FOOD COMPOSITION AND ADDITIVES

A Novel Method for Determination of Protein in Human Serum Utilizing the Trihydroxylphenylfluorone-Molybdenum(VI) Complex as a Probe by Fluorescence Spectrometry

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The fluorescence intensity of the trihydroxylphenylfluorone-molybdenum(VI) [Mo(VI)] complex is quenched by protein. Based on this, a novel method for protein assay in aqueous solution was developed. With pH 3.75 acetic acid–sodium acetate buffer solution, in the presence of *p*-octyl polyethylene glycol phenyl ether microemulsion, the quenched fluorescence intensity is proportional to the concentration of bovine serum albumin (BSA) in the range of 0–7.00 μg/mL, and the detection limit of BSA is 5.65 ng/mL. There is no interference from amino acids and most metal ions. The method developed in this paper has been used for the successful determination of protein in human serum.

Protein analysis is an active and important area of investigation in the fields of life science, clinical, chemical, and biochemical analysis. Routine biochemical methods have been developed, such as the Lowry et al. (1) and Bradford (2) assays and the bromophenol blue (3) and bromocresol green (4) methods. In addition, many other methods have been published in recent years, such as those based on spectrophotometry (5–7), resonance light-scattering spectrometry (8, 9), fluorometry (10–12), electrochemistry (13), and chemiluminescence (14). In recent years, fluorometry has become one of the most important means in the quantitative determination of protein due to its advantages in terms of high speed, sensitivity, and relatively few interfering substances. There are many types of probes for the quantitative determination of protein (such as a dye-metal probe), which have received much attention in the determination of protein at trace level with high sensitivity, long stability, and good selectivity.

Phenylfluorone (PF) is one kind of conjugated π-bond-system molecule with a rigid and planar configuration. Because of its characteristic high sensitivity, PF has become an important analytical reagent. In the past, this sort of PF dye has been widely applied in the spectrophotometric analysis of metals (15, 16), and recently it has also been used in biomacromolecular analysis, such as protein determination by spectrophotometry (17, 18) and resonance light-scattering spectrometry (19, 20).

Microemulsions are transparent or translucent, low viscosity, homogeneous, and thermodynamically stable systems that are composed of surfactant, water, oil, and cosurfactant in appropriate ratios. Compared with micellar systems, microemulsions have lower surface tension and strong solubilization ability for organic and inorganic substances (21). Our previous studies have applied to the determination of many ions and proteins by different methods, and all of the results obtained were satisfactory (22–31).

In this paper, a novel dye-metal probe for protein has been developed. Trihydroxylphenylfluorone (TH-PF; Figure 1) with molybdenum (VI) [Mo(VI)] was first used in the fluorometric analysis of protein in microemulsion medium. Compared with other kinds of dye-metal probes, the TH-PF-Mo(VI) complex is characterized by high sensitivity, good selectivity, and stability. *p*-Octyl polyethylene glycol phenyl ether (OP) microemulsion has been introduced into the system, which has significantly increased the sensitivity of the system. The method developed in this paper was then used successfully to determine the amount of protein in human serum.

Experimental

Apparatus

Fluorescence spectra and intensities were measured on a Model LS-55 Spectrofluorometer (Perkin-Elmer, Shelton, CT) equipped with 1 cm quartz cell. Absorption spectra were obtained with a Model UV-3101PC spectrophotometer (Shimadzu, Columbia, MD). A PHS-3B pH meter was used in determining pH values (Shanghai Precision and Scientific Instrument Co., Ltd., PRC).

Reagents

Unless otherwise specified, all chemicals were of analytical grade and doubly distilled water was used throughout this experiment.

