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Spectrophotometric Determination of Trace Protein Using a New Inhibitive-Kinetic Indication Reaction System Protein-Tribromoarsenazo-Potassium Periodate

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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 tribromoarsenazo (TBA) by potassium periodate (KIO_4) in the medium of Clark-Lubs buffer solution in this study. The maximum absorption peak of the BSA-TBA-KIO_4 system locates at 398 nm. The absorbance difference (ΔA) is linearly related to the concentration of bovine serum albumin over the range of 5-100 μg/mL and fitted the equation: ΔA = 0.0119 C (μg/mL) + 0.0737, with a correlation coefficient γ = 0.9991. The detection limit of the method is 4.996 μg/mL. The present method has been successfully used in the determination of the content of protein in soy milk and milk samples.

Key Words: Protein, Bovine serum albumin, Inhibitive kinetic spectrophotometry, Potassium periodate, Tribromoarsenazo.

INTRODUCTION

Protein is the important substance of life. It is closely related to alimentation, growth, descendiblity, metabolism life activity[1]. At present, the quantitative analytical methods of protein deal with spectrophotometry, fluorophotometry, resonance scattering photometry, high performance liquid chromatography and capillary electrophoresis analytical method[2]. Among these methods, for photometry the need of instrumentation is relatively simple and the process of operation is simple and convenient. The methods for the spectrophotometric determination of protein include ultraviolet-visible absorption spectrometry, dye probe method and dye-metal complex probe method[2,3]. Azo dye probe is one of the commonly used reagents in the photometric determination of protein. The reported reagents dealt with thorin I, chlorosulfophenol S, arsenazo K[2]. Some investigations on triphenyl methane type dye, azo type dye, porphyrin type dye probes for the determination of protein have been made. The application of some dye probes including Coomassie brilliant blue G-250, bromcresol blue, methyl orange, eosin B, bromcresol green has already been gained in the determination of protein[4]. Although these methods have their characteristics, they have their own respective defects. The determination of protein by kinetic spectrophotometry[5] has the advantages of sensitivity highness, detection limit lowness and this caused involved researchers concern and interest. Tribromoarsenazo (TBA) is an azo agent and has been used in the determination of rare earth[6]. Based on the principle that in the Clark-Lubs buffer solution an oxidation decolouring reaction between potassium and tribromoarsenazo takes place and protein can inhibit the decolouring reaction. This paper proposed a novel method for the determination of trace protein. The method has been used in the determination of practical samples and satisfactory results were obtained.

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.

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

0.5 % (v/v) Emulsifier OP solution: 0.5 mL of OP (Fifth Reagent Plant of Shenyang, China) was dissolved in 100 mL of water. Tribromoarsenazo (TB-ASA) solution (5.0×10^{-4} mol/L): 0.0480 g of tribromoarsenazo (Shanghai Changke Research Institute of Reagent, China) was dissolved in 100 mL of water. pH 3.2 Clark-Lubs buffer solution: 7.45 mL of 0.2 mol/L HCl (Beijing Chemical Plant, China) and 25.00 mL of 0.2 mol/L potassium hydrogen phthalate (Tianjin Kemio Exploitation Center for Chemical Reagent, China) were mixed and then diluted to 100 mL with water. Bovine serum albumin (BSA) aqueous solution (0.5 mg/mL):an amount of 50 mg BSA (Biochemical reagent, Beijing Aoboxing Biotechnology

Co., Ltd., China) was weighed, placed into a 100 mL calibrated flask and diluted up to the mark with water to get 0.5 mg/mL bovine serum albumin working solution, kept in cold storage at 2 ºC. KIO_4 solution (1.0×10^{-2} mol/L): 0.2300 g of KIO_4 (Beijing Chemical Plant, China) was prepared by dissolution of in 100 mL of water. Anhydrous ethanol (Beijing Chemical Plant, China) was used.

