

# Langmuir Aggregation of Chromophore in Biomacromolecule and its Application: Interaction of Picramine CA (PCA) with Proteins

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The microphase adsorption-spectral correction (MPASC) technique has been described. The formation of microelectrostatic fields in macromolecule is proposed and it causes the aggregation of a chromophore in protein. We have studied the interaction of picramine CA (PCA) with five proteins: bovine serum albumin (BSA), human  $\gamma$ -globulin ( $\gamma$ -G), bovine hemoglobin (Hb), horse myoglobin (Mb) and ovalbumin (OVA). Results show that at pH 2.21, the adsorption ratios of PCA to BSA,  $\gamma$ -G, Hb, Mb and OVA are 46, 54, 35, 15 and 21, respectively, their adsorption constants  $3.17 \times 10^5$ ,  $6.58 \times 10^4$ ,  $1.40 \times 10^5$ ,  $8.99 \times 10^4$  and  $1.68 \times 10^5$  and their absorptivities  $3.56 \times 10^5$ ,  $3.06 \times 10^5$ ,  $2.91 \times 10^5$ ,  $1.10 \times 10^5$  and  $1.97 \times 10^5 \text{ l mol}^{-1} \text{ cm}^{-1}$  at 630 nm. The analysis of samples has given the satisfactory result.

**Keywords:** MPASC technique; Protein; Picramine CA (PCA); Microelectrostatic field; Langmuir aggregation

## INTRODUCTION

At present, more and more biochemists are interested in research on supramolecular chemistry. Understanding the interaction between protein and a chromophore is important in biochemistry and quantitative determination of proteins is useful in clinical tests and laboratory practice. The conventional methods include the Biuret [1], Bradford [2], Lowry [3], Bromocresol green [4], and resonance light scattering (RLS) technique [5–8]. However, the interaction between biological macromolecules and organic dyes has not been elucidated satisfactorily and earlier observations have not been explained

clearly and reasonably, e.g. the Pesavento equation [9], and Scatchard model [10].

Some chromophores, e.g. Bromophenol blue, Neutral red as the adsorption indicators are usually used in the determination of halogen anions. The same color change was observed when we used proteins in place of halogen anions. Consequently, the interaction of biological macromolecules with chromophore has the same mechanism as the precipitation adsorption reaction. Commonly, the biological macromolecule contains a complex spatial structure. The winding, folds, coils and other arrangement lead to many holes, gullies and helix grooves. Many secondary bonds are close to each other, such as van der Waals bonds, salt bonds, hydrogen bonds. The charge density becomes very high around the holes, grooves and gullies. The like charges aggregate together to form many microelectrostatic fields as shown in Fig. 1(1). They will selectively attract charged organic compounds till kinetic equilibrium (Fig. 1(2)). The existence of a microelectrostatic field is the basis of the adsorption of a chromophore in macromolecules. In addition, we found that the equilibrium equation of the conventional biochemical reaction is same as the Langmuir adsorption equation [11]. The microelectrostatic field is so narrow that organic molecules (with charge) were adsorbed only in a monolayer. Therefore, the aggregation of a chromophore in macromolecule is regarded as monolayer adsorption of the microelectrostatic field. Such an adsorption equilibrium occurs:  $L(\text{aqueous phase}, C_L) \rightleftharpoons ML_N(\text{macromolecular phase}, C_M)$  in L–M solution. The

