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Study of the Inhibitive Kinetic Spectrophotometry of Protein-Arsenazo III-Potassium Periodate

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Quantitative determination of protein is an important parameter and indicator of other nature and function investigation of proteins. A novel kinetic spectrophotometric method for the determination of protein is proposed based on the inhibitive effect of bovine serum albumin (BSA) on the oxidation reaction of arsenazo III (ASA III) by potassium periodate in the medium of the Clark-Lubs of pH 3.0. The maximum absorption peak of BSA-ASA III-KIO_4 system locates at 552 nm. The absorbance difference (ΔA) is linearly related with the concentration of bovine serum albumin over the range of 8-60 μg mL^{-1} and fitted the equation: $\Delta A = 9.832 \times 10^{-3}$ C (C: μg mL^{-1})-0.0797, with a correlation coefficient $\gamma = 0.9932$. The detection limit of the method was 4.8 μg mL^{-1}. The method was successfully used to determine protein content in milk, soybean milk, soybean powder and egg white samples, with relative standard deviations of less than 5 % for 13 replicate determinations. The recovery of the method was 100.7-103.7 %.

Key Words: Protein, Potassium periodate, Arsenazo III, Inhibitive kinetic spectrophotometry.

INTRODUCTION

Protein is closely related to the origin of life, existence and evolution. Determination of protein involves many fields of production and scientific research[1]. Protein levels directly determine the nutritional value of food. Protein plays a vital role in catalyzing different reactions of living organisms, regulating metabolism, resisting the invasion of foreign substances and controlling genetic information[2]. Chemists and biologists have been concerning study on the analysis of protein and determination of protein has great importance to biochemical analysis and clinical analysis[2]. At present, there are some methods for the determination of protein content. Spectrophotometric methods including Folin phenol method (Lowry method), biuret method, Coomassie brilliant blue G-250 method and UV absorption method, *etc*.[1]. The Lowry method[3] has certain defects of time consuming for colouring, low sensitivity and poor stability. The Coomassie brilliant blue method[4,5] shows large deviation used for the determination of different proteins because of the difference of arginine and aromatic amino acid contents in various proteins. The biuret method[4] is more practical for the determinations which need to be quick but not precise, as Cu^{2+} is susceptible to interference and reduced. Kinetic spectrophotometric method is widely used in trace analysis because of its high sensitivity and simple apparatus features[6]. Arsenazo III contains -N=N- group, which itself can produce colour. The -N=N- group is damaged by oxidation and reduction, which will make the colour of solution become light or even colourless. This research discovers that bovine serum albumin has an inhibitory effect on the oxidation of arsenazo III by KIO_4 and based on this a new method for determining protein is proposed. The method is simple concerning operation, sensitive and general laboratory can meet its testing requirements. It has been successfully used in determination of the protein content in milk and egg white.

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): Weigh 50 mg of BSA and dissolve it in amount of 100 mL of water to obtain a final concentration of 500 mg L^{-1} standard solution. It was kept in the icebox at 2 ºC. Arsenazo III solution (ASA III, Shanghai Reagent Third Factory): Weigh accurately 0.0388 g of ASA III and dissolve it in 100 mL of water to obtain a concentration of 5.0×10^{-4} mol L^{-1} solution. KIO_4 solution: Weigh 0.2300 g of KIO_4 and dissolve it in 100 mL of water to get 1.0×10^{-2} mol L^{-1} solution. A Clark-Lubs buffer solution at pH 3.0: Take 10.2 mL of 0.2 mol L^{-1} HCI, 25.0 mL of 0.2 mol L^{-1} potassium hydrogen phthalate to be diluted to 100 mL with water. Emulsifier OP solution (Shenyang Reagent Fifth

Factory): Weighed 0.5 mL of OP and dissolved it in 100 mL water to get 0.5 % (v/v) OP solution. Anhydrous ethanol and other reagents were of analytical grade and the water was deionized water unless specified otherwise.

