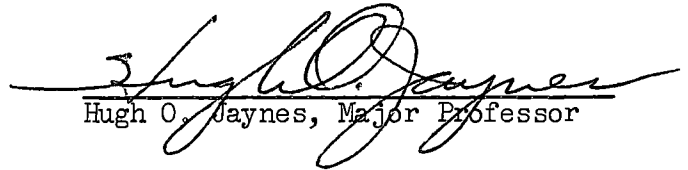
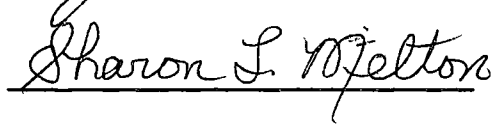


To the Graduate Council:

I am submitting herewith a thesis written by Shya Ling Hu entitled "A Comparison of Two Methods for Estimating Milk Proteins by Ultraviolet Spectrophotometry." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.


Hugh O. Jaynes, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:


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A COMPARISON OF TWO METHODS FOR ESTIMATING MILK PROTEINS
BY ULTRAVIOLET SPECTROPHOTOMETRY

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee

Shya Ling Hu

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ABSTRACT

The purpose of this study was to compare two ultraviolet spectrophotometric methods for the determination of milk proteins: the difference in absorbance at 215 and 225 nm, and the absorbance at 280 nm. Seven purified milk proteins were dried to constant weight under vacuum, dissolved, and diluted to five concentration levels in 0.9% NaCl, phosphate buffer and sodium dodecyl sulfate buffer. Duplicate concentration series were measured spectrophotometrically and the results were analyzed by regression.

From the absorbances for the series of seven proteins studied, the 215-225 nm measurements were from 2 to 16 times as sensitive as those at 280 nm. For both methods, precision was about 5%, and the accuracy was around 5%, also. When the same protein was measured in different solvents at 215-225 nm, the absorbances among different solutions of the same protein showed a slightly significant difference.

The error for estimating different proteins by using one standard at 215-225 nm was less than at 280 nm, but the error was too large to make that technique practical.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION.	1
II. REVIEW OF LITERATURE.	3
Milk Proteins	3
Caseins	3
Whey Protein.	5
Quantitative Determination and Separation of Milk Proteins	
by Column Chromatography and Spectrophotometric Methods .	6
Column Chromatography	6
Spectrophotometric Methods for Estimating Protein	
Concentrations.	7
III. MATERIALS AND METHODS	14
Milk Proteins	14
Solvents.	14
Concentrations of Milk Proteins	15
Preparation of Protein Solutions.	15
Ultraviolet Absorbance.	19
Experimental Procedures	20
Statistical Analysis.	20
Regression equations and lines.	20
Sensitivity	21
Precision	21

CHAPTER	PAGE
III. (Continued)	
Accuracy.	22
The Number of Peptide Bonds and Aromatic Amino Acid in Milk Proteins	22
IV. EXPERIMENTAL RESULTS AND DISCUSSION	27
Moisture Content of Milk Protein Samples.	27
Absorbances of The Three Solvents Over The Ultraviolet Light Range	27
Comparison of Results Between Different Methods and Solvents.	29
Sensitivity	29
Precision.... .	34
Accuracy.	35
Variance of Regression Lines:	37
The Interaction Between Solvents and Proteins	46
V. SUMMARY	52
LITERATURE CITED.	54
VITA.	59

LIST OF TABLES

TABLE	PAGE
1. Amino Acid Composition of Seven Milk Proteins.	12
2. Concentration Levels for Protein Determination at 215-225 nm and 280 nm	16
3. Residues of Amino Acids Per Molecule Protein	23
4. Peptide Bonds and Aromatic Amino Acids of Milk Proteins. . . .	25
5. Moisture Contents of Milk Protein Samples.	28
6. Sensitivity Value, M, of Each Protein in Each Solvent at Both Methods.	32
7. Regression Coefficient of Each Regression Line	33
8. Coefficient of Variation of Each Regression Line (%)	36
9. "A" Value of Each Regression Line.	38
10. Covariance Analysis of Regression Lines in 0.9% NaCl Solvent at 215-225 nm.	39
11. Covariance Analysis of Regression Lines in Phosphate Solvent at 215-225 nm.	40
12. Covariance Analysis of Regression Lines in SDS Solvent at 215-225 nm	41
13. Covariance Analysis of Regression Lines in 0.9% NaCl Solvent at 280 nm.	42
14. Covariance Analysis of Regression Lines in Phosphate Solvent at 280 nm.	43

TABLE

PAGE

15.	Covariance Analysis of Regression Lines in SDS Solvent at 280 nm	44
16.	Correspondence Between The Content of Peptide Bonds in Seven Milk Proteins and The Regression Coefficients at 215-225 nm	47
17.	Correspondence Between The Content of Aromatic Amino Acids in Seven Milk Proteins and The Regression Coefficients at 280 nm	48
18.	Factorial Test Between Proteins and Solvents With The 215-225 nm Method.	49
19.	Factorial Test Between Proteins and Solvents With The 280 nm Method.	51

CHAPTER I

INTRODUCTION

Ultraviolet absorption spectrophotometry has been widely used in the quantitative estimation of protein concentrations. For most proteins, a distinct absorption peak is exhibited at 280 nm which is related to the presence of tyrosine, tryptophan, and phenylalanine in the protein molecule (6). The intensity of absorption at this wavelength depends on the proportions of these amino acids in the protein. Since the tyrosine, tryptophan and phenylalanine content of various proteins varies within fairly reasonable limits, the absorption peak at 280 nm has been used as a rapid and a fairly sensitive measure of protein concentration. But it is recognized that the absorption at 280 nm is interfered with seriously by the presence of even small amounts of nucleic acids or nucleotides. The varying aromatic amino acid content of different proteins also makes it unreliable for the measurement of impure or uncharacterized proteins. For these reasons, methods based on the ultraviolet absorption of the peptide bond in the low ultraviolet wavelength region have been developed. The total peptide bonds per unit weight differ relatively little compared to the aromatic amino acids among milk proteins. This led us to propose that the use of a lower wavelength method might make it possible to determine protein concentrations in dilute solutions of different proteins by a single standard

regression line generated by one protein. In addition, the low wavelength methods are sensitive enough to measure concentrations in the dilute solutions frequently encountered in column chromatography of proteins (42).

Waddell (38) developed a procedure for determining the concentration of human plasma proteins at 215 and 225 nm. He derived the protein concentrations from the difference between the absorbance at 215 nm and at 225 nm. The use of the difference, or slope function, minimized errors from the non-protein constituents of plasma. His results showed that the difference in absorbance at 215 nm and 225 nm could be used to measure protein concentrations as low as 20 $\mu\text{g/ml}$.

The purpose of this experiment was to compare these two spectrophotometric methods for the quantitative estimation of milk protein concentrations; the frequently used absorbance at 280 nm which is based on the contribution of aromatic side chains, and the difference in absorbance at 215 nm and 225 nm which is based on absorbance by the peptide bonds. The sensitivity, precision and accuracy of both methods were investigated. The possibility of using one milk protein as a regression standard to measure seven milk proteins in dilute solutions was tested in three different solvents. The interaction relation of milk protein absorption between different milk proteins and solvents was also tested.

CHAPTER II

REVIEW OF LITERATURE

I. MILK PROTEINS

Milk proteins have been divided into two general groups, the caseins and the remainder of the proteins which are called whey proteins. Most fractionation procedures begin with the precipitation of casein from skim milk by the addition of acid. Other methods, however, may be used for the separation of casein from whey proteins. There are approximately 30 grams of protein per liter of milk and about 25 grams of this is casein. Almost all of the non-casein proteins are in the whey and there are some minor proteins associated with the lipids (5).

Milk is one of the classical examples of a biological colloid due to the presence of micelles of the caseins, the main protein group of milk. Other proteins are complexed or aggregated in the casein micelles and not all of the casein is in the micelles. The formation and structure of the micelles is not completely understood even today (19).

Caseins

Caseins occur in milk as a colloidal calcium phosphate complex which contains about 7% inorganic material (28). They are involved in complex interactions in the micelle and tend to associate with themselves

or other proteins. For a long time, acid precipitated casein was believed to be a pure protein. This was due to a large extent to the very low solubility of casein at its isoelectric point in water and organic solvents, which made fractionation difficult by the frequently used method of dissolving in alkali and reprecipitating with acid (4, 20, 39).

