutoAnalyzer: 1, 3/4, 2/3, 3/5, 1/2, 1/3, 1/4, and 1/7 (Table 2).

Figure 2. Percent full scale vs percent non-casein protein for establishing linearity of automated Lowry method

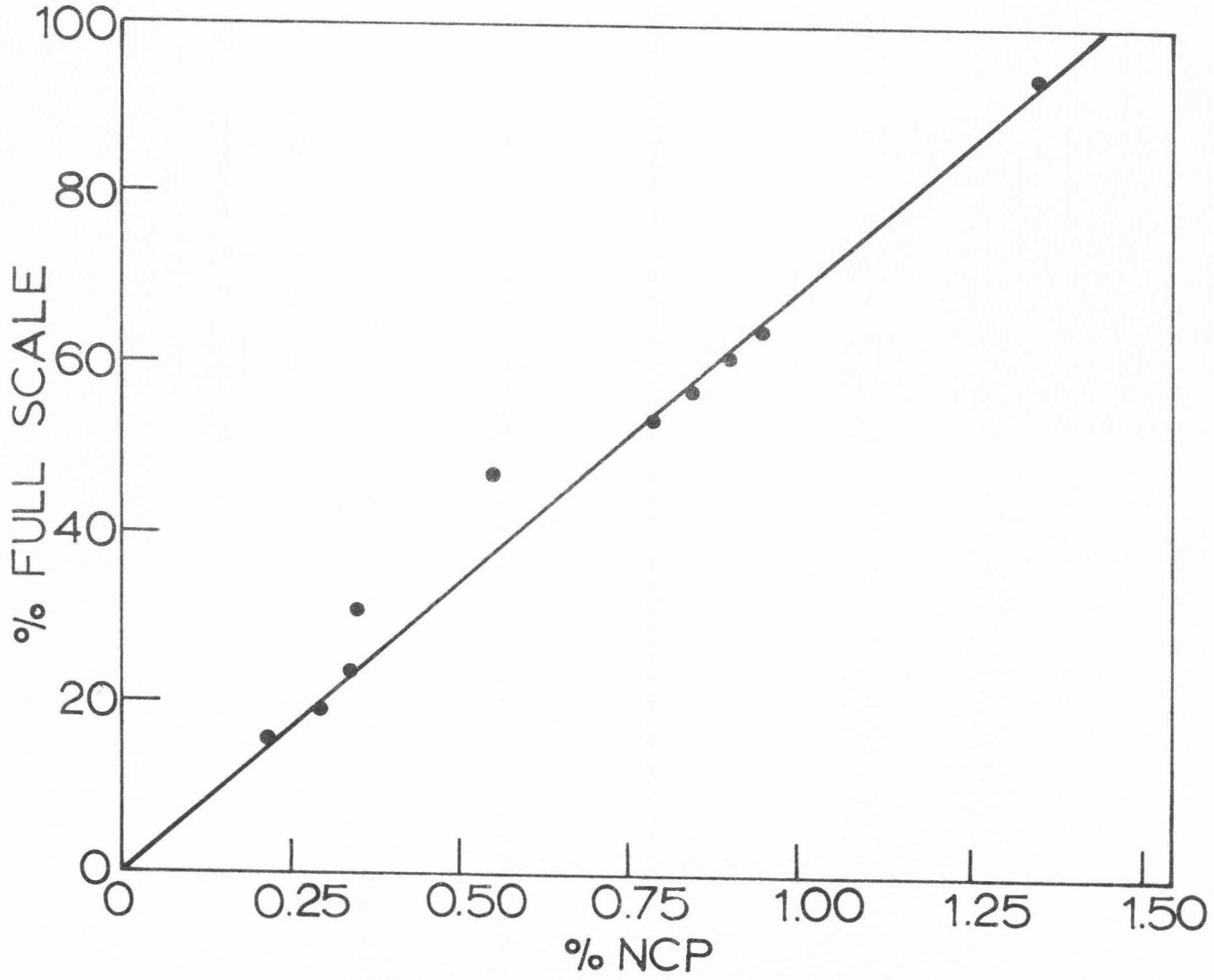

100
80
60
40
20
0
% FULL SCALE
0
0.25
0.50
0.75
1.00
1.25
1.50
% NCP

Table 2. Data from tyrosine linearity study

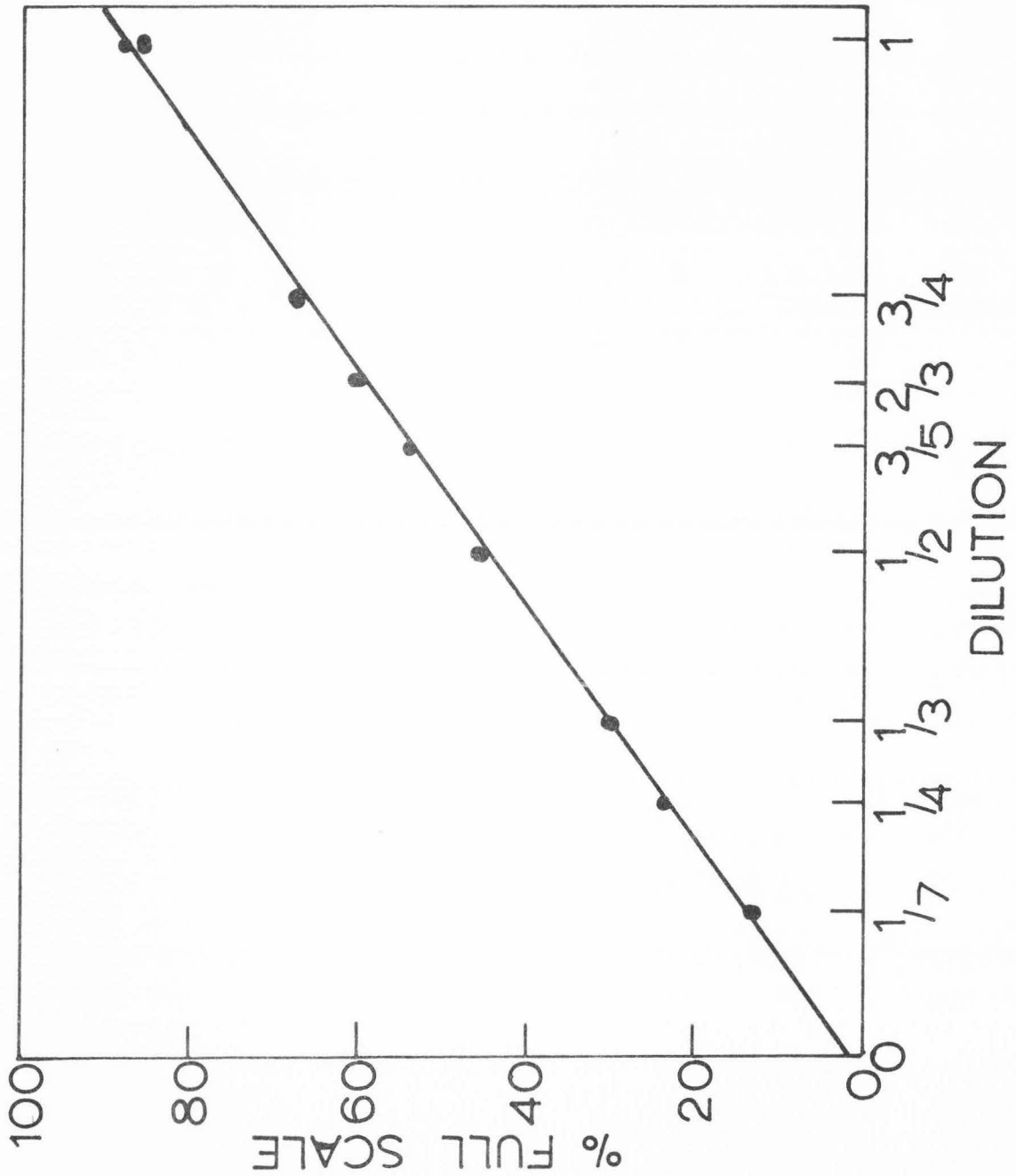
Dilutions	% Full Scale		
	1st Pass	2nd Pass	3rd Pass
1	88.5	86.5	86.5
3/4	68.0	67.0	67.5
2/3	61.5	60.5	59.5
3/5	55.0	55.0	55.0
1/2	46.5	45.0	45.0
1/3	31.5	30.0	31.0
1/4	24.5	24.0	24.0
1/7	14.0	13.5	14.0

This data is shown graphically in Figure 3.