Procedure: Take two 10 mL calibrated flasks. 1.2 mL of 500 mg L^{-1} BSA was placed into one flask (as inhibitory system), while in the other without BSA (as non-inhibitory system). 0.5 mL of 0.5 % emulsifier OP solution, 0.5 mL of 5.0×10^{-4} mol L^{-1} ASA III solution, 0.8 mL of pH 3.0 Clark-Lubs buffer solution, 0.5 mL of anhydrous ethanol and 1.5 mL of 1.0×10^{-2} mol L^{-1} KIO_4 solution were placed into both flasks in succession, diluted up to the mark with water. Shake up the mixed solution. The solution was heated for 5 min using 60 ºC water bath, then quickly taken out and cooled by running water for 20 min. Using 1 cm cell, with water as reference, measure the absorbance A of inhibitory system and the absorbance A_0 of non-inhibitory system at 552 nm and calculate $\Delta A = (A - A_0)$.

RESULTS AND DISCUSSION

Absorption spectra: Arsenazo III presents purple in the Clark-Lubs solution. Fading reaction is initiated when the oxidant potassium periodate is added. The system discolouring is inhibited after adding BSA. In Fig. 1, curve A showed that the maximum absorption wavelength of inhibitory system is 552 nm. Curve B showed that the maximum absorption wavelength of non-inhibitory system ASA III + KIO_4 against water is 560 nm. Curve C showed the absorbance difference ΔA between inhibitory and non-inhibitory reaction. ΔA has a peak value at 552 nm and the experimental sensitivity is the highest. So this work chose 552 nm as the measurement wavelength.

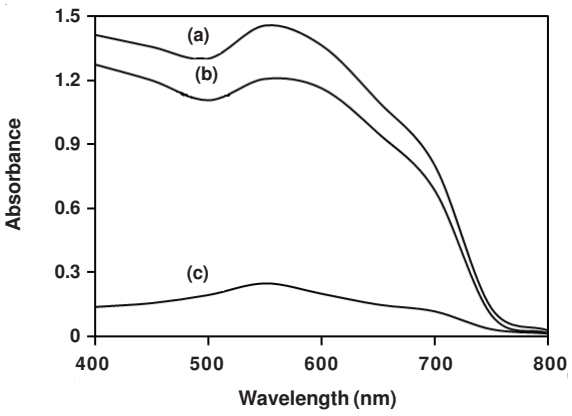

Fig. 1. Absorption spectra: (a) BSA + ASA III + KIO_4 (against water)-inhibitory reaction A; (b) ASA III + KIO_4 (against water)-non-inhibitory reaction A_0; (c) Absorbance difference between inhibitory and non-inhibitory reaction-ΔA; [BSA] = 8.8×10^{-9} mol L^{-1}; [ASA III] = 2.5×10^{-5} mol L^{-1}; [KIO_4] = 1.5×10^{-3} mol L^{-1}; [OP] = 0.5 % (v/v); [C_2H_5OH] = 8.5×10^{-1} mol L^{-1}; pH = 3.0; heating temperature T = 60 ºC; heating time t = 5 min

Effect of pH value: The study investigated the effect of pH values on the kinetic reaction. By comparison, Fig. 2 indicated that ΔA changed little at pH values ranging from 2.4-3.4, which allowed the choice of pH 3.0 as the measurement value.

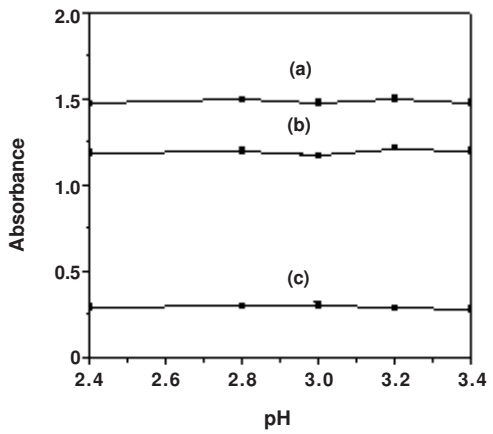

Fig. 2. Effect of acidity: (a) BSA + ASA III + KIO_4 (against water)-inhibitory reaction A; (b) ASA III + KIO_4 (against water)-non-inhibitory reaction A_0; (c) Absorbance difference between inhibitory and non-inhibitory reaction-ΔA; [BSA] = 8.8×10^{-9} mol L^{-1}; [ASA III] = 2.5×10^{-5} mol L^{-1}; [KIO_4] = 1.5×10^{-3} mol L^{-1}; [OP] = 0.5 % (v/v); [C_2H_5OH] = 8.5×10^{-1} mol L^{-1}; heating temperature T = 60 ºC; heating time t = 5 min

Effect of the amount of Clark-Lubs buffer solution: The study investigated effect of the amount of Clark-Lubs buffer solution under the optimal conditions. By comparision, it was found (Fig. 3) that ΔA increased when the amount of Clark-Lubs buffer solution was in the range of 0.2-0.8 mL and got a maximum value at 0.8 mL, but decreased in the range of 0.8-1.2 mL. This work chose 0.8 mL as the measurement amount.

