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Study on the Reaction of Proteins with 5′-Nitrosalicylfluorone-molybdenum(VI) Complex by Spectrophotometry in PVA 124 Microemulsion

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The application of 5′-nitrosalicylfluorone (5′-NSF)-molybdenum(VI) complex as a spectroscopic probe was studied. In the buffer medium of HOAc-NaOAc at 3.45 and in the presence of PVA 124 microemulsion, 5′-NSF-Mo(VI) complex combines protein rapidly to form a stable compound, leading to an absorbance decrease at 525 nm of 5′-NSF-Mo(VI) complex. According to this change, microdetermination of protein has been described. Bovine serum albumin (BSA) could be determined in the linear range of 0—16 μg•mL^{-1} with the detection limit of 11 ng•mL^{-1}. Many amino acid and metal ions studied do not interfere with the assay. The method possesses high sensitivity as well as high selectivity. It can be used to determine protein in human urine and milk powder successfully. The relative standard deviations are in all instances less than 4.7%, and the recoveries are between 97.6% and 106.0%. Moreover, the binding number of BSA with the complex, which is determined by using molar ratio, Rosenthanl graphic and slope ratio methods, is in good agreement with each other.

Keywords protein, spectrophotometry, 5′-NSF-Mo(VI) complex, microemulsion

Introduction

Microemulsion, which consists of surfactant, consurfactant, water and oil at appropriate ratios, is colorless, transparent or subtransparent, low viscosity and thermodynamically stable system. Compared with micellar system, microemulsion has lower surface tension and strong soluble power to organic and inorganic substances.[1] Microemulsion, as the media, is not only applied to the determination of many metal ions by different methods[2-5] in our previous research, but also firstly introduced to protein determination.

Proteins are very important in living beings and they take part in almost all of the life processes. They have much in common with metabolism, immunity and life evolution. At the same time, they can offer us lots of information about ourselves. So the quantitative study of protein is very important and valuable in biochemical and clinical test as well as food test.

Many methods for the determination of proteins have been put forward, including resonance light scattering, chemiluminescence, electrochemical assays, Kjeldahl, *etc*.[6-10] In recent years, spectroscopic probe has been widely applied in protein determination. There are three kinds of probes for spectrophotometric method, which refer to metal probe, dye probe and dye-metal complex probe. Metal probe methods, such as silver staining,[11] Lowry[12] and so on, have some limitations. For example, the Lowry assay is poorly sensitive and has serious interference from coexisting substances. The silver staining method suffers multiple steps, high background and toxicity of formaldehyde. Hence, dye and dye-metal complex probes have been developed. Dye probe approaches include Bradford,[13] Arsenazo M,[14] Chromotrope 2R,[15] *etc*. Considering the advantages and disadvantages related to the above assays, dye-metal complex probe of protein, which has high sensitivity, long stability and good selectivity, has been considerably noticed. Arsenazo K-Cu(II), chromazurol-Al(III), and so forth have also been reported.[16-18]

Trihydroxyfluorone reagents are very sensitive to some metals.[19-23] However, much attention has not been paid on them in terms of determination of protein. Fujita[24] and Shen[25] have proposed spectrophotometric methods for protein determination using *o*-sulfophenylfluorone-U(VI) and 4-azochromotropic acid phenylfluorone-Mo(VI). Up to date, 5′-nitrosalicylfluorone(5′-NSF)-Mo(VI) complex as a spectroscopic probe for protein is not available. The structure of 5′-NSF is presented in Figure 1. In this paper, based on the interaction of proteins with 5′-NSF-Mo(VI) complex as a probe, a novel determination method has been devel-

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oped in PVA 124 microemulsion. Microemulsion has increased the sensitivity of this system with its sensitization and solubilization. It has higher molar absorptivity than that of micelle media. This assay is much more sensitive than most of reported dye and dye-metal complex probe methods.

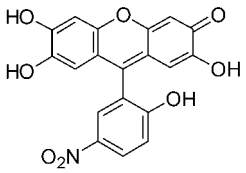

Figure 1 Structure of 5′-NSF.

The precision and the accuracy of the proposed method are achieved by analysis of human urine and milk powder samples with satisfactory results.

Experimental

Apparatus

A Shimadzu Model UV-3000 spectrophotometer with 1.0 cm-quartz cells was employed for absorption spectral records and absorbance measurements. A PHS-3B acidimeter was used in determining pH values.

