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An ultraviolet spectrophotometric method to determine milk protein content in alkaline medium

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An ultraviolet (UV) spectrophotometric method to estimate the total milk protein content is proposed. The method is based on the capacity of strong alkaline solutions to change the spectrum of the amino acid tyrosine to higher wavelength values in the UV region. In the range between 248 and 256 nm the absorbance is a linear function of the wavelength and the slope coefficient is directly proportional to the protein concentration. Thus, the measurements of absorbance at two wavelengths were used to estimate the protein content. For samples where the ratio of lipid to protein was ≤0.05, the following general expression can be used:

$$[\text{Protein}]\ (\text{g litre}^{-1}) = (A_{248} - A_{256})/(\mathring{A}_{248} - \mathring{A}_{256})$$

where A_{248} and A_{256} are the absorbance values of the sample at 248 and 256 nm and $\mathring{A}_{248}$ and $\mathring{A}_{256}$ are the values for the standard protein at the same wavelengths. Due to the interference of the fat globules, when the ratio lipid/protein is ≥ 0.05, it is necessary to introduce a correction coefficient, as follows:

$$[\text{Protein}] = \{(A_{248} - A_{256})/(\mathring{A}_{248} - \mathring{A}_{256})\} \cdot \{1/[1 + a \cdot \exp(b(A_{256}/A_{248}))]\}$$

where $a = 1.76 \times 10^5$ and $b = 12.29$

INTRODUCTION

Spectrophotometric methods to determine protein contents using ultraviolet (UV) spectra are based on the capacity of the aromatic amino acids to absorb light in this range. One method that uses a measurement of absorbance at 280 nm has a good reproducibility, high sensitivity (0.01 to 1 g litre^{-1}), and is not influenced by temperature variation. However, the presence of aromatic compounds, which absorb light or radiation, and of dispersed particles (fat globules, casein, insoluble salts, etc.) which cause dispersion of the radiation, overestimates protein contents and the samples should be purified (Nakai *et al.*, 1964; Nakai & Chile 1970).

At present, a large variety of spectrophotometric protein estimation methods exists (Whitaker & Granun, 1980; Wolf, 1983; Iersel *et al.*, 1985), in which other wavelengths of the UV spectrum are used. Kalb and Bernlohr (1977) estimated the concentration of proteins in cellular extracts and proposed the measurement of absorbance at 230 and 260 nm, after pH adjustment (6.85–7.00) and purification of the medium. They suggested the following expression:

$$[\text{Protein}](\text{mg litre}^{-1}) = 183A_{230} - 75.8A_{260}$$

where A_{230} and A_{260} are the absorbance values of the sample at 230 and 260nm. Thus, the interference due to nucleic acids is corrected by measurements of absorbance at 260 nm.

In a study of the reaction between sodium hydroxide solutions and the deposits (mainly proteins) of the heat exchange surfaces in milk pasteurizers, Kuaye (1988) showed the differences in absorbance spectra of reaction products due to the alkaline medium.

In this paper, the influence of pH upon the UV spectra of the milk proteins, and a factor responsible for this variation, are shown. A new UV spectrophotometric assay is developed to estimate milk protein contents in alkaline solutions.

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MATERIAL AND METHODS

Standard samples of proteins

The standard stock solutions of proteins (1 g litre^{-1}) were prepared with: β-lactoglobulin (Sigma L0130), casein (Merck 2242), α-bovine lactalbumin (Sigma 6010), bovine albumin (Sigma A2153). The solutions were made with sodium hydroxide (NaOH, 0.25M) and sodium phosphate buffer ($Na_2HPO_4.12H_2O$ 0.06M + $NaH_2PO_4.2H_2O$ 0.05M) as solvents. The concentrations of the standard protein solutions were confirmed by measuring the absorbances at 280 nm in buffer solutions (pH 7.0) and comparing with theoretical values (Sober, 1970; Iersel *et al.*, 1985).

Readings of absorbance were made in the range 230–310nm, after filtering (0.22 μm Millipore) to remove suspended particles and diluting the protein solutions (1 : 4 v/v).

Standard protein samples with calcium salts

To β-lactoglobulin alkaline solutions (0.23 g litre^{-1}) were added variable quantities of calcium salts ($Ca_3(PO_4)_2$). During absorbance readings, the samples were stirred by a magnetic system to avoid deposition of insoluble salts.