Standard stock solutions of protein (100 μg/mL) were prepared by dissolving 0.0100 g commercial products in water and diluting to 100 mL in a volumetric flask; they were

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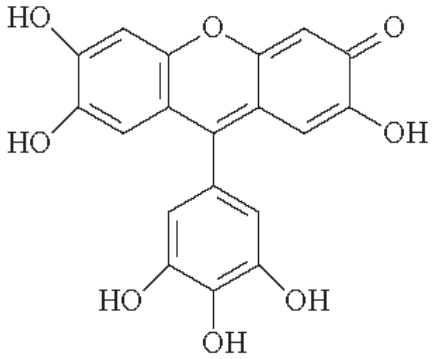

Figure 1. Structure of TH-PF.

stored at 0–4°C. The working solutions (10 μg/mL) were diluted from the standard stock solution directly. Proteins used in this study included bovine serum albumin (BSA), human serum albumin (HSA), γ-globulin (γ-G), and ovalbumin (Ova), which were purchased from Sigma (St. Louis, MO).

TH-PF was purchased from Shanghai Changke Reagent Graduate School (PRC). TH-PF solution (1.00×10^{-4} M) was prepared by dissolving 0.0037 g TH-PF in pure ethanol in a 100 mL volumetric flask.

A Mo(VI) stock solution (0.1000 M) was prepared by dissolving 1.4394 g of spectroscopically pure (99.999%) MoO_3 in 20 mL concentrated HCl with heating. After being cooled, the solution was diluted to 100 mL and stored. The Mo(VI) working solutions were prepared from the stock solution as needed. MoO_3 was purchased from Tianjin Fuchen Chemical Reagent Factory (PRC).

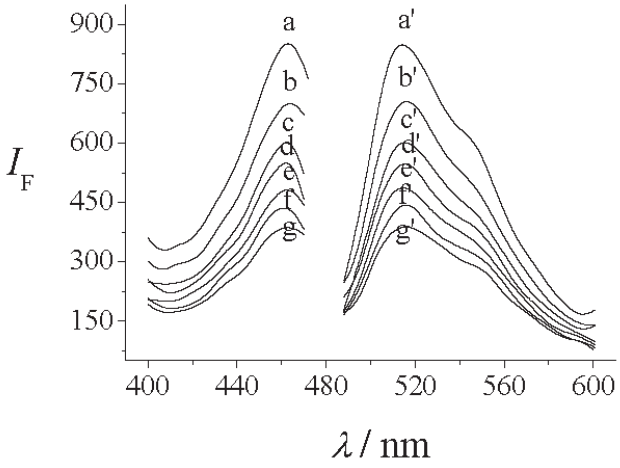

Figure 2. Fluorescence excitation and emission spectra. Excitation spectra of complex with different concentrations of BSA (a–g): 0, 0.10, 0.20, 0.30, 0.40, 0.50, 0.60 ($\times 10^{-5}$) M; a′–g′: emission spectra with the same concentration of BSA conditions: pH = 3.75, $V_{OP\ microemulsion}$ = 1.50 mL, $c_{Mo(VI)} = 1.00 \times 10^{-6}$ M, $c_{TH\text{-}PF} = 1.00 \times 10^{-6}$ M, λ_{ex} = 463 nm, λ_{em} = 516 nm, and slit widths = 5/15 nm.

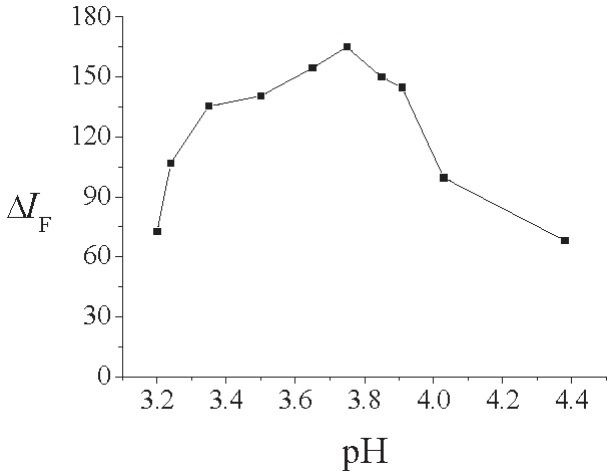

Figure 3. Effect of different buffer pH solutions. Conditions: $V_{OP\ microemulsion}$ = 1.50 mL, $c_{Mo(VI)} = 1.00 \times 10^{-6}$ M, $c_{TH\text{-}PF} = 1.00 \times 10^{-6}$ M, λ_{ex} = 463 nm, λ_{em} = 516 nm, and slit widths = 5/15 nm.