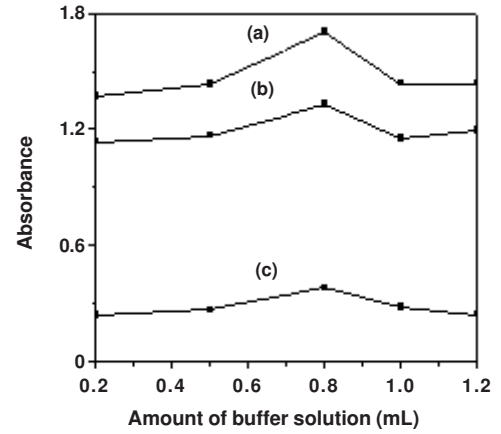

Fig. 3. Effect of the amount of buffer solution: (a) BSA + ASA III + KIO_4 (against water)-inhibitory reaction A; (b) ASA III + KIO_4 (against water)-non-inhibitory reaction A_0; (c) Absorbance difference between inhibitory and non-inhibitory reactions-ΔA; [BSA] = 8.8×10^{-9} mol L^{-1}; [ASA III] = 2.5×10^{-5} mol L^{-1}; [KIO_4] = 1.5×10^{-3} mol L^{-1}; [OP] = 0.5 % (v/v); [C_2H_5OH] = 8.5×10^{-1} mol L^{-1}; pH = 3.0; heating temperature T = 60 ºC; heating time t = 5 min

Effect of the amount of arsenazo III: The study investigated effect of the amount of chromogenic reagent under the optimal conditions. By comparision, it was found (Fig. 4) that with an increase in the amount of ASA III, ΔA increased in the range of 0.2-0.5 mL, obtaining an maximum value at 0.5 mL, but decreased in the range of 0.5-2.0 mL. This work chose 0.5 mL as the measurement amount. The concentration of ASA III solution in 10 mL system was 5.0×10^{-5} mol L^{-1}.

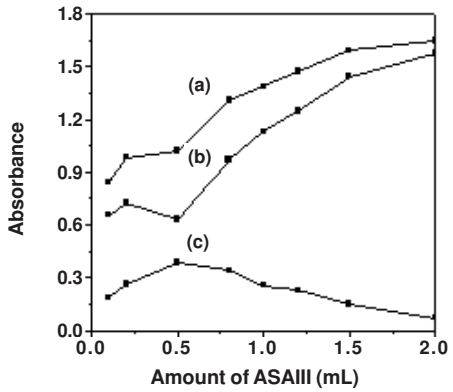

Fig. 4. Effect of the amount of ASA III: (a) BSA + ASA III + KIO_4 (against water)-inhibitory reaction A; (b) ASA III + KIO_4 (against water)-non-inhibitory reaction A_0; (c) Absorbance difference between inhibitory and non-inhibitory reactions-ΔA; [BSA] = 8.8×10^{-9} mol L^{-1}; [KIO_4] = 1.5×10^{-3} mol L^{-1}; [OP] = 0.5 % (v/v); [C_2H_5OH] = 8.5×10^{-1} mol L^{-1}; pH = 3.0; heating temperature T = 60 ºC; heating time t = 5 min

Effect of the amount of ethanol: The study investigated effect of the amount of ethanol under the optimal conditions. The results (Fig. 5) showed that the absorbance difference between inhibitory and non-inhibitory reactions increased over the range of 0-0.5 mL. ΔA was the highest at 0.5 mL, but decreased over the range of 0.5-1.0 mL. This work chose 0.5 mL of ethanol as the measurement amount.