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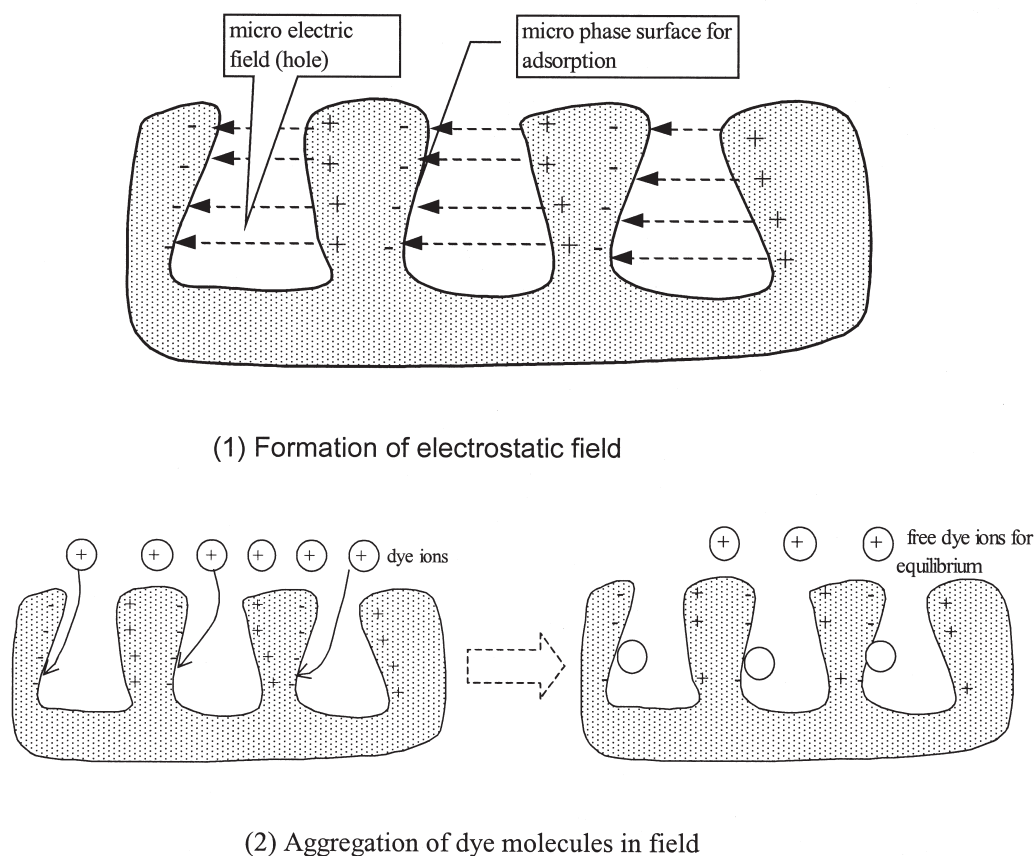


FIGURE 1 (1) The formation of microelectrostatic field in macromolecule and (2) the adsorption process of dye molecules in microphase surface.

Langmuir adsorption equation may be used as follows

$$\gamma = \frac{KNC_L}{1 + KC_L} \quad (1)$$

where  $K$  is the equilibrium constant and  $C_L$ , the concentration of the excess  $L$ .  $N$  indicates the maximal adsorption ratio of  $L$  to  $M$  and  $\gamma$  the molar ratio of  $L$  adsorbed to  $M$ .  $K$  is calculated from Eq. (1). Both  $C_L$  and  $\gamma$  are calculated by means of [12]:

$$\gamma = \eta \times \frac{C_{L0}}{C_M} \quad (2)$$

$$C_L = (1 - \eta)C_{L0} \quad (3)$$

where

$$\eta = \frac{A_c - \Delta A}{A_0} \quad (4)$$

where both  $C_M$  and  $C_{L0}$  are the concentration of the  $M$  and  $L$  added initially and  $\eta$  indicates the effective fraction of  $L$ .  $A_c$ ,  $A_0$  and  $\Delta A$  are the real absorbance of the  $M-L$  product, the measurement absorbance of the reagent blank against water and that of the  $M-L$  solution against reagent blank directly measured at the peak wavelength  $\lambda_2$ . Within increase in  $L$  concentration,  $\gamma$  will approach a maximum  $N$ . The