OP microemulsion was prepared as OP–*n*-butanol–*n*-heptane–water (5.0 + 3.3 + 0.8 + 90.9, w/w/w/w).

A pH 3.75 acetic acid (HAc)–sodium acetate (NaAc) buffer solution was used to control the acidity of the tested solutions.

Procedures

Solutions were added in the following order to a 10 mL colorimetric tube: 1.50 mL OP microemulsion, 2.00 mL pH 3.75 buffer solution, 0.10 mL of 1.00×10^{-4} M Mo(VI), 0.10 mL 1.00×10^{-4} M TH-PF, and an appropriate volume of protein working solution or sample solution. Then the mixture was diluted to 10 mL. After 15 min, the fluorescence intensity

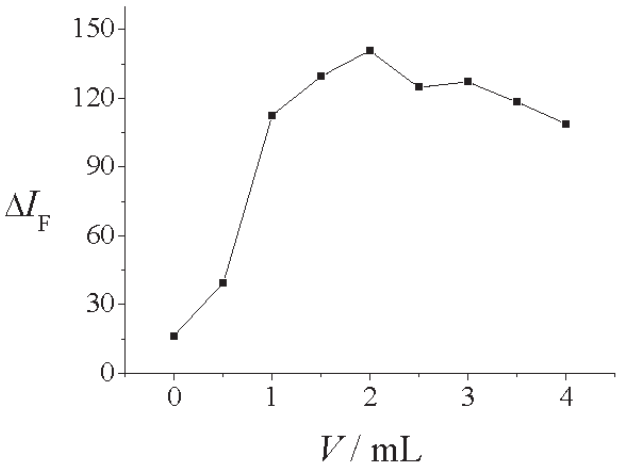

Figure 4. Effect of the dosage of buffer solution. Conditions: pH = 3.75, $V_{OP\ microemulsion}$ = 1.50 mL, $c_{Mo(VI)} = 1.00 \times 10^{-6}$ M, $c_{TH\text{-}PF} = 1.00 \times 10^{-6}$ M, λ_{ex} = 463 nm, λ_{em} = 516 nm, and slit widths = 5/15 nm.

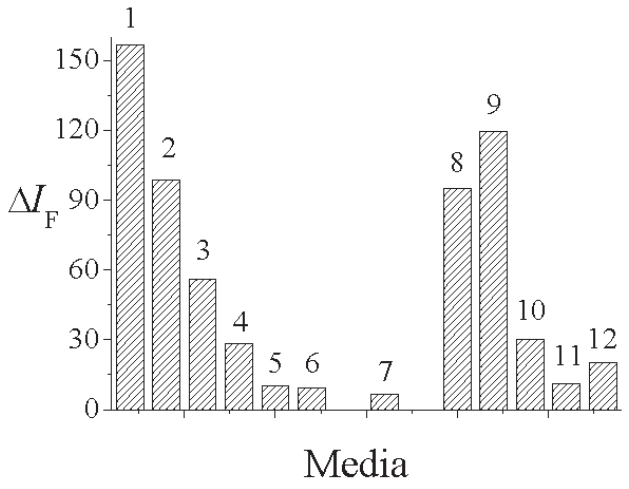

Figure 5. Effect of different media. 1–6 were OP, dodecyl phenol polyethoxy (TX)-10, sodium bis-(2-ethylhexyl) sulfosuccinate (AOT), Triton X-100, cetyltrimethyl ammonium bromide (CTAB), and Tween-80 microemulsion; 7 was water; 8–12 were OP, TX-10, Triton X-100, cetylpyridinium bromide (CPB), and Tween-80 micelle. Conditions: pH = 3.75, $c_{Mo(VI)}$ = 1.00 × 10^{-6} M, $c_{TH\text{-}PF}$ = 1.00 × 10^{-6} M, λ_{ex} = 463 nm, λ_{em} = 516 nm, and slit widths = 5/15 nm.