In 1918, Osborne and Wakeman (22) isolated a small amount of casein with unique properties from the alcohol extracts obtained in drying casein. This fraction differed from ordinary casein in its high solubility in 50% alcohol and its low phosphorus content, only 0.1% as compared with 0.85% in unfractionated casein. Later, Hipp et al. (9) devised a method of preparing an alcohol soluble γ -casein which resembled in composition and properties the low phosphorus casein fraction prepared by Osborne and Wakeman.

Mellander (21), using the method of electrophoretic analysis of proteins, found that casein was composed of three electrophoretic components which he designated α -casein, β -casein and γ -casein in the order of decreasing mobility. Another method has been described for separating these three caseins by fractional precipitation from alcohol solutions and urea solutions by Hipp et al. (10). The order of precipitation of the casein components by both of these methods is α -casein, β -casein and γ -casein. These methods involve conditions which minimize interaction among the three caseins. Whole casein contains about 75% α -casein, 22% β -casein, and 3% γ -casein by electrophoretic analysis (3).

Precise quantitative fractionations have not been attained.

Von Hippel and Waugh (37) developed a method of fractionation of casein with calcium chloride. Their results indicated interaction of two fractions which they designated α_s casein and k-casein. The complexity of casein was illustrated through the electrophoretic analysis of casein by Wake and Baldwin (40) using starch gel containing urea. They concluded that casein could contain as many as 20 components instead of the four or five previously believed present.

Whey Protein

In the early investigation of milk protein, little attention was given to the whey after the removal of casein. In 1885, Sebelien (33) demonstrated these proteins in the milk whey. He obtained a globulin fraction by maturation with magnesium sulfate and an albumin fraction from globulin supernatant by acidification.

Palmer 1934 (23) isolated a crystalline "globulin" which was later named β -lactoglobulin from the albumin fraction of milk, β -lactoglobulin, shown to be the most abundant of the whey proteins, has been the subject of many physical chemical investigations and exists as polymers and genetic variants (41). α -lactalbumin is the second most abundant protein of milk whey and can also be crystallized from the lactalbumin fraction (7). The function of α -lactalbumin is as a component of the enzyme, lactose synthetase (46). The β -protein was designated as globulin because of its insolubility near the isoelectric point in the absence of salt and the α -protein as an albumin because

of its source in the "lactalbumin" fraction of whey (19).

Bovine serum albumin, a protein apparently identical to bovine blood serum albumin, has been isolated from cow's milk by repeated fractionation of the mother liquor which remained after crystallization of β -lactoglobulin from the crude lactalbumin fraction (25).

II. QUANTITATIVE DETERMINATION AND SEPARATION OF MILK PROTEINS BY COLUMN CHROMATOGRAPHY AND SPECTROPHOTOMETRIC METHODS

Column Chromatography

Ion exchange and gel chromatography are probably used more frequently than any other fractionation procedures to separate total skim milk protein, casein, whey proteins, and enzymes. Both have made possible the isolation and purification of many previously unrecognized milk proteins (17, 18, 30), and now are considered the essential research techniques in milk protein analysis.

The common chromatographic media for the separation of milk proteins are readily divided into two classes: (a) Ion exchange media which separate proteins in relation to a net charge effect, and (b) Gel filtration media which separate proteins on the basis of size (15).

Chromatography of proteins on ion exchangers involves the establishment of multiple electrostatic bonds between ionized groups on the surface of the exchanger and opposite charges in the proteins, followed by selective release of these bonds by changes in the concentration or pH of the eluent (12). In gel chromatography, molecules within a specific

size range penetrate some gel pores and emerge later, followed by small molecules and ions that freely penetrate the gel pores (27). The chromatographic methods, combined with conventional fractionation procedures, might eventually enable researchers to isolate all the individual components of casein and the whey protein. There are several other relatively new techniques such as preparative gel electrophoresis, electrofocusing, column electrophoresis, and membrane ultrafiltration (2, 16, 24), which are also useful for separating milk proteins.

Spectrophotometric Methods For Estimating Protein Concentrations

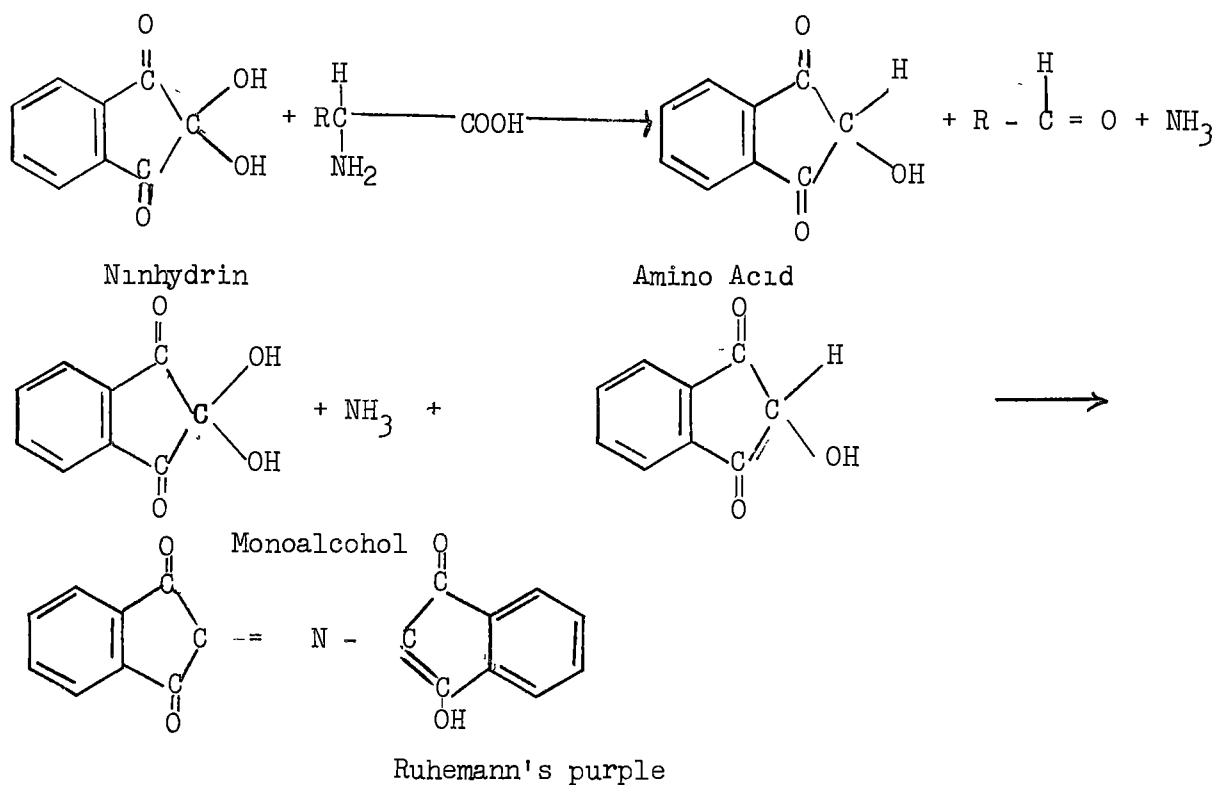
There are several spectrophotometric methods widely used for the quantitative estimation of protein concentration in protein chemistry:

The Folin-Lowry Method. This method described by Lowry et al. (14), has become commonly used in chemistry and biochemistry where its primary use has been in the estimation of total protein solution. The final color is a result of: (a) biuret reaction of protein with copper ion in alkali, and (b) reduction of the phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophan present in the treated protein. It is convenient and sensitive; however, there are two major disadvantages of this method. The amount of color varies with different proteins, and the color is not strictly proportional to concentration.

The Biuret Method. Copper reacts with proteins in alkaline solution to produce a purplish color. The sensitivity is not high, the color yield is variable, and opalescence is often difficult to prevent in the

final solution, especially when lipids and carbohydrates are present. Also, more material is required for assay by the biuret method (29).

The Ninhydrin Method. This reaction, discovered by Ruhemann in 1911 (31), is used by many workers in analysis. It gives a strong reaction with amino acids, but a weak reaction with proteins. The schematic for reactions with an amino acid is shown below.