$A_c$  is calculated by means of [13]:

$$A_c = \frac{\Delta A - \beta \Delta A'}{1 - \alpha \beta} \quad (5)$$

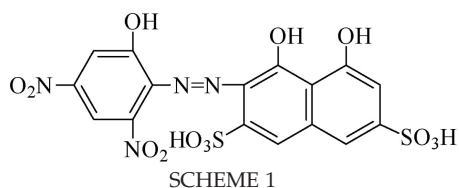
where  $\Delta A'$  indicates the absorbance of the  $M-L$  solution measured respectively at the valley absorption wavelength  $\lambda_1$ . Usually,  $\alpha$  and  $\beta$  are the correction constants [13] and they are calculated by measuring directly  $ML_N$  and  $L$  solutions. In addition, the absorptivity (real  $\epsilon_r^{\lambda_2}$  not apparent  $\epsilon_a^{\lambda_2}$ ) of the adsorption product  $ML_N$  at  $\lambda_2$  is also directly calculated by the means of:

$$\epsilon_r^{\lambda_2} = \frac{NA_c}{\delta \gamma C_M} \quad (6)$$

where  $\delta$  is the cell thickness (cm) and the others have the same meaning as in the equations above. In addition, the adsorption constant ( $K$ ) of the aggregate  $ML_N$  can also be calculated by the relation:

$$K = \frac{N_\gamma}{(N - \gamma)(NC_{L0} - \gamma C_M)} \quad (7)$$

We find that the Pesavento hypothesis and Scatchard model are same as the Langmuir adsorption Eq. (1). Consequently, in fact, both, the Pesavento hypothesis and Scatchard model are just microelectrostatic



field's adsorption between macromolecules and small dye molecules. The combination of both the Langmuir adsorption and the spectral correction technique will provide a very helpful experimental strategy for study of aggregation of chromophore on surfactant micelle. The method is called microphase adsorption-spectral correction technique (MPASC). In the present report, we have studied the interaction between proteins and picramine CA (PCA). PCA is one of chromotropic acid azo derivant, which was ever used in the determination of some organic metal ions, e.g. Zr, Sc, rare earth and so on. The structure of the chromophore is shown in Scheme 1

It is sensitive to aggregate in protein. The aggregation of PCA in protein (BSA, Hb, Mb, OVA and  $\gamma$ -G) accords with the Langmuir adsorption. Results showed that at pH 2.21, the maximal adsorption ratios of BSA,  $\gamma$ -G, Hb, Mb and OVA to PCA are 46, 48, 35, 15 and 13, respectively, and their adsorption constants  $K_{\text{BSA-PCA}} = 3.17 \times 10^5$ ,  $K_{\gamma\text{-G-PCA}} = 6.58 \times 10^4$ ,  $K_{\text{Hb-PCA}} = 1.40 \times 10^5$ ,  $K_{\text{Mb-PCA}} = 8.99 \times 10^4$  and  $K_{\text{OVA-PCA}} = 1.68 \times 10^5$ .

## RESULTS AND DISCUSSION

### Spectra

The adsorption between PCA and proteins (BSA as representative) was carried out. At pH 2.21, the absorption spectra of BSA-PCA solution is shown in Fig. 2, where 0.50 mg of BSA and 0.50  $\mu\text{mol}$  of PCA were added. From curves 1 and 2, we find that the peak of PCA is located at 550 nm and that of BSA-PCA product at 590 nm. We observe that the product has a longer peak wavelength than PCA. The spectral red shift is only 40 nm. Therefore, the excessive PCA will interfere with the real absorbance measurement of the product. From curve 3, representing the relative absorption spectrum of PCA-protein solution against PCA solution, we observe that the peak is located at 630 nm and the valley at 510 nm. So such two wavelengths were used in this work. From curves 1 and 2, the correction coefficients were calculated to be  $\beta = 0.239$  and  $\alpha = 0.714$ . Because of high  $\beta$ , the spectral correction method should be used in place of the ordinary spectrophotometry.

