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Inhibitive Kinetic Spectrophotometric Determination of Protein Using Sulfonazo(III)-Potassium Periodate System

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Quantitative analysis of protein is often involved in biochemistry and other biological disciplines. It is also an important indicator of clinical laboratory diagnosis of diseases and checking the treatment effect, but also the test item of separation and purification of many biological drugs in food quality testing and analysis. This paper puts forward a new method of kinetic spectrophotometric determination of protein based on the principle that potassium periodate oxidizes sulfonazo(III) fading in the Clark-Lubs buffer medium at pH 2.2 and bovine serum albumin has an inhibitive effect on this reaction. The maximum absorption wavelength of system is 570 nm. The absorbance difference (ΔA) is linearly related with the concentration of bovine serum albumin over the range of 2-48 μg/mL and fitted the equation: ΔA = 0.0307 C (C: μg/mL) + 0.1781, with the correlation coefficient of 0.9930. The detection limit of the method was 1.6 μg/mL. The method has been successfully used to determine protein content in soybean milk and soybean powder samples, with the relative standard deviations of 3.32 and 1.70 % calculated from 11 determinations. Method recoveries were 102.3 and 102.5 %, respectively.

Key Words: Protein, Inhibition kinetic spectrophotometry, Sulfonazo(III), Potassium periodate.

INTRODUCTION

Proteins are essential components consisting of biological cells and tissues. Protein in food is the only source of nitrogen in human body, which can not be replaced with sugar and fat[1]. Protein has high correlation with nutrient metabolism, cell structure, enzyme, hormone, virus, immunity, material transformation, genetic factor, *etc*. Therefore, to make research on protein separation, qualitative and quantitative analysis of it is the most important work in biochemistry and other biological disciplines, food inspection, clinical examination and disease diagnosis, separation and purification of biological drugs and quality inspection[2]. At present, there are many methods for determination of protein[3-5], such as Kjeldahl method, isoelectric point precipitation method, UV absorption method, biuret method, Coomassie Brilliant Blue staining method, *etc*. Now there are many advanced automatic or semi-automatic Kjeldahl apparatuses, but they are expensive and require special reagents, which prohibits their widespread use[6]. Dye probe is one of the most commonly used probes for the analysis of protein, which can be divided into triphenylmethanes, azo dyes, azo chromotropic acids and porphyrin dyes based on their molecular structures[5]. Azo dye probes such as methyl orange, amido black 10B, zincon have been reported, but they have lower detection sensitivity[4]. Azo chromotropic acid probes like arsenazo(III), acid chrome blue K, sulfochlorophenol S and nitrosulfophenol C have been reported[4,5] and they are more sensitive than azo dyes, some of them with the colour contrast of more than 80 nm, having some practical value.

Many scholars have been doing research on new methods that are more sensitive, convenient and economical[3-5], of which spectrophotometry with simplicity, rapidity and reproducibility has attracted more attention[7-10]. Kinetic spectrophotometric method has high theory value and application value because of its high detection sensitivity and low detection limit characteristics. This paper presents a new method of inhibitive kinetic spectrophotometric determination of protein using sulfonazo(III) -potassium periodate system with sulfonazo(III) as indicator based on inhibitive kinetic spectrophotometric method. This method combines the advantages of simple operation and rapidity in spectrophotometric method with the advantage of high sensitivity in kinetic method with sulfonazo(III) as indicator, which is of great value to the determination of protein. It has been successfully used to determine protein content in soybean milk and soybean powder samples.

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, Beijing Aoboxing Biotechnology Co., Ltd., China) 0.2 mg/mL solution: an amount of 0.020 g of bovine serum albumin was placed into a 100-mL calibrated flask and diluted up to the mark with water to get 0.2 mg/mL bovine serum albumin working solution, kept in cold storage at 2 ºC. Sulfonazo(III) (AS(III), Shanghai Changke Reagent Research Institute, China) solution (5.0×10^{-4} mol/L): 0.0389 g of sulfonazo(III) was dissolved in water, which was placed into a 100 mL calibrated flask to get 5.0×10^{-4} mol/L sulfonazo(III) solution. KIO_4 solution (1.0×10^{-2} mol/L): 0.2300 g of KIO_4 was placed into a 100 mL calibrated flask to get 1.0×10^{-2} mol/L solution. pH 2.2 Clark-Lubs buffer solution was prepared. The reagents were of analytical grade and the water was deioned water.