The dilution of 1/3 is the same concentration of tyrosine as that in the standard reference solution. The standard tyrosine solution is defined to be 30.0% full scale which represents 0.72% NCP. Thus from Figure 3 it is apparent that the automated Lowry method is linear up to approximately 2.10% NCP, about three times that to be expected in normal milk.

The linear correlation coefficient for the data was 0.9993. The linear regression curve was found to be $y = 86.1x + 2.4$.

Figure 3. Percent full scale vs dilution for establishing linearity of the automated Lowry method using tyrosine solution


100
80
60
40
20
0
% FULL SCALE
0
1/7
1/4
1/3
1/2
3/5
2/3
3/4
1
DILUTION

Interference of chemical preservatives with the automated Lowry method

The possible interference of the chemical preservatives HCHO, $HgCl_2$ and $K_2Cr_2O_7$ with the automated Lowry method was investigated. The control was USU 2% milk. Each preservative was added in increments of 0.05% (w/w) up to the maximum percentage suggested by the AOAC (eleventh edition, 1970, page 245). Readings were taken after zero and two days at 25C.

Mercuric chloride was found to interfere significantly with color development in a negative manner (Table 3). An initial drop in reading was noted and this was more pronounced following storage. A slight reduction was noted with the other preservatives. However, further work would be needed to establish significance.

Table 3. Data from preservative study

Sample	% Full Scale	
	Day 0	Day 2
Control	36.0	35.0
0.05% HCHO	36.0	34.0
0.10% "	35.0	34.0
0.05% $HgCl_2$	33.0	33.5
0.10% "	30.0	30.0
0.15% "	30.0	29.0
0.20% "	28.0	27.0
0.25% "	28.0	26.0
0.30% "	26.0	23.0
0.05% $K_2Cr_2O_7$	35.0	33.5
0.10% "	35.0	34.0
0.15% "	35.5	34.0
0.20% "	35.5	34.0
0.25% "	37.5	34.0
0.30% "	37.0	34.5

Calibration of the AutoAnalyzer with Kjeldahl nitrogen

USU 2% milk was used as the calibration standard. The casein was precipitated according to AOAC method 16.041 (eleventh edition, 1970), the precipitate collected on S&S filter paper #596 blue ribbon, and the non-casein protein content of the filtrate determined by Kjeldahl nitrogen digestion. On the basis of six replicate samples and using the 6.38 factor to convert non-casein nitrogen to non-casein protein, 0.902% non-casein protein was found in the filtrate.

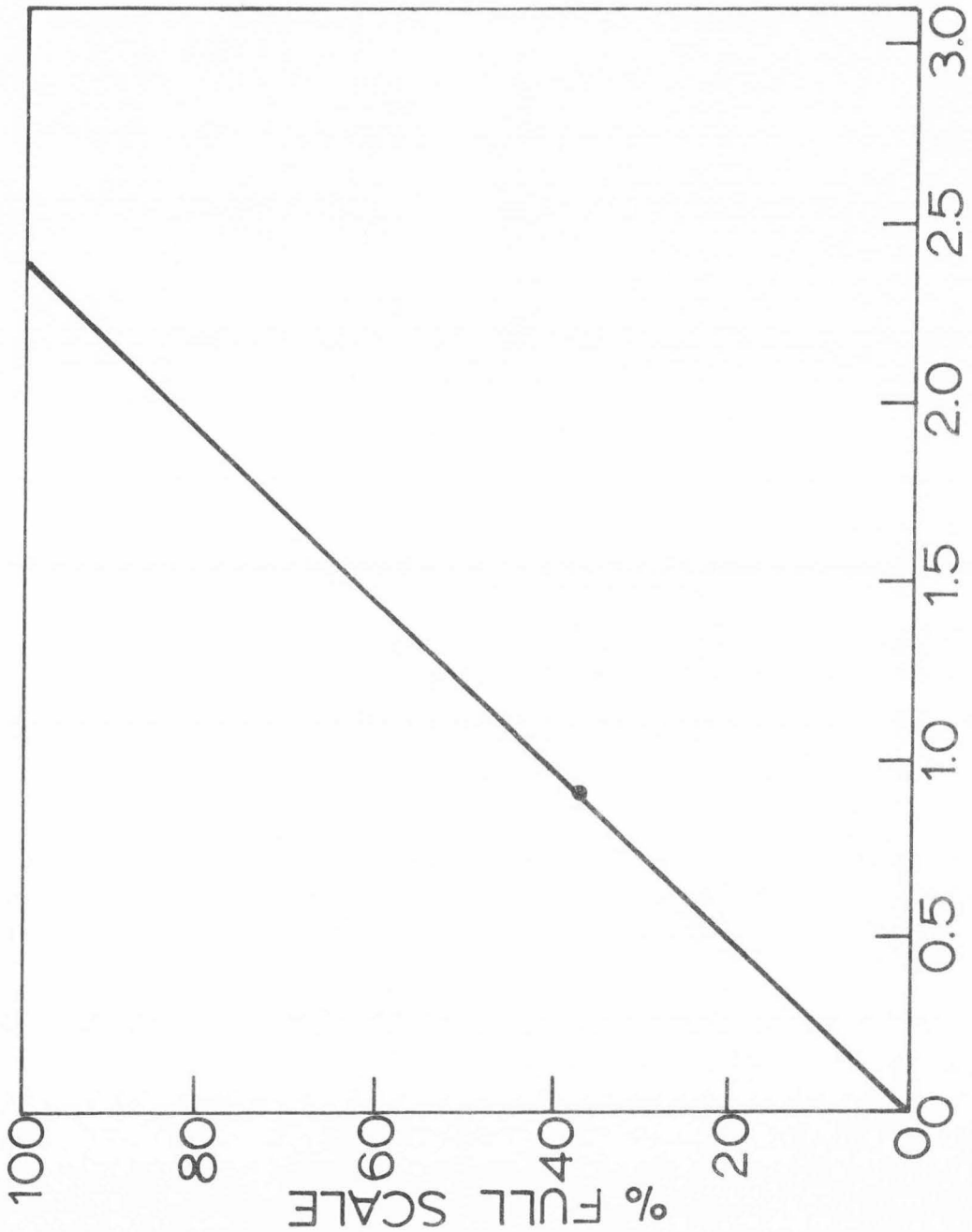
Using the same USU 2% milk as above, twenty-five replicate samples were assayed on the AutoAnalyzer. The mean readout value was 37.5% full scale with a coefficient of variability of 0.90. This is the same data found in the second reproducibility study on page 35.

On the basis of this data and the linearity of automated analytical system, a standard curve was constructed (Figure 4). When the standard tyrosine solution is calibrated to read out at 30.0% full scale, 37.5% full scale is equivalent to 0.90% non-casein protein.

Analysis of individual milk samples using the automated Lowry method

Using the flow diagram of Figure 1, USU dairy herd individual samples collected on 19 November 1972 were assayed for % NCP. The milk samples had been preserved with less than 0.05% $K_2Cr_2O_7$ using a series of $K_2Cr_2O_7$ preserved milks as the basis for visual comparison.