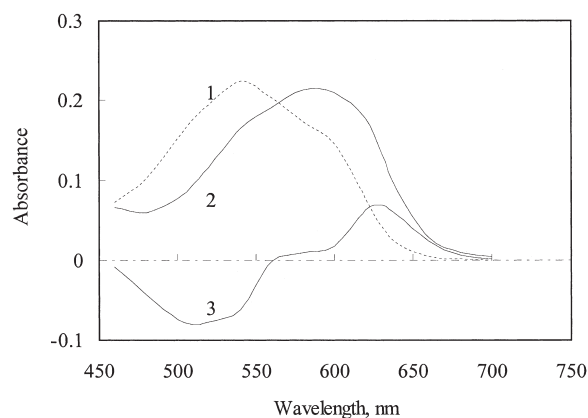


FIGURE 2 Absorption spectra of PCA and its BSA solutions at pH 2.21: (1) 0.200  $\mu\text{mol}$  of PCA, (2) solution initially containing 0.200  $\mu\text{mol}$  of PCA and 10 mg of BSA, (3) solution initially containing 0.200  $\mu\text{mol}$  of PCA and 0.500 mg of BSA. Both 1 and 2 against water and 3 against the blank.

### Effect of PH

By varying pH of solution, the absorption of the BSA-PCA solution was measured and the ratio of PCA to BSA is shown in Fig. 3. We observe that lower the pH changes, higher the adsorption ratio becomes. This is because the protonation reaction of PCA occurs in acidic solution. PCA cations are attracted easily and closely on microphase surface in proteins.

### Effect of Ion Strength, Surfactants, Temperature and Interaction Time

The influence of ionic strength of solution on binding ratio is shown in Fig. 4(1). Between the ionic strength 0 and 0.4 M, the ratio of PCA to BSA becomes lower with increase in ionic strength. As more ions were attracted into microelectrostatic fields to take up the microphase surface, this causes decrease of the adsorption ratio.

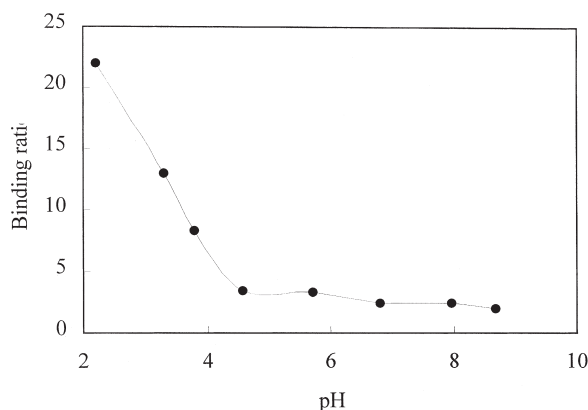


FIGURE 3 Effect of pH on  $\gamma$  (solution initially containing 0.200  $\mu\text{mol}$  of PCA and 0.50 mg of BSA).