Reagents

Unless otherwise mentioned, all chemicals were of analytical reagent grade and doubly distilled water was used.

Standard stock solutions of proteins were prepared by dissolving commercial products in doubly distilled water and stored at −4 ℃. Proteins used in this study include bovine serum albumin (BSA), human serum albumin (HSA), Ovalbumin (Ova) and Lysozyme (Lys), which were purchased from Sigma. The concentrations of their working solutions were 100 μg•mL^{-1}.

5′-NSF solution (3.0×10^{-4} mol•L^{-1}) was prepared by dissolving 0.0114 g of 5′-NSF in ethanol containing several drops of 1∶1 sulfuric acid and brought to 100 mL in a volumetric flask with pure ethanol.

PVA 124 microemulsion was prepared with PVA 124∶*n*-butanol∶*n*-heptane∶water=5.0∶3.3∶0.8∶90.9 (mass ratio).

A Mo(VI) stock solution (0.1 mol•L^{-1}) was prepared by dissolving 1.4394 g of spectroscopically pure MoO_3 in 20 mL of concentrated HCl, under heating. After cooling, the solution was diluted to 100 mL and stored. The Mo(VI) working solutions were prepared from stock as needed.

A pH 3.45 of Michaelis buffer was obtained by mixing 0.1 mol•L^{-1} NaOAc and 0.1 mol•mL^{-1} HOAc at a 1∶16 (volume ratio), and adjusting to pH 3.45.

Procedure

The following solutions were successively added into a 10 mL-colorimetric tube: 1.5 mL of PVA 124 microemulsion, 2.0 mL of Michaelis buffer (pH=3.45), 1.0 mL of 3.0×10^{-4} mol•L^{-1} Mo(VI), 1.0 mL of 3.0×10^{-4} mol•L^{-1} 5′-NSF ethanolic solution and an appropriate volume of protein or sample working solution. Then, the mixture was diluted to the mark with doubly distilled water and mixed thoroughly. After lying aside for 10 min at room temperature, the absorbance was measured with 1.0 cm-cell at 525 nm against reagent blank.

Results and discussion

Absorption spectral characteristics

The absorption spectra of 5′-NSF, 5′-NSF-BSA, 5′-NSF-Mo(VI) and 5′-NSF-Mo(VI)-BSA in PVA 124 microemulsion medium at pH 3.45 are shown in Figure 2.

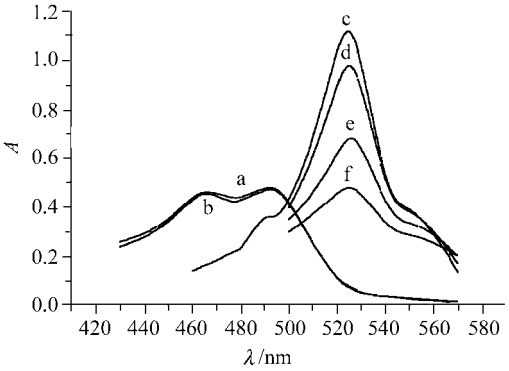

Figure 2 Absorption spectra of (a) 5′-NSF; (b) 5′-NSF-BSA; (c) 5′-NSF-Mo(VI); (d)—(g) 5′-NSF-Mo(VI)-BSA, conditions: [5′-NSF]=1.5×10^{-5} mol•L^{-1}, [Mo(VI)]=1.5×10^{-5} mol•L^{-1}, $V_{PVA\ 124}$=1.5 mL, pH=3.45; (d) [BSA]: 2 μg•mL^{-1}; (e) [BSA]: 4 μg•mL^{-1}; (f) 6 μg•mL^{-1}.

It could be seen that BSA did not react with 5′-NSF to form a binary complex, since the spectral of 5′-NSF and 5′-NSF-BSA systems overlapped almost completely. But the absorption spectra above changed significantly with Mo(VI) in solution, which gave maximum absorption peak at 525 nm with 65 nm of red shift. Meanwhile, with the increase of concentration of BSA, the absorbance at 525 nm decreased and was proportional to it. Therefore 5′-NSF-Mo(VI) complex could serve as a sensitive and valid spectroscopic probe for protein and the quantification of microamounts of protein was feasible.

