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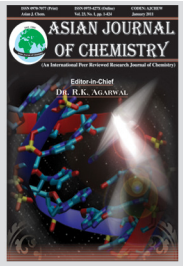

Sensitive Kinetic-Spectrophotometric Determination of Protein Based on its Inhibitive Effect on the Decolorization of Dibromo-*p*-methylsulfonazo

HUI YU, QING-ZHOU ZHAI*, WEI-HUA HU and SONG-BAI XIANG

Research Center for Nanotechnology, Changchun University of Science and Technology, Changchun 130022, P.R. China

*Corresponding author: Fax: +86 431 85383815; Tel: +86 431 85583118; E-mail: zhaiqingzhou@163.com; zhaiqingzhou@hotmail.com

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A novel kinetic spectrophotometric method for the determination of protein is developed on the basis of kinetic effect of bovine serum albumin (BSA) on the inhibitory effect on the oxidation reaction of dibromo-*p*-methylsulfonazo by potassium periodate. The absorbance differences (ΔA) between inhibitive and non-inhibitive systems are maximum at 558 nm. The absorbance difference is linearly related with the concentration of bovine serum albumin over the range of 5.0-55.0 μg/mL and fitted the equation: $\Delta A = 8.988 \times 10^{-3}$ C (C: μg/mL) + 0.0185, with a regression coefficient of 0.9960 at the wavelength. The detection limit of the method was 1.55 μg/mL. The method was used to determine protein in pure milk. The relative standard deviation was 1.58 and 1.49 % for 13 replicate determinations. The recovery of the standard addition was 101.1 and 99.33 %.

Key Words: Inhibitive kinetic spectrophotometry, Bovine serum albumin, Protein, Dibromo-*p*-methylsulfonazo, Potassium periodate, Milk.

INTRODUCTION

Protein is the substance undertaker of descendiblity information of a biological body and is one of the major contents in the studies of biochemistry. Protein is the most important substance of human body *i.e.*, without protein means without life[1]. Protein consists of more than 20 kinds of amino acids according to different amount and different order to combine and is formed. Small protein molecule consists of a few hundreds amino acids and big one consists of a few ten thousand amino acids. Protein can constitute and repair the tissues of human body, enzyme and hormone, composes antibody, adjusts the equilibrium of body fluid, transfers various substances, maintains the natural functions of nervous system and provides heat energy, *etc.*[2]. Therefore, the quantitative determination of protein is sufficiently important. Analytical methods of protein are many, such as nephelometry, Kjeldahl method, spectrophotometry, fluorophotometry, resonance scattering photometry, high performance liquid chromotography and capillary electrophoresis analytical method[3]. In recent years, photometry has been applied to the determination and study of biological macromolecules as a spectral analytical technique where operation is simple and convenient, interference factors are samll, repeatability is good[4-8]. Spectrophotometric determination is rapid, operation is simple and convenient, instrumentation is simple and price is cheap. Almost micro-amount component of all inorganic substances and many organic can be determined using this method. The spectrophotometric methods for the determination of protein include violet absorption spectrometry, metal probe method, dye probe method and dye-metal complex probe method. In the methods of determination of protein, dye combination method is one of the commonly used methods[9-11]. Dye combination method is in virtue of letdown of dye colour or variational extent to determine the content of protein[12]. The commonly used dye probes mainly have triphenyl methane kind dye, azo kind dye, porphyrin kind dye[1]. Besides the above-stated dye probes, the other probe reagents reported again have chloranil[13], fuchsine acid[14], alizarin red S[15], cyanine dye[16]. Although these methods have some characteristics, they also have their own defetcts. For instance, the selectivities of these methods was poor at large and the detection limits await to be improved. Kinetic method has the advantages of detection limit lowness, sensitivity highness, but the study of its use in the determination of protein is little seen[17].

In this study, dibromo-*p*-methylsulfonazo(3-[(2,6-dibromo- 4-methyl-phenyl)azo]-6[(2-sulfophenyl)azo]-4,5-dihydoxynaphthalene-2,7-disulfonic acid; DBMAS) is used as colour reagent. In the aryl of DBMAS with -N=N- and many ligands containing N and O, the reagent not only has strong ability of chelation and can chelate with metallic ions to form various water soluble complexes, but also DBMAS contains

-N=N- groups which themselves can produce colour. When -N=N- group is oxidized or reduced, it will be damaged. This makes the colour of solution become weak or even colourless[18]. This paper discovered that in a Clark-Lubs buffer solution medium of pH = 2.2 protein can inhibit the discolouring reaction of DBMAS oxidized by KIO_4 and a novel method for the determination of protein was developed. The sensitivity of the present method is high, the operation is simple and the analytical cost is low. It has been applied to the determination of protein in milk samples with satisfactory results.