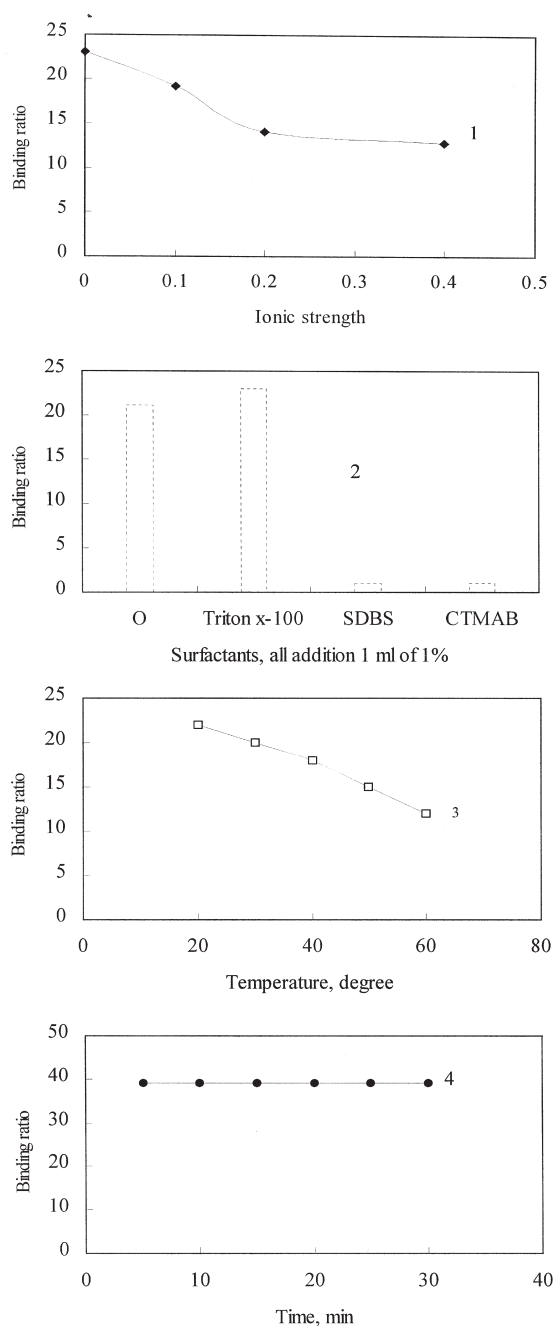


FIGURE 4 Effect of ionic strength (1), surfactants (2), temperature (3) and the interaction time (4) on  $\gamma$  (solutions initially all containing  $0.200 \mu\text{mol}$  of PCA and  $0.50 \text{ mg}$  of BSA).

The effect of various surfactants on the binding ratio of PCA to BSA is shown in Fig. 4(2). The use of ionic surfactants CTMAB and SDBS decreases the adsorption ratio because they can also be adsorbed closely in place of PCA molecules. The non-ionic surfactant Triton x-100 does not affect the binding ratio because it is neutral.

At various temperatures, the relationship of the binding ratio with PCA is shown in Fig. 4(3). The ratio remains almost constant with increase in temperature. It indicates that the adsorption of

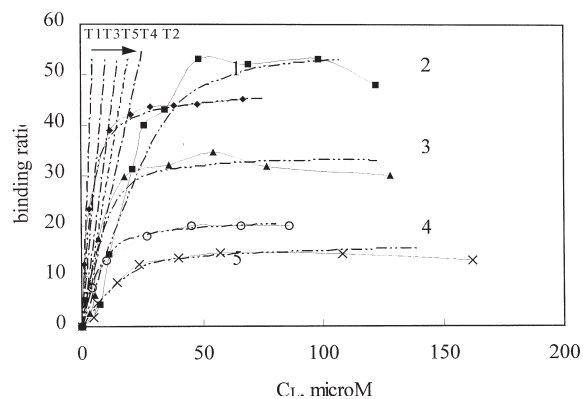


FIGURE 5 Relationship between  $\gamma$  and  $C_L$ . Solutions initially containing: (1)  $0.500 \text{ mg}$  of BSA, (2)  $1.00 \text{ mg}$  of  $\gamma\text{-G}$ , (3)  $0.500 \text{ mg}$  of Hb, (4)  $0.500 \text{ mg}$  of Mb, (5)  $0.500 \text{ mg}$  of OVA.

PCA is too close and strong for higher temperature to desorb it from protein macromolecule. So the formed aggregate is quite stable.

From Fig. 4(4), at room temperature, the interaction between BSA and PCA is complete in 5 min. So, the monolayer adsorption of PCA in protein is rapid.