Procedure: In 10 mL calibrated flasks, 1.0 mL of 0.5 % (v/v) OP solution, 0.5 mL of 5.0×10^{-4} mol/L TB-ASA solution, 2.0 mL of pH 3.2 Clark-Lubs buffer solution, 2.0 mL of ethanol, 1.0 mL of 0.5 mg/mL BSA standard solution, 1.2 mL of 1.0×10^{-2} mol/L KIO_4 solution were added in turn. The content was diluted to the mark with water. After the mixture was heated for 12 min at a water bath of 60 ºC, it was cooled for 10 min using running tap water. Using 1 cm cell, the absorbance of the inhibitive system containing protein and the absorbance of non-inhibitive system containing no protein were respectively determined at 392 nm with water as reference and marked as A_1, A_2, respectively. Then, $\Delta A = (A_1\text{-}A_2)$ was calculated.

RESULTS AND DISCUSSION

Absorption spectra: Fig. 1 shows the absorption spectra of corresponding systems. The results show that the maximun absorbance difference, ΔA, appeared at 392 nm. At this wavelength the senstivity for the determination of protein is the highest. Hence, 392 nm was selected as the optimum measurement wavelength in this work.

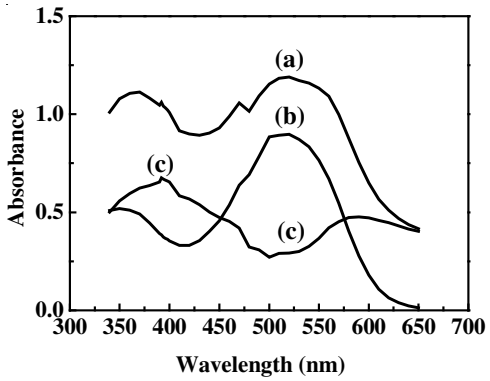

Fig. 1. Absorption spectra: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [TBA] = 2.5×10^{-5} mol/L, [BSA] = 0.05 mg/mL, [KIO_4] = 1.2×10^{-2} mol/L, [OP] = 0.5 % (v/v), pH = 3.2, heating temperature: T = 60 ºC, heating time: t = 12 min

Optimization of experimental variable

Effect of pH: When other experimental conditions were kept optimum, the effect of pH was determined over the pH range of 2.4, 2.8, 3.0, 3.2, 3.4. Fig. 2 shows that the maximum ΔA appeared at pH 3.2. At this time, the sensitivity for the determination of protein is the highest. Thus, pH was selected to be 3.2.

Effect of the amount of buffer solution: According to the procedure, the effect of amount of buffer solution was studied using 1.0, 1.5, 2.0, 2.5, 3.0 mL. Fig. 3 indicate that the maximum ΔA emerged at 2.0 mL. At this time the sensitivity is the highest. Therefore, the amount of buffer solution was ascertained to be 2.0 mL.

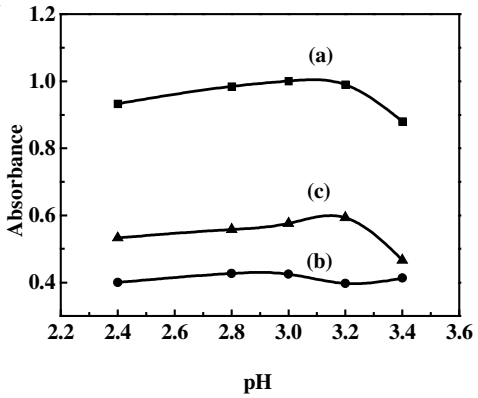

Fig. 2. Effect of pH: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [TBA] = 2.5×10^{-5} mol/L, [BSA] = 0.05 mg/mL, [KIO_4] = 1.2×10^{-2} mol/L, [OP] = 0.5 % (v/v), heating temperature: T = 60 ºC, heating time: t = 12 min