The Turbidimetric Method. The turbidity produced when protein is mixed with low concentrations of any of the common protein precipitants can be used as an index of protein concentration. This method is rapid and convenient, but it gives different values with different proteins. It does not permit differentiation between protein and acid-insoluble compounds such as nucleic acids (13).

Ultraviolet Absorption Method. About one hundred years ago, investigators noted that the presence of certain groups appeared to be associated with the appearance of color in organic molecules. These were called color bearers or chromophores (18). Since then, chemists have made extensive studies of the ultraviolet and visible electronic spectra of organic molecules, and the absorption properties of chromophores have become well defined. Today, a chromophore is defined as a group that has an absorption band in any region of the electromagnetic spectrum. The use of absorption spectra in the ultraviolet and visible region is the basis of many common analytical procedures in chemistry and biochemistry. The molar absorptivity of a protein at specific wavelengths in the ultraviolet region is used in the determination of its concentration in solution (18).

The two fundamental laws for the practice of spectrophotometric analysis are those of Bouguer and Beer (26).

a. Bouguer's law:

$$\log (I_0/I) = kb \quad (1)$$

I_0 : The incident intensity.

I : The transmitted intensity.

b : The path length of the transmitted light.

k : Constant.

This law states that when a beam of parallel monochromatic light enters an absorbing medium at right angles to plane parallel surfaces of the medium, the layer of the medium decreases the intensity of the beam entering that layer by a constant fraction.

b. Beer's Law:

$$\log (I_0/I) = k'c \quad (2)$$

where c: The concentration of the absorbing solution.

This gives the relationship between the intensity of the light beam and the concentration of the absorbing solution.

The combined Bouguer-Beer relationship is:

$$\log (I_0/I) = abc = A \quad (3)$$

Where a is a constant depending only on the wavelength of the radiation and the nature of the absorbing solution, and A is referred to as the absorbance.

Ultraviolet absorption methods are convenient, rapid and are used as routine methods for quantification of protein in column chromatographic methods. They do not destroy the protein components by reacting them with reagents as in the methods described above.

Estimation of protein by ultraviolet absorption has been the subject of considerable study. The light absorbance at 280 nm has been used frequently (1, 15, 35). Absorbance at this wavelength is due primarily to the presence of residues of the aromatic amino acids phenylalanine, tryptophan and tyrosine. The intensity of absorption at this wavelength depends on the proportions of these amino acids in the polypeptide (1, 15). Since the content of such side chains varies considerably from protein to protein, it is necessary to construct a separate standard curve for each species of protein if the proteins are to be analyzed as separated components of mixtures. In addition,

measurement at 280 nm is insensitive to protein concentrations lower than about 100 µg/ml (42). This limits its usefulness for the low concentrations which are frequently encountered in the eluates from column chromatography of proteins. For these reasons there have been several attempts to develop methods which are based on the ultraviolet absorption of the peptide bond (6, 8, 36, 38). This would be relatively free of side chain contributions and should yield a more uniform measure of the protein content.

Basically, the proposition to use one protein as a regression standard to measure several proteins by ultraviolet absorption assumes uniformity of the content of absorbing groups among the proteins. Goldfarb (6) and Waddell (38) observed that the absorption slope functions generated by different proteins in the range of 200 to 230 nm were similar. This indicated that the "concentrations" of peptide bonds in different proteins were fairly uniform. Table 1 lists the amino acid composition of the seven milk proteins used in this study (11, 41). This data was used to calculate the ratios of peptide bonds and aromatic amino acid residues per unit weight of protein among the various milk proteins. The variations between the ratios will be shown in the Materials and Methods Chapter.

In addition, peptide bond absorbance is a sensitive measure of lower protein concentration (6, 42, 43, 44). The absorptivity of protein in the 200-250 nm region increases rapidly with decreasing wavelength. In this spectral range, the intensities are several times greater than

Table 1. Amino Acid Composition of Seven Milk Proteins.

Amino Acid	Proteins ^a					Serum Albumin
	α -Casein	β -Casein	γ -Casein	K-Casein	α -lactal- bumin	β -Lacto- globulin
	g. of amino acid per 100 g. protein					
Aspartic A.	8.4	4.9	4.0	7.3	18.7	11.4
Threonine	4.9	5.1	4.4	6.6	5.5	5.0
Serine	6.3	6.8	5.5	6.1	4.8	4.0
Gultamic A.	22.5	23.2	22.9	17.4	12.9	19.3
Proline	7.5	15.1	17.0	8.8	1.5	5.1
Glycine	2.3	1.6	1.5	1.3	3.2	1.4
Alanine	3.8	2.0	2.3	5.4	2.1	7.0
Cystine	0.43	0.0	0.0	1.4	6.4	3.4
Valine	6.3	10.2	10.5	5.1	4.7	6.1
Methionine	2.5	3.4	4.1	1.0	1.0	3.2
Isoleucine	6.4	5.5	4.4	6.1	6.8	6.9
Leucine	7.9	11.6	12.0	6.1	11.5	15.5
Tyrosine	8.1	3.2	3.7	7.4	5.4	3.7
Phenylalanine	4.6	5.8	5.8	4.1	4.5	3.5
Lysine	8.9	6.5	6.2	5.8	11.5	11.8
Histidine	2.9	3.1	3.7	1.7	2.9	1.6
Arginine	4.3	3.4	1.9	4.0	1.2	2.8
Tryptophan.	2.2	0.8	1.2	1.1	7.0	2.7

^aK-Casein (44); all others from (43).

those at 280 nm (1, 6). The absorption peak for the peptide bond has been shown to fall at about 186 nm (6, 8), but there are severe limitations on the capacity of conventional spectrophotometers to operate in this range, such as the optical limitations of the machine, the absorption of oxygen has required nitrogen purging of the light path (43), and the contribution of stray light leads to spurious absorption peaks (32).

Tombs et al. (36) recommended the spectrophotometric estimation of protein at a wavelength of 210 nm. Woods and O'Bar (43) reported that measurements of protein concentration at 187 nm had good sensitivity when the sample temperature was lowered to 6°C and oxygen was eliminated. Measurement at 205 nm (6) has been proposed also. Waddell 1956 (38) used the difference in absorbance at 215 and 225 nm to estimate the concentration of proteins in solution. Using the difference instead of the absorbance at only one wavelength minimized the error from non-protein constituents, so, because the concentration of protein was so high relative to interfering materials, he used a slope function.

CHAPTER III

MATERIALS AND METHODS

I. MILK PROTEINS

Seven purified milk proteins were used in this work: α -casein, β -casein, γ -casein, k-casein, α -lactalbumin, β -lactoglobulin and bovine serum albumin. These were purchased from supply houses; α -lactalbumin from Sigma Chemical Company, St. Louis, Missouri and the other six milk proteins from Nutritional Biochemicals Corporation, Cleveland, Ohio. Purity statements were 90 to 95%. The protein samples were stored in the refrigerator at 4°C. Before use, the samples were dried at 60°C in a vacuum oven to constant weight (drying time, 14 hr.) then kept in a desiccator over phosphorus pentoxide. The moisture content of each protein was measured by weighing the sample before and after drying.

II. SOLVENTS

Three different solvents which have been used as eluants for separating milk proteins by column chromatography were used to dissolve each protein (4, 37, 39, 45, 46).

- A. No buffer, dilution in 0.9% NaCl, pH 7.0.
- B. Phosphate buffer, 0.02 M, pH 7.0.
- C. 0.02 M Phosphate buffer of pH 7.6 with 0.02 M sodium dodecyl sulfate (SDS).

These solvents were scanned against a water blank from 190 to 300 nm to measure their absorbance in the 215 to 225 and 280 nm area. This was done to check for solvent interferences in the UV range.

III. CONCENTRATIONS OF MILK PROTEINS

Five concentration levels of each protein in each solvent were used for both 215-225 nm and 280 nm absorption measurements. These are shown in Table 2.

IV. PREPARATION OF PROTEIN SOLUTIONS

Stock solutions of each protein were prepared and the various concentration levels were made by diluting the appropriate stock solution with the appropriate solvent.

- A. The concentration levels for 215-225 nm (except β - and γ -casein; see IV, C, and D.)
 - 1. Dissolved 20 mg of milk protein in each solvent and made it to 100 ml for the stock solution.
 - 2. Diluted 2, 3, 4, 5 and 6 ml of stock solution with the solvent to a total of 20 ml to get 20, 30, 40, 50 and 60 $\mu\text{g/ml}$ concentration levels.
- B. The concentration levels at 280 nm:
 - 1. α -casein
 - a. Dissolved 100 mg of protein with solvent to a volume of 40 ml for the stock solution.