#### Effect of PCA Concentration

By varying the addition of PCA solution, the absorption of the various protein solutions was measured. Calculated  $\gamma$  and  $C_L$  of each solution. Their relationship is shown in Fig. 5, where the dashed lines indicated the Langmuir regression adsorption, solid lines, the measured absorption and dotted lines, the tangent of grid origin. We find that the measurement points are close to the Langmuir adsorption curve. Therefore, the aggregation of PCA in proteins agrees to the monolayer adsorption. To analyze the extended constant of curves 1–5, we obtain the binding ratios of PCA to BSA,  $\gamma\text{-G}$ , Hb, Mb and OVA to be 46, 54, 35, 15 and 21, respectively. From the tangent lines T1–T5 of curves at O point, we calculate the binding constants of products of PCA with BSA, OVA, Hb,  $\gamma\text{-G}$  and Mb to be  $K = 3.56 \times 10^5$ ,  $1.68 \times 10^5$ ,  $1.10 \times 10^5$ ,  $5.75 \times 10^4$  and  $8.88 \times 10^4$ , respectively. Additionally, we prepared the solutions (given in Table I) for another determination [13] of  $K$  and real absorptivity ( $\epsilon$ ) of the product from Eqs. (6) and (7). The results are given in Table I. We find that the mean  $K$  of six replicated determinations of each solution in Table I is similar to that above. To see Table I, the binding constants of products arrange as BSA, OVA, Hb, Mb and  $\gamma\text{-G}$  in high to low order. From  $\epsilon$  in Table I, the higher the absorptivity of the product became, larger the binding ratio  $N$  is. In the determination of the adsorption ratio and equilibrium constant, the spectral correction method had special advantage in operation and principle by contrast to classical methods such as molar ratio [14],

TABLE I Determination of the binding ratio, equilibrium constant and real asborptivity of the adsorption products at pH 2.21 (six replicated determination)

Interaction	$\gamma/N$	K	$\epsilon$ ( $1 \text{ mol}^{-1} \text{ cm}^{-1}$ at 630 nm)
BSA (0.500 mg)-PCA (0.200 $\mu\text{mol}$ )	(23.4 $\pm$ 1.4)/48	(3.17 $\pm$ 0.51) $\times 10^5$	3.56 $\times 10^5$
OVA (0.500 mg)-PCA (0.200 $\mu\text{mol}$ )	(13.2 $\pm$ 0.81)/21	(1.68 $\pm$ 0.33) $\times 10^5$	1.97 $\times 10^5$
Hb (0.500 mg)-PCA(0.200 $\mu\text{mol}$ )	(17.5 $\pm$ 1.6)/35	(1.40 $\pm$ 0.26) $\times 10^5$	2.91 $\times 10^5$
$\gamma$ -G (1.00 mg)-PCA (0.400 $\mu\text{mol}$ )	(31.4 $\pm$ 2.3)/54	(6.58 $\pm$ 1.02) $\times 10^4$	3.06 $\times 10^5$
Mb (0.500 mg)-PCA (0.400 $\mu\text{mol}$ )	(8.7 $\pm$ 0.42)/15	(8.99 $\pm$ 1.13) $\times 10^4$	1.10 $\times 10^5$

TABLE II The linear regression equations for determination of proteins with PCA at pH 2.21 and in the presence of EDTA

Protein	Linear scope (mg/10ml)	Regression equation at 630 nm	Correlation coefficient
BSA	0-1.5	$A_c = 0.231x + 0.004$	0.9947
OVA	0-1.2	$A_c = 0.256x - 0.005$	0.9980
Hb	0-2.0	$A_c = 0.217x - 0.017$	0.9998
$\gamma$ -G	0-1.2	$A_c = 0.267x + 0.006$	0.9989
Mb	0-2.0	$A_c = 0.313x - 0.018$	0.9990

continuous variation [15] and equilibrium movement [16].

### Calibration Graph and Precision

The standard series of proteins were prepared and measured at pH 2.21 where 1.0 ml of PCA solution was added. The linear regression equations are given in Table II. The slopes of the equations are between 0.217 and 0.313 and the mean is 0.257. The maximal deviation is only 20%. It indicates that the absorption per milligram of protein is almost similar despite of various structures. So constant number of amino acid residues consists of one microelectrostatic field by folding. It is convenient for total proteins in samples to be determined only by one calibration graph, e.g. the OVA-PCA regression equation. Additionally, the adsorption ratio ( $\gamma$ ) and the effective fraction ( $\eta$ ) of PCA in each solution above are shown in Fig. 6. All  $\gamma$  reach peak after the addition of 0.80 mg of

proteins. The peak  $\gamma$  increases with increase in Dalton formula weight of proteins. This also confirms that the aggregation of PCA in proteins is in relation to only the number of amino acid residues in protein.