Figure 4. Percent full scale vs percent non-casein protein for establishing standard curve to be used in determination of the casein content of milk by difference


100
80
60
40
20
0
% FULL SCALE
0
0.5
1.0
1.5
2.0
2.5
3.0
% NCP

Percent NCP was determined using the standard curve (Figure 4). Percent casein was calculated as the difference between % total protein (% TP) and % NCP. The % TP readings were obtained by Dr. N. R. Gandhi (unpublished studies) using the infrared milk analyzer (IRMA).

The data gathered is displayed in Table 4.

Table 4. Individual milk samples analyzed for non-casein protein (NCP) and casein

SAMPLE #	% FULL SCALE	% NCP	% TP (TOTAL PROTEIN)	% TP-NCP or % CASEIN	% $\frac{\text{CASEIN}}{\text{TP}}$
2118	62.5	1.50	3.96	2.46	62.1
2146	42.5	1.02	3.47	2.45	70.6
2276	43.5	1.05	3.78	2.73	72.2
2488	69.75	1.68	4.22	2.54	60.2
2540	60.5	1.45	4.38	2.93	66.9
2580	39.5	0.95	3.44	2.49	72.4
2658	39.0	0.94	2.92	1.98	67.8
2664	44.5	1.07	3.80	2.73	71.8
2676	50.0	1.20	3.81	2.61	68.5
2704	51.0	1.23	4.34	3.11	71.6
2712	41.5	1.00	3.34	2.34	70.0
2714	41.0	0.99	3.64	2.65	72.8
2744	45.5	1.09	3.94	2.85	72.3
2746	34.5	0.83	3.35	2.52	75.2
2758	47.5	1.14	4.37	3.23	73.9
2768	48.5	1.17	3.23	2.06	63.8
2842	41.25	0.99	3.19	2.20	69.0
2880	61.0	1.47	3.95	2.48	62.8
2912	(2X)69.0	(2X1.66)3.32	5.62	2.30	41.0
2920	59.0	1.42	5.53	4.11	74.3
2930	49.5	1.19	3.98	2.79	70.1
2942	47.5	1.14	4.40	3.26	74.1
2948	40.5	0.97	4.28	3.31	77.3
2962	56.0	1.35	3.52	2.17	61.6
2982	54.5	1.31	3.75	2.44	65.1
2998	29.5	0.71	2.59	1.88	72.6
3002	49.0	1.18	3.77	2.59	68.7

Table 4. Continued

SAMPLE #	% FULL SCALE	% NCP	% TP (TOTAL PROTEIN)	% TP-NCP or % CASEIN	% $\frac{\text{CASEIN}}{\text{TP}}$
3018	38.75	0.93	3.63	2.70	74.4
3060	34.75	0.84	3.81	2.97	77.9
3086	37.0	0.89	3.83	2.94	76.8
3090	37.5	0.90	3.19	2.29	71.8
3108	42.0	1.01	3.64	2.63	72.2
3116	37.25	0.90	3.52	2.62	74.4
3120	50.5	1.21	3.54	2.33	65.8
3136	51.0	1.23	4.13	2.90	70.2
3140	37.0	0.89	3.40	2.51	73.8
3142	72.0	1.73	5.28	3.55	67.2
3154	45.5	1.09	3.43	2.34	68.2
3166	37.0	0.89	3.19	2.30	72.1
3170	50.0	1.20	4.38	3.18	72.6
3178	50.5	1.21	5.63	4.42	78.5
3192	34.5	0.83	2.98	2.15	72.1
3196	48.5	1.17	4.23	3.06	72.3
3256	55.0	1.32	4.50	3.18	70.7
3272	45.0	1.08	3.18	2.10	66.0
3274	47.0	1.13	3.25	2.12	65.2
3276	39.0	0.94	3.50	2.56	73.1
3278	45.0	1.08	3.69	2.61	70.7
3282	42.0	1.01	3.95	2.94	74.4
3286	36.0	0.86	3.07	2.21	72.0
3288	50.5	1.21	2.99	1.78	59.5
3298	50.5	1.21	4.75	3.54	74.5
3304	55.0	1.32	4.31	2.99	69.4
3306	54.5	1.31	4.18	2.87	68.7

Table 4. Continued

SAMPLE #	% FULL SCALE	% NCP	% TP (TOTAL PROTEIN)	% TP-NCP or % CASEIN	% $\frac{\text{CASEIN}}{\text{TP}}$
3308	41.75	1.00	4.19	3.19	76.0
3316	34.0	0.82	3.47	2.65	76.4
3348	47.0	1.13	4.27	3.14	73.5
3352	55.5	1.33	5.07	3.74	73.8
3358	42.0	1.01	3.99	2.98	74.7
3362	34.5	0.83	3.09	2.26	73.1
3366	37.0	0.89	3.34	2.45	73.3
3374	36.5	0.88	3.48	2.60	74.7
3390	45.0	1.30	4.19	2.89	69.0
3394	38.5	0.93	3.60	2.67	74.2
3400	47.5	1.14	4.59	3.45	75.2
3404	48.5	1.17	4.26	3.09	72.5
3414	48.0	1.15	4.21	3.06	72.7
3424	40.5	0.97	3.66	2.69	73.5
3454	35.0	0.84	4.00	3.16	79.0
3456	36.0	0.86	3.32	2.46	74.1
3462	43.5	1.05	4.30	3.25	75.6
3466	38.5	0.93	4.21	3.28	77.9
3470	40.5	0.97	4.03	3.06	75.9
3480	41.5	1.00	3.85	2.85	74.0
3482	39.0	0.94	3.63	2.69	74.1
3484	35.5	0.85	3.23	2.38	73.7
3494	58.5	1.41	4.37	2.96	67.7
3498	37.5	0.90	3.63	2.73	75.2
3506	37.5	0.90	3.30	2.40	72.7
3508	70.0	1.68	5.87	4.19	71.4
3526	42.5	1.02	3.55	2.53	71.3

Table 4. Continued

SAMPLE #	% FULL SCALE	% NCP	% TP (TOTAL PROTEIN)	% TP-NCP or % CASEIN	% $\frac{\text{CASEIN}}{\text{TP}}$
3532	39.0	0.94	3.73	2.79	74.8
3538	39.0	0.94	3.72	2.78	74.7
3542	44.5	1.07	4.62	3.55	76.8
3558	56.5	1.36	5.32	3.96	74.4
3562	37.5	0.90	3.56	2.66	74.7
3564	34.0	0.82	3.21	2.39	74.4
3568	33.0	0.79	3.30	2.51	76.1
3574	48.5	1.17	4.64	3.47	74.8
3578	46.5	1.12	3.95	2.83	71.6
3580	47.0	1.13	3.76	2.63	69.9
3584	37.5	0.90	3.71	2.81	75.7
3586	39.5	0.95	3.67	2.72	74.1
3604	39.0	0.94	4.22	3.28	77.7
3608	33.0	0.79	3.13	2.34	74.8
3614	34.0	0.82	3.43	2.61	76.1
3616	47.5	1.14	5.01	3.87	77.2
3618	39.5	0.95	4.12	3.17	76.9
3624	48.5	1.17	4.42	3.25	73.5
3632	40.5	0.97	3.87	2.90	74.9
3634	39.5	0.95	4.09	3.14	76.8
3638	44.0	1.06	4.23	3.17	74.9
3640	39.5	0.95	4.14	3.19	77.0
3644	35.0	0.84	3.24	2.40	74.1
3646	36.5	0.88	3.67	2.79	76.0
3648	33.0	0.79	3.57	2.78	77.9
3650	41.0	0.99	3.96	2.97	75.0
3656	34.0	0.82	3.13	2.31	73.8