Table 2. Concentration Levels for Protein Determination. at 215-225 nm and 280 nm.

Protein	Concentration Range ($\mu\text{g/ml}$)		Increments ($\mu\text{g/ml}$)	
	215-225 nm	280 nm	215-225 nm	280 nm
α -casein	20-60	100-500	10	100
β -casein	20-60	200-1000	10	200
γ -casein	20-60	200-1000	10	200
k-casein	20-60	200-1000	10	200
α -lactalbumin	20-60	100-500	10	100
β -lactoglobulin	20-60	200-1000	10	200
Bovine serum albumin	20-60	200-1000	10	200

- b. Diluted 2, 4, 6, 8 and 10 ml of the stock solution to 50 ml with the solvent to get 100, 200, 300, 400, and 500 $\mu\text{g/ml}$ concentration levels.

2. α -lactalbumin

- a. Dissolved 20 mg of α -lactalbumin with solvent to 40 ml for the stock solution.
- b. Diluted 2, 4, 5 and 8 ml of stock solution to 10 ml with the solvent to get 100, 200, 300 and 400 $\mu\text{g/ml}$ levels; the stock solution served as the 500 $\mu\text{g/ml}$ level.

3. β -casein, k-casein, β -lactoglobulin and bovine serum albumin

- a. Dissolved 40 mg sample with the solvent to 40 ml for the stock solution.
- b. Diluted 2, 4, 6 and 8 ml stock solution with the solvent to 10 ml to give 200, 400, 600 and 800 $\mu\text{g/ml}$ concentration levels; the stock solution served as the 1000 $\mu\text{g/ml}$ level.

C. γ -casein

This protein was used at the same levels as β -casein. Only 184 mg was available, so a different scheme was used.

1. Dissolved 17 mg γ -casein with solvent to 17 ml volume for the stock solution (see IV, D, 2.).
2. Diluted 1, 2, 3, 4 and ml stock solution to 5 ml to get 200, 400, 600 and 800 $\mu\text{g/ml}$ concentration level. The

1000 $\mu\text{g/ml}$ level was the stock solution. These levels were used at 280 nm.

3. Five ml of the 280 nm stock solution was diluted to 50 ml for the stock solution used at 215-225 nm. The concentration was 100 $\mu\text{g/ml}$.

4. Diluted 2, 3, 4, 5 and 6 ml stock solution (215-225 nm) to 10 ml with the solvent to get 20, 30, 40, 50 and 60 $\mu\text{g/ml}$ concentration levels.

D. β -casein and γ -casein dissolved in the phosphate and phosphate-SDS solutions, but would not dissolve in the 0.9% NaCl, pH 7.0, solvent. The stock solution of these two proteins were put into NaCl solution by the following scheme.

1. β -casein

a. 280 nm stock solution

(i). 40 mg β -casein was dissolved in 0.1N NaOH, and the solution was neutralized to pH 7.0 with 1N HCl.

(ii). Dry NaCl was added to the neutralized solution to make it 0.9% NaCl solvent and the solution was made to 40 ml with the 0.9% NaCl solvent.

(iii). The stock solution contained 1000 $\mu\text{g/ml}$.

b. 215-225 nm stock solution

(i). 20 mg β -casein was dissolved in 0.1N NaOH, and the solution was neutralized with 1N HCl to pH 7.0.

(ii). Dry NaCl was added to make the neutralized solution 0.9% NaCl, then the solution was made to 100 ml with 0.9% NaCl solvent.

(iii). The stock solution contained 200 $\mu\text{g}/\text{ml}$.

2. γ -casein

a. 280 nm stock solution

(i). 17 mg γ -casein was dissolved in 0.1N NaOH, and the solution was neutralized to pH 7.0 with 1N HCl.

(ii). Dry NaCl was added to make the neutralized solution 0.9% NaCl, then the solution was made to 17 ml with 0.9% NaCl solvent.

(iii). The stock solution contained 1000 $\mu\text{g}/\text{ml}$.

b. 215-225 nm stock solution.

5 ml of the 280 nm stock solution was diluted to 50 ml to be the 215-225 nm stock solution.

V. ULTRAVIOLET ABSORBANCE

The absorbance of each protein at 215, 225 and 280 nm solution was measured with the double-beam grating spectrophotometer, Model 124 (Hitachi, Ltd., Tokyo, Japan). Its specifications include: wavelength accuracy 0.5 nm; stray light-less than 0.1% at 220 nm; three fixed slit widths 0.5, 1, and 2.0 nm. The slit setting of 1.0 nm was used for measuring all the milk protein absorbances.

VI. EXPERIMENTAL PROCEDURES

- A. Each concentration series consisted of five concentrations.
- B. The concentration series of one protein in one solvent was a treatment. Each treatment was duplicated.
- C. There were 3 solvents x 7 proteins = 21 treatments to be analyzed by regression and comparison of slope values.
- D. In order to secure the best analytical results and to minimize the errors, samples were measured in the spectrophotometer within 20 minutes after the concentration series were prepared.

VII. STATISTICAL ANALYSIS

Regression Equations and Lines

The duplicate absorbance data of each concentration series of each protein in each solvent was fitted to a regression equation.

$$Y = a + bX \quad (4)$$

where Y is the concentration of protein.

X is the estimated absorbance.

b is the regression coefficient or slope.

a is the protein concentration when $X = 0$, the intercept.

There were 2 replications x 3 solvents x 7 proteins = 42 regression lines in this experiment.

Sensitivity

The sensitivity (M) of each method was measured by the ratio of the mean of the protein concentration series to the mean of absorbance and by the regression coefficient (b).

$$M = \frac{\bar{Y}}{\bar{X}} \quad \begin{array}{l} \text{(Mean of protein concentration series)} \\ \text{(Mean of absorbance)} \end{array} \quad (5)$$

$$b = \frac{\sum xy}{\sum x^2} \quad (6)$$

where

$$\begin{aligned} \sum xy &= \sum XY - \frac{\sum X \sum Y}{n} \\ \sum x^2 &= \sum X^2 - (\sum X)^2 / n \end{aligned}$$

Precision

The coefficient of variation of regression for each protein in each solvent was calculated and compared to the others.

$$C.V. = \frac{S}{Y} \quad (7)$$

where

$$S_{yx} = \sqrt{S_{yx}^2}$$

$$S_{yx}^2 = \sum d_{yx}^2 / n-2 \quad (n \text{ is the number of absorbances})$$

$$\sum d_{yx}^2 = \sum Y^2 - \frac{(\sum xy)^2}{\sum x^2}$$

$$\sum y^2 = \sum Y^2 - \frac{(\sum Y)^2}{n}$$

Accuracy

The accuracy was shown by the absolute value of the intercept value against the mean of the protein concentration.

$$A \text{ (accuracy) } \% = (|a|/\bar{Y}) \times 100 \quad (8)$$

E. The significant difference test of seven milk proteins absorptivity in each solvent was analyzed by the covariance method (35).

F. The factorial design was used for analysis of the relationship between the protein contribution and the solvent contribution and the interactions between them.

