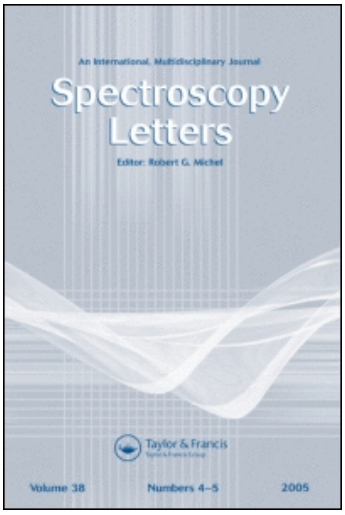

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Spectroscopic Determination of Cysteine with Alizarin Red S and Copper

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ABSTRACT The interaction between metal complex Cu^{2+}–ARS (Alizarin Red S) and l-cysteine was investigated via fluorescence and absorption spectroscopies. In pH 5.2 Britton–Robinson buffer, the addition of L-cysteine into Cu^{2+}–ARS system resulted in a fluorescence enhancement because cysteine reduced Cu^{2+} to Cu^{+}, which led to Cu^{2+}–ARS decompound, and ARS was released. The result was also supported by absorption spectroscopy change. A good linear response of fluorescence intensity as a function of cysteine concentration was obtained ranging from 1.0×10^{-6} to 4.0×10^{-5} mol L^{-1} with the detection limit as 1.08×10^{-7} mol L^{-1}. The introduced method has high selectivity over other amino acids such as cystine, tyrosine, tryptophan, methionine, and glycine. It was applied to determine cysteine in protein hydrolysate of fresh pig blood with recovery of 88.4–100.2%.

KEYWORDS Cu^{2+}–ARS complex, fluorescence spectroscopy, L-cysteine, oxidation-reduction reaction

INTRODUCTION

Thiol-containing L-cysteine (Cys) plays important roles in biological systems by its oxidation–reduction reaction.[1] It has been widely used in pharmaceutical products, food additives, and cosmetics. Many methods have been developed for the determination of Cys, which include high-performance liquid chromatography (HPLC),[2–4] capillary electrophoresis,[5] chemiluminescence,[6,7] electrochemistry,[8,9] spectrophotometry,[10–18] and spectrofluorimetry.[19–24] Among them, spectroscopic methods have advantages such as sensitivity, high selectivity, and easy operation. The determination of Cys using a copper (II)–catalyzed redox reaction has been previously reported.[13] Off-the-shelf and water-soluble Alizarin Red S (ARS) is a good chelating agent for metal ions like Cu^{2+}, Fe^{3+}, and Al^{3+}.[25,26] Some groups studied the interaction between ARS and BSA or DNA via electrochemistry method.[27,28] Obviously, ARS is an efficient photochemistry and electrochemistry reagent and has potential application in biological systems. In this paper, a convenient assay of Cys was established based on Cu^{2+}–ARS. Cu^{2+}–ARS emits weaker fluorescence than does ARS alone. The addition of Cys to Cu^{2+}–ARS solution caused the release of ARS due to the redox reaction between Cys and Cu^{2+}. The method has high selectivity toward Cys over other amino acids like cystine, tyrosine, tryptophan, methionine,

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and glycine due to the redox property of Cys. To our knowledge, it is the first time that Cu^{2+}–ARS complex was used in determining amino acids.

MATERIALS AND METHODS

Apparatus

All fluorescence measurements were carried out on a F-4500 spectrofluorimeter (Hitachi, Tokyo, Japan) equipped with a xenon lamp source and a 1.0-cm quartz cell, and the scan speed was $240\,nm\,min^{-1}$. Absorption spectra were recorded on a Shimadzu-2501 UV-Vis spectrophotometer (Kyoto, Japan) using a 1.0 cm quartz cell. All pH measurements were made with a pHS-3C digital pH-meter (Shanghai REX Instrument Factory, Shanghai, China) with a combined glass–calomel electrode.

Reagents

All chemicals were of analytical grade and were used without further purification. All solution was prepared using doubly distilled water.