Table 4. Continued

SAMPLE #	% FULL SCALE	% NCP	% TP (TOTAL PROTEIN)	% TP-NCP or % CASEIN	% $\frac{\text{CASEIN}}{\text{TP}}$
3658	38.5	0.93	3.72	2.79	75.0
3662	39.5	0.95	3.85	2.90	75.3
3668	41.5	1.00	3.84	2.84	73.9
3674	41.0	0.99	4.11	3.12	75.9
3676	49.75	1.20	3.63	2.33	66.0
3680	34.0	0.82	3.19	2.37	74.3
3682	35.5	0.85	3.35	2.50	74.6
3684	39.0	0.94	3.52	2.58	73.3
3690	35.0	0.84	4.49	3.65	81.3
3692	35.5	0.85	3.58	2.73	76.2
3696	41.0	0.99	3.35	2.36	70.4
3702	35.0	0.84	3.68	2.84	77.2
3704	27.5	0.66	2.83	2.17	76.7
3706	30.5	0.73	3.12	2.39	76.6
3710	33.5	0.81	3.15	2.34	74.3
3712	39.5	0.95	3.47	2.52	72.6
3714	36.0	0.86	3.74	2.88	77.0
3716	37.0	0.89	3.59	2.70	75.2
3722	40.0	0.96	3.43	2.47	72.0
3724	36.0	0.86	3.63	2.77	76.3
3726	30.0	0.72	2.94	2.22	75.5
3728	35.5	0.85	3.53	2.68	75.9
3734	34.5	0.83	3.40	2.57	75.6
3736	39.25	0.94	3.20	2.26	70.6
3738	41.5	1.00	3.43	2.43	70.8
3742	33.5	0.81	3.10	2.29	73.9
3748	38.0	0.91	3.49	2.58	73.9

Table 4. Continued

SAMPLE #	% FULL SCALE	% NCP	% TP (TOTAL PROTEIN)	% TP-NCP or % CASEIN	% $\frac{\text{CASEIN}}{\text{TP}}$
3750	33.5	0.81	3.11	2.30	73.9
3752	35.0	0.84	3.79	2.95	77.8
3764	30.0	0.72	3.52	2.79	79.5
3768	35.0	0.84	3.36	2.52	75.0
3772	42.75	1.03	4.20	3.17	75.5
3778	33.5	0.81	3.54	2.73	77.1
3780	30.0	0.72	3.39	2.67	78.8
3782	70.5	1.69	4.89	3.20	65.4
3784	35.5	0.85	3.37	2.52	74.8
BULK	41.0	0.99	3.56	2.57	72.2

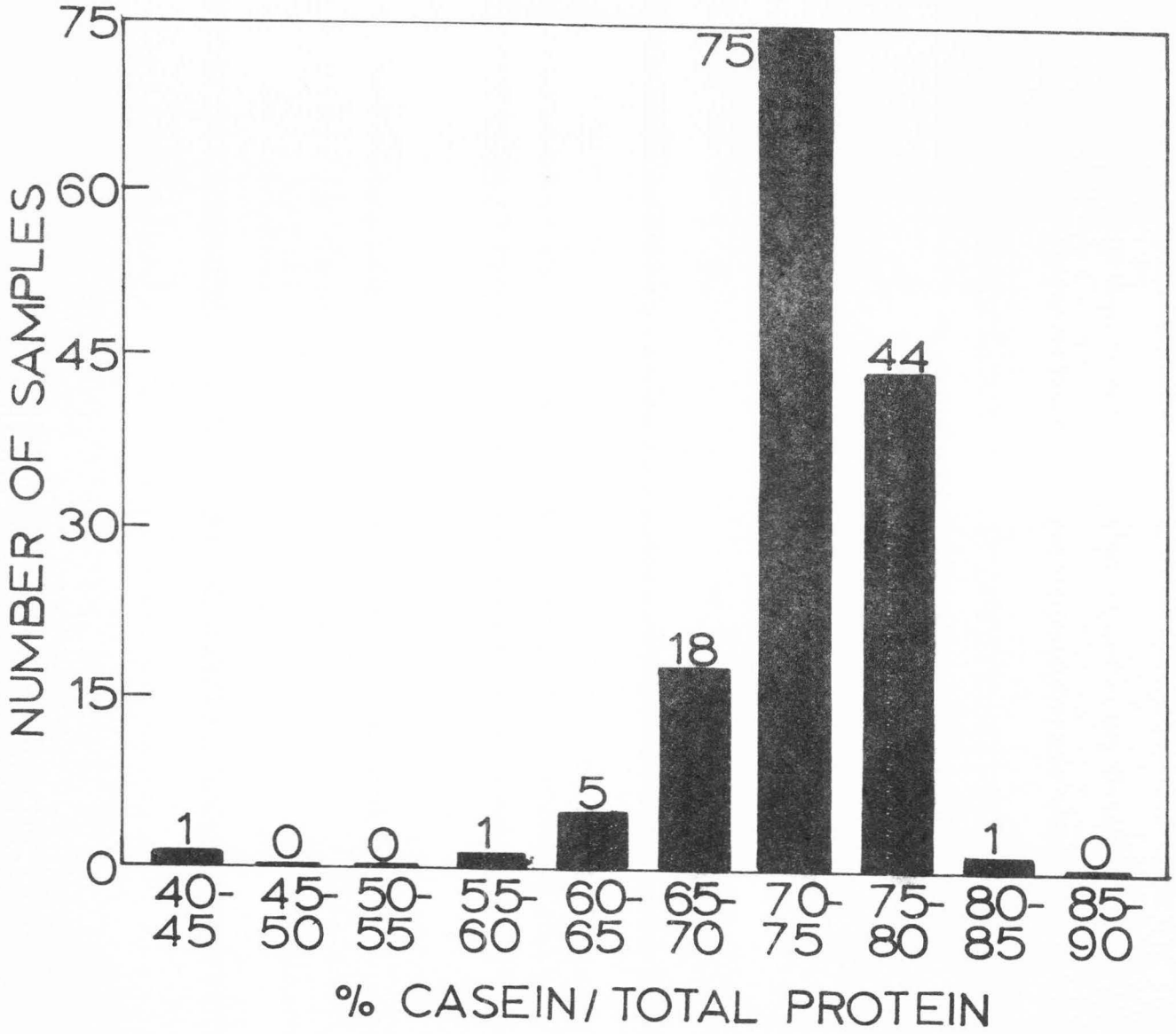
The casein levels of Table 4 were lower than some earlier reported findings. Only one sample had as much as 80% casein contrary to Rowland's (99) claims that casein constitutes over 80% of the total protein of normal milk. The range of casein expressed as a percentage of total protein was 41.0-81.3% with a mean value of 72.8%. This average was below those reported by Shahani and Sommer (102), Rowland (99), Davies (24), and Golding (42). Seventy-five samples of the 145 samples assayed lay within the 70-75% grouping and another 44 samples between 75-80%. Twenty-five samples contained less than 70% (Figure 5).

However, the range of casein expressed as a percentage of total protein agrees well with that found by Cziardo, Irvine, and Biggs (unpublished studies) who reported a range of 59-82%. If one eliminates the 41.0% casein reading in this study and uses the next lowest reading for the lower limit of the range, the range becomes 59.5-81.3% which is in excellent agreement with Cziardo, Irvine, and Biggs. They reported average casein levels of 73-74% which is very consistent with the 72.8% found in this study.

The reason for varying casein content expressed as a percentage of total protein is probably due to one or more factors: 1) genetics, 2) presence of disease, most notably mastitis, 3) the total errors of the IRMA and Technicon systems, and 4) the need for further optimizing of the NCP methodology.