EXPERIMENTAL

A 722S spectrophotometer (Shanghai Lingguang Technique Co. Ltd., China) and a HH4-digital thermostat water bath kettle (Jiangsu Jintan Ronghua Apparatus Manufacture Co. Ltd., China) were used for absorbance measurement and temperature control, respectively.

Bovine serum albumin (BSA, biochemical reagent) 0.5 mg/mL aqueous solution: An amount of 50 mg BSA was weighed, placed into a 100 mL calibrated flask and diluted up to the mark with water to get 0.5 mg/mL BSA working solution, kept in cold storage at 2 ºC.

Dibromo-*p*-methylsulfonazo (DBMAS, 5.0×10^{-4} mol/L) solution: 0.0390 g of DBMAS was dissolved in 100 mL of water.

KIO_4 solution (1.0×10^{-2} mol/L): 0.2300 g of KIO_4 was dissolved in 100 mL of water.

Clark-Lubs buffer solution (pH = 2.2): 46.24 mL of 0.2 mol/L KCl and 7.52 mL of 0.2 mol L^{-1} HCl were mixed and diluted to 100 mL with water.

The reagents were of analytical grade and the water was deionized water.

Procedure: Two 10 mL comparison tubes were taken. In turn were added 0.5 mL of pH 2.2 Clark-Lubs buffer solution, 0.6 mL of 5.0×10^{-4} mol/L DBMAS solution. A definite amount of bovine serum albumin solution was added to one of them (inhibitive system), while no bovine serum albumin solution was added to another one (non-inhibitive system). 2.5 mL of 1.0×10^{-2} mol /L KIO_4 solution was, respectively added and the mixture was diluted to the mark with water, shaken well. A water bath of 100 ºC was used to heat for 4.5 min and the solutions were rapidly taken out and were cooled by running water for 10 min. Using 1 cm cells, the absorbance of non-inhibitive system (A_0) and the absorbance (A) of inhibitive system were measured at 558 nm with water as reference. Then $\Delta A = A - A_0$ was calculated.

RESULTS AND DISCUSSION

Absorption spectra: DBMAS presents violet in the Clark-Lubs buffer solution. After oxidant KIO_4 was added, a decolouring reaction occurred. After BSA was added, decolouration of the system was inhibited. Curve (a) in Fig. 1 is absorption spectrum of BSA + DBMAS + KIO_4 inhibitive system; curve (b) is absorption spectrum of DBMAS + KIO_4 non-inhibitive system; curve (c) is absorbance difference ΔA of inhibitive system and non-inhibitive system. At 558 nm, ΔA was maximum and sensitivity was the largest. Thus, the present study selected 558 nm as the measurement wavelength.

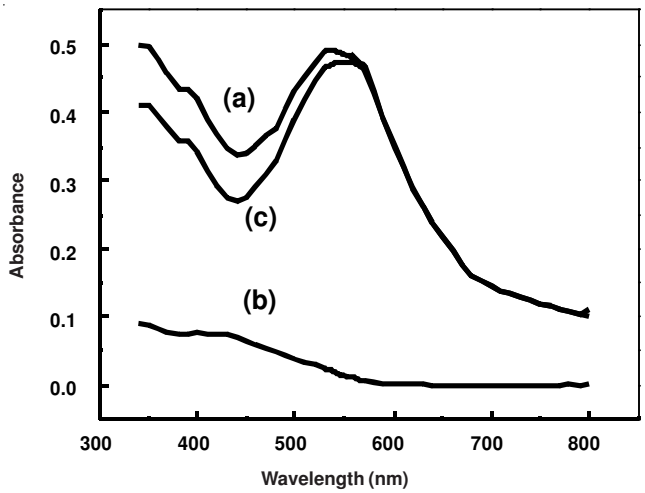

Fig. 1. Absorpion spectra: (a) BSA + DBMAS + KIO_4 (against water), inhibitive reaction; (b) DBMAS + KIO_4 (against water), non-inhibitive reaction; (c) absorbance difference of inhibitive system and non-inhibitive system, net inhibitive reaction ΔA; [BSA] = 50 μg/mL; [DBMAS] = 3.0×10^{-5} mol/L; [KIO_4] = 2.5×10^{-3} mol/L; pH = 2.2; T = 100 ºC, t = 4.5 min