Selection of medium

PVA 124 micell solution, PVA 124 microemulsion, Tween 20 micell solution, Tween 20 microemulsion and so on were chosen as developing medium, respectively. Absorbance of different media above at pH=3.45 was measured at various wavelengths. The results were given in Figure 3.

Obviously, each microemulsion was more sensible than its corresponding micelle. For microemulsion media, their sensibilities were as follows:

PVA 124>Tween 20>Triton X-100>Tween 80

Hence, PVA 124 microemulsion was optimum medium for further study.

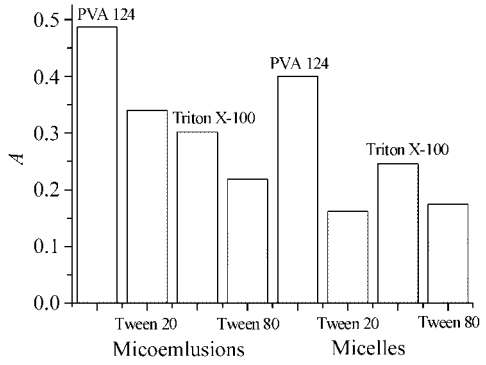

Figure 3 Effect of various microemulsions and micelles on *A*, condition: [5′-NSF]=3.0×10^{-5} mol•L^{-1}, [Mo(VI)]=3.0×10^{-5} mol•L^{-1}, pH=3.45, [BSA]=4 μg•mL^{-1}.

In the case of PVA 124 microemulsion medium, the effect of amounts of microemulsion was studied by varying microemulsion volume. The results showed that the maximum and constant absorbance was attained when PVA 124 microemulsion was added in volumes of 1.2—1.8 mL. Thus 1.5 mL was the preferred additive.

Effect of pH

The influence of pH on this assay was investigated from pH values of 2.15—3.85. The absorbance of the system was greatly affected by pH. When the pH of NaOAc-HOAc buffer solution varied in the range of 2.85—3.70, the absorbance reached the maximum value and remained constant, after that the absorbance sharply decreased. Hence, Michaelis buffer solution NaOAc-HOAc (pH = 3.45) was accepted to throughout the study.

Effect of 5'-NSF and Mo(VI)

Mo(VI) was superior to the metal ions such as U(V), Sn(IV), Ga(III) and Ti(IV) in aspects of sensitivity.

The influence of molar ratio of 5′-NSF to Mo(VI) was tested by changing the concentration of 5′-NSF and keeping those of Mo(VI) and BSA at 3.0×10^{-5} mol•L^{-1} and 4 μg•mL^{-1}, respectively. The absorbance recorded as a function of 5′-NSF amount was displayed. From the experiment, the maximal and stable absorbance was found to be in the range of 0.9—1.1. That is to say, 1∶1 ratio of 5′-NSF to Mo(VI) was suitable as a result of its benefit for the binding reaction of BSA.

On the other hand, the concentration of 5′-NSF-Mo(VI) complex had an effect on the sensitivity at constant BSA concentration. For 4 μg•mL^{-1} BSA, the highest sensitivity was reached when the complex concentration was between 2.7×10^{-5} mol•L^{-1} and 3.3×10^{-5} mol•L^{-1}. Hence, 3.0×10^{-5} mol•L^{-1} was selected for further study which was about 510 times higher than that of BSA.

Reaction of time and stability

The interaction between 5′-NSF-Mo(VI) complex and BSA reached the equilibrium in 5 min, and the system was stable for 3 h at room temperature. After that the absorbance decreased gradually while standing for longer time, even the precipitation appeared. So there was enough time to make the measurements.

Effect of ionic strength

The effect of NaCl concentration on the interaction was examined. The result showed that the absorbance of the solution was stable when the ionic strength was lower than 2.0 g•L^{-1} (NaCl), and dropped when it was high than 2.0 g•L^{-1}. This phenomenon was ascribed to the decrease of the interaction between 5′-NSF-Mo(VI) and BSA because of the shielding effect of the charges on BSA with increasing ionic strength. Thus, in this work, no NaCl solution was recommended.

Effect of protein denaturation and organic solvent

A portion of BSA working solution was heated in a boiling water bath for 30 min, then cooled to room temperature. It was found that the absorbance caused by denatured BSA (4 μg•mL^{-1}) fell approximately 15.7%, suggesting that the secondary and tertiary structures of BSA contribute partly to the binding of 5′-NSF-Mo(VI) complex and BSA.