The automated Lowry method for quantitating % NCP for indirect % casein has been shown to be practical, rapid, and accurate. However, improvements and modifications on the system remain to be made. For

Figure 5. Distribution of casein expressed as percentage of total protein in Utah State University dairy herd


NUMBER OF SAMPLES
75
60
45
30
15
0
1
0
0
1
5
18
75
44
1
0
40-45
45-50
50-55
55-60
60-65
65-70
70-75
75-80
80-85
85-90
% CASEIN/TOTAL PROTEIN

example, while the sampling rate used in this work was 20 sph, it may be possible to accelerate the rate of analysis.

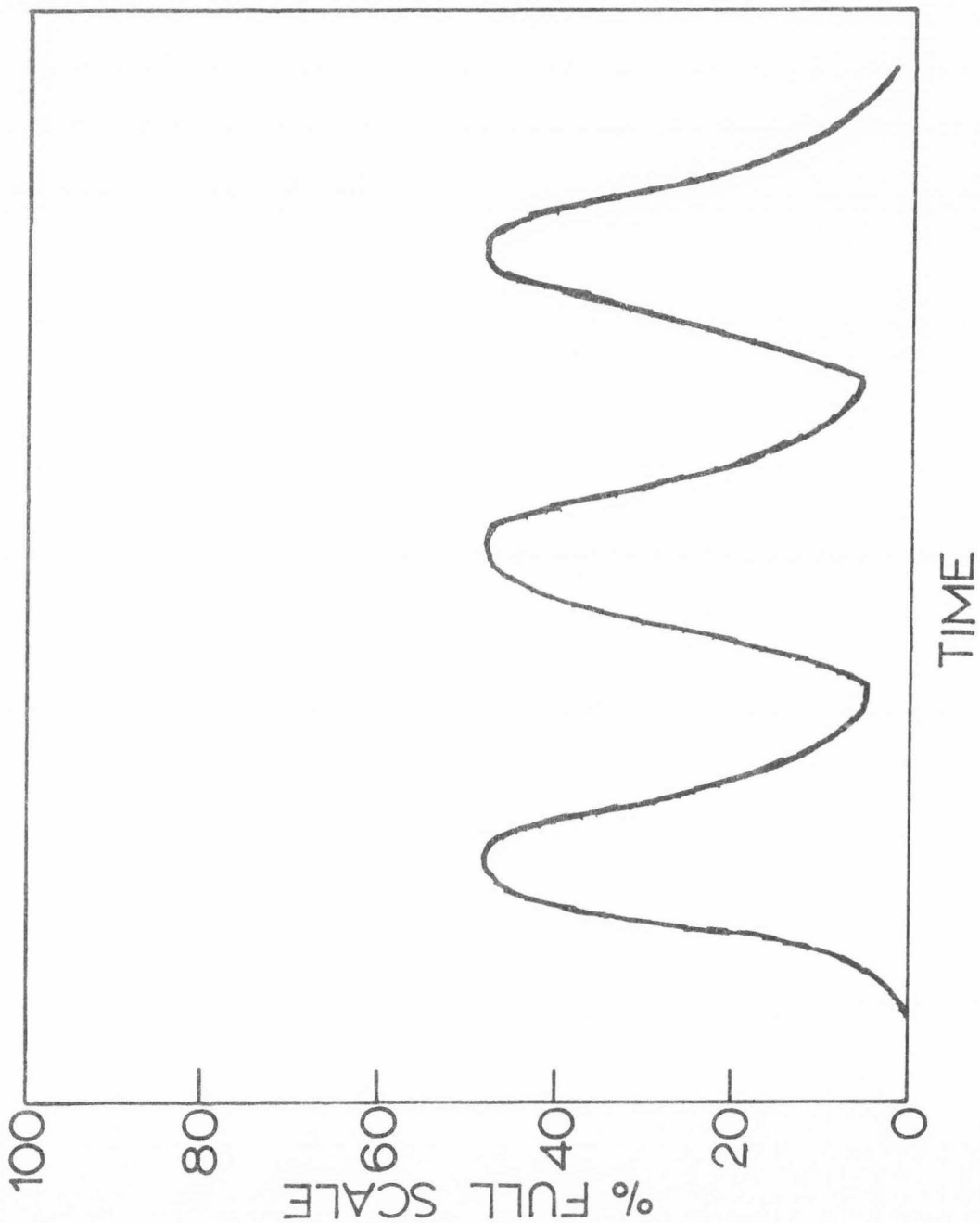
It is evident from the typical readout curves (Figure 6) that the wash period may be reduced. Using glass transmission tubing throughout the flow system and in as short lengths as possible may improve the wash characteristics and enable even further reduction of the wash phase.

Due to the viscosity and inherent nature of milk, a film was left by the milk in the transmission tubing as it passed from the sampler to the proportioning pump. The air bubble which enters the line between sampling and wash phases sweeps off the film but this residue creates a "higher-than-has-to-be" wash valley. A segmentatinh probe would improve this aspect of the wash phase.

The slight dip observed on some readout peaks may be eliminated if an intersample air compression (ISAC) fitting is incorporated into the flow system. This is essential if a digital printer is going to be used.

Nonetheless, this AutoAnalyzer procedure for non-casein protein quantitation and subsequent calculation of the casein content eliminates the tedious, repititious, and time-consuming manual methods for quantitation of casein. This automated procedure is strongly recommended for future milk analysis. This method would lend itself to large-scale, centrally located, analytical milk laboratories. With further development, analysis might be extended to other fluid dairy products such as cream and half and half. Chocolate milk might even be assayed if it were found that the brown pigmentation had no effect on the chemistry involved.

Figure 6. Readout pattern of replicate milk samples


100
80
60
40
20
0
% FULL SCALE
TIME

Quantitation of non-casein protein at 280 nm needs to be re-examined. This readout method would probably have been more effective had the continuous filter system been in use when absorption at 280 nm was being evaluated. Use of the temporary filter was shown to produce erratic readout patterns. A shorted electrical cable which rendered the Beckman DBG spectrophotometer inoperable compounded the problem.

CONCLUSIONS

1. The Technicon AutoAnalyzer II analytical system has been shown to be adaptable to the assay of non-casein protein in milk following on-stream precipitation of casein. This method is rapid, practical, and accurate.

2. The calculation of the casein content in milk by difference using total protein data from infrared milk analysis (IRMA) and non-casein protein data is easily accomplished.

3. The Lowry method for protein quantitation is suitable for quantitation of non-casein protein in a continuous manner with the Technicon AutoAnalyzer II. The automated Lowry method obeys Beer's law.

4. The percent non-casein protein (NCP) and casein varies greatly from animal to animal.

5. Casein as a percent of the total protein in the milk samples analyzed varied from 41.0 to 81.3% in the Utah State University dairy herd.

6. The automated assay for non-casein protein would lend itself well to a large, centrally located, analytical laboratory facility.

7. Interference of the chemical preservative $HgCl_2$ with the automated Lowry method warrants further investigation. The effects of the chemical preservatives HCHO and $K_2Cr_2O_7$ over longer periods of time also demand more attention.

8. Adaptation of the automated Lowry method to other fluid dairy products such as cream and half and half is an area for further research.

9. Quantitation of non-casein protein at 280 nm needs to be re-examined using the continuous filter system for collection of the casein precipitate.

10. The possibility of an increased sampling rate is a matter for further study. It is recommended that as short lengths of glass transmission tubing as possible be used as well as a segmentating sample probe. Increased detergent in the wash solution may also help minimize precipitate build up in the filter block.

BIBLIOGRAPHY

1. Alais, C., and P. Jolles. 1964. Action of acetic and trichloroacetic acids on casein. Ann. Biol. Anim. Biochim. Biophys. 4:79. (Original not seen; abstracted in Chem. Abstr. 61:10891b.)