Procedure: Take two 10 mL calibrated flasks. 0.2 mL of pH 2.2 Clark-Lubs buffer solution, 2.2 mL of 5.0×10^{-4} mol/L sulfonazo(III) chromogenic reagent, 1 mL of 0.2 mg/mL bovine serum albumin solution and 0.5 mL of 1.0×10^{-2} mol/L KIO_4 solution were subsequently added into one flask, with water to a constant volume of 10-mL. Shake the mixed solution well to serve as inhibitory system. To other flask was added the same solutions except bovine serum albumin solution, as non-inhibitory system. They were quickly put into boiling water at 100 ºC for 14 min, then removed and cooled by running water for 6 min. In 1 cm cells against water, the absorbance values of both non-inhibitory reaction (A_0) and inhibitory reaction (A) were measured at 570 nm with the spectrophotometer and $\Delta A = (A - A_0)$ was calculated.

RESULTS AND DISCUSSION

Absorption spectra: Under the optimum experimental conditions, measure the absorption spectra of the different solutions (Fig. 1). The maximum absorption wavelength of the inhibitory reaction system was 568 nm, while the non-inhibitory reaction system was 530 nm. The ΔA of their maximum absorbance difference between inhibitory and non-inhibitory systems was at 570 nm, reached the highest sensitivity, thus 570 nm was selected as the measurement wavelength.

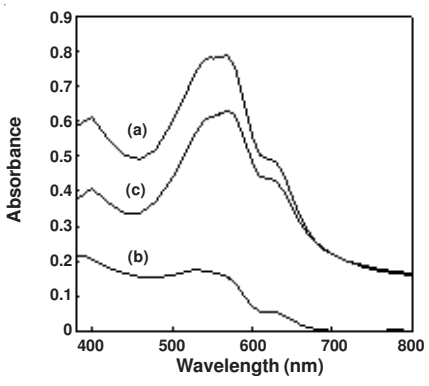

Fig. 1. Absorption spectra: (a): BSA + AS(III) + KIO_4 (against water)-inhibitory reaction A; (b): AS(III) + KIO_4 (against water)-non-inhibitory reaction A_0; (c): net inhibitory reaction-ΔA; [BSA] = 20 μg/mL; [AS(III)] = 1.1×10^{-4} mol/L; [KIO_4] = 5×10^{-4} mol/L; pH = 2.2; reaction temperature T = 100 ºC; heating time t = 14 min

Effect of acidity: The effect of different acidity on the reactions was studied, respectively. Experimental results (Fig. 2) showed that in the inhibitory system (curve a) ΔA got the largest value at pH = 2.2, while in the non-inhibitory system (curve b) became a small valley at pH = 2.2. In net inhibitory reaction (curve c) ΔA got its maximum at pH = 2.2. Thus the pH 2.2 Clark-Lubs buffer solution was selected.

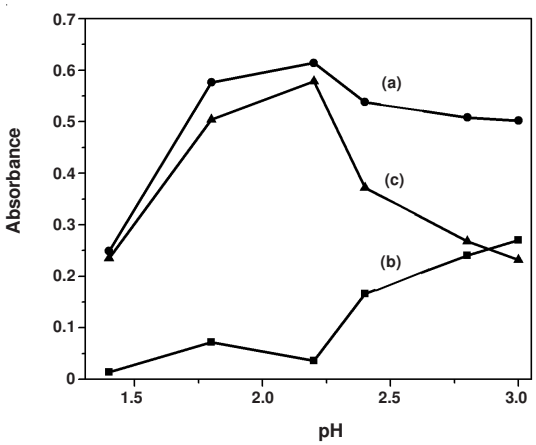

Fig. 2. Effect of pH: (a): BSA + AS(III) + KIO_4 (against water) - inhibitory reaction A; (b): AS(III) + KIO_4 (against water) - non-inhibitory reaction A_0; (c): net inhibitory reaction-ΔA; [BSA] = 20 μg/mL; [AS(III)] = 1.1×10^{-4} mol/L; [KIO_4] = 5×10^{-4} mol/L; reaction temperature T = 100 ºC; heating time t = 14 min

Under the optimum conditions, effect of the buffer solution on absorbance was measured (Fig. 3). The experimental results indicated that the value of ΔA gradually ascended with the increase in amount of the buffer solution over the range of 0.10-0.20 mL, descended within a range of 0.20-2.0 mL and reached a maximum at 0.20 mL. This work chose 0.20 mL of buffer solution with pH value of 2.2.