For 0.010 of  $A_c$ , the detection limit was calculated to be 0.04 mg of protein in 10 ml of solution. Six replicated determinations of 1.00 mg of BSA were carried out. The mean was  $1.06 \pm 0.03$  mg and the recovery of standard BSA 106% with the relative standard deviation (RSD) 2.43%.

### Effect of Foreign Ions

By adding EDTA- $\text{Na}_2$  solution (0.5 ml of 5%) in protein solution, the influence of foreign substances including ions and organic compounds, on the determination of protein was tested at pH 2.21. Because EDTA may complex most of metal ions, the high concentration of metal ions may be allowed. None of the following ions affected the direct determination of 0.50 mg of BSA (less than 10% error): 1 mg of  $\text{K}^+$ ,  $\text{Ca(II)}$ ,  $\text{SO}_4^{2-}$ ,  $\text{CO}_3^{2-}$ , 0.5 mg of  $\text{Cl}^-$ ,  $\text{F}^-$ ,  $\text{PO}_4^{3-}$ ,  $\text{NO}_3^-$ ,  $\text{SO}_3^{2-}$ ,  $\text{C}_2\text{O}_4^{2-}$ , triton x-100,  $\text{Ac}^-$ , ctDNA, yRNA, glucose, amino acid,  $\text{Mg(II)}$ , 0.1 mg of acetone, ethanol,  $\text{NH}_4^+$ ,  $\text{Mn(II)}$ ,  $\text{Zn(II)}$ ,  $\text{Ti(IV)}$ ,  $\text{Pb(II)}$ ,  $\text{Ni(II)}$ ,  $\text{Co(II)}$ ,  $\text{Sn(II)}$ ,  $\text{Cd(II)}$ , 0.02 mg of  $\text{Fe(III)}$ ,  $\text{Al(III)}$ ,  $\text{Cu(II)}$ ,  $\text{Hg(II)}$ . The ion surfactants, e.g. CTMAB and SDBS can bring the negative interference.

### Sample Analyzed

Two samples were determined. The first sample was prepared by diluting 1.00 ml of Duoqi Children Drink to 100 ml with deionized water and the other was prepared with drinking water as background aqueous by adding drops of protein and the following compounds or ions: 1 mg of  $\text{Ca(II)}$ ,

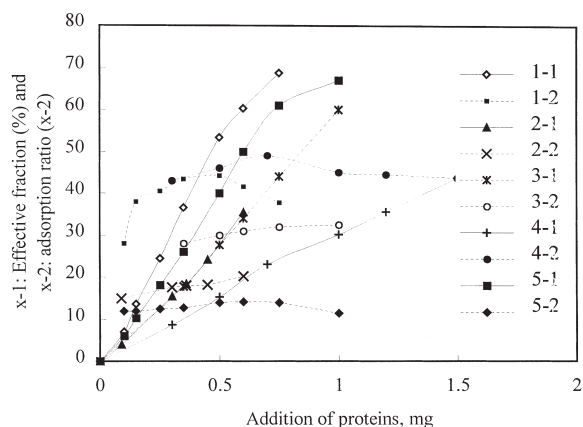


FIGURE 6 Effect of protein concentration on  $\eta$  ( $x-1$ ) and  $\gamma$  ( $x-2$ ) where 1.00  $\mu\text{mol}$  of PCA was added initially: (1) BSA, (2) OVA, (3) Hb, (4)  $\gamma$ -G, (5) Mb.

TABLE III Determination of proteins in samples in the presence of EDTA (rec indicates recovery)