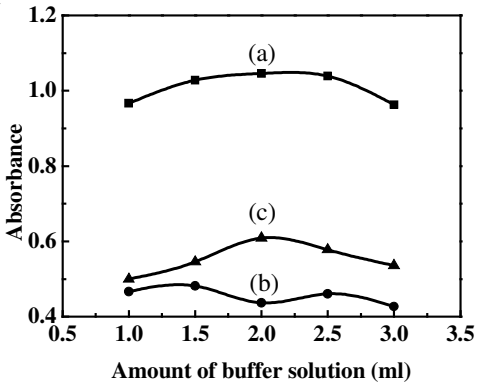

Fig. 3. Effect of the amount of buffer solution: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [TBA] = 2.5×10^{-5} mol/L, [BSA] = 0.05 mg/mL, [KIO_4] = 1.2×10^{-2} mol/L, [OP] = 0.5 % (v/v), pH = 3.2, heating temperature: T = 60 ºC, heating time: t = 12 min

Effect of the amount of tribromoarsenazo: According to the procedure, absorbances of the systems were respectively determined when the amount of TBA was 0.1, 0.3, 0.5, 1.0, 1.5, 2.0 mL. Fig. 4 shows that the maximum ΔA appeared at 0.5 mL. At this time, the sensitivity of determiantion of protein is the highest. Thus, the amount of TBA was chosen to be 0.5 mL.

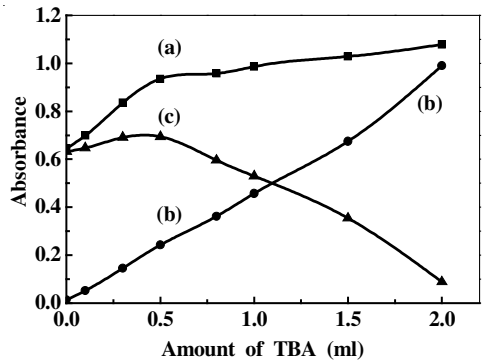

Fig. 4. Effect of the amount of TBA: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [BSA] = 0.05 mg/mL, [KIO_4] = 1.2×10^{-2} mol/L, [OP] = 0.5 % (v/v), pH = 3.2, heating temperature: T = 60 ºC, heating time: t = 12 min

Effect of the amount of KIO_4: According to the procedure, the effect of the amount of KIO_4 was studied using 0.5,

0.8, 1.0, 1.2, 1.5, 2.0 mL. The results (Fig. 5) indicate that ΔA showed a maximum at 1.2 mL. At this time the sensitivity is the highest. Thus, 1.2 mL of KIO_4 solution was selected.

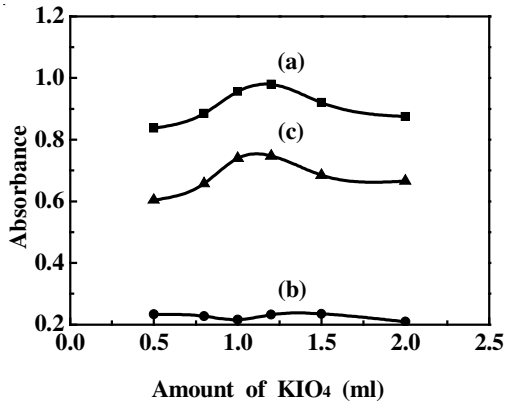

Fig. 5. Effect of the amount of KIO_4: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [TBA] = 2.5×10^{-5} mol/L, [BSA] = 0.05 mg/mL, [OP] = 0.5 % (v/v), pH = 3.2, heating temperature: T = 60 ºC, heating time: t = 12 min

Effect of the amount of OP: The experimental results (Fig. 6) indicate that the maximum ΔA appeared at the OP amount of 1.0 mL. At this time the sensitivity is the highest. Therefore, 1.0 mL of OP was ascertained. The addition of OP could increase the stability of system.

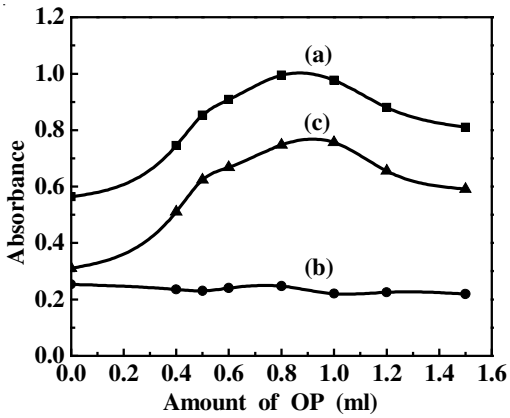

Fig. 6. Effect of the amount of OP: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [TBA] = 2.5×10^{-5} mol/L, [BSA] = 0.05 mg/mL, [KIO_4] = 1.2×10^{-2} mol/L, pH = 3.2, heating temperature: T = 60 ºC, heating time: t = 12 min