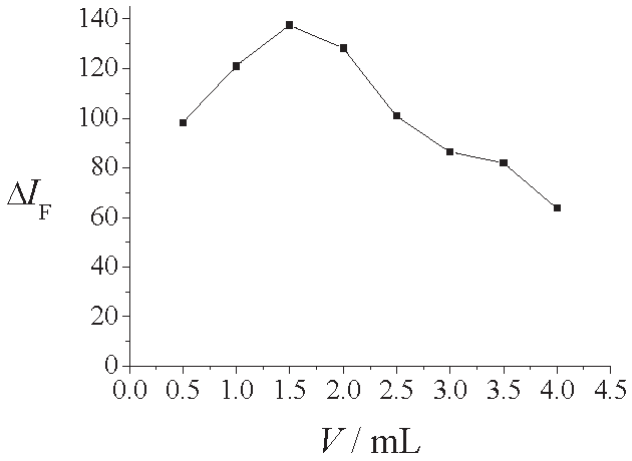

Figure 6. Effect of amount of OP microemulsion. Conditions: pH = 3.75, $c_{Mo(VI)}$ = 1.00 × 10^{-6} M, $c_{TH\text{-}PF}$ = 1.00 × 10^{-6} M, λ_{ex} = 463 nm, λ_{em} = 516 nm, and slit widths = 5/15 nm.

of the mixture was measured with the following settings of the spectrofluorometer: excitation wavelength 463 nm; emission wavelength 516 nm; excitation and emission slit widths were 5 and 15 nm, respectively. The sensitivity of this system was expressed by:

$$\Delta I_F \ (\Delta I_F = I_{F0} - I_F)$$

where I_{F0} is the fluorescence intensity of the mixture and I_F is the fluorescence intensity of the solution to which protein was added.

Results and Discussion

Spectral Characteristics

The fluorescence spectra of TH-PF-Mo(VI) and TH-PF-Mo(VI)-BSA at pH 3.75 are shown in Figure 2. TH-PF-Mo(VI) has a fluorescence emission spectrum with its maximum located at 516 nm and an excitation spectrum with its maximum wavelength at 463 nm. When BSA was added into the TH-PF-Mo(VI), the excitation and emission wavelengths were unchanged while the intensities of the peaks decreased significantly. The decrease depended on the concentration of protein. It can be concluded that the TH-PF-Mo(VI) complex can be used as a sensitive and valid fluorescence probe for protein.

Effect of Acidity

The effect of acidity on the system was investigated, and the results are shown in Figure 3. It can be seen that the acidity of the system has a significant effect on the fluorescence intensity, ΔI_F. ΔI_F reached its maximum and remained constant at pH 3.50–3.90. Therefore, pH 3.75 HAc–NaAc buffer solution was chosen for this assay. This choice can be explained as follows: BSA is charged positively and reacts with TH-PF-Mo(VI) complex by electrostatic forces. As the pH increases, the more negative charge on the TH-PF-Mo(VI) complex enhances the interaction between TH-PF-Mo(VI) complex and BSA. However, the increase in pH also causes an increase in negative charge on BSA, which in contrast weakens the binding of TH-PF-Mo(VI) complex and BSA. These 2 opposite effects of pH result in the strongest binding and the maximum ΔI_F at pH 3.75.

It was found that the amount of buffer solution also has an effect on ΔI_F, and the results are displayed in Figure 4. ΔI_F