Alizarin Red S (Shanghai Chemical Corp, Shanghai, China) solution was prepared by dissolving 0.0180 g ARS in 50 mL water to give a concentration of $1.0 \times 10^{-3}\,mol\,L^{-1}$. L-Cysteine, L-cystine, L-tyrosine, L-tryptophan, L-methionine, and glycine were all purchased from Sigma (St. Louis; MO). Cys solution with a concentration of $1.0 \times 10^{-2}\,mol\,L^{-1}$ was prepared with water daily. Cu^{2+} solution with the concentration of $1.0 \times 10^{-3}\,mol\,L^{-1}$ was prepared by dissolving 0.0250 g copper sulfate pentahydrate (Jiangsu Reagent Factory of Jintan County, Jiangsu, China) in 100 mL water. Cu^{+} solution with the concentration of $2.0 \times 0^{-4}\,mol\,L^{-1}$ was prepared by dissolving 0.0010 g CuCl in 50 mL acetonitrile.

Britton–Robinson (B-R) buffer was prepared by adding 2.71 mL 85% phosphoric acid, 2.36 mL acetic acid, and 2.4700 g boric acid to a 1000-mL flask and diluting the mixture with water to the mark and adjusting the pH value with $0.20\,mol\,L^{-1}$ NaOH.

Samples

Eight grams of fresh pig blood was dispersed in 100 mL water. AS 1.398 neutral protease was added to a proportion of 8000 U per gram of pig blood. The solution at pH 7.5 was hydrolyzed for 7 h in a 40°C water bath. It was decolored at 70°C using 2.0 g active carbon. Nitrogen gas was used throughout to prevent oxidizing. The filtered hydrolysate was stored at 0 ~4°C.

Procedure

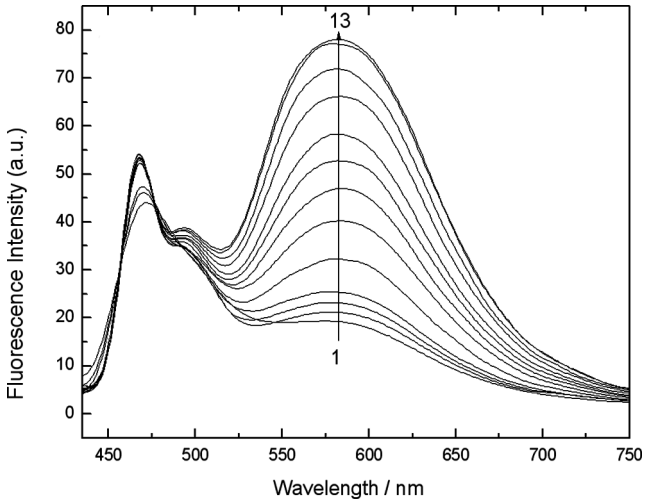
To a 10-mL colorimetric tube a certain amount of ARS and Cu^{2+} solution were added and stabilized for 0.5 h. The molar concentration ratio for ARS and copper sulfate was kept as 2:1. Subsequently, 1.0 mL B-R buffer solution with a pH value of 5.2 was added, which was followed by the addition of a standard solution of amino acid. Then the mixture was diluted to the mark with water. After 5 mins reaction at room temperature, fluorescence spectra were measured at excitation wavelength of 425 nm and the band slits of both excitation and emission were set as 5.0 nm. Absorption spectra were collected for the solution prepared the same way as those for fluorescence studies. When the real sample was determined, the standard solution of amino acid was substituted by the sample.

RESULTS AND DISCUSSION

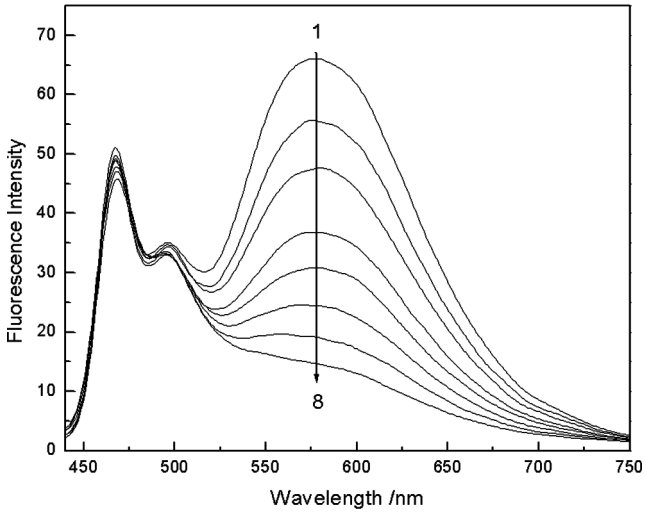
Fluorescence Titration of ARS–Cu(II) with Cys