Effect of the amount of ethanol: Fig. 7 shows that when the amount of ethanol was 2.0 mL, the best sensitivity for the determination of protein could be obtained. Thus, the experimental amount of ethanol was confirmed to be 2.0 mL for further study.

Effect of temperature: The experimental results (Fig. 8) indicate that as the temperature of reaction increased over the range of 20-60 ºC, the maximum ΔA showed at 60 ºC. The sensitivity is the highest at this time. Then, ΔA gradually decreased. Therefore, the reactive temperature was confirmed to be 60 ºC. Fig. 9 shows a linear relationship between ln (ΔA) and 1/T and the regression equation is: ln (ΔA) = -836.5137 (1/T) + 2.2494, with a correlation coefficient of γ = 0.9955. The activation energy was calculated to be E_a = 6.95 kJ/mol.

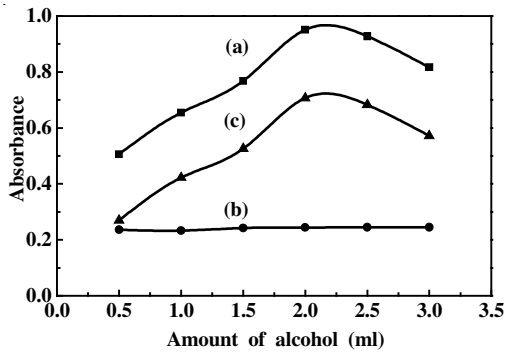

Fig. 7. Effect of the amount of ethanol: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [TBA] = 2.5×10^{-5} mol/L, [BSA] = 0.05 mg/mL, [KIO_4] = 1.2×10^{-2} mol/L, [OP] = 0.5 % (v/v), pH = 3.2, heating temperature: T = 60 ºC, heating time: t = 12 min

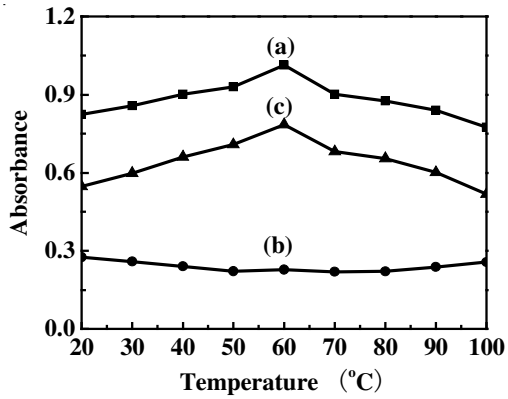

Fig. 8. Effect of temperature: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [TBA] = 2.5×10^{-5} mol/L, [BSA] = 0.05 mg/mL, [KIO_4] = 1.2×10^{-2} mol/L, [OP] = 0.5 % (v/v), pH = 3.2, heating time: t = 12 min

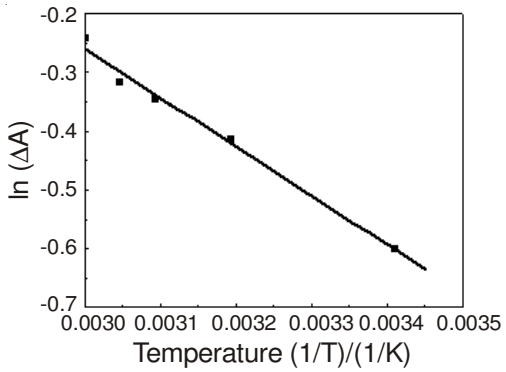

Fig. 9. Effect of temperature: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [TBA] = 2.5×10^{-5} mol/L, [BSA] = 0.05 mg/mL, [KIO_4] = 1.2×10^{-2} mol/L, [OP] = 0.5 % (v/v), pH = 3.2, heating time: t = 12 min

Effect of reactive time: The experimental results (Fig. 10) show that ΔA achieved a maximum when reactive time was 12 min. The sensitivity is the highest at this time. Thus, the reactive time was confirmed to be 12 min. Fig. 11 shows a linear relationship between ln (ΔA) and heating time t (min) over 1-12 min. Its regression equation calculated is as follows: ln (ΔA) = 0.0237t (min) - 0.5105, with a correlation coefficient of γ = 0.9912. The half-period was $t_{1/2}$ = 29.24 min.