VIII. THE NUMBER OF PEPTIDE BONDS AND AROMATIC AMINO ACID IN MILK PROTEINS

The residues of amino acids and aromatic acids were calculated from the data in Table 1, page 12. They are listed in Table 3.

$$\text{Residues of amino acids / molecule protein} = \quad (9)$$

$$\frac{(\text{g. of A. A./100g. protein}) \times (\text{residue w./mol. w.}) \times (\text{mol. w. of protein})}{\text{Residue w. of amino acid}}$$

$$\frac{\text{Number of aromatic amino acids}}{\text{molecule protein}} = \text{Number of (tyrosine + phenylalanine + tryptophan) residues} \quad (10)$$

The peptide bonds and aromatic amino acids per molecule and per mole of protein for each protein are listed in Table 4. The ratios of peptide bonds and aromatic amino acids are listed on the last two lines. The ratios were established by setting the protein with the smallest number

Figure 3. (Continued)

Amino Acid	Res. ^a		α -Casein		β -Casein		γ -Casein		k-Casein		α -Lactal- bumin		β -Lacto- globulin		Bovine Serum Albumin	
	Mol. Wt.	Res. Wt.	Mol. Wt.	Res. Wt.	Mol. Wt.	Res. Wt.	Mol. Wt.	Res. Wt.	Mol. Wt.	Res. Wt.	M. W.	M. W.	M. W.	M. W.	M. W.	M. W.
residues per molecule																
Isoleucine	131.2	113.2	0.86	11.2	11.1	7.1	12.0	7.5	18.8	13.6						
Leucine	131.2	113.2	0.86	13.8	21.2	19.2	12.0	12.6	42.4	64.5						
Tyrosine	181.2	163.2	0.90	10.3	4.2	4.3	10.6	4.3	7.4	19.4						
Phenyl- alanine	165.2	147.2	0.89	6.4	8.5	7.4	6.4	3.9	7.7	27.5						
Lysine	146.2	128.2	0.88	14.1	10.7	9.0	10.4	11.4	29.1	60.6						
Histidine	155.2	137.2	0.88	4.3	4.8	5.0	2.8	2.6	3.7	17.7						
Arginine	174.2	156.2	0.90	5.7	4.7	2.3	6.0	1.0	5.9	23.5						
Tryptophan	204.2	186.2	0.91	2.5	1.0	1.3	1.4	4.9	4.7	1.9						
Total				195.3	206.9	178.7	190.9	132.9	299.1	575.4						
Number of Aromatic Amino Acid				19.2	13.7	13.0	18.4	13.1	19.8	48.8						

^aReference 18.

Table 4. Peptide Bonds and Aromatic Amino Acids of Milk Proteins.

	α -Casein	β -Casein	γ -Casein	k-Casein	α -lactal- bumin	β -Lacto- globulin	Bovine Serum Albumin
P. B./mole- cule ^a	194	206	178	190	132	298	574
Aro. A. A./ molecule ^b	19	14	13	18	13	20	49
P. B./Mole ^c	116788x10 ²¹	124012x10 ²¹	107156x10 ²¹	114380x10 ²¹	79464x10 ²¹	179396x10 ²¹	345548x10 ²¹
Aro. A. A./ Mole ^d	11438x10 ²¹	8428x10 ²¹	7826x10 ²¹	10836x10 ²¹	7826x10 ²¹	12040x10 ²¹	29298x10 ²¹
P. B./g. protein	5078x10 ¹⁸	5167x10 ¹⁸	5103x10 ¹⁸	4399x10 ¹⁸	5518x10 ¹⁸	4983x10 ¹⁸	5008x10 ¹⁸
Aro. A. A./ g/protein	497x10 ¹⁸	351x10 ¹⁸	373x10 ¹⁸	417x10 ¹⁸	543x10 ¹⁸	334x10 ¹⁸	428x10 ¹⁸
Ratio of P. B.	1.15	1.17	1.16	1.00	1.25	1.13	1.14
Ratio of Aro. A. A.	1.49	1.05	1.12	1.25	1.63	1.00	1.28

^aFrom Table 3, page 24. Total amino acids - 1

^bFrom Table 3, page 24. Total aromatic amino acids.

^cP. B./molecule x Avagadro's number (6.02 x 10²³ molecules/g. mol. wt.)

^dAro. A. A./molecule x Avogadro's number.

of peptide bonds or aromatic amino acids per unit weight of protein equal to 1.00. These were used to determine if the absorbances at 215-225 nm and 280 nm corresponded to the "concentrations" of peptide bonds and aromatic amino acids, respectively.

CHAPTER IV

EXPERIMENTAL RESULTS AND DISCUSSION

I. MOISTURE CONTENT OF MILK PROTEIN SAMPLES

The seven purified proteins purchased from biochemical companies were considered as standards. The samples had to be dried before use to determine actual weight to be analyzed, so the moisture contents were measured.

Milk protein samples from 100 mg to 300 mg were weighed out according to the quantities which were needed in the experiment. The samples were dried at 60°C in a vacuum oven to constant wt. (drying time, 14 hr.), then weighed again. The calculated moisture content of each protein sample is listed in Table 5. The average moisture content was 6.81% and the range was from 3.97% (α -lactalbumin) to 13.70% (k-casein).

II. ABSORBANCES OF THE THREE SOLVENTS OVER THE ULTRAVIOLET LIGHT RANGE

The sensitivity of the spectrophotometer would vary in the wavelength range of interest (215-280 nm) if there was significant absorbance present from the solvents employed. Therefore, the three solvents were scanned against a water blank from 190 to 300 nm to determine the absorbance of each solvent in the ultraviolet light range before they were used for dissolving and diluting the milk proteins. The scanning

Table 5. Moisture Contents of Milk Protein Samples.

Protein	Moisture Content (%)
α -casein	7.62
β -casein	5.26
γ -casein	7.68
k-casein	13.70
α -lactalbumin	3.97
β -lactoglobulin	5.49
Bovine serum albumin	3.93

speed was 60 nm/min. and the ultraviolet light range was from 190 to 300 nm. The absorbance of each solvent in this spectral range is shown in Figure 1. It shows that the absorbance of the three solvents from 215 to 225 nm and 280 nm were minimal. The absorbances increased rapidly at wavelengths below 215 nm. The sensitivity of the spectrophotometer should be fairly constant among the three solvents in the wavelength range of interest.

III. COMPARISON OF RESULTS BETWEEN DIFFERENT METHODS AND SOLVENTS

The results of this work were represented and compared by sensitivity, precision and accuracy among methods and solvents. Figure 2 illustrates sensitivity, precision and accuracy of two regression lines from α -lactalbumin and γ -casein in SDS solvent with 215-225 nm method. These regression lines demonstrate the typically high linearity correlation relationship between absorbances and protein concentration.

Sensitivity

Since varied milk protein concentration levels were measured in the optimum absorbance scale by two different methods, there are two parameters determining the sensitivity of methods: (a) M, the protein concentration measured by each absorbance unit, and (b) the slope of the regression line. These two results are listed in Table 6 and Table 7 along with data from the other proteins, and indicated on Figure 2.