Figure 1a shows emission spectral changes of Cu^{2+}–ARS upon addition of Cys. Cu^{2+}–ARS emitted weak fluorescence in pH 5.2 B-R buffer. However, an enhancement in fluorescence intensity was observed in the presence of Cys. ARS is a good fluorescence compound whose maximum emission wavelength centered at 580 nm in pH 5.2 B-R buffer media. The presence of Cu^{2+} led the fluorescence intensity of ARS decrease, and the spectral changes are shown in Fig. 1b. The fluorescence quenching was ascribed to the Cu^{2+}–ARS formation. It was clear that the fluorescence spectral profiles of Figs. 1a, b were very similar. Obviously, the fluorescence increase was possibly due to the release of ARS when Cys was added. The process of change is depicted in Scheme 1. The decomposition of Cu^{2+}–ARS was owing to Cys as an efficient reductant to reduce Cu^{2+} to Cu^{+}.

Fluorescent titration results displayed a linear response as a function of Cys concentration. The linear relationship between Cys concentration and

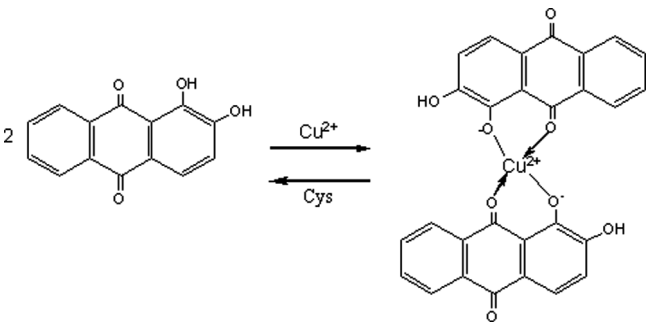


(a)



(b)

FIGURE 1 **(a) Fluorescence spectra of Cu^{2+}–ARS (2.5×10^{-5} mol L^{-1}) in the presence and absence of Cys in pH 5.2 B-R buffer. The concentrations of Cys for curves 1 to 13 were 0, 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0 ($\times 10^{-5}$ mol L^{-1}), respectively. The excitation wavelength was set at 425 nm. (b) Fluorescence spectra of ARS (5.0×10^{-5} mol L^{-1}) upon addition of Cu^{2+} in pH 5.2 B-R buffer. The concentrations of Cu^{2+} for curves 1 to 8 were 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.5, 4.5 ($\times 10^{-5}$ mol L^{-1}), respectively. The excitation wavelength was set at 425 nm.**



SCHEME 1 **Metal complex formation and decompound.**

fluorescence enhancement ranging from 1.0×10^{-6} to 4.0×10^{-5} mol L^{-1} was obtained and the regression equation was as follows: $I_{\Delta F} = 19.5 + 1.319 \times 10^6\ C_{cys}$ ($R = 0.9994$, $n = 9$, SD $= 0.686$). The limit of detection (LOD) was given as 1.08×10^{-7} mol L^{-1} by equation LOD $= KS_0/S$, where K is a numerical factor chosen according to the confidence level desired, S_0 is the standard deviation (SD) of the blank measurements ($n = 9, K = 3$), and S is the slope of calibration curve.