Sample	Added	Found (mg)
1# from Duoqi Drink (protein content about 2%)	0.40 ml	0.912 ± 0.021 RSD. 2.87%
	0.500 mg of OVA	1.448 ± 0.050 rec.107.3%
2# Drinking water background (three replicated determinations of 5.0 ml)	0.500 mg of BSA	0.487 ± 0.021 rec. 97.4%
	1.00 mg of $\gamma$ -G	0.938 ± 0.040 rec. 93.8%
	0.500 mg of Hb	0.465 ± 0.024 rec.93.0%
	0.500 mg of Mb	0.492 ± 0.018 rec.98.4%
	0.500 mg of OVA	0.511 ± 0.022 recovery 102.2%

ethanol, acetate, ctDNA, yRNA, glucose and  $\text{PO}_4^{3-}$ , 0.5 mg of Ca(II), Mg(II),  $\text{Cl}^-$ , 0.1 mg of  $\text{F}^-$ , Cu(II), Mn(II), Zn(II) and 0.02 mg of Pb(II). The determination of samples and recovery of proteins are given in Table III. Calculated the protein content of the original drink to be 2.3% and it is close to the "about 2% protein" marked the container. The recovery of OVA added in the first sample was 107.3% together with the relative standard deviation 2.87%. The recovery of various proteins added in second sample is between 93.0 and 102.2%.

The investigation to the aggregation of PCA and the other chromophores [17] in proteins supports the monolayer adsorption and the hypothesis of the microelectrostatic field. Though MPASC technique has not given the higher sensitivity than other methods such as RLS [8], it meets precision and accuracy criteria and offers the additional benefits of simplicity and versatility. We understand that the classical method can still play an important role in the macromolecular behaviour and the determination of the property constants.

## MATERIALS AND METHODS

### Materials

Absorption spectra were recorded with a UV/VIS 265 spectrophotometer (Shimadzu, Kyoto, Japan). DDS-11A conductivity meter (Tianjin Second Analytical Instrument Works) was used to measure conductivity together with a DJS-1 conductivity immersion electrode (electrode constant 0.98) (Shanghai Tienkuang Device Works) in the production of deionized water of  $0.5\text{--}1\ \mu\Omega^{-1}\text{cm}^{-1}$ . The pH of the solution was measured with a pHs-2C acidity meter (Leici Instrument, Shanghai, China) and Model 620D pH Pen (Shanghai Ren's Electrostatic). The temperature was adjusted and remained constant in a Model 116R, electrostatically heated

thermostatic bath (Changjiang Test Instrument of Tongjiang, China).

Stock standard solutions of proteins were prepared by dissolving the commercial bovine serum albumin (BSA) (Beitai Biochemical, Chinese Academy Sciences, Beijing, China), human  $\gamma$ -globulin ( $\gamma$ -G), (Serva, Heidelberg, Germany), bovine hemoglobin (Hb, Dongfeng Biological Technological), horse myoglobin (Mb) (Shanghai Chemical Reagents Center) and ovalbumin (OVA) (Shanghai Chemical Reagents Center) in non-ionic water. The  $\gamma$ -G solution is  $1.00\ \text{mg ml}^{-1}$  and the others  $0.500\ \text{mg ml}^{-1}$ .

PCA solution (1.00 mM) was prepared by dissolving 0.2650 g of PCA (purified and provided by Changke Regent Institute, Shanghai) in 500 ml of deionized water. The Britton–Robinson buffer solutions between pH 2.21 and 8.69 were prepared to control the acidity of the interaction solution. 2 M NaCl was used to adjust the ionic strength of the aqueous solutions.  $\text{Na}_2\text{-EDTA}$  solution (5%) was prepared to mask the foreign metallic ions co-existed possibly in the practical samples. All reagents were of analytical grade and used without further purification. The surfactant solutions, Triton x-100, sodium dodecylbenzene sulfonate (SDBS) and cetyltrimethylammonium bromide (CTMAB) (all 1%), were prepared for testing their effect on the sensitivity.

### Methods

Into a 10 ml calibrated flask were added an appropriate working solution of proteins or proteins, 1.0 ml of Britton–Robinson buffer solution and appropriate PCA solution. The mixture was then diluted to 10 ml with deionized water and mixed thoroughly. All the absorption measurements were obtained against the blank treated in the same way without proteins.

In the determination of proteins in samples, the masking reagent, 0.5 ml of Na<sub>2</sub>-EDTA solution (5%) was added to complex metal ions, e.g. Pb(II), Cd(II), Hg(II) and so on. The next successive operation is same as the above procedure.

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