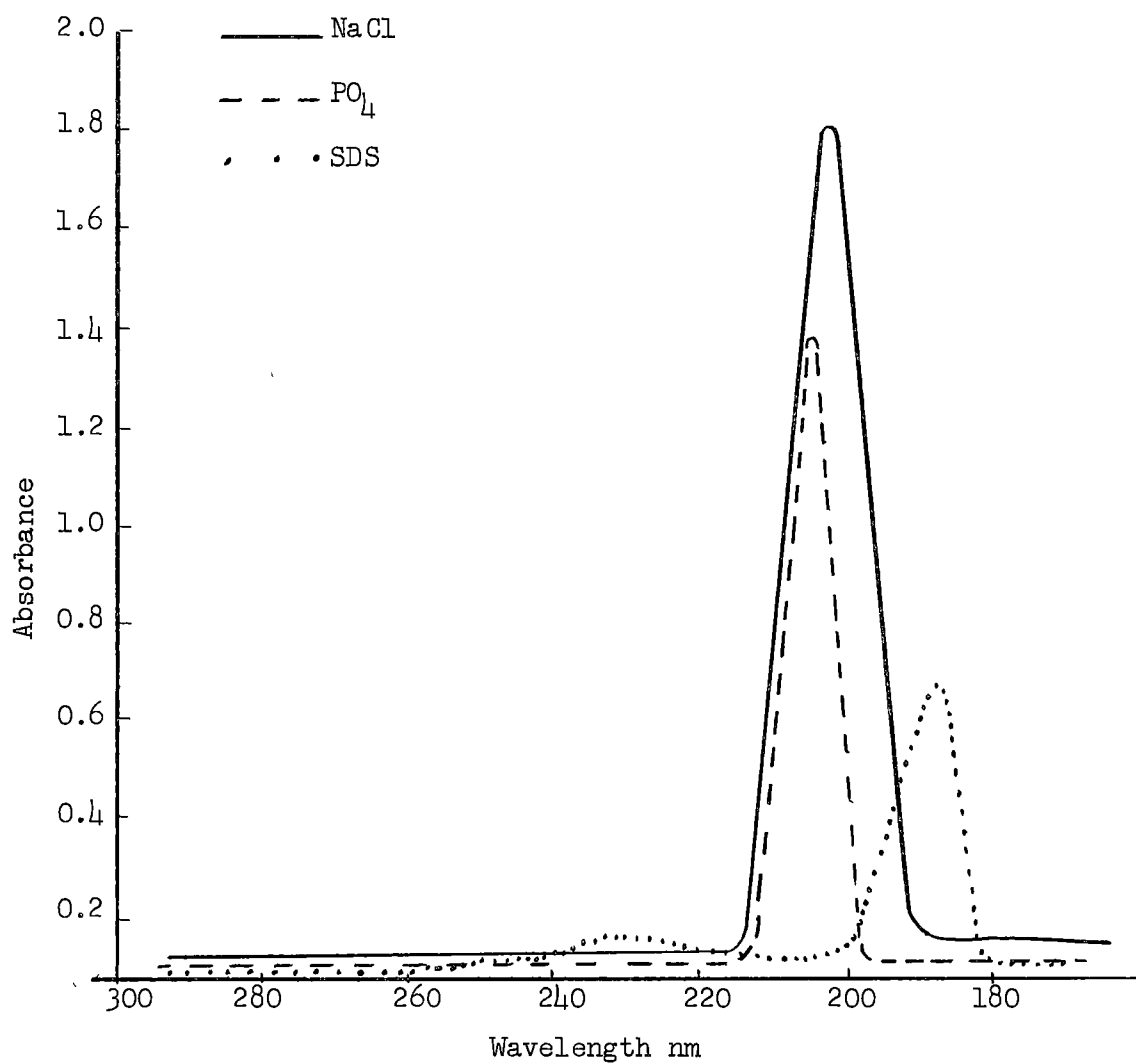


Figure 1. Absorbances of Three Solvents Against A Water Blank from 190 to 300 nm.

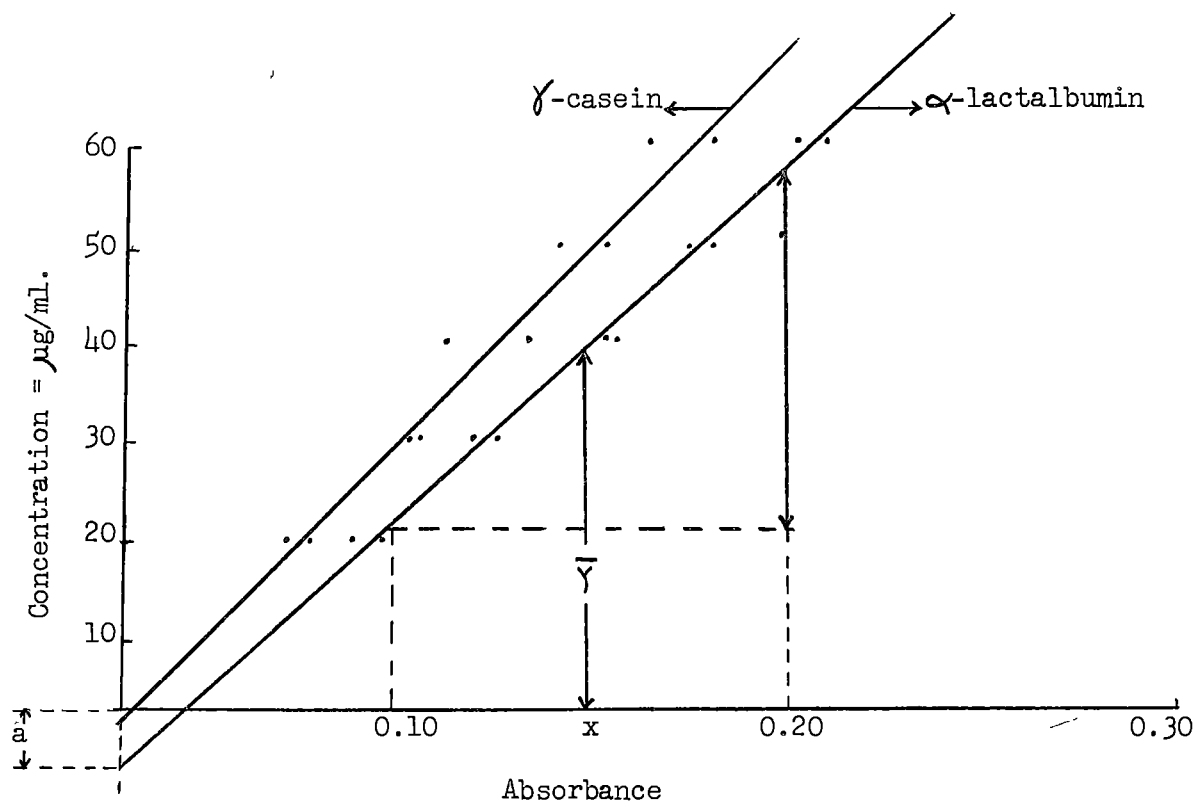


Figure 2. Sensitivity, Precision and Accuracy of The Regression Line.

Sensitivity =

$$1. \quad M = \frac{\bar{X}}{\bar{Y}}$$

$$2. \quad b \text{ (slope)} = \frac{\sum xy}{\sum x^2}$$

Precision =

$$c v \% = (S_{y.x} / \bar{Y}) \times 100$$

Accuracy

$$A\% = (|a| / \bar{Y}) \times 100$$

Table 6. Sensitivity Value, M, of Each Protein in Each Solvent at Both Methods.^a

Protein	Method	Solvent		NaCl		POL ₄		SDS	
				215-225	280	215-225	280	215-225	280
α -casein				19.5	64.9	19.5	57.7	20.6	49.1
β -casein				12.1	206.4	12.2	200.2	12.3	196.5
κ -casein				27.5	316.8	23.4	236.7	31.7	355.9
κ -casein				13.4	113.9	14.0	111.9	14.5	108.5
α -lactalbumin				25.6	50.6	27.1	48.6	26.2	46.9
β -lactoglobulin				18.8	83.6	18.7	103.4	20.3	104.2
Bovine serum albumin				16.4	149.1	16.3	146.3	15.2	152.2

$$a_M = \frac{\bar{Y} \text{ (Mean of protein conc.)}}{\bar{X} \text{ (Mean of absorbance)}} = \frac{\text{Protein } \mu\text{g/ml}}{0.1 \text{ absorbance unit}}$$

Table 7. Regression Coefficient^a of Each Regression Line.

Protein	Method	Solvent		NaCl		PO ₄		SDS	
		215-225	280	215-225	280	215-225	280	215-225	280
α -casein		20.7	63.5	20.4	59.5	21.3	51.4		
β -casein		12.9	214.5	12.7	209.3	13.1	206.9		
γ -casein		24.8	260.7	24.7	238.0	33.0	386.0		
k-casein		14.6	119.7	14.8	116.7	15.5	113.9		
α -lactalbumin		29.9	50.4	29.3	50.6	30.0	51.7		
β -lactoglobulin		20.2	84.1	18.7	107.2	20.2	108.6		
Bovine serum albumin		17.4	154.4	17.0	152.4	16.1	158.1		

$$a_b = \frac{\text{The protein concentration change } \mu\text{g/ml}}{0.1 \text{ absorbance change}}$$

Since the data was transformed to agree with the general regression equation

$$Y = a + bX$$

where Y is protein concentration and X is absorbance, a smaller number for sensitivity and slope indicates a higher degree of actual sensitivity. The transformation was made to facilitate the calculation of protein in terms of absorbance, rather than the inverse in which form the data was collected.

In Table 6, the smallest difference between the two methods is that for α -lactalbumin, for which the sensitivity of the 280 nm method is two times less than the 215-225 nm method in all solvents. The largest difference between the methods is for β -casein; the 280 nm method is sixteen times lower than the sensitivity of 215-225 nm method. The regression coefficients for 280 nm in Table 7 are higher than the regression coefficients for 215-225 nm, and the biggest difference between them is about 10 fold (for γ -casein). The lower the sensitivity number, the smaller the protein quantity measureable in the ultraviolet spectrophotometer.

Precision

Precision is related to the difference between the values obtained when the same quantity is measured in replicate. If such differences are small, the precision is high. If an infinite number of samples were measured, the means of all the repeated data would fall on the regression

line. The precision of each method is represented by the ratio of the standard error of each regression line to the mean of the protein concentration series. The coefficient of variation of a regression line is the percentage of this value, and these are shown in Table 8.