Milk samples and protein determination

Sterilized commercial milk samples (skimmed and whole milk) were filtered (Whatman GF/C) after samples had been removed for the determination of protein and fat by reference methods (Kjeldahl (FIL-IDF 20, 1962) and Rose-Gottlieb (FIL-IDF 1A, 1969) respectively). Samples with different fat and protein ratios (*L/P*) were obtained by mixing whole and skimmed milk. The absorbance readings were taken for diluted filtrated solutions (1:500; NaOH 0.25 M). The protein contents in the UV 280 nm method were determined by measuring the absorbance in diluted samples and the values were reported to specific coefficient ($A_{280}^{0.1\%}$) obtained with the standard solutions.

Absorbance measurements

Absorbance measurements and spectrum scans were made using a ultraviolet Kontron spectrophotometer, Uvikon-860 model, using cuvettes with a 1.0-cm light path.

RESULTS AND DISCUSSION

Influence of the pH in the protein spectra

Figure 1 shows a strong pH influence in the spectra of the standard proteins in the UV range. By raising the pH from neutral to alkaline the absorbance spectra were displaced, the absorbance values were higher and the 280 nm absorbance peaks were displaced to around

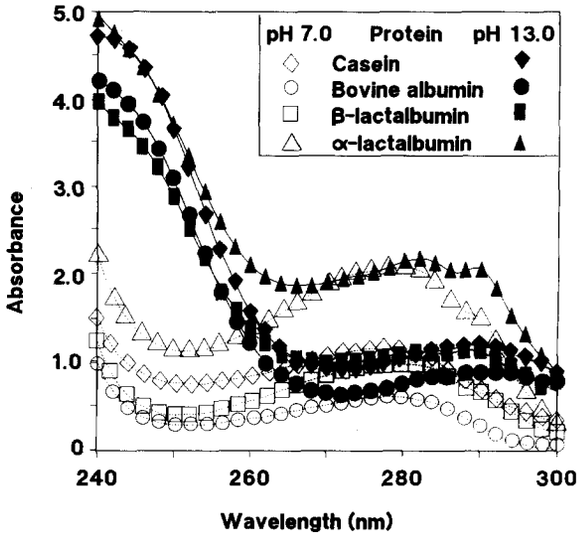

Fig. 1. Absorption spectra of standard protein solutions (1g litre^{-1}) at pH 7·0 (·····) and 13·0 (——).

290 nm. More changes in the spectra took place in wavelengths lower than 280 nm for which the absorbance was higher. This phenomenon was due principally to the change in the spectrum of the amino acid tyrosine. In fact, this variation is due to the ionization of the phenol group of tyrosine at pH values over 12 (Wetlaufer, 1962).

Expression for proteins estimation in an alkaline medium

The protein standard spectra curves obtained with alkaline solutions revealed a straight line correlation between the absorbance values and the protein concentration in all UV spectrum regions (230–310 nm). Specially, between 248 and 256 nm, as shown in Fig. 2, the absorbance is a linear function of the wavelength, and the slope coefficient is directly proportional to the protein concentration. The results led the author to establish the following general expression:

$$[\text{Proteins}]\ (\text{g litre}^{-1}) = (A_{248} - A_{256})/(\mathring{A}_{248} - \mathring{A}_{256}) \quad (1)$$

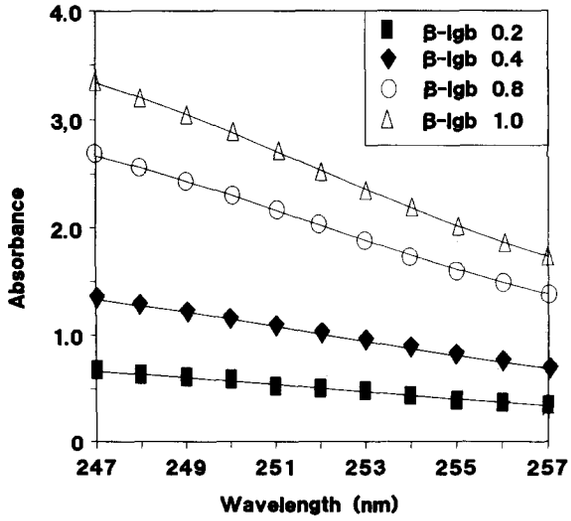

Fig. 2. Absorption spectra of β-lactoglobulin solutions (g litre^{-1}) at pH 13·0.