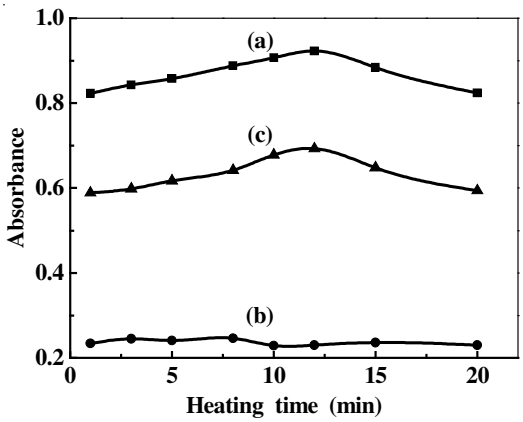

Fig.10. Effect of reaction time: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [TBA] = 2.5×10^{-5} mol/L, [BSA] = 0.05 mg/mL, [KIO_4] = 1.2×10^{-2} mol/L, [OP] = 0.5 % (v/v), pH = 3.2, heating temperature: T = 60 ºC

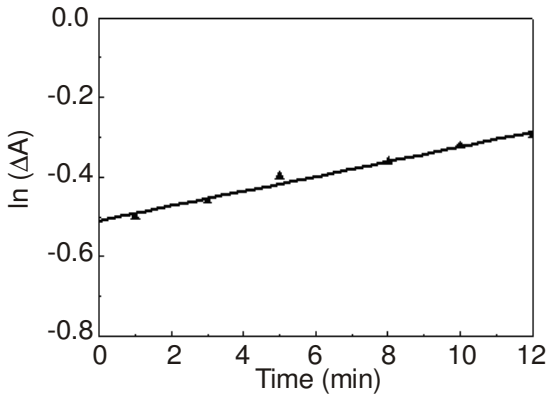

Fig. 11. Effect of heating time: [TBA] = 2.5×10^{-5} mol/L, [BSA] = 0.05 mg/mL, [KIO_4] = 1.2×10^{-2} mol/L, [OP] = 0.5 % (V/V), pH = 3.2, heating temperature: T = 60 ºC

System stability: Under the optimum conditions, the stability of system was determined using the standard that a relative error of ΔA did not exceed 5 %. The results indicated that within 5.5 h the variation of ΔA did not exceed 5 %.

Working curve and analytical characteristics: A definite amount of bovine serum albumin (BSA) was transferred into a series of 10 mL calibrated flasks. Then, the operation was made according to the recommended procedure. A linear relationship between the ΔA and final concentrations of BSA over the range of 5-100 μg/mL was found with an equation ΔA = 0.0119C (μg/mL) + 0.0737 (γ = 0.9991), where C is the concentration of BSA in μg/mL and γ is the correlation coefficient, respectively. The detection limit of BSA determination was found to be 4.996 μg/mL, which was calculated by multiplying the standard deviation of eleven replicate measurements of absorbance for the reagent blank by three and dividing by the slope of the linear calibration curve. The relative standard deviation for eleven replicate determinations of 50 μg/mL of BSA was 1.3 %. This indicates that the method has very good reproducibility.