Because γ -casein was the smallest amount of protein dissolved in a small amount of solvent used in this experiment for making the stock solution, the errors of weighing and diluting gave γ -casein the highest value in the table, or the lowest precision. The mean coefficient of variation of all the regression lines in PO_4 and SDS solvents for the 215-225 nm method is higher than the mean at 280 nm. However, in 0.9% NaCl solvent, the mean coefficient of variation at 215-225 nm method is lower than the mean at 280 nm method. Thus, it appears that the precision depends mostly upon random errors from weighing, diluting samples and laboratory techniques. Neither method was clearly more precise than the other. Except for γ -casein most values in Table 8 are lower than 5%.

Accuracy

Accuracy is related to the degree to which a measurement agrees with the "true" value; the closer the agreement with the true value, the higher the accuracy. Since the regression line of each protein in each solvent represents the protein concentrations estimated by the absorbance at 215-225 nm and 280 nm, one estimate of the true value is at the intercept where protein concentration should equal zero when the absorbance is zero. The accuracy of the experiment is measured by A,

Table 8. Coefficient of Variation^a of Each Regression Line (%)

Protein \ Method	Solvent	NaCl		PO ₄		SDS	
		215-225	280	215-225	280	215-225	280
α -casein		2.48	11.68	4.33	2.27	3.73	1.21
β -casein		3.03	3.95	1.98	1.30	2.10	0.86
γ -casein		18.60	22.44	6.67	6.26	7.25	6.60
k-casein		2.53	2.05	1.90	1.22	1.80	0.97
α -lactalbumin		3.38	3.93	3.63	2.48	3.43	1.97
β -lactoglobulin		0.88	8.04	2.55	0.72	4.33	1.68
Bovine serum albumin		1.18	0.74	4.10	0.49	1.85	0.65
Mean		4.58	7.55	3.59	2.11	3.50	1.99

$$^a \text{ C.V. \%} = \frac{S_{yx}}{\bar{Y}} \times 100 = \text{Precision}$$

$$S_{yx} = \sqrt{\frac{\sum Y^2 - (\sum xy)^2 / \sum x^2}{n-2}}$$

which is the absolute value of the intercept value "a" in the regression equation divided by the mean of the protein concentration series, as shown in Figure 2, page 31. Table 9 lists the A values calculated from each regression line.

The results show that γ -casein in NaCl solvent at 280 nm, and α -lactalbumin in NaCl and SDS solvent at 215-225 nm had the largest differences between the estimated protein concentration and the "true" value. The intercept of other regression lines were around 5% of the mean protein concentration. β -lactoglobulin had the highest accuracy among all treatments.

IV. VARIANCE OF REGRESSION LINES

The aim of this investigation was to test if all the seven slopes of regression lines which the milk proteins generated by the ultraviolet absorptions in each solvent by each method were the same. In other words, if such were the case, the seven regression lines in each solvent would be parallel. This condition would allow us to use one protein as a standard to represent the relationship between absorption and concentration of all the milk proteins. The covariance analysis of the seven regression coefficients in each solvent is summarized in Tables 10, 11, and 12 for the 215-225 nm method and in Tables 13, 14 and 15 for the 280 nm method.

For all the data the test of hypothesis is that the slopes of the seven regression lines are the same, $b_1 = b_2 = b_3 = b_4 = b_5 = b_6 =$

Table 9. "A" Value^a of Each Regression Line.

Protein \ Method	Solvent		NaCl		PO ₄		SDS	
	215-225	280	215-225	280	215-225	280	215-225	280
α-casein	6.28	2.14	4.50	3.10	3.52	4.79		
β-casein	6.60	3.94	4.35	3.85	6.63	5.36		
γ-casein	9.58	17.69	5.98	0.55	4.05	8.47		
k-casein	8.83	5.01	5.83	4.25	7.90	4.99		
α-lactalbumin	17.00	0.36	8.23	4.04	14.23	4.22		
β-lactoglobulin	6.95	0.58	0.40	3.74	0.05	4.26		
Bovine serum albumin	6.35	3.56	3.78	4.16	5.95	3.93		

$$^aA\% = (|a|/\bar{Y}) \times 100.$$

a: intercept as on Figure 2, page 31.

$\bar{Y}$: Mean protein concentration.

Table 10. Covariance Analysis of Regression Lines in 0.9% NaCl Solvent at 215-225 nm.

	d.f.	$\sum x^2$	$\sum xy$	$\sum y^2$	b	d.f.	Deviation from Regression		
							$\sum d_{yx}^2$	M.S.	F.
α -casein	9	46359	9610	2000	20.7	8	7.89		
β -casein	9	119908	15440	2000	12.9	8	11.86		
γ -casein	9	25252	6270	2000	24.8	8	443.19		
k-casein	9	93818	13670	2000	14.6	8	8.71		
δ -lactalbumin	9	22140	6630	2000	29.9	8	14.61		
β -lactoglobulin	9	49228	9920	2000	20.2	8	1.02		
Bovin serum albumin	9	65954	11480	2000	17.2	8	1.80		
Within						56	489.08	8.73	
Regression Coefficient						6	895.74	149.29	17.1**
Common-	63	422659	73020	1400	17.3	62	1384.82	22.34	

Table 11. Covariance Analysis of Regression Lines in Phosphate Solvent at 215-225 nm.

Deviation from Regression									
	d.f.	$\sum x^2$	$\sum xy$	$\sum y^2$	b	d.f.	$\sum d_{yx}^2$	M.S.	F
α -casein	9	47616	9700	2000	20.4	8	23.97		
β -casein	9	123714	15710	2000	12.7	8	5.05		
γ -casein	9	31720	7850	2000	24.7	8	57.31		
k-casein	9	91204	13490	2000	14.8	8	4.69		
α -lactalbumin	9	23044	6760	2000	29.3	8	16.98		
β -lactoglobulin	9	56738	10630	2000	18.7	8	8.46		
Bovine serum albumin	9	68837	11670	2000	17.0	8	21.58		
Within						56	138.04	2.47	
Regression Coefficient						6	884.98	147.50	59.72**
Common	63	442873	75810	15000	17.1	62	1023.02	16.50	

Table 12. Covariance Analysis of Regression Lines in SDS Solvent at 215-225 nm.

	d.f.	$\sum x^2$	$\sum xy$	$\sum y^2$	b	d.f.	Deviation from Regression		
							$\sum d_{yx}^2$	M.S.	F
α -casein	9	43731	9310	2000	21.3	8	17.95		
β -casein	9	115393	15170	2000	13.1	8	5.69		
γ -casein	9	17710	5850	2000	33.0	8	67.62		
k-casein	9	81580	12760	2000	15.5	8	4.20		
α -lactalbumin	9	22078	6620	2000	30.0	8	15.06		
β -lactoglobulin	9	48157	9750	2000	20.2	8	23.98		
Bovine serum albumin	9	77052	12400	2000	16.1	8	4.48		
Within						56	138.98	2.48	
Regression Coefficient						6	1132.78	188.80	76.13**
Common	63	405701	71860	14000	17.7	62	1271.76	20.51	

Table 13. Covariance Analysis of Regression Lines in 0.9% NaCl Solvent at 280 nm.

	d.f.	$\sum x^2$	$\sum xy$	$\sum y^2$	b	d.f.	Deviation from Regression		
							$\sum d_{yx}^2$	M.S.	F
α -casein	9	471983	299600	200000	63.5	8	9823		
β -casein	9	172838	370800	800000	214.5	8	4500		
γ -casein	9	96344	251200	800000	260.7	8	145043		
k-casein	9	557960	667600	800000	119.7	8	1216		
α -lactalbumin	9	782104	394400	200000	50.4	8	1112		
β -lactoglobulin	9	1104543	929000	800000	84.1	8	18644		
Bovine serum albumin	9	335470	51800	800000	154.4	8	156		
Within						56	180494	3223	
Reg. Coef.						6	877215	146203	45.36**
Common	63	3521242	3430600	4400000	97.4	62	1057709	17060	

Table 14. Covariance Analysis of Regression Lines in Phosphate Solvent at 280 nm.

	d.f.	$\sum x^2$	$\sum xy$	$\sum y^2$	b	d.f.	Deviation from Regression		
							$\sum d_{yx}^2$	M.S.	F
α -casein	9	564188	335600	200000	59.5	8	373		
β -casein	9	182516	382000	800000	209.3	8	487		
γ -casein	9	139249	331400	800000	238.0	8	11295		
k-casein	9	587532	685400	800000	116.7	8	429		
α -lactalbumin	9	780270	394600	200000	50.6	8	442		
β -lactoglobulin	9	695400	745800	800000	107.2	8	148		
Bovine serum albumin	9	344298	524800	800000	152.4	8	68		
Within						56	13242	236	
Reg. Coef.						6	877590	146265	620**
Common	63	3293453	3399600	4400000	103.2	62	890832	14368	