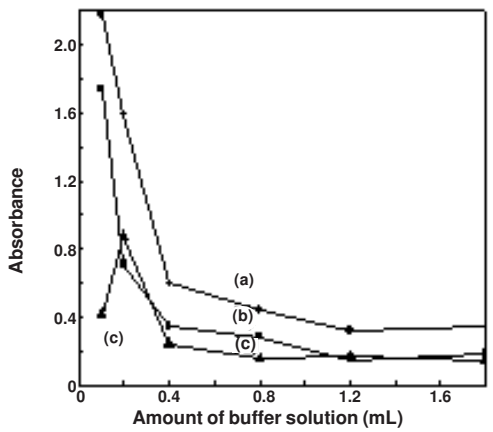

Fig. 3. Effect of the amount of buffer solution: (a): BSA + AS(III) + KIO_4 (against water)-inhibitory reaction A; (b): AS(III) + KIO_4 (against water)-non-inhibitory reaction A_0; (c): net inhibitory reaction-ΔA; [BSA] = 20 μg/mL; [AS(III)] = 1.1×10^{-4} mol/L; [KIO_4] = 5×10^{-4} mol/L; pH = 2.2; reaction temperature T = 100 ºC; heating time t = 14 min

Effect of the amount of sulfonazo(III): The experimental results (Fig. 4) on effect of the amount of sulfonazo(III) showed that with the increase in amount of sulfonazo(III) solution the

difference of absorbance of two reactions and net inhibitory sensitivity increased within a range of 0.8-2.0 mL and ΔA was the largest and constant in a range of 2.0-2.5 mL, but decreased in a range of 2.5-3.0 mL. When the amount of sulfonazo(III) solution was 2.2 mL, ΔA reached its maximum. 2.2 mL of 1.1×10^{-4} mol/L sulfonazo(III) solution was selected and the concentration of sulfonazo(III) solution in 10 mL system was 1.1×10^{-4} mol/L.

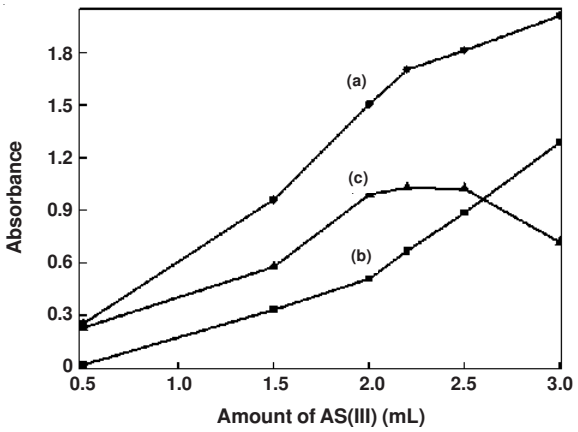

Fig. 4. Effect of the amount of AS(III): (a): BSA + AS(III) + KIO_4 (against water) - inhibitory reaction A; (b): AS(III) + KIO_4 (against water) - non-inhibitory reaction A_0; (c): net inhibitory reaction-ΔA; [BSA] = 20 μg/mL; [KIO_4] = 5×10^{-4} mol/L; pH = 2.2; reaction temperature T = 100 ºC; heating time t = 14 min

Effect of the amount of KIO_4: The experimental results (Fig. 5) on effect of the amount of KIO_4 showed that with the increase in amount of KIO_4 solution the difference of absorbance of two reactions increased within a range of 0.1-1.0 mL and decreased in a range of 1.0-3.0 mL. When the amount of KIO_4 solution was 1.0 mL, ΔA reached its maximum. 1.0 mL of 1.0×10^{-2} mol/L KIO_4 solution was selected and the concentration of KIO_4 solution in 10 mL system was 5.0×10^{-4} mol/L.