To examine the effect of organic solvent concentration, different volumes of absolute methanol, ethanol as well as acetone were added just after the addition of 5′-NSF. It could be observed that the absorbance was influenced by additional organic solvent. This may be due to a change in micro environment for the binding reaction.

Working curves and sensitivity

Under the conditions given above, a linear relationship was obtained between the absorbance and the concentration of proteins such as BSA, HSA, Ova and Lys. All the analytical parameters were summarized in Table 1. Their sensitivities had some differences probably because of the difference numbers of amino group in different proteins.

In Table 2, characteristics of the method were compared with those of similar reported spectrophotometries for protein determination. This assay proposed was sensitive, simple and rapid. It did not require heating and standing for a long time. The sensitivity was much higher than that of those currently used and reported dye and dye-metal complex spectrophotometric methods, including those using other complexes of trihydroxyfluorone reagents.

Tolerance for foreign substances

A number of coexisting substances including amino acids and metal ions were estimated for their effects on the determination of 4 μg•mL^{-1} BSA. As displayed in Table 3, there were few tested-ions and amino acids interfering with this assay. Therefore, no special preparation was needed to perform before sample determination.

Table 1 Parameters for proteins determination

Proteins	Linear regression equation [ρ/(μg•mL^{-1})]	Linear range/ (μg•mL^{-1})	r	ε/ (L•mol^{-1}•cm^{-1})
BSA	$A=0.121+0.0934\rho$	0—16	0.990	8.41×10^6
HSA	$A=0.193+0.0887\rho$	0—16	0.989	9.31×10^6
Lys	$A=0.198+0.0794\rho$	0—16	0.988	1.84×10^6
Ova	$A=-0.0606+0.0478\rho$	0—16	0.989	1.40×10^6

Table 2 Comparison of spectrophotometric methods for protein determination

Method	Linear range/ (μg•mL^{-1})	Sensitivity Times[a]	Reaction time/ min	Reference
Pyogallol red-Mo(VI)	0—40	1	20	31
Pyrocatechol violet-Mo(VI)	0—30	1.2	15	28
3,4,5,6-Tetrachlorogallein-Mo(VI)	0—15	3	10 (50 ℃)	29
p-Chlorophenylfluorone-Mo(VI)	0—12	4.5	3	26
2-(5-Bromo-2-pyridylazo)-5-(*N*-phenyl-*N*-sulfopropylamino)phenol-Co(II)	0—7.0	5	10 (50 ℃)	30
Dibromohydroxyphenylfluorone-Mo(VI)	0—8.0	5	10	27
4-Azochromotropic acid phenyl-fluorone-Mo(VI)	0—18	0.8	10	25
Arsenazo K-Cu(II)	10—140	0.3	20	16
Chromotrope 2R	0—100	0.2	20	15
Chromazurol-Al(III)	1.25—25	0.9	30	17
p-Chloranil	0—120	0.5	5 (37 ℃)	32
Arsenazo III-Yb(III)	1—35	1.3	1	18
Sulfochlorophenol S	0—140	0.2	5	33
Dibromophenylfluorone-Ti(IV)	1—6	1.9	15 (40—45 ℃)	34
Dibromohydroxyphenylfluorone-Mo(VI)	0—20	0.8	5	35
5′-Nitrosalicylfluorone-Mo(VI)	0—16	6.2	5	This work

[a] The sensitivity was expressed relative to that of the Pyogallol red-Mo(VI) method.

Table 3 Effect of foreign substances on the determination of 4 μg•mL^{-1} BSA

Interfering substances	Added/ μg	Relative error/%	Interfering substances	Added/ μg	Relative error/%
Glucose	250	2.16	*L*-His	200	5.79
L-Try	100	5.34	*L*-Arg	200	−5.97
L-Met	200	−4.37	*DL*-Val	200	−6.47
L-Leu	100	5.76	*DL*-*α*-Ala	100	4.34
L-Lys	50	2.96	Zn(II)	50	6.45
Cr(III)	50	1.38	Ni(II)	50	2.87
Co(II)	50	−1.65	Cu(II)	50	4.91

Measurement of binding numbers

To some extent, the binding number can reflect on the binding ability and the sensitivity.[31] Three methods were applied to measure the binding number of 5′-NSF-Mo(VI) complex and BSA. The result determined by three methods was in good agreement with each other.