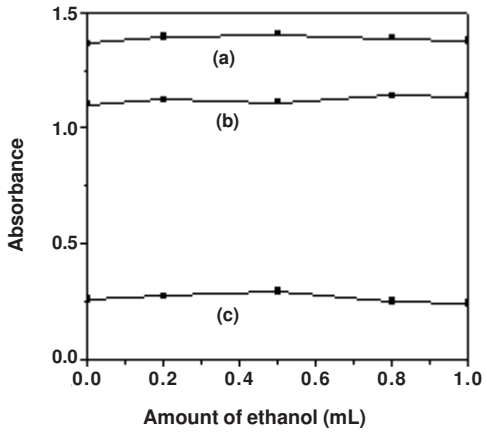

Fig. 5. Effect of the amount of ethanol: (a) BSA + ASA III + KIO_4 (against water)-inhibitory reaction A; (b) ASA III + KIO_4 (against water)-non-inhibitory reaction A_0; (c) Absorbance difference between inhibitory and non-inhibitory reactions-ΔA; [BSA] = 8.8×10^{-9} mol L^{-1}; [ASA III] = 2.5×10^{-5} mol L^{-1}; [KIO_4] = 1.5×10^{-3} mol L^{-1}; [OP] = 0.5 % (v/v); pH = 3.0; heating temperature T = 60 ºC; heating time t = 5 min

Effect of the amount of potassium periodate: The study investigated effect of the amount of potassium periodate under the optimal conditions. By comparision, it was found (Fig. 6) that ΔA increased when the amount of potassium periodate was in the range of 0.5-1.5 mL and became the highest at 1.5 mL, but decreased in the range of 1.5-2.5 mL. This work chose 1.5 mL of 1.0×10^{-2} mol L^{-1} KIO_4 solution as the measurement amount.

Effect of the amount of OP: The study investigated effect of the amount of OP under the optimal conditions. By comparision, it was found (Fig. 7) that ΔA increased when the amount of OP was in the range of 0-0.5 mL and became the highest at 0.5 mL, but decreased in the range of 0.5-1.2 mL. The present studies selected 0.5 mL of 0.5 % emulsifier-OP solution as the measurement amount.

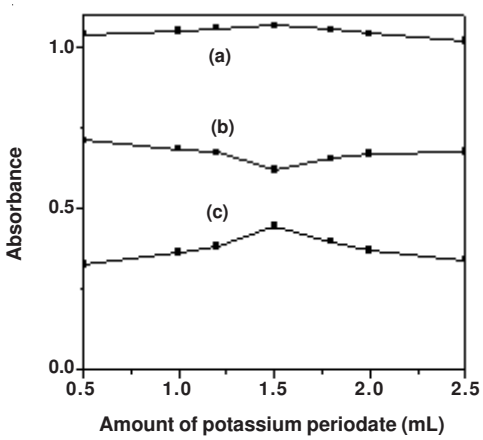

Fig. 6. Effect of the amount of KIO_4: (a) BSA + ASA III + KIO_4 (against water)-inhibitory reaction A; (b) ASA III + KIO_4 (against water)-non-inhibitory reaction A_0; (c) Absorbance difference between inhibitory and non-inhibitory reactions-ΔA; [BSA] = 8.8×10^{-9} mol L^{-1}; [ASA III] = 2.5×10^{-5} mol L^{-1}; [OP] = 0.5 % (v/v); [C_2H_5OH] = 8.5×10^{-1} mol L^{-1}; pH = 3.0; heating temperature T = 60 ºC; heating time t = 5 min

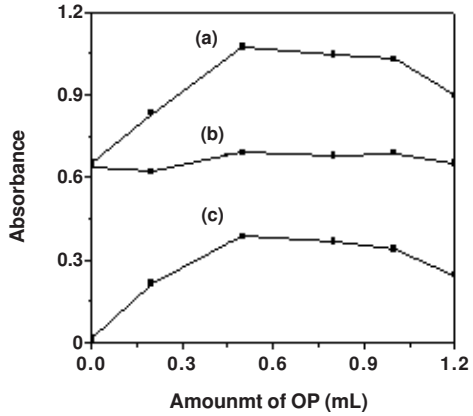

Fig. 7. Effect of the amount of OP: (a) BSA + ASA III + KIO_4 (against water)-inhibitory reaction A; (b) ASA III + KIO_4 (against water)-non-inhibitory reaction A_0; (c) Absorbance difference between inhibitory and non-inhibitory reactions-ΔA; [BSA] = 8.8×10^{-9} mol L^{-1}; [ASA III] = 2.5×10^{-5} mol L^{-1}; [KIO_4] = 1.5×10^{-3} mol L^{-1}; [C_2H_5OH] = 8.5×10^{-1} mol L^{-1}; pH = 3.0; heating temperature T = 60 ºC; heating time t = 5 min