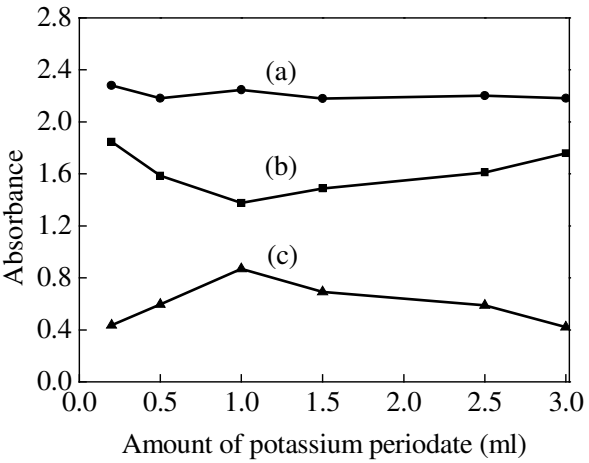

Fig. 5. Effect of the amount of KIO_4: (a): BSA + AS(III) + KIO_4 (against water) - inhibitory reaction A; (b): AS(III) + KIO_4 (against water) - non-inhibitory reaction A_0; (c): net inhibitory reaction-ΔA; [BSA] = 20 μg/mL; [AS(III)] = 1.1×10^{-4} mol/L; pH = 2.2; reaction temperature T = 100 ºC; heating time t = 14 min

Effect of temperature: Under the optimum experimental conditions, effect of temperature was carried on. The results (Fig. 6) showed that ΔA increased gradually with temperature, presented a linear increase over the range 70-100 ºC. ΔA got a maximum when the temperature reached 100 ºC. Therefore, 100 ºC was selected as optimum experimental temperature. The data measured over the range 70-100 ºC was processed by regression and the linear regression equation obtained was as follows (Fig. 7): $\log (A/A_0) = - 356.908/T(K) + 1.091$, $\gamma = 0.9939$. The apparent activation energy calculated by the slope of the equation was $E_a = 6.17$ kJ/mol.

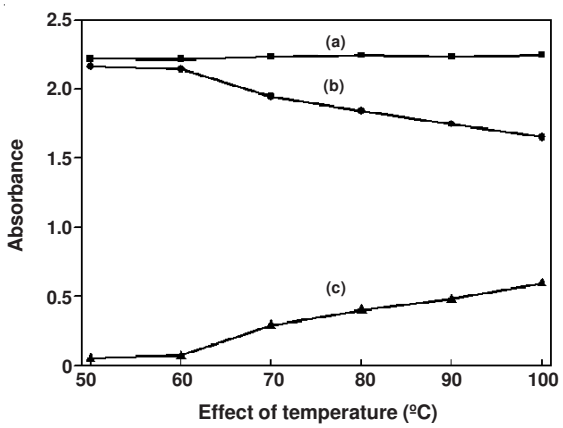

Fig. 6. Effect of heating temperature: (a): BSA + AS(III) + KIO_4 (against water) - inhibitory reaction A; (b): AS(III) + KIO_4 (against water)- non-inhibitory reaction A_0; (c): net inhibitory reaction-ΔA

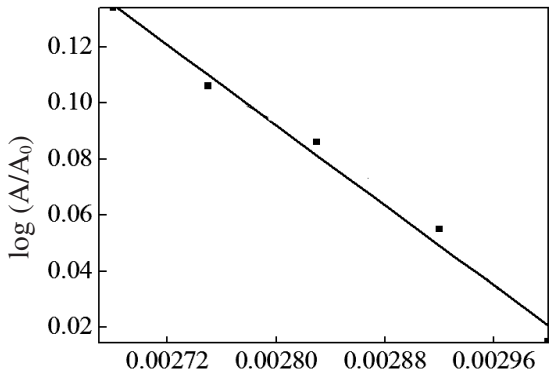

Fig. 7. Effect of heating temperature: [BSA] = 20 μg/mL; [AS(III)] = 1.1×10^{-4} mol/L; [KIO_4] = 5×10^{-4} mol/L; pH = 2.2; heating time t = 14 min

Effect of heating time: Experiments on the effect of heating time were conducted when other conditions were at optimal level. The results (Fig. 8) showed that ΔA presented a linear relation with the time over the range 2-14 min. The linear regression equation was: ΔA = 0.0781t (min) - 0.0869, $\gamma = 0.9949$. ΔA reached a maximum value at 14 min and reduced gradually over the range 14-18 min. 14 min was selected as optimum time in the experiment. Draw a graph of $\log (A/A_0)$ with t (Fig. 9). The linear regression equation was: $\log (A/A_0) = 0.0781t - 0.0869$, $\gamma = 0.9949$. The reactive rate constant calculated was $k = 7.53 \times 10^{-2}$ s^{-1} and the half-life period was $t_{1/2} = 2.11$ min.