Table 15. Covariance Analysis of Regression Lines in SDS Solvent at 280 nm.

	d.f.	$\sum x^2$	$\sum xy$	$\sum y^2$	b	Deviation from Regression		
						d.f.	$\sum d_{yx}^2$	M.S.
F								
α -casein	9	775058	388500	200000	51.5	8	105	
β -casein	9	186680	386400	800000	207.0	8	211	
γ -casein	9	52848	204000	800000	386.0	8	12540	
k-casein	9	616563	702200	800000	113.9	8	268	
α -lactalbumin	9	746800	386200	200000	51.7	8	281	
β -lactoglobulin	9	677070	735600	800000	108.6	8	810	
Bovine serum albumin	9	319840	505800	800000	158.1	8	120	
Within						56	14335	256
Reg. Coef.						6	1141827	190305
Common	63	3374859	3308700	4440000	98.0	62	1156162	118648

b7. This test would be made by

$$F = \frac{\text{Mean Square for Regression Coefficients}}{\text{Mean Square Within Samples}} \quad (11)$$

$$\text{d.f.} = 6, 56$$

The F values of the three solvents at 215-225 nm method are 17.1, 59.72 and 76.13, which are very significant compared to the $F = 3.12$ at the 0.01 level of probability from the F value distribution table. The results in Tables 13, 14 and 15 at the 280 nm indicate the same conclusion.

It appears from these results that the regression lines of the seven proteins in each solvent are not parallel since the difference in their slopes are very significant for either the 215-225 nm method or the 280 nm method. This says that neither the 280 nm method, based on the amount of aromatic amino acids, nor the 215-225 nm method, based on the total protein peptide bonds, could be used with a single standard to estimate the other proteins.

From the literature, one would expect certain relationships between ultraviolet absorbances and the amino acid content of the different protein polypeptides. If there is a higher ratio of peptide bonds per unit weight of protein, then we would expect a higher slope function for that protein at 215-225 nm. In the same way, if there is a higher content of aromatic amino acid residues in the protein polypeptide, we would expect a higher slope function at 280 nm. The order of the "concentration" of peptide bonds in seven milk proteins and the order

of their regression coefficients in NaCl solvent by the 215-225 nm method from high to low are listed in Table 16. The order of the "concentration" of aromatic amino acids and the regression coefficients in NaCl solvent at 280 nm are listed in Table 17. The sequences in these two tables show that the protein which had more protein peptide bonds, calculated from Table 4, page 25 does not necessarily have a higher regression coefficient at 215-225 nm method. The protein which contains more aromatic amino acids as shown in Table 17, calculated from Table 4, page 25 does not follow higher regression coefficients at 280 nm either.

The same unpredictability was shown in the other two solvents. These results are not easily explained. Evidently, internal shielding or other factors related to primary and secondary structure were operating. The SDS solvent did not reduce structural influences enough to produce the hoped for result.

V. THE INTERACTION BETWEEN SOLVENTS AND PROTEINS

Both solvents and proteins influenced the ultraviolet absorptions in each method. The interaction, which means that the absorption of one protein is very strong in one solvent but weaker in another solvent, can be detected by the factorial test (34). Table 18 shows that for the 215-225 nm method, there is no interaction effect between the protein and solvent. The differences of the estimated milk protein concentration among different proteins are very significant, but the estimated

Table 16. Correspondence Between The Content of Peptide Bonds in Seven Milk Proteins and The Regression Coefficients at 215-225 nm.

Proteins	Peptide Bonds		Regression Coefficient	
	Ratio Value	Sequence	Ratio Value	Sequence
α -lactalbumin	1.25	1	29.9	1
β -casein	1.17	2	12.9	7
γ -casein	1.16	3	24.8	2
α -casein	1.15	4	20.7	3
Bovine serum albumin	1.14	5	17.4	5
β -lactoglobulin	1.13	6	20.2	4
k-casein	1.00	7	14.6	6

Table 17. Correspondence Between The Content of Aromatic Amino Acids in Seven Milk Proteins and The Regression Coefficients at 280 nm.

Proteins	Aromatic Amino Acid		Regression Coefficient	
	Ratio Value	Sequence	Ratio Value	Sequence
α -lactalbumin	1.63	1	50.4	7
α^i -casein	1.49	2	63.5	6
Bovine serum albumin	1.28	3	154.4	3
k-casein	1.25	4	119.7	4
γ -casein	1.12	5	260.7	1
β -casein	1.05	6	214.5	2
β -lactoglobulin	1.00	7	84.1	5

Table 18. Factorial Test Between Proteins and Solvents With The 215-225 nm Method.

Variance	d.f.	SS	MS	F
Treatment	20	(191,086)	9,554	
Protein	6	180,957	30,160	53.19**
Solvent	2	2,412	1,206	2.13
Pro. x Sol.	12	7,717	643	1.13
Error	21	11,916	567	
Total	41	203,002		

concentrations of the same milk protein among different solvents are not significantly different even at the 5% level of probability. In Table 19, it is indicated that there is a slightly significant interaction effect between the protein and the solvent. The difference of estimated protein concentrations among different solvents, in Table 19, is not significant even at 5% level of probability, but the difference among the estimated protein concentrations from different proteins is very significant. Both results from the 215-225 nm and 280 nm methods lead to the conclusion that the regression lines estimated from different proteins have different slopes. Hence, this experiment indicated that we could not use one standard regression line to estimate the amounts of other proteins in solution by either method. The suggestion that the ultraviolet absorption at 215-225 nm is solely and simply related to the number of peptide bonds and that at 280 nm to the number of aromatic amino acids was also not borne out in this work. The relationship between ultraviolet absorption and the composition of protein polypeptides is evidently not a simple one.

To measure different milk proteins by ultraviolet spectrophotometry, one would still need to determine the adsorptivities of each protein. The errors involved with attempting to use the 215-225 nm method were smaller than those at 280 nm, but still were too large for satisfactory analytical results.

Table 19. Factorial Test Between Proteins and Solvents With The 280 nm Method.

Variance	d.f.	SS	MS	F
Treatment	20	(35,240,340)		
Protein	6	32,826,022	5,471,004	111.40**
Solvent	2	309,837	154,919	3.15
Pro. x Sol.	12	2,104,481	175,373	3.57**
Error	21	1,031,378	49,113	
Total	41	36,271,718		

$$F_{2,21}^{0.05} = 3.47$$

$$F_{2,21}^{0.01} = 5.78$$

$$F_{6,21}^{0.01} = 3.81$$

$$F_{12,21}^{0.05} = 2.25$$

$$F_{12,21}^{0.01} = 3.17$$

CHAPTER V

SUMMARY

Seven purified milk proteins were dried to constant weight under vacuum, dissolved, and diluted to five concentration levels in 0.9% NaCl, phosphate buffer, or sodium dodecyl sulfate buffer. These three solvents are among those commonly used in column chromatography for separating milk proteins. The concentration series of seven proteins in three solvents were analyzed spectrophotometrically in duplicate by two methods: the difference in absorbance at 215 and 225 nm and the absorbance at 280 nm. The results established a population of 42 regression lines. The sensitivity, precision, accuracy of protein concentration measurements, analysis of variation among regression coefficients, and the interaction between proteins and solvents at 215-225 nm and 280 nm were compared and tested.

Based on the results of this study, the following conclusions were reached.

1. The sensitivities of measurements at 215-225 nm were from two to sixteen times higher than 280 nm measurements among the different proteins and solvents.
2. Precision was about 5%, with no advantage to either method or any particular solvent.
3. Except for α -lactalbumin measurements in NaCl and SDS solvents at 215-225 nm and β -casein measurements in NaCl solvent at

280 nm, the accuracy errors were around 5%.

4. If seven proteins were measured by one standard, the error for measurements at 215-225 nm would be smaller than for measurements at 280 nm, but the slope variations among the proteins were too large to make either method feasible with one standard protein.

5. At 215-225 nm, different solvents didn't influence the measurements. But there was a slightly significant difference among different solvents at 280 nm.

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