2. Aleshin, S. N. 1962. Calculation of the amounts of casein from the fat content of milk. Molochnaya Prom. 23:11. (Original not seen; abstracted in Chem. Abstr. 58:4971e.)

3. Anderson, A. K. 1947. Essentials of physiological chemistry. John Wiley and Sons, Inc., New York. 395 p.

4. Anonymous. 1965. Automated analyses monitor bacterial growth. Chem. Eng. News 43:38.

5. Arny, H. V., and H. H. Schaefer. 1914. The ferric alum estimation of casein. J. Ind. Eng. Chem.6:748.

6. Ashworth, U. S., R. Seals, and R. E. Erb. 1960. An improved procedure for the determination of milk proteins by dye binding. J. Dairy Sci. 43:614.

7. 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.

8. Bannenberg, H. J., and W. van den Hoek. 1949. Formol titration. A practical method for the determination of total protein and casein in cow milk. Neth. Milk Dairy J. 3:162.

9. Block, R. J., and D. Bolling. 1945. The amino acid composition of proteins and foods. Charles C. Thomas, Springfield, Illinois. 398 p.

10. Block, R. J., and K. W. Weiss. 1956. Amino acid handbook. Charles C. Thomas, Springfield, Illinois. 386 p.

11. Brereton, J. G., and P. F. Sharp. 1942. Refractometric determination of casein in skimmilk. Ind. Eng. Chem. Anal. Ed. 14:872.

12. Brisson, G. J. 1965. Determination of total nitrogen in milk using a Technicon AutoAnalyzer system. p. 54 to 56. In L. T. Skeggs, Jr. (Ed.). Automation in analytical chemistry, 1965. Mediad, Inc., New York.

13. Brunner, J. R., C. A. Ernstrom, R. A. Hollis, B. L. Larson, R. McL. Whitney, and C. A. Zittle. 1960. Nomenclature of the proteins of bovine milk--first revision. J. Dairy Sci. 43:901.

14. Buruiana, L. 1933. A new method for determining the protein content of milk. Lait 13:1214. (Original not seen; abstracted in Chem. Abstr. 28:1413.)

15. Buruiana, L. 1936. Methods for colorimetric determination of protein. Bull. Soc. Chim. Romania 18A:191. (Original not seen; abstracted in Chem. Abstr. 32:1736.)

16. Buruiana, L. M. 1958. The determination of proteins by means of the xanthoproteic reaction. Naturwissenschaften 45:339.

17. Cerbulis, J., and C. A. Zittle. 1965. Lipids associated with acid-precipitated casein. J. Dairy Sci. 48:1154.

18. Chibnall, A. C., M. W. Rees, and E. F. Williams. 1943. The total nitrogen content of egg albumin and other proteins. Biochemical J. 37:354.

19. Churina, N. 1948. Determination of casein in milk. Molochnaya Prom 9:25. (Original not seen; abstracted from Chem. Abstr. 43:7598h.)

20. Cluskey, F. J., E. J. Thomas, and S. T. Coulter. 1969. Precipitation of milk proteins by sodium carboxymethyl cellulose. J. Dairy Sci. 52:1158.

21. Cohn, E. J. 1925. The physical chemistry of the proteins. Physiol. Rev. 5:349.

22. Colowick, S. P., and N. O. Kaplan. (Ed.). 1955. Methods in enzymology. Vol. I. Academic Press, Inc., New York. 835 p.

23. Conetta, A., L. Stookey, and H. Zehnder. 1970. An automated system for the determination of milkfat, protein, and lactose in milk, p. 81 to 85. In Advances in automated analysis, Vol. II, 1970. Thurman Associates, Miami.

24. Davies, W. L. 1936. Investigations on the milk of a typical herd of shorthorn cows. III. Nitrogen distribution, chloride, lactose, copper, and iron content over a period of two years. J. Dairy Res. 6:363.

25. Dawson, R. M. C., D. C. Elliott, and W. H. Elliott. 1969. Data for biochemical research. Oxford University Press, New York. 325 p.

26. DeMediros, G. 1971. Experiences with and suggestions for multiple component pricing plans. Paper presented at the Western Dairy Conference Symposium, Seattle, Washington.

27. Deniges, G. 1931. Rapid nephelometric examination of milk. Bull. Soc. Pharm. Bordeaux 69:1. (Original not seen; abstracted in Chem. Abstr. 26:4385.)

28. Dordevic, J. 1953. Comparison of the accuracy of four different methods of casein determination. Arh. Poljopr. Jauk 6:149. (Original not seen; abstracted in Dairy Sci. Abstr. 16:936.)

29. Dumas, B. R. 1968. Simultaneous determination of α_s-, β-, and κ- caseins in whole casein using carboxypeptidase A. Biochim. et Biophys. Acta. 168:274.

30. Dumazert, C., and M. Bozzi-Tichadou. 1962. Determination of casein on its electrophoretic properties. Compt. Rend. Soc. Biol. 146:468. (Original not seen; abstracted in Chem. Abstr. 47:709a.)

31. El-Negoumy, A. M. 1963. Effect of method of preparing isoelectric casein on its electrophoretic properties. J. Dairy Sci. 46:768.

32. Fleury, P. F., and R. Eberhard. 1953. Gravimetric and colorimetric determination of casein in cow milk. Ann. Pharm. Franc. 11:183. (Original not seen; abstracted in Chem. Abstr. 47:7129f.)

33. Fleury, P. F., and R. Eberhard. 1956. Photometric determination of proteins in biological fluids by biuret reaction. Pharm. Biol. 1:24. (Original not seen; abstracted in Chem. Abstr. 51:16668h.)

34. Folin, O., and W. Denis. 1912. On phosphotungstic-phosphomolybdic compounds as color reagents. J. Biol. Chem. 12:239.

35. Folin, O., and V. Ciocalteau. 1927. On tyrosine and tryptophane determinations in proteins. J. Biol. Chem. 73:627.

36. Fraenkel-Conrat, H., and M. Cooper. 1944. The use of dyes for the determination of acid and basic groups in proteins. J. Biol. Chem. 154:239.

37. Ganguli, N. C., O. N. Agarwala, and V. R. Bhalerao. 1965. Colorimetric method for estimation of proteose-peptone in milk. J. Dairy Sci. 48:674.

38. Garza, A. C. 1965. The determination of proteins in brewing materials, p. 61 to 65. In L. T. Skeggs, Jr. (Ed.). Automation in analytical chemistry, 1965. Mediad, Inc., New York.

39. Gehrke, C. W., L. L. Wall, Sr., J. S. Killingley, and K. Inada. 1970. Missouri automated nitrogen method (MANM), p. 29 to 45. In Advanced in automated analysis, Volume II, 1970. Thurman Associates, Miami.

40. Gehrke, C. W., and L. L. Wall. 1970. Automated TNBS method for protein analysis in forages and grain, p. 93 to 97. In Advances in automated analysis, Volume II, 1970. Thurman Associates, Miami.

41. Goiffon, E. 1949. Colorimetric determination of casein, lactalbumin, and the polypeptides of milk. Lair 29:231. (Original not seen; abstracted in Chem. Abstr. 45:1263c.)

42. Golding, J., J. Mackintosh, and E. C. V. Mattick. Investigations on the milk of a typical herd of shorthorn cows, I. J. Dairy Res. 4:48.

43. Gorbach, G., G. Dedic, and E. H. Philippi. 1955. Determination of free amino acids in biological material. Z. Physiol. Chem. 301:185.

44. Gorbacheva, A. P. 1940a. A new gravimetric method for determination of casein. Dairy Sci. Abstr. 2:181. (Original not seen; abstracted from Chem. Abstr. 36:3345.)

45. Gorbacheva, A. P. 1940b. Simple method of casein determination in cow milk and regeneration of the solvent. Molochnaya Prom. 7:10. (Original not seen; abstracted from Chem. Abstr. 35:6681.)