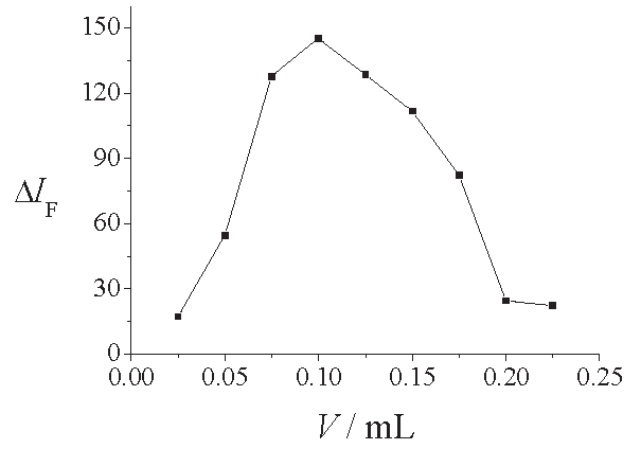

Figure 7. Effect of amount of TH-PF-Mo(VI) with fixed ratio of $n_{TH\text{-}PF}$:$n_{Mo(VI)}$ = 1:1. Conditions: pH = 3.75, $V_{OP\ microemulsion}$ = 1.50 mL, λ_{ex} = 463 nm, λ_{em} = 516 nm, and slit widths = 5/15 nm.

Table 1. Working curves of some proteins

Protein	Regression equation (ρ = μg/mL)	Correlation coefficient (r)	Linear range, μg/mL	Detection limit, ng/mL
BSA	$\Delta I_F = 33.71\ \rho + 109.76$	0.995	0–7.00	5.65
HSA	$\Delta I_F = 34.66\ \rho + 63.96$	0.995	0–9.00	5.50
Ova	$\Delta I_F = 60.68\ \rho + 16.79$	0.996	0–2.50	3.14
γ-G	$\Delta I_F = 24.24\ \rho + 0.54$	0.988	0–5.00	7.86
H-γ	$\Delta I_F = 31.22\ \rho + 42.70$	0.983	0–7.00	6.10

reached its maximum when 1.50–2.50 mL pH 3.75 HAc–NaAc buffer solution was added into the system. Thus, 2.00 mL of pH 3.75 HAc–NaAc buffer solution was chosen for use in the method.

Effect of Different Media

In order to increase the sensitivity of the system, various surfactants were chosen for investigating the effect of media on ΔI_F. As seen in Figure 5, the influence of various surfactants on the sensitivity of the system is different. The effect of OP microemulsion was the most remarkable.

The amount of OP microemulsion had a substantial effect on the sensitivity of the system due to its different sensitization and solubilization by changing the microenvironment. With the procedures described above, the effect of different amounts of OP microemulsion on the ΔI_F was investigated, and the results are shown in Figure 6. For 2.00 μg/mL BSA, maximum ΔI_F was obtained when the OP microemulsion was in the range of 1.40–1.70 mL. Therefore, 1.50 mL was chosen for the method.

Effect of the Proportion and Amount of Complex Probe

The effect of the proportion of complex probe, that is, the proportion of the amount of TH-PF to the amount of Mo(VI), was examined by changing the concentration of one component of the complex while keeping the other constant. The results indicated that ΔI_F reached maximum value when the proportion was 1:1.

At the same time, the amount of the complex probe had a remarkable effect on the intensity of fluorescence of the system (Figure 7). It was found that ΔI_F reached a maximum value and remained constant in the range of 0.09–0.12 mL TH-PF-Mo(VI). Therefore, the optimum amount of complex probe for this assay was 0.10 mL.

Reaction Time and Stability

At room temperature, TH-PF-Mo(VI) complex combined with protein rapidly to form a stable compound, and ΔI_F reached a maximum 10 min after all of the reagents had been added and remained stable for about 2 h. ΔI_F gradually decreased after 2 h. Therefore, 15 min was set as the standard for measurements.

Influence of Protein Denaturation

To be thermally denatured, a portion of a working solution of protein in a colorimetric tube was heated in a boiling water bath for 30 min and then cooled rapidly in ice water to room temperature. The denaturation of protein may be due to the breach of the secondary bond that holds the structure together; it is known that the strenuous vibration between molecules caused by high temperature may lead to the breach of the secondary bond. It was found that ΔI_F for denatured BSA was distinctly larger than that of corresponding native protein at the same level. At the same time, the fluorescence intensity of denatured protein gradually decreased with time owing to the relatively poor stability. This can be explained as follows: the structure of the denatured protein is loose, and, therefore, hydrophilic groups are exposed to the outside, making the reaction easy and enabling more TH-PF-Mo(VI) complex to