One of them is the molar ratio method. The binding number was examined by varying the concentration of 5′-NSF-Mo(VI) complex when the concentration of BSA remained constant (4 μg•mL^{-1}). And the other procedures followed the previously described procedure. Then the absorbance as a function of 5′-NSF-Mo(VI) concentration was obtained. From the experimental result, it was obvious that the graph had a point of intersection. Hence, the ratio of 5′-NSF-Mo(VI) complex concentration corresponding to it to BSA concentration was the binding number. The values of *n* for BSA and Ova were 102 and 88, respectively.

Another method is slope ratio method. Changing the concentration of BSA, keeping that of 5′-NSF-Mo(VI) complex constant (3.0×10^{-5} mol•L^{-1}) and sufficient excess, then one linear section, whose slope was k_1, could be gained. On the contrary, another was obtained in the same way when BSA concentration was kept at 3.0×10^{-7} mol•L^{-1} and its slope was k_2. Then, the ratio of k_2 to k_1 was the binding number. Therefore, the binding number of 5′-NSF-Mo(VI) complex and BSA was 100.

The last method is the Rosenthanl graphic method, which is considered as a modification of the Scatchard method.[36] From the slope and intercept of the linear section, *n* and *K* were gained to be 101 and 7.23×10^5 L•mol^{-1} correspondingly.

Application to sample analysis

A certain amount of fresh urine sample was directly transferred to 10 mL-color comparison tube without pre-treatment and total protein was measured. As for milk powder sample, it was pretreated in the following way prior to the determination of protein. Weigh out 0.2 g of the sample accurately and place in a mortar followed by the addition of 4 mL of HOAc-NaOAc buffer solution. Then the mixture was ground for 5 min. After that, the aqueous phase was transferred into a 100 mL-calibrated flask and diluted to the mark with doubly distilled water. An appropriate amount of the sample solution was transferred to 10 mL-color comparison tube after being mixed thoroughly and determined as described above. The results are given in Tables 4 and 5.

Table 4 Results for the determination of protein in urine

Samples[a]	Found value/ ($\mu g \cdot mL^{-1}$)	Mean/ ($\mu g \cdot mL^{-1}$)	Recovery/ %	*R.S.D*/ %
$\mathbf{A_1}$	92.00, 90.82, 90.18, 91.47, 92.46	91.39	97.6	1.0
$\mathbf{A_2}$	86.54, 84.61, 83.86, 86.33, 87.18	85.70	106.0	1.6
$\mathbf{A_3}$	81.29, 80.22, 79.58, 82.47, 83.86	81.48	102.4	2.1

[a] $\mathbf{A_1}$, $\mathbf{A_2}$ and $\mathbf{A_3}$ were taken from fresh human urine samples.

Table 5 Analytical results for milk powder sample

Samples[a]	Found value/ %	Mean/ %	Recovery/ %	*R.S.D*/ %
B	17.75, 19.11, 18.61, 16.90, 17.88	18.05	103.4	4.7

[a] **B** was milk powder sample.

For each sample, five parallel experiments were conducted and the maximum relative standard deviation (*R.S.D*) was 4.7%. The recovery of the sample has also been determined. Fixed amounts of BSA standard were added to five samples of known BSA content and the mixtures were analyzed, respectively. The recovery was in the range of 97.6%—106.0%. All these presented sufficient precision and high accuracy.

Conclusion

The spectrophotometric determination method of protein has been investigated using 5′-NSF-Mo(VI) complex as a probe. From the results of analysis it is suggested that the method is selective, accurate and stable. Moreover, the method is more sensitive, especially by introducing PVA 124 microemulsion to the system. Hence, the assay is worthy of use for the determination of biological samples.