46. Hansson, E. 1959. Determination of casein content in milk. Svenska Mejeritidn 49:277. (Original not seen; abstracted from Chem. Abstr. 52:20724c.)

47. Harland, H. A., S. T. Coulter, and R. Jenness. 1952. The effect of the various steps in manufacture on the extent of serum protein denaturation on nonfat dry milk solids. J. Dairy Sci. 35:363.

48. Harland, H. A., S. T. Coulter, and R. Jenness. 1955. Natural variation of milk serum proteins as a limitation of their use in evaluating the heat treatment of milk. J. Dairy Sci. 38:858.

49. Harris, L. J. 1924. The combination of proteins, amino-acids, etc., with acids and alkalis, and their combining weights as determined by physico-chemical measurements. Proc. Roy. Soc. London--series B 97:364.

50. Hart, E. B. 1907. A simple test for casein in milk and its relation to the dairy industry. Wis. Agr. Expt. Sta. Bull. 156:1.

51. Hart, E. B. 1909. A volumetric method for the estimation of casein in milk. J. Biol. Chem. 6:445.

52. Hiller, A., J. Plazin, and D. D. Van Slyke. 1948. A study of conditions for Kjeldahl determination of nitrogen in proteins. Description of methods with mercury catalyst and titrimetric and gasometric measurements of the ammonia formed. J. Biol. Chem. 176:1401.

53. Hipp, N. J., M. L. Groves, J. H. Custer, and T. L. McMeekin. 1950. Separation of α- casein. J. Amer. Chem. Soc. 72:4928.

54. Hipp, N. J., M. L. Groves, J. H. Custer, and T. L. McMeekin. 1952. Separation of α-, β-, and γ-casein. J. Dairy Sci. 35:272.

55. Holsinger, V. H., L. P. Posati, and M. J. Pallansch. 1970. Anomalous results obtained in the determination of available lysine in casein using 2,4,6-trinitrobenzenesulfonic acid. J. Dairy Sci. 53:1638.

56. Horlamus, J. 1971. Experiences with and suggestions for multiple component pricing plans. Paper presented at the Western Dairy Conference Symposium, Seattle, Washington.

57. Jakubowski, J., A. Sienkiewicz, and E. Nowak. 1958. Rapid determination of casein in milk. Nature 181:1277.

58. Jenness, R., B. L. Larson, T. L. McMeekin, A. M. Swanson, D. H. Whitnah, and R. McL. Whitney. 1956. Nomenclature of the proteins of bovine milk. J. Dairy Sci. 39:536.

59. Joe, M. M., D. Sakai, and R. A. Moffitt. 1966. Simultaneous determination of nitrogen, calcium, and phosphorus in fluid milk products. Paper presented at the Technicon Symposium, New York, New York.

60. Joost, K. 1958. Accuracy of Hansson's refractometric determination method. Svenska Mejeritdn 50:39. (Original not seen; abstracted from Chem. Abstr. 53:15401h.)

61. Joslyn, M. A. 1970. Methods in food analysis. Academic Press, New York. 845 p.

62. Kober, P. A. 1913. Nephelometric determination of proteins: casein, globulin, and albumin in milk. J. Amer. Chem. Soc. 35:1585.

63. Kofranyi, E. 1950. A rapid method for the determination of the protein content of cow milk. Milchwissenschaft 5:51. (Original not seen; abstracted from Chem. Abstr. 44:4158f.)

64. Larson, B. L., and G. D. Rolleri. 1955. Heat denaturation of the specific serum proteins in milk. J. Dairy Sci 38;351,

65. Leali, L. 1958. Estimation of total protein and casein at the isoelectric point. Atti. Soc. Ital. Sci. Vet. 12:378. (Original not seen; abstracted from Chem. Abstr. 57:11610a.)

66. Lebermann, F. 1929. The precipitation of casein at the isoelectric point. Biochem. Z. 206:56. (Original not seen; abstracted from Chem. Abstr. 23:2995.)

67. Lento, H. G., and C. E. Daugherty. 1970. The automated protein-nitrogen analysis of foods, p. 75 to 80. In Advances in automated analysis, Volume II, 1970. Thurman Associates, Miami.

68. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

69. MacRae, H. F., and B. E. Baker. 1958. Application of electrophoresis on paper to the estimation of alpha-, beta-, and gamma- casein. J. Dairy Sci. 41:233.

70. Mann, G. 1906. Chemistry of the proteins. The Macmillan Co., New York. 606 p.

71. McDowall, F. H., and R. M. Dolby. 1936. The Walker method of estimation of casein in milk and its application to preservatized composite samples. New Zeal. J. Sci. Technol. 17:619. (Original not seen; abstracted from Chem. Abstr. 30:3897.)

72. McDowall, F. H., and A. K. R. McDowell. 1936. Determination of casein by formol titration after precipitation with acid. An improved technic. Analyst 61:824.

73. McMeekin, T. L., N. J. Hipp, and M. L. Groves. 1959. The separation of the components of α- casein. Arch. Biochem. Biophys. 83:35.

74. Mascre, M., and E. Bouchara. 1930. Influence of formaldehyde on the preparation of milk protein material. Bull. Soc. Chim. Biol. 12:994. (Original not seen; abstracted from Chem. Abstr. 25:1916.)

75. Matthaiopoulos, G. T. 1908. Determination of the equivalent weight of casein and a new method for the determination of casein. Z. Anal. Chem. 47:492. (Original not seen; abstracted from Chem. Abstr. 2:2961.)

76. Menefee, S. G., and O. R. Overman. 1940. A semimicro-Kjeldahl method for the determination of total nitrogen in milk. J. Dairy Sci. 23:1177.

77. Menefee, S. G., O. R. Overman, and P. H. Tracy. 1941. The effect of processing on the nitrogen distribution in milk. J. Dairy Sci. 24:953.

78. Michaelis, L., and A. V. Szent-Gyorgyi. 1920. The influence of salts on the precipitation optimum of casein. Biochem. Z. 103:178. (Original not seen; abstracted from Chem. Abstr. 14:2572.)

79. Moir, G. M. 1931a. The determination of the milk proteins. I. The chemistry of the separation of casein. Analyst 56:1.

80. Moir, G. M. 1931b. The determination of the milk proteins. II. The identity of the casein precipitate. Analyst 56:73.

81. Moir, G. M. 1931c. The determination of the milk proteins. III. Proposed modified method for casein. Analyst 56:147.

82. Moore, H. P., and R. E. Kelly. 1970. Total nitrogen in fertilizer and feed by means of the AutoAnalyzer with continuous digestion, p. 47 to 51. In Advances in automated analysis, Volume II, 1970. Thurman Associates, Miami.

83. Muenchberg. F., R. Leskova, and D. Svastics. 1969. Refractometric determination of casein and total protein in milk. Milchwissenschaft 24:65.

84. Neurath, H., and K. Bailey. 1953. The proteins: chemistry, biological activity, and methods. Volume I, Part A. Academic Press, Inc., New York. 548 p.

85. Novy, O. 1953. Quick method for determination of milk protein. Sbornil Ceskoslov. Akad. Zemedelske Ved. 26A:483. (Original not seen; abstracted from Chem. Abstr. 48:4717h.)

86. Orlova, N., A. Mizyakina, N. Popova, and M. Chistyakova. 1965. Determination of protein and casein in milk. Molochnaya Prom. 26:18. (Original not seen; abstracted from Chem. Abstr. 66:94044e.)

87. Perov, S. S. 1931. Casein. Trudui Belkova Lab., Sbornik. 82 p. (Original not seen; abstracted from Chem. Abstr. 29:6612.)