Stability of system: For 20 μg/mL of bovine serum albumin, ΔA was determined by changing cooling time. The cooling time was 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 min, respectively. The

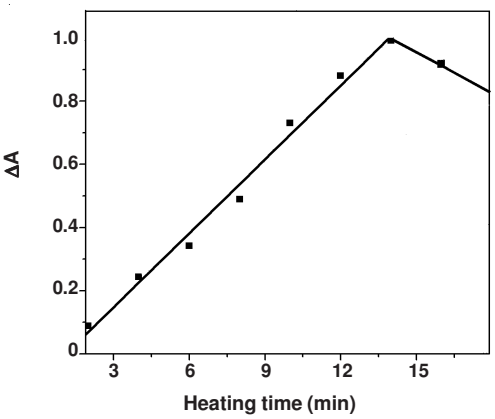

Fig. 8. Effect of heating time

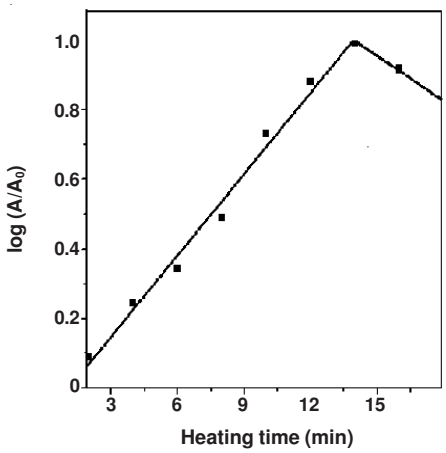

Fig. 9. Effect of heating time: [BSA] = 20 μg/mL; [AS(III)] = 1.1×10^{-4} mol/L; [KIO_4] = 5×10^{-4} mol/L; pH = 2.2; heating temperature T = 100 ºC

results showed that the value of ΔA became constant after 6 min and the change of ΔA was within 5 % in 100 min. The system was remained stable.

Effect of co-existing ions: Conduct the experiment on the effect of co-existing ions under the optimal conditions. In 10 mL system, 0.2 mg BSA was determined with the relative error ≤ ± 5 % and the tolerance limits of the co-existing ions tested were summarized as follow (μg): Li^+, Zn^{2+} (1000), Mg^{2+}, Ba^{2+} (20), Co^{2+} (100); Al^{3+} (10); Ce^{4+}, Ti^{4+}, Mn^{2+}, Cr^{6+}, Ni^{2+}, Fe^{2+} (2); Eu^{3+}, Mo^{6+} (1); Hg^{2+}, La^{3+}, Cu^{2+}, Fe^{3+}, B^{3+}, Sr^{2+} (0.2); Bi^{3+} (0.02); VO_3^- (20); S^{2-} (2); I^-, PO_4^{3-} (1); Br^-, MnO_4^- (0.2); vitamin C, oxalic acid (100); malic acid (20); citric acid, SiO_3^{2-}, glucose (10); EDTA, W^{6+}, glycine, acetic acid (2); urea (0.4); Th^{4+} (0.2).

Working curve: Under the optimum experimental conditions the linear range was measured. The results showed that ΔA presented a good linear relation with bovine serum albumin in 10 mL solution system over the range 20-480 μg (2.0-48.0 μg/mL) and the linear regression equation was ΔA = 0.0307 C (C: μg/mL) + 0.1781 with a regression coefficient γ = 0.9930. The relative standard deviation was RSD = 1.59 % for 13 parallel determinations of 20 μg/mL of bovine serum albumin. The reagent blank was determined 11 times and the standard deviation was S_{blank} = 0.98 % and the detection limit calculated was 1.6 μg/mL by 3S/K method (S is the standard deviation and K is the slope of the working curve).