Table 1. Absorbance measurements[a] ($A^{0.1\%}$) for standard milk protein solutions (g litre^{-1}), pH 13

Protein	Wavelength (nm)			
	280	248 (A_1)	256 (A_2)	A_1–A_2
Casein	0·98	3·96	2·15	1·81
Bovine albumin	0·76	3·42	1·82	1·60
β-lactoglobulin	1·13	3·21	1·87	1·34
α-lactalbumin	2·24	4·18	2·68	1·50

[a] Means of duplicate analysis.

Table 2. Absorbance measurements[a] ($A^{0.1\%}$) for individual and total milk protein at pH 13

Protein		Wavelength		Ratio
	(%)	280	A_{248}–A_{256}	A_{248}/A_{256}
Casein	79·5	0·779	1·440	0·542
Bovine albumin	5·1	0·039	0·082	0·531
β-lactoglobulin	11·1	0·125	0·148	0·583
α-lactalbumin	4·3	0·096	0·065	0·639
Total	100·0	1·039	1·736	0·550

[a] Means of duplicate analysis.

where A_{248}, and A_{256} are the absorbance values of samples, measured at 248 and 256 nm and $\mathring{A}_{248}$ and $\mathring{A}_{256}$ are the absorbance values of standard samples at 1 g litre^{-1}, at these same wavelengths. Table 1 shows that the absorbance values for the standard solutions of milk proteins were higher at wavelengths lower than 280 nm, and thus the differential method (eqn 1) would be more sensitive than the method using measurements of absorbance at 280 nm.

Insoluble salts interference

The presence of insoluble calcium salts ($Ca_3(PO_4)_2$) in alkaline milk protein solutions, for a ratio between the salt contents and proteins up 0.25, causes an increase of the 280 nm absorbance values with increasing salt concentration. At the highest level of calcium salt used, the overestimation was about 33%. However, a negligible overestimation of about 2% upon the difference absorbance values (A_{248}–A_{256}) of β-lactoglobulin solutions was observed.

Lipids interference

Initially, the milk absorbance values were calculated using the absorbance values determined experimentally on the individual proteins (Table 1) and the mean composition of proteins in milk (Dalgleish, 1982) (Table 2). For this estimation only the four main protein groups were considered (caseins, β-lactoglobulin, α-lactalbumin and bovine albumin), which represent more than 90% of the total. The fraction corresponding to other proteins was assimilated by bovine albumin. The absorbances ($A_{280}{}^{0.1\%}$=1.039; $\mathring{A}_{248}$–$\mathring{A}_{256}$=1.736) of the total milk protein solution were used to calculate the amount of protein in the milk samples.

In Table 3, the interference of fat globules upon the protein spectrophotometric methods can be seen. When comparing the values of protein contents obtained by absorbance measures at 280 nm (P^*) to those by the Kjeldahl method (P), high overestimation factors ($F = P^*/P$) are observed, even for samples in which the ratio between the lipid contents (L) and protein content (P) is low. For example, in the skimmed milk (L/P = 0.05), the amount of protein is overestimated by about 52%. Thus, the result from this method in samples having fat globules, as shown by Guillou *et al.* (1976), would not be accurate, and it is necessary to use certain additional treatments to determine proteins in the presence of fat globules. Even if the differential method (P^{**}) results in smaller overestimation factors ($F = P^{**}/P$) of the protein contents, its utilization can be only accepted if the ratio (L/P) is less than 0.05 (overestimation = 3%).

Correction factor to eliminate the scattering interference

The assays with standard proteins show that the ratio $\phi = A_2/A_1$ ($A_1 > A_2$), in the range 248–256 nm, was constant for each individual protein independent of the concentration. Besides, as shown in Table 2, this ratio is nearly constant for the four different proteins at $A^{0.1\%}$.

Table 3. Determination of protein concentration[a] (g litre^{-1}) in milk samples by three methods and compared to the Kjeldahl method

Milk samples	P[b]	L[c]	L/P[d]	P^*[e]	$F(P^*/P)$[f]	P^{**}[g]	$F(P^{**}/P)$[f]
Skimmilk A	31·1	1·5	0·05	47·2	1·52	32·0	1·03
Skimmilk B	31·3	29·6	0·94	207·9	6·64	41·2	1·32
Whole A	31·0	69·5	2·24	438·4	14·14	53·3	1·72
Whole B	31·1	71·3	2·29	421·6	13·56	51·3	1·65

[a] Means of duplicate analysis.
[b] P: Protein—Kjeldahl method.
[c] L: Lipid—Rose-Gottlieb method.
[d] L/P: Ratio (lipid/protein).
[e] P^*: Protein—Ultraviolet method (280 nm).
[f] $F = P^*/P$ or $F = P^{**}/P$: Overestimation factors related to the Kjeldahl method.
[g] P^{**}: Protein—differential method.