88. Petersen, W. E. 1936. Report on [the determination of] milk proteins. J. Assoc. Offic. Agr. Chem. 19:383.

89. Peterson, R. C. 1966. Stability of the folin phenol reagent. J. Pharm. Sci. 55:523.

90. Pfyl, B., and R. Turner. 1914. The volumetric determination of casein in milk. Arb. Kais. Gesundh. 47:347. (Original not seen; abstracted from Chem. Abstr. 8:3082.)

91. Probst, A., J. Osterholzer, and F. Kiemeier. 1966. On the accuracy of the refractometric determination of casein in milk. Milchwissenschaft 21:708.

92. Puri, B. R., L. R. Sharma, and R. Chandra. 1957. Physiochemical properties of milk. VIII. Heat of flocculation of milk at the isoelectric point. Indian J. Dairy Sci. 10:79. (Original not seen; abstracted from Chem. Abstr. 52:4047f.)

93. Pyne, G. T. 1932. Determination of milk proteins by formaldehyde titration. Biochemical J. 26:1006.

94. Robertson, T. B. 1909. Rapid method of determining the percentage of casein in milk. J. Ind. Eng. Chem. 1:723.

95. Rolleri, G. D., B. L. Larson, and R. W. Touchberry. 1956. Protein production in the bovine. Breed and individual variations in the specific constituents of milk. J. Dairy Sci. 39:1683.

96. Rose, D., D. T. Davies, and M. Yaguchi. 1969. Determination of of major components of casein mixtures by column chromotography on DEAE-cellulose. J. Dairy Sci. 52:8.

97. Rowland, S. J. 1938a. The precipitation of the proteins in milk. I. Casein II. Total proteins III. Globulin IV. Albumin and proteose-peptone. J. Dairy Res. 9:30.

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

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

100. Schober, R., W. Christ, and W. Niclaus. 1954. Refractometric determination of casein in milk. Z. Lebensm. -Untersuch. u. -Forsch. 99:299. (Original not seen; abstracted from Chem. Abstr. 49:1232g.)

101. Shahani, K. M., and H. H. Sommer. 1951a. The protein and non-protein fractions in milk. I. Method of analysis. J. Dairy Sci. 34:1003.

102. Shahani, K. M., and H. H. Sommer. 1951b. The protein and non-protein nitrogen fractions in milk. II. Their content in fresh raw milk. J. Dairy Sci. 34:1010.

103. Southward, C. R., and R. M. Dolby. 1968. Seasonal variation in the viscosity index and adhesive strength of casein from the milk of individual cows. J. Dairy Res. 35:25.

104. Starkenburg, R. 1971. Multiple component pricing; why be concerned. Paper presented at the Western Dairy Conference Symposium, Seattle, Washington.

105. Thompson, M. P., N. P. Tarassuk, R. Jenness, H. A. Lillevik, U. S. Ashworth, and D. Rose. 1965. Nomenclature of the proteins of cow's milk--second revision. J. Dairy Sci. 48:159.

106. Thompson, M. P. 1970. Phenotyping milk proteins: a review. J. Dairy Sci. 53:1341.

107. Timita, Shigeru. 1963. The fluorometric analysis of polyelectrolytes. I. Proteins. Tokyo Kogyo Shikenso Kokoku. 58:99. (Original not seen; abstracted from Chem. Abstr. 62:8109f.)

108. Treece, J. M., L. O. Gilmore, and N. S. Fechheimer. 1959. A comparison of the Orange G dye and Kjeldahl protein method for determining milk protein. J. Dairy Sci. 42:367.

109. Trembath, R. H. 1951. The Walker casein test for cheese milk. Australia J. Dairy Technol. 6:133. (Original not seen; abstracted from Chem. Abstr. 56:4138d.)

110. Udy, D. C. 1956. A rapid method for estimating total protein in milk. Nature 178:314.

111. Van Slyke, L. L., and A. W. Bosworth. 1909a. A volumetric method for the determination of casein in milk. Ind. Eng. Chem. 1:768.

112. Van Slyke, L. L., and A. W. Bosworth. 1909b. A volumetric method for the determination of casein in milk. New York Agr. Expt. Sta. Tech. Bull. 10.

113. Van Slyke, L. L., and A. W. Bosworth. 1910. A simple method for the determination of casein in cow's milk. New York Med. J. 10:542. (Original not seen; abstracted from Chem. Abstr. 4:351.

114. Vereshchagina, V., and E. Meshcheryakova. 1958. Rapid method for determination of total protein, casein, and phosphorus in milk with help of perhydrol. Molochnaya Prom 19:35. (Original not seen; abstracted from Chem. Abstr. 52:8406c.)

115. Vereshchagina, V. I., and E. D. Meshcheryakova. 1959. Rapid determination of proteins and phosphorus in milk. Byull. Nauch. -Tekh, Inform. Sibirsk. Nauch. Issledovatel. Inst. Zhivotnovodstva #3:48. (Original not seen; abstracted from Chem. Abstr. 56:12043b.)

116. Von Hippel, P. H., and D. F. Waugh. 1955. Casein: monomers and polymers. J. Amer. Chem. Soc. 77:4311.

117. Walker, W. O. 1914. Rapid method for determining percentage of casein in milk. J. Ind. Eng. Chem. 6:131.

118. Warner, R. C. 1944. Separation of α- and β- casein. J. Amer. Chem. Soc. 66:1725.

119. Waterman, H. C. 1926. Determination of casein in milk. J. Assoc. Offic. Agr. Chem. 9:246.

120. Waterman, H. C. 1927. The determination of casein in milk by an approximately isoelectric precipitation. J. Assoc. Offic. Agr. Chem. 10:259.

121. Waterman, H. C. 1928. Report on milk proteins (determination of casein). J. Assoc. Offic. Agr. Chem. 11:298.

122. Waterman, H. C. 1930. Report on milk proteins (determination of casein). J. Assoc. Offic. Agr. Chem. 13:254.

123. Waterman, H. D. 1931. Report on milk proteins (determination of casein). J. Assoc. Offic. Agr. Chem. 14:246.

124. Weichselbaum. T. E. 1946. An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. Amer. J. Clin. Pathol. (Tech. Sec.) 10:40.

125. West, E. S., W. R. Todd, H. S. Mason, and J. T. Van Bruggen. 1966. Textbook of biochemistry. The Macmillan Company, New York. 1595 p.

126. Wilcox, C. J., S. N. Gaunt, and B. R. Farthing. 1971. Genetic interrelationships of milk composition and yield. Paper presented at the Western Dairy Conference Symposium, Seattle, Washington.

127. Zhebrovskii, L. S. and T. A. Pavlyuchenko. 1968. Rapid determination of protein fractions in milk by dye adsorption. Zhivotnovodstvo 30:69. (Original not seen; abstracted from Chem. Abstr. 69:9776q.)

128. Zittle, C. A. 1966. Precipitation of casein from acidic solutions by divalent anions. J. Dairy Sci. 49:361.

VITA

Robert Steven White

Candidate for the Degree of

Master of Science

Thesis: Continuous Flow Analysis of Non-casein Protein in Milk

Major Field: Nutrition and Food Sciences

Biographical Information:

Personal Data: Born at Omaha, Douglas, Nebraska, December 22, 1946, son of Robert Nelson and Frances Meidlinger White; married Connie King, June 4, 1971.

Education: Attended elementary schools in Lyons, Illinois, Overland Park, Kansas, and Omaha, Nebraska; graduated from Pulaski High School, Milwaukee, Wisconsin in 1964; received the Bachelor of Arts degree from Carleton College Northfield, Minnesota in 1968 with a major in chemistry and a minor in history; completed requirements for the Master of Science degree in Nutrition and Food Sciences at Utah State University in 1972.

Professional Experience: Summer, 1967, research chemist for Dairyland Food Laboratories, Waukesha, Wisconsin; 1972, associate member of American Chemical Society.