Table 2. Effect of coexisting substances

Foreign substance	Added, μg	Relative error, %
L-Tryptophan	200	–0.33
L-Histidine	100	–4.66
L-Arginine	100	7.21
L-Methionine	40	–8.00
L-Leucine	200	–2.60
L-Cysteine	200	–9.99
L-Tyrosine	40	–4.5
Glycine	200	5.37
DL-Valine	200	–1.41
L-Glutamic acid	40	5.46
L-Lysine	40	–8.9
DL-α-Alanine	200	–6.22
Glucose	200	–5.87
Al^{3+}	50	–6.84
Cu^{2+}	20	–7.3
Fe^{3+}	10	9.2
Zn^{2+}	100	8.73
Ca^{2+}	18	–6.80

Table 3. Results for the determination of protein in human serum samples

Sample[a]	Found (n = 11), g/L	Mean[b], g/L	RSD, %	Recovery, %	Bradford (ref. 2) method, g/L
1	68.44, 67.73, 68.23, 68.20, 69.77, 70.60, 68.48, 69.27, 70.13, 70.20, 69.55	69.15 ± 1.18	1.41	97.6	68.53
2	70.73, 70.69, 71.42, 72.91, 70.32, 73.12, 72.43, 71.84, 72.17, 71.85, 72.60	71.73 ± 1.11	1.33	102.8	71.26
3	83.17, 84.66, 84.52, 83.87, 81.85, 82.96, 82.47, 81.93, 83.44, 81.66, 82.07	82.96 ± 1.08	1.29	99.07	82.15

[a] 1–3 were 3 different samples of human serum.

[b] Results expressed as: $\bar{x} \pm st / \sqrt{n}$ (n = 11), where $\bar{x}$ is the mean of n observations of x, s is the standard deviation, and t is distribution value chosen for the desired confidence level. Theoretical value at 95% confidence limit: t = 2.78.

bind with protein. Furthermore, the size of the TH-PF-Mo(VI)-BSA compound becomes much larger, which would lead to the decrease of fluorescence intensity.

Calibration Curves and Sensitivity

Under the optimum conditions selected above, a linear relationship was obtained between ΔI_F and the concentration of proteins such as BSA, HSA, γ-G, and Ova. As shown in Table 1, ΔI_F is in proportion to the concentration of BSA in the linear range of 0–7.00 μg/mL, and the detection limit of BSA is 5.65 ng/mL. The linear ranges of HSA, γ-G, and Ova are 0–9.00, 0–5.00, and 0–2.50 μg/mL, respectively. The detection limits are 5.50, 7.86, and 3.14 ng/mL, respectively.

Influence of Coexisting Substances

Under the optimum conditions, the influence of various substances, including metal ions and amino acids, on the system was tested. The results are summarized in Table 2. None of the substances interfered with the determination at a relative error level of less than ±10%, indicating that the method has a good selectivity. Therefore, no special sample preparation is needed before determination.

Analysis of Real Samples

The present method was applied to determine protein in human serum samples. Human serum was mainly composed of HSA and γ-G with a mass ratio of 2:1. It can be seen from Table 1 that HSA and γ-G have different responses, so the standard protein (H-γ) solution was obtained according to the following mass ratio: HSA:γ-G = 2:1, and the regression equation of H-γ was used (*see* Table 1). An appropriate amount of the solution was transferred to a 10 mL color comparison tube and determined using the regression equation of H-γ. The results are shown in Table 3. It can be seen that the results were in good agreement with those obtained by the Bradford method (2).

As can be seen from Table 3, the recovery of protein was 97.6–02.8% and the relative standard deviation (RSD) values in all instances were less than 1.5%. Therefore, this method is fit for routine analysis of protein in human serum with high precision and accuracy.

Conclusions

A novel fluorescence probe for the determination of protein was developed based on the change of fluorescence caused by protein. The technique is useful as a quantitative method in the chemical, biochemical, and food fields. This method has the following advantages: high sensitivity, rapid reaction, simple methodology, and relatively few interfering substances.