Effect of heating temperature: The study investigated the effect of heating temperature under the optimal conditions. The experiments showed that ΔA increased when the temperature was 30-60 ºC and ΔA obtained a maximum value at 60 ºC, but decreased over 60-100 ºC. The paper chose 60 ºC as the measurement temperature. The data which were tested over 30-60 ºC were processed by regression and the linear regression equation was: $\log (A/A_0) = 216.6/T(K) - 0.455$, $\gamma = 0.9935$. The activation energy of this inhibitory reaction which was calculated by the slope of the equation was $E_a = 1.892$ kJ mol^{-1}.

Effect of heating time: The study investigated the effect of heating time under the optimal conditions. The experiments showed that ΔA increased linearly with the time in the range of 1-5 min and decreased after 5 min. The linear regression

equation was: ΔA = 0.0391t (min) + 0.1336, γ = 0.9946. ΔA became the highest at 5 min. 5 min was selected as the measurement value. Draw a diagram of log (A/A_0) with t, whose linear regression equation was: log (A/A_0) = 0.0405t + 0.1630, γ = 0.9927. The reactive rate constant was $K = 6.52 \times 10^{-4}$ s^{-1} and half-life period was $t_{1/2}$ = 1.85 min.

Cooling time and stability of system: The study investigated the effect of cooling time under the optimal conditions. By comparision, it was found that ΔA got the highest value when the cooling time was 20 min. Thus 20 min was selected for the measurement.

Under the optimal conditions, 50 μg mL^{-1} bovine serum albumin system was measured. The results showed that when the relative error of ΔA was less than 5 %, the system could remain stable for 140 min.

Effect of co-existing ions: Experiments of the influence of co-existing ions were conducted under the optimal conditions. In 10 mL system, 500 μg of BSA was determined and the relative error was not more than 5 %. The tolerant amount of co-existing ions was as follows (μg): Li^+ (2); Co^{2+} (0.5); Mn^{2+} (10); Pb^{2+}, Hg^{2+}, Fe^{2+}, Ba^{2+} (5); Ca^{2+}, Mg^{2+} (50); Cu^{2+} (100); Sr^{2+}, Ni^{2+} (250); Zn^{2+} (1000); La^{3+}, Bi^{3+} (0.5); Fe^{3+}, Cr^{3+} (5); Al^{3+} (50); Ce^{4+}, Ti^{4+} (0.5); Cr^{6+}, Mo^{6+} (5); I^- (0.5); MnO_4^-, Br^- (5); VO_3^- (50); S^{2-} (0.5); SiO_3^{2-} (50); PO_4^{3-} (100); W^{6+} (5); EDTA (0.5); acetic acid (0.5); citric acid (5); urea (5); glucose (50); oxalic acid (50); glycine (50); alanine (50); ascorbic acid (250); malic acid (500).

Working curve: Under the optimal conditions linear range was measured. The results showed that the BSA in 10 mL solution presented a good linear relation with ΔA in the range of 80-600 μg (8-60 μg mL^{-1}) and its regression equation was: $\Delta A = 9.832 \times 10^{-3}$ C (C: μg mL^{-1}) - 0.0797, γ = 0.9932. The relative standard deviation for 50.0 μg mL^{-1} BSA was 1.48 % (n = 13). The relative standard deviation for the reagent blank was 0.16 % (n = 11). The detection limit which was calculated by 3 S/K (S = standard deviation and K = slope of regression equation of the working curve, respectively) was 4.8 μg mL^{-1}.

Preliminary discussion on reaction mechanism: In the aryl of ASA III 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 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. 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 ASA III occurred due to electrostatic attraction. As the amido was distributed uniformly in protein, ASA III interacted with amido to go into the protein structure, which protected chromophore -N=N- and inhibited the process of fading. The first structure of protein and the structure of ASA III are seen in Fig. 8 and Fig. 9, respectively. The oxidation reaction is seen in Fig. 10. The inhibitive reaction is shown in Fig. 11.