Effect of acidity: Effect of different acidity conditions on the reactive system was respectively reviewed. Other experimental conditions being kept optimum, according to the procedure the buffer solutions of pH = 1.4, 1.8, 2.2, 2.4, 2.8, 3.0 were, respectively added for test. The results are shown as Fig. 2. The absorbance difference of inhibitive system and non-inhibitive system was the largest at pH = 2.2. Therefore, the present study selected the Clark-Lubs buffer solution of pH = 2.2.

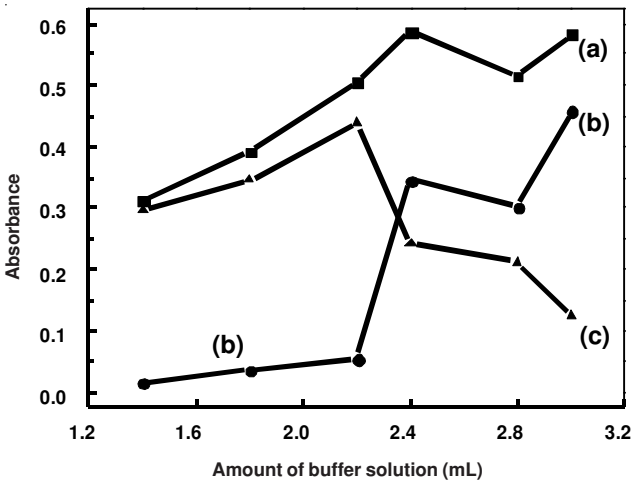

Fig. 2. Effect of acidty: (a) BSA + DBMAS + KIO_4 (against water), inhibitive reaction; (b) DBMAS + KIO_4 (against water), non-inhibitive reaction; (c) absorbance difference of inhibitive system and non-inhibitive system-net inhibitive reaction ΔA; [BSA] = 50 μg/mL; [DBMAS] = 3.0×10^{-5} mol/L; [KIO_4] = 2.5×10^{-3} mol/L; T = 100 ºC, t = 4.5 min

Effect of the amount of buffer solution: The other condition being kept unchanged, according to the procedure the pH 2.2 buffer solutions of 0.2, 0.5, 0.8, 1.0, 1.5 mL were, respectively added for test. The results are shown as Fig. 3. The absorbance difference had a maximum value at the buffer solution

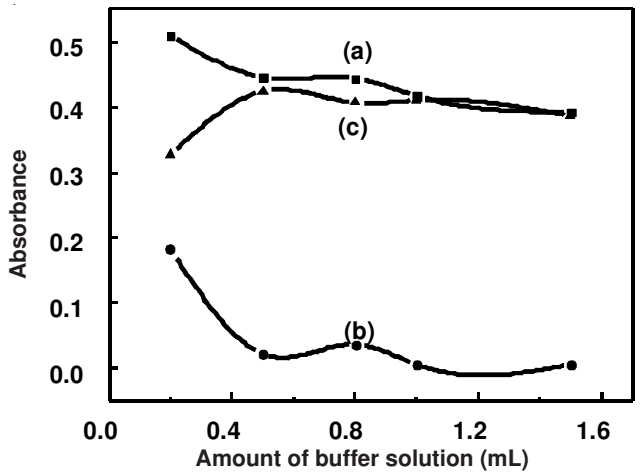

Fig. 3. Effect of the amount of buffer solution: (a) BSA + DBMAS + KIO_4 (against water), inhibitave reaction; (b) DBMAS + KIO_4 (against water), non-inhibitive reaction; (c) absorbance difference of inhibitive system and non-inhibitive system-net inhibitive reaction ΔA; [BSA] = 50 μg/mL; [DBMAS] = 3.0×10^{-5} mol/L; [KIO_4] = 2.5×10^{-3} mol/L; pH = 2.2; T = 100 ºC, t = 4.5 min

amount of 0.5 mL. Thus, the present study selected the buffer solution to be 0.5 mL.