Acknowledgments

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References

(1) Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265–275

(2) Bradford, M.M. (1976) *Anal. Biochem.* **72**, 248–254

(3) Flores, R. (1978) *Anal. Biochem.* **88**, 605–611

(4) Rodkey, F.L. (1965) *Clin. Chem.* **11**, 478–487

(5) Hu, Q.L., Zhao, F.L., & Li, K.A. (2002) *Chin. Chem. Lett.* **13**, 71–72

(6) Fujita, Y., Mori, I., & Kitano, S. (1983) *Bunseki Kagaku* **32**, E379–E386

(7) Fujita, Y., Mori, I., & Matsuo, T. (1995) *Bunseki Kagaku* **44**, 733–738

(8) Hu, X.L., & Feng, P. (1998) *J. SW China Normal Univ.* **23**, 561–566

(9) Huang, C.Z., Li, Y.F., & Feng, P. (2001) *Fresenius J. Anal. Chem.* **371**, 1034–1036

(10) Zhang, X.W., Zhao, F.L., & Li, K.A. (2001) *Microchem. J.* **68**, 53–59

(11) Ma, C.Q., Li, K.A., & Tong, S.Y. (1996) *Anal. Chim. Acta* **333**, 83–88

(12) Qin, W.W., Gong, G.Q., & Song, Y.M. (2000) *Spectrochim. Acta A* **56**, 1021–1025

(13) Zhang, H.M., Zhu, Z.W., & Li, N.Q. (1999) *Fresenius J. Anal. Chem.* **363**, 408–412

(14) Sun, C.X., Yang, J.H., Li, L., Wu, X., Liu, Y., & Liu, S.F. (2004) *J. Chromatogr. B* **803**, 173–190

(15) Wei, Q., Yang, J.H., Zhong, Y., Chang, G.H., & Du, B. (2002) *Talanta* **58**, 419–426

(16) Du, B., Yang, J.H., Wei, Q., & Chang, G.H. (2002) *Anal. Lett.* **35**, 895–908

(17) Guo, Z.X., Hao, Y.M., Cong, X., & Shen, H.X. (2000) *Anal. Chim. Acta* **403**, 225–233

(18) Guo, Z.X., & Shen, H.X. (1999) *Anal. Chim. Acta* **396**, 83–90

(19) Guo, Z.X., & Shen, H.X. (1999) *Spectrochim. Acta A* **55**, 2919–2925

(20) Guo, Z.X., & Shen, H.X. (2000) *Anal. Chim. Acta* **408**, 177–182

(21) Schwuger, M.J., Stickdorn, K., & Schmaker, R. (1995) *Chem. Rev.* **95**, 849–864

(22) Du, B., Wei, Q., Wang, S.R., & Yu, W.L. (1997) *Talanta* **44**, 1803–1806

(23) Wei, Q., & Du, B. (1998) *Talanta* **45**, 957–961

(24) Wei, Q., Yang, J.H., Zhong, Y., Chang, G.H., & Du, B. (2002) *Talanta* **58**, 419–426

(25) Wei, Q., Yan, L.G., Chang, G.H., & Ou, Q.Y. (2003) *Talanta* **59**, 253–259

(26) Du, B., Wei, Q., & Xu, G.Y. (1999) *Anal. Lett.* **32**, 1011–1021

(27) Du, B., Yang, J.H., Wei, Q., & Chang, G.H. (2002) *Anal. Lett.* **35**, 895–908

(28) Wei, Q., Wu, D., Du, B., Zhang, H., & Ou, Q.Y. (2004) *Chin. J. Chem.* **22**, 714–718

(29) Wei, Q., Wu, D., Du, B., & Ou, Q.Y. (2004) *Chin. Chem. Lett.* **15**, 667–670

(30) Wei, Q., Du, B., Wu, D., & Ou, Q.Y. (2004) *J. Anal. Chem.* **32**, 370–373

(31) Wei, Q., Du, B., Wu, D., Zhang, H., Li, Y., & Ou, Q.Y. (2004) *J. Anal. Chem.* **32**, 1509–1512