References

1 Schwuger, M. J.; Stickdorn, K.; Schmacke, R. *Chem. Rev.* **1995**, *95*, 849.
2 Du, B.; Wei, Q.; Wang, S. R.; Yu, W. L. *Talanta* **1997**, *44*, 1803.
3 Wei, Q.; Du, B. *Talanta* **1998**, *45*, 957.
4 Du, B.; Wei, Q.; Xu, G. Y. *Anal. Lett.* **1999**, *32*, 1011.
5 Wei, Q.; Yan, L.G.; Chang, G. H.; Ou, Q. Y. *Talanta* **2003**, *59*, 253.
6 Huang, C. Z.; Li, Y. F.; Feng, P.; Li, M. *Fresenius J. Anal. Chem.* **2001**, *371*, 1034.
7 Tsukagoshi, K.; Tanaka, A.; Nakajima, R.; Hara, T. *Anal. Sci.* **1996**, *12*, 525.
8 Johll, M. E.; Willimas, D. G.; Johnson, D. C. *Electroanalysis* **1997**, *9*, 1397.
9 Chen, L. H.; Zhao, F. L.; Li, K. A. *Chin. J. Chem.* **2002**, *20*, 368.
10 Jiang, Z. L; Peng, Z. Li.; Liu, S. P. *Chin. J. Chem.* **2002**, *20*, 1566.
11 Oshawa, K.; Ebata, N. *Anal. Biochem.* **1983**, *135*, 409.
12 Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
13 Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
14 Hu, Q. L.; Zhao, F. L.; Li , K. A. *Chin. Chem. Lett.* **2002**, *13* , 71.
15 Hu, Q. L.; Zhao, F. L.; Li , K. A. *Chem. J. Internet* **2000**, *2*, 9.
16 Hu, Q. L.; Zhao, B. T.; Shen, Y. Y. *Chem. J. Internet* **2001**, *3*, 23.
17 Satio, Y.; Kanetsuna, A.; Okuka, H.; Mifune, M.; Odo, J.; Tanaka, Y. *Chem. Pharm. Bull.* **1986**, *34*, 746.
18 Ying, Y.; Huang, F. D.; Gao, Q. *Chin. J. Anal. Chem.* **2001**, *29*, 1205 (in Chinese).
19 Du, B; Yang, J. H.; Wei, Q.; Chang, G. H. *Anal. Lett.* **2002**, *35*, 895.
20 Wei, Q.; Yang, J. H.; Zhong, Y.; Chang, G. H.; Du, B. *Talanta* **2002**, *58*, 419.
21 Huang, B. H.; Luo, Z. M.; Zhong, L. F.; Yang, S. N. *Yejin Fenxi* **1998**, *18*, 7 (in Chinese).
22 Wang P.; Liu X. G.; Yang W. H. *Guangpu Shiyanshi* **1998**, *15*, 55 (in Chinese).
23 Al′bota, L. A.; Gutsulyak, R. B. *Ukr. Khim. Zh.* (*Russ. Ed.*) **1983**, *49*, 385.
24 Fujita, Y.; Mori, I.; Toyada, M.; Kato, K.; Nakamura, M.; Nakanishi, T. *Chem. Pharm. Bull.* **1990**, *38*, 956.
25 Guo, Z. X.; Shen, H. X. *Anal. Chim. Acta* **1999**, *396*, 83.
26 Guo, Z. X.; Shen, H. X. *Analyst* **1999**, *124*, 1093.
27 Guo, Z. X.; Hao, Y. M.; Cong, X.; Shen, H. X. *Anal. Chim. Acta* **2000**, *403*, 225.
28 Fujita, Y.; Mori, I.; Kitano, S. *Chem. Pharm. Bull.* **1984**, *32*, 4161.
29 Fujita, Y.; Mori, I.; Ikuta, K.; Nakahashi, Y.; Kato, K. *Chem. Pharm. Bull.* **1989**, *37*, 2452.
30 Mori, I.; Taguchi, K.; Fujita, Y.; Matsuo, T. *Anal. Lett.* **1995**, *28*, 225.
31 Fujita, Y.; Mori, I.; Kitano, S. *Bunseki Kagaku* **1983**, *32*, E379.
32 Zaia, D. A. M.; Verri, Jr, W. A.; Zaia, C. T. B. V. *Talanta* **1999**, *49*, 373.
33 Hu, Q. L.; Li, N.; Zhao, F. L.; Li, K. A.; Tong, S. Y. *Chin. J. Anal. Chem.* **1999**, *27*, 166 (in Chinese).
34 Tang, G. N.; Wei, W.; Wang, B. N. *Chin. J. Anal. Chem.* **1993**, *21*, 831 (in Chinese).
35 Chen, H. Q. *J. Northwest Normal Univ.* **2000**, *36*, 53 (in Chinese).
36 Rosenthanl, H. R. *Anal. Biochem.* **1967**, *20*, 525.

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