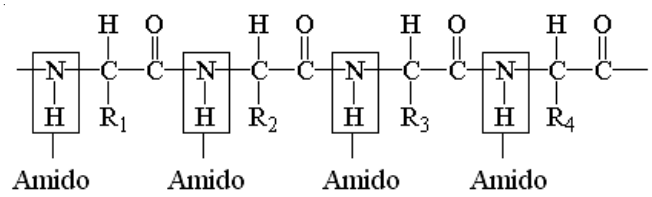

Fig. 8. First structure of protein

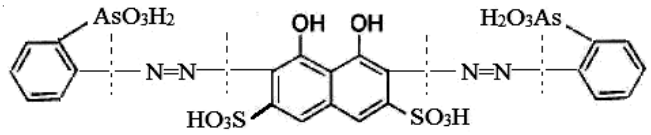

Fig. 9. Structure of arsenazo III

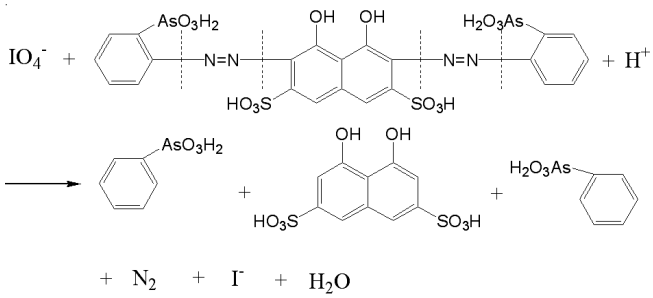

Fig. 10. Oxidative reaction

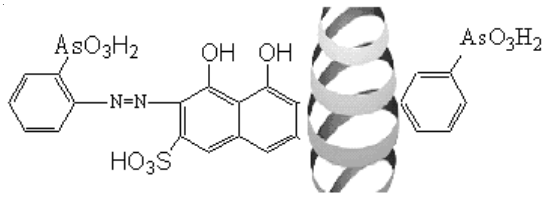

Fig. 11. Inhibitive reaction

TABLE-1
ANALYTICAL RESULTS OF SAMPLES

Sample	Found (mg mL^{-1})	Average (mg mL^{-1})	RSD (%)	Added (mg mL^{-1})	Recovered (mg mL^{-1})	Recovery (%)	*m*-Acetylchlorophosphonazo-KIO_4 kinetic spectrophotometric contranst method (mg mL^{-1})
Milk	34.36, 34.46, 34.66, 34.15, 34.66, 34.26, 34.05, 34.26, 35.17, 33.95, 34.15, 34.56, 34.15	34.36	1.26	10.00	10.37	103.7	34.36
Egg white	64.18, 62.75, 66.21, 65.40, 67.03, 65.40, 65.40, 65.40, 67.03, 66.21, 67.44, 67.85, 65.40	65.81	4.25	10.00	10.07	100.7	65.80

Method for analysis of milk: Take 1 mL of milk and place it into a 100-mL calibrated flask. The sample was diluted up to the mark with water to get working solution. 1 mL of this sample solution was used for the determination according to the experimental procedure. The results are given in Table-1.

Method for analysis of egg white: Take 0.5 mL of egg white and place it into a 100-mL calibrated flask. The sample was diluted up to the mark with water. Take 25 mL of this dilution and place it into a 50-mL calibrated flask to be diluted with water again as sample solution. 1 mL of this sample solution was used for the determination according to the experimental procedure. The results are given in Table-1.

Conclusion

The paper established the optimal conditions of BSA-KIO_4-ASA III inhibitive kinetic reaction. The maximum absorption wavelength of the system was at 552 nm. Protein presented the good linear relation with ΔA over the range of 8-60 μg mL^{-1}. The linear regression equation was: $\Delta A = 9.832 \times 10^{-3}$ C (C: μg mL^{-1}) - 0.0797, $\gamma = 0.9932$. The detection limit of the method was 4.8 μg mL^{-1}. This method has been successfully applied to determine the protein content in milk and egg white and satisfactory results were obtained.

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