Effect of the amount of DBMAS: The other condition being kept unchanged, according to the procedure 0, 0.2, 0.5, 0.6, 0.8, 1.0, 1.5 mL of 5.0×10^{-4} mol/L DBMAS solutions were, respectively added for test. The results are shown as Fig. 4. The absorbance difference was the largest when the amount of DBMAS solution was 0.6 mL. The present study chose the amount of DBMAS solution to be 0.6 mL. At this time, in the 10-mL system the concentration of DBMAS was 3.0×10^{-5} mol/L.

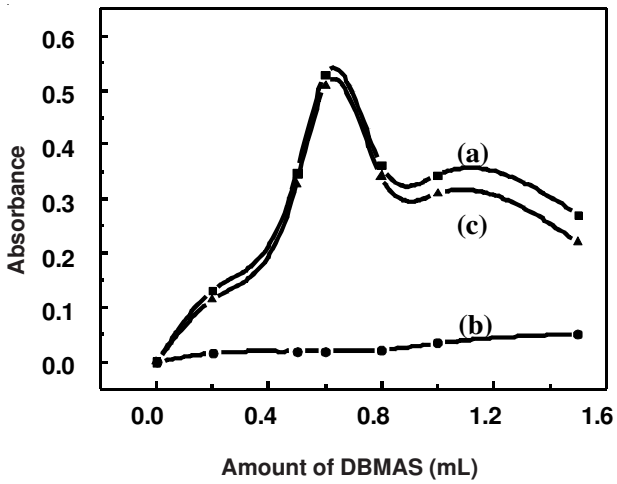

Fig. 4. Effect of the amount of DBMAS: (a) BSA + DBMAS + KIO_4 (against water), inhibitave reaction; (b) DBMAS + KIO_4 (against water), non-inhibitive reaction; (c) absorbance difference of inhibitive system and non-inhibitive system-net inhibitive reaction ΔA; [BSA] = 50 μg/mL; [KIO_4] = 2.5×10^{-3} mol/L; pH = 2.2; T = 100 ºC, t = 4.5 min

Effect of the amount of KIO_4: The other condition being kept unchanged, according to the procedure 0.2, 0.5, 1.0, 1.5, 2.0, 2.2, 2.5, 3.0 mL of 1.0×10^{-2} mol/L KIO_4 solutions were, respectively added for test. The results are shown as Fig. 5. The absorbance difference ΔA attained the largest when the amount of KIO_4 solution was 2.5 mL. The present study selected the amount of 1.0×10^{-2} mol/L KIO_4 to be 2.5 mL. At this time, in the 10-mL system the concentration of KIO_4 was 2.5×10^{-3} mol/L.

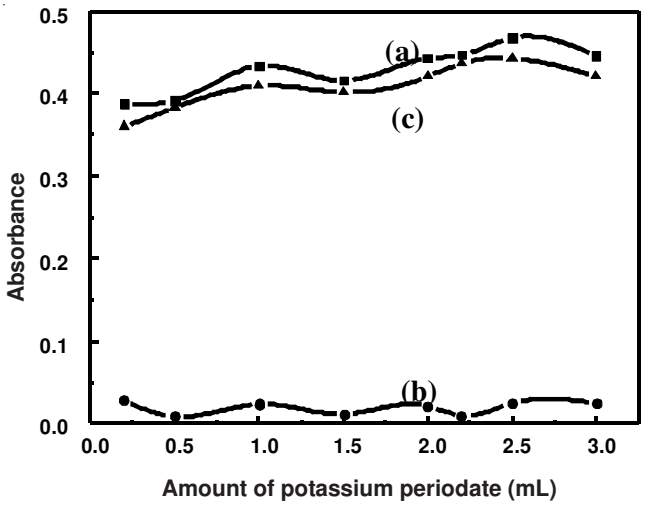

Fig. 5. Effect of the amount of potassium periodate: (a) BSA + DBMAS + KIO_4 (against water), inhibitave reaction; (b) DBMAS + KIO_4 (against water), non-inhibitive reaction; (c) absorbance difference of inhibitive system and non-inhibitive system-net inhibitive reaction ΔA; [BSA] = 50 μg/mL; [DBMAS] = 3.0×10^{-5} mol/L; pH = 2.2; T = 100 ºC, t = 4.5 min

Effect of heating temperature: The other conditions were kept optimum,a water bath of 20, 40, 50, 60, 70, 80, 90, 95, 100 ºC was, respectively used to heat and according to the procedure the effect of temperature was made using blank reagent as contrast. The experimental results are shown in Fig. 6. The experimental results showed that as the temperature of reaction increased, ΔA gradually increased. At 100 ºC, ΔA was the largest. The present method selected the water bath of 100 ºC for heating. A regression process of the data obtained between 40-100 ºC was made and a regression equation was obtained: log (A_0/A) = 1675.4 /T (K) - 5.292; with a correlation coefficient of γ = 0.9910. Based on the slope of the equation the activation energy of the inhibitive reaction was obtained to be Ea = 32.079 kJ/mol.