Table 4. Determination of protein concentration[a] (mg litre^{-1}) in mixed milk samples and compared to the Kjeldahl method

Samples	P^b	L^c	L/P^d	ϕ^e	$(A_{248}-A_{256})^f$	P^{**g}	F^h
1	62·2	3·0	0·05	0·604	0·111	64·0	1·03
2	62·2	8·4	0·14	0·673	0·116	66·8	1·07
3	62·4	13·6	0·22	0·720	0·122	70·4	1·13
4	62·4	16·2	0·26	0·740	0·125	72·0	1·15
5	62·4	19·0	0·30	0·754	0·128	73·8	1·18
6	62·6	24·2	0·39	0·781	0·133	76·6	1·22
7	62·2	29·6	0·48	0·794	0·139	80·2	1·29
8	62·0	36·2	0·58	0·804	0·147	84·8	1·37
9	62·0	43·0	0·69	0·819	0·153	88·2	1·42
10	62·0	56·2	0·91	0·841	0·166	95·6	1·54
11	62·0	69·6	1·12	0·859	0·185	106·6	1·72

[a] Means of duplicate analysis.
[b] P: Protein—Kjeldahl method.
[c] L: Lipid—Rose-Gottlieb method.
[d] L/P: Ratio (lipid/protein).
[e] ϕ: Ratio (A_{256}/A_{248}).
[f] $(A_{248} - A_{256})$: Absorbance difference.
[g] P^{**}: Protein—differential method.
[h] $F = P^{**}/P$: Overestimation factor related to the Kjeldahl method.

Table 4 presents the results of protein (P) and lipid (L) estimations by the reference methods, proteins $(P)^{**}$ by the differential method, the ratios ϕ and the overestimated factors F. For these samples of milk mixtures in which the lipid concentration varies in relation to a constant concentration of protein, the results show the direct influence of the lipid content over the factors F. In whole milk (sample 11), the overestimation reaches a level of approximately 72%.

The interrelations between the variables in Table 4 were verified, using linear and exponential functions. It was verified that the overestimation factor F by the differential method was directly proportional to the concentration of lipid and to the ratio (P/L) (Table 5). The comparison between the ϕ and F factors revealed a good correlation (0.995) represented by an exponential type function. This led the author to the following equation for the estimation of proteins in presence of lipids.

$$[P]=\{(A_{248}-A_{256})/(\mathring{A}_{248}-\mathring{A}_{256})\}\cdot\{1/[1+a\cdot\exp(b(A_{256}/A_{248}))]\} \quad (2)$$

where, for milk samples, the parameters a and b were determined to be 1.76×10^5 and 12.29, respectively.

Table 5. Interrelation among variables and correlation analyses

Variables		Equation	Regression Coefficients
X	Y		
L	F	$F = 0{\cdot}988 + 0{\cdot}020\ L$	0·997
L/P	F	$F = 0{\cdot}989 + 0{\cdot}633\ (L/P)$	0·997
ϕ	F	$F = 1 + [1{\cdot}76 \times 10^5 \times \exp(12{\cdot}29\phi)]$	0·995

P: Proteins.
L: Lipids.
ϕ: Ratio (A_{256}/A_{248}).
F: Overestimation factor.

CONCLUSION

The method proposed in this research reveals a viability for the determination of proteins in sterilised (homogenised) milk with the advantage of utilisation of samples having particles in dispersion (fat globules, insoluble salts). However, its use in other proteinaceous foods should be tested individually. In such cases, some conditions of the samples to be analysed need to be verified: (i) solubility of the proteins in alkaline medium (pH≥12); (ii) absence of substances that absorb the light in the UV spectrum; (iii) previous knowledge of the proteins in the product to prepare standard solutions. Besides, the samples could be filtered to promote partial elimination of particles in dispersion and to reduce the interference of the size distribution variation of fat globules.

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