Interference study: The selectivity of the method was tested by studying the effect of various ions on the determination of 50.0 μg/mL of BSA. The tolerance limits were taken for a maximum error of ± 5 %. The interference effect of the tested ions are as follows (μg): Li^+, Zn^{2+}, PO_4^{3-} (250); ascorbic acid (100); Mg^{2+}, citric acid (50); Ni^{2+}, acetic acid, alanine, glucose, malic acid (25); Mn^{2+}, SiO_3^{2-}, oxalic acid (10); Sr^{2+}, Ca^{2+}, Ba^{2+}, Pb^{2+}, Br^-, VO_3^-, WO_4^{2-}, MoO_4^{2-}, urea (5); Cu^{2+} (1); Fe^{2+}, Hg^{2+}, Bi^{3+}, Cr^{3+}, Al^{3+}, Th^{4+}, Ti^{4+}, Ce^{4+}, Cr^{6+}, I^-, MnO_4^-, S^{2-}, EDTA (0.5); Fe^{3+} (0.25).

Primary discussion on 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 aryl group of TBA 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 TBA also contains -N=N- groups which themselves can produce colour. 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 TBA occurred due to electrostatic attraction. As the amido was uniformly distributed in protein, TBA interacted with amido group into the protein structure, which protected the chromophore -N=N- and inhibited the process of fading. The oxidation reaction is shown in Fig. 12. The inhibitive reaction is seen in Fig. 13.

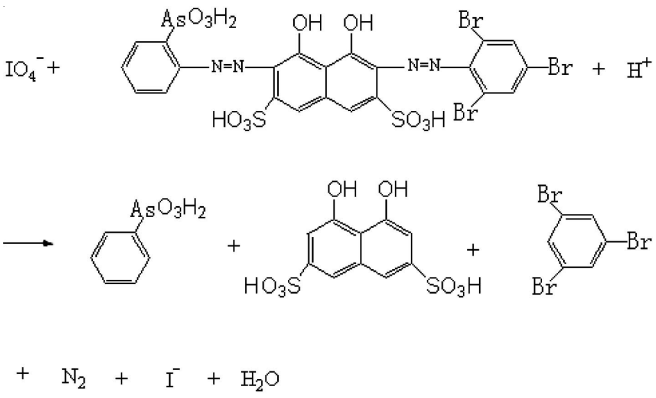

Fig. 12. Oxidation reaction

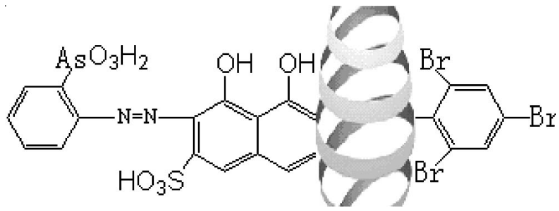

Fig. 13. Inhibitive reaction

Analysis of sample: 1 mL of soy milk or milk sample was taken, placed in a 100-mL calibrated flask and diluted by water to the mark. 1 mL of the sample was taken and determined according to the standard procedure. The analytical results are seen in Table-1. Table-1 indicates that the analytical results of present method were satisfactory.

Conclusion

This paper established a method for the inhibitive kinetic spectrophotometric determination of protein using protein-tribromoarsenazo-potassium periodate system and confirmed the optimum experimental conditions. The linear range of the determination of protein was 5-100 μg/mL at 392 nm and its

TABLE-1
ANALYTICAL RESULTS OF SAMPLES

Sample	Found (mg/mL)		Average (mg/mL)	Relative standard deviation (%)	Added (mg/mL)	Recovered (mg/mL)	Recovery (%)	*m*-Acetylchlorophosphonazo inhibitive kinetic spectrophotometric contrast method (mg/mL) [Ref. 5]
Soy milk	17.89 18.09 18.81 18.81 19.33 19.53 19.63	19.84 20.05 20.05 20.05 20.46 20.97	19.53	4.60	1.000	0.973	97.30	19.52
Milk	34.26 34.68 35.36 35.44 35.69 35.86 36.03	36.20 36.37 36.70 36.96 37.63 38.05	36.33	3.38	1.000	1.032	103.2	36.33

regression equation was: $\Delta A = 0.0119C$ (μg/mL) + 0.0737,with a correlation coefficient of $\gamma = 0.9991$. The detection limit of the method was 4.996 μg/mL. The contents of protein in soy milk and milk samples have been determined with satisfactory results.

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