Reaction mechanism: A class of derivatives containing azo (-N=N-) is known as azo reagent. Because most of organic compounds containing azo structures have colour, azo groups are also called chromophores. The reagent has the strong ability of chelate and can chelate with metallic ion to form various aqueous complexes. The -N=N- group itself can produce colour and the colour of solution will become weak even colourless when -N=N- group is oxidized or reduced. In this paper, sulfonazo(III) was oxidized by KIO_4. However, under acidic conditions, the amido of the side chain in bovine serum albumin is protonated, then association reaction between the protonated protein with positive charge and sulfonazo(III) through electrostatic attraction occurred. As the amido of protein was of uniform distribution within protein, sulfonazo(III) interacted with amido to go into the protein structure, which gave protection to the chromophore -N=N- and inhibited the process of discolouring. The primary structure of protein and the structure of sulfonazo(III) were respectively shown in Figs. 10 and 11. Oxidation reaction is shown in Fig. 12 and inhibitive reaction is shown in Fig. 13.

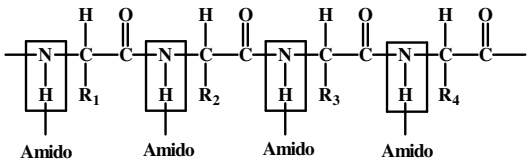

Fig. 10. Primary structure of protein

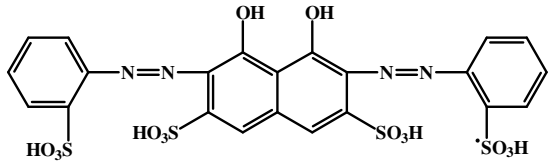

Fig. 11. Structure of sulfonazo(III)

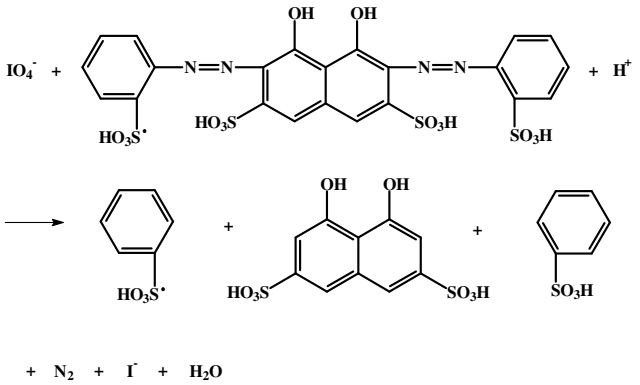

Fig. 12. Oxidation reaction

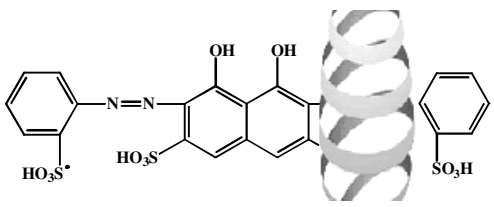

Fig. 13. Inhibitive reaction

TABLE-1
ANALYTICAL RESULTS OF SAMPLES

Sample	Found	Average	RSD (%)	Added	Recovered	Recovery (%)	Dibromo-*p*-sulfonic acid arsenazo spectrophotometry [Ref. 7]
Soybean milk (mg/mL)	23.1, 23.5, 23.6, 23.9 24.7, 23.8 23.8, 24.1 24.1, 25.3 25.7	24.1	3.32	10.00	10.23	102.3	24.0
Soybean powder (g/g)	0.270, 0.273, 0.268, 0.293 0.269, 0.308 0.300, 0.298 0.316, 0.280 0.278	0.287	1.70	10.00	10.25	102.5	0.287

Analysis of sample

Soybean milk: 1 mL of soybean milk was placed into a 100 mL calibrated flask, diluted up to the mark with water to get sample working solution. 0.6 mL of working solution was used for the determination according to the experimental procedure. The results are shown in Table-1.

Soybean powder: 0.2500 g of soybean powder was dissolved in a definite amount of water and then placed into a 100-mL calibrated flask, diluted up to the mark with water, from which 10 mL was taken and put into another 100 mL flask. Water was added to the mark to get sample working solution. 0.4 mL of this sample solution was used for the determination according to the experimental procedure. The results are shown in Table-1.

Conclusion

The paper studied the inhibitive effect of protein on the fading reaction of sulfonazo(III) oxidized by potassium periodate and the optimum experimental conditions. Protein content presents good linear relation with ΔA over the range of 2-48 μg/mL at 570 nm. Its linear regression equation is: ΔA = 0.0307 C (C: μg/mL) + 0.1781, $\gamma = 0.9930$ and the detection limit of the method is 1.6 μg/mL. This method has been successfully applied to the analysis of protein content in the soybean milk and soybean powder.

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