Effect of heating time: The other conditions were kept constant, heating was made for 0.25, 0.5, 1, 2, 3, 3.5, 4, 4.5, 5, 6 min and the effect experiments of heating time were made. The results showed that ΔA and heating time have a good linear relationship over 0.5-4.5 min and linear regression equation was: ΔA = 0.0915t (t: m) + 0.1166,with a correlation coefficient of γ = 0.9932. At 4.5 min, ΔA was a maximum. The paper chose heating time to be 4.5 min. Plotting of log A/A_0 *versus* t was made and its linear regression equation obtained was: log (A/A_0) = 0.3024 t (t: min) - 0.0090; with a correlation coefficient of γ = 0.9952. The reactive rate constant was K = 3.947×10^{-2} (s^{-1}) and the half-period was 2.573 min.

Stability of system: Under the optimum experimental conditions, for the determination of 50 μg/mL BSA, the results showed that within 1 h, ΔA was stable and the variation of ΔA was within ± 5 %.

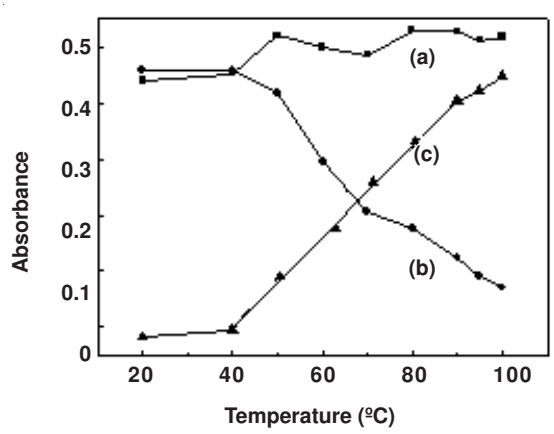

Fig. 6. Effect of temperature: (a) BSA + DBMAS + KIO_4 (against water), inhibitave reaction; (b) DBMAS + KIO_4 (against water), non-inhibitive reaction; (c) absorbance difference of inhibitive system and non-inhibitive system-net inhibitive reaction ΔA; [BSA] = 50 μg/mL; [DBMAS] = 3.0×10^{-5} mol/L; [KIO_4] = 2.5×10^{-3} mol/L; pH = 2.2; T = 100 ºC, t = 4.5 min

Selectivity of method: The effect of a series of diverse substances on the determination of 50.0 μg/mL BSA was checked. The tolerance limits (mass multiple, m/m) of the common ions tested (causing < ± 5 % relative error) are summarized as follows: Li^+, Zn^{2+} (2); Ni^{2+}, alanine (1); oxalic acid, acetic acid (0.5); Cu^{2+}, urea, malic acid, citric acid (0.2); VO_3^-, WO_4^-, Ba^{2+}, Pb^{2+}, Fe^{3+}, lysine, ascorbic acid (0.1); Mg^{2+}, Si^{4+}, (0.05); Fe^{2+}, Sr^{2+}, La^{3+}, Mo^{6+} (0.02); Sr^{2+}, Mn^{2+}, Cr^{3+}, PO_4^{3-}, Ce^{4+}, Cr^{6+} (0.01); I^-, Ca^{2+}, Al^{3+}, EDTA (0.005); Hg^{2+}, Co^{2+}, Bi^{3+}, Ti^{4+} (0.002); MnO_4^-, S^{2-} (0.001); Th^{4+} (0.0005).

Working curve: Under the optimum experimental conditions, the experiments of linear range were made. A definite amount of BSA standard solution was respectively added and blank reagent was used for contrast. The results showed that under the optimum conditions the concentration of BSA over the range of 5.0-55.0 μg/mL with ΔA presented a linear relationship and its linear regression equation was: $\Delta A = 8.988 \times 10^{-3}$ C (μg/mL) + 0.0185, with a correlation coefficient of γ = 0.9960. Thirteen parallel determinations of 50.0 μg/mL BSA were made and a relative standard deviation (RSD) = 0.50 % was found. Eleven determinations of a blank reagent were made and the standard deviation obtained was 0.460 %. The detection limit of the method 3S/K was 1.55 μg/mL (S is the standard deviation of deteminations of 11 blank reagent, K is the slope of working curve).

Discussion on reaction mechanism: Because most of the organic compounds containing azo structures have colour, azo groups are also called chromophores. In the aryl of DBMAS with -N=N- and many ligands containing N and O, the reagent not only has strong ability of chelation and can chelate with metallic ions to form various water soluble complexes. When -N=N- group is oxidized or reduced, it is damaged. This makes the colour of solution become weak or even colourless. Under acidic conditions, the amido of the side chain in BSA is protonated and for the protonated protein with positive charge an association reaction with DBMAS occurred due to electrostatic attraction. As the amido was uniformly distributed in protein, DBMAS interacted with amido to go into the protein structure, which protected the chromophore -N=N- and inhibited the process of fading. The oxidation reaction is presented in Fig. 7. The inhibitive reaction is shown in Fig. 8.

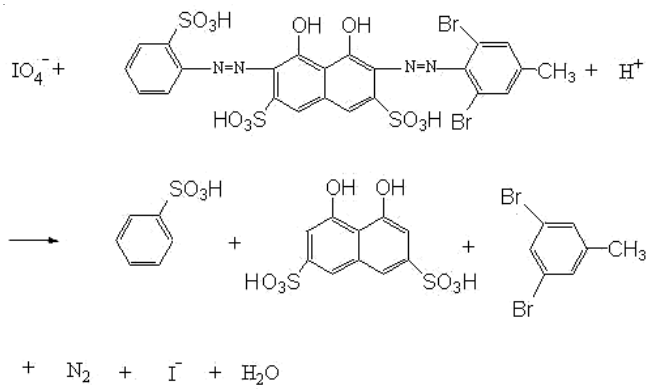

Fig. 7. Oxidation reaction

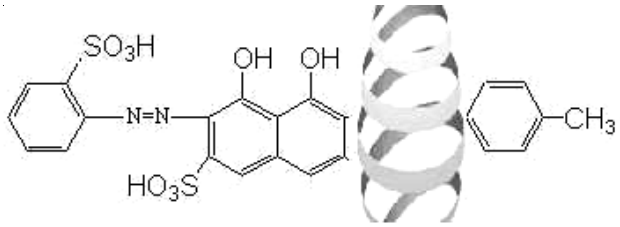

Fig. 8. Inhibition reaction

Analysis of sample: The present research system was used to determine the protein contents in two kinds of milk samples. Analytical method of the sample was as follows: 1 mL of milk was taken and placed in a 100 mL calibrated flask. Water was used to dilute to the mark and the working solution of the sample was obtained. 1 mL of the sample working solution was taken and determined according to the recommended procedure. The determined results are see in Table-1.

Conclusion

The bovine serum albumin inhibitive decolouring reaction of DBMAS oxidized by KIO_4 and its optimum experimental

TABLE-1
ANALYTICAL RESULTS OF MILK SAMPLES

Milk	Found (mg/mL)	Average (mg/mL)	RSD (%)	Added (mg/mL)	Recovered (mg/mL)	Recovery (%)	Contrast method* (mg/mL)[17]
1	35.21, 35.32, 35.54, 33.76, 35.10, 34.77, 35.21, 35.21, 35.21, 33.99, 34.21, 35.21, 34.87	34.88	1.49	0.1500	0.1490	99.33	34.86
2	37.88, 37.99, 38.88, 37.88, 37.10, 37.77, 37.32, 37.10, 38.21, 37.55, 38.77, 37.43, 38.99	37.88	1.58	0.1500	0.1520	101.1	37.89

*Contrast method was *p*-acetylchlorophosphonazo indicator kinetic spectrophotometry.

conditions were studied. Protein can protect the -N=N of DBMAS from KIO_4 oxidation. In pH 2.2 Clark-Lubs buffer medium,the maximum absorption peak of BSA-DBMAS-KIO_4 system is at 558 nm. At the wavelength, a good linear relationship was presented between the range of 5.0-55.0 μg/mL for BSA and the absorance difference. The linear regression equation of the method was: $\Delta A = 8.988 \times 10^{-3}$ C (C: μg/mL) + 0.0185, with a correlation coefficient of $\gamma = 0.9960$. The present method has been successfully applied to the determination of proteins in milks.

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