Absorption Titration of Cu^{2+}–ARS with Cys

Figure 2a exhibited the absorption spectrum of ARS in the presence and absence of Cu^{2+}. The maximum absorption wavelength of ARS in pH 5.2 B-R buffer aqueous solution centered at 438 nm. When Cu^{2+} was added, the peak shifted to 501 nm along with color changing from yellow to red. The spectral change was owing to 1:2 (Cu^{2+}:ARS) model complex formation,[25] and Job plot curve also provided positive evidence. In Cu^{2+}–ARS solution, the addition of Cys resulted in dramatic spectral changes, which are shown in Fig. 2b. The spectral recovery of ARS was observed, and the color changed from red to yellow as well. It can be concluded that Cu^{2+}–ARS complex decompounded and ARS was released. However, upon addition of other amino acids such as cystine, tyrosine, tryptophan, methionine, and glycine, neither spectral nor color changes were observed, which implied high selectivity for cysteine over other amino acids. The result was consistent with that of fluorescence titration.

At the same time, it was found that the absorbance change at 501 nm of Cu^{2+}–ARS also exhibited a linear response to Cys concentration ranging from 1.2×10^{-6} to 5.4×10^{-5} mol L^{-1}. The regression equation was $A_{501\,nm} = 0.2545 - 2625C_{cys}$ ($R = 0.996$ $n = 7$, SD $= 0.005$) with the limit of detection as 1.20×10^{-7} mol L^{-1}. The calculation method was the same as for fluorescence assay. Therefore, dual spectral response could be used for determining the concentration of Cys.

In order to explain the mechanism, absorption spectral properties of Cu^{+}–ARS and the effect of Cys on the complex (Cu^{+}–ARS) were also investigated under identical experimental condition. Figure 3a, b shows the absorption spectra of ARS, Cu^{+}–ARS, and

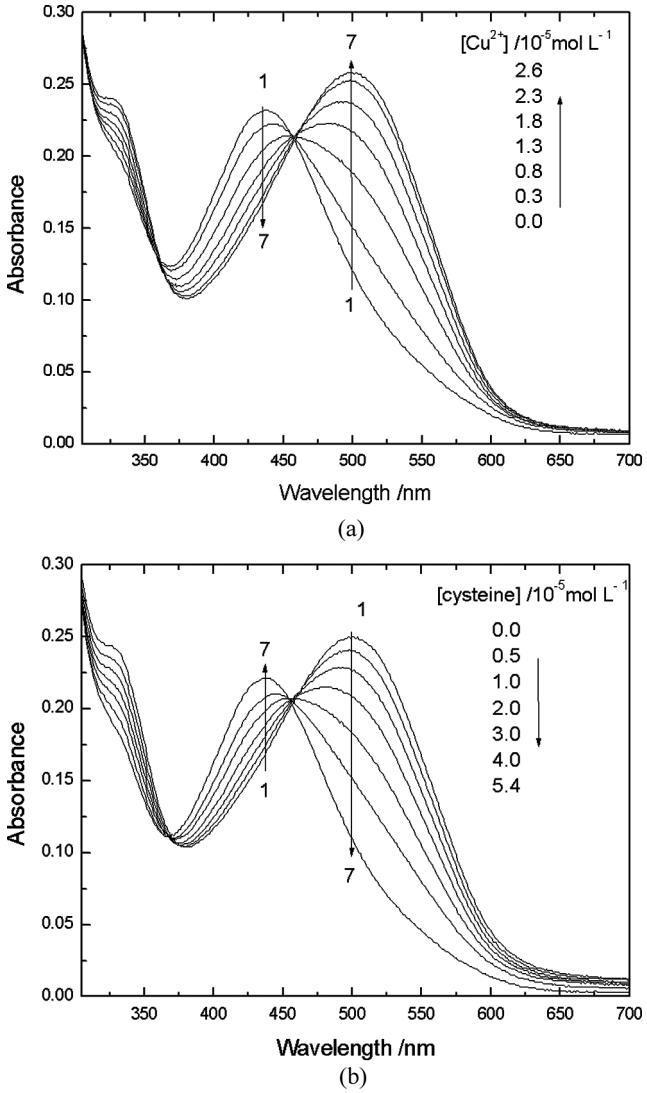


FIGURE 2 **(a) Absorption spectra of ARS (5.0×10^{-5} mol L^{-1}) in the presence and absence of Cu^{2+} in pH 5.2 B-R buffer. (b) Absorption spectral change of Cu^{2+}–ARS (2.5×10^{-5} mol L^{-1}) in the presence and absence of Cys in pH 5.2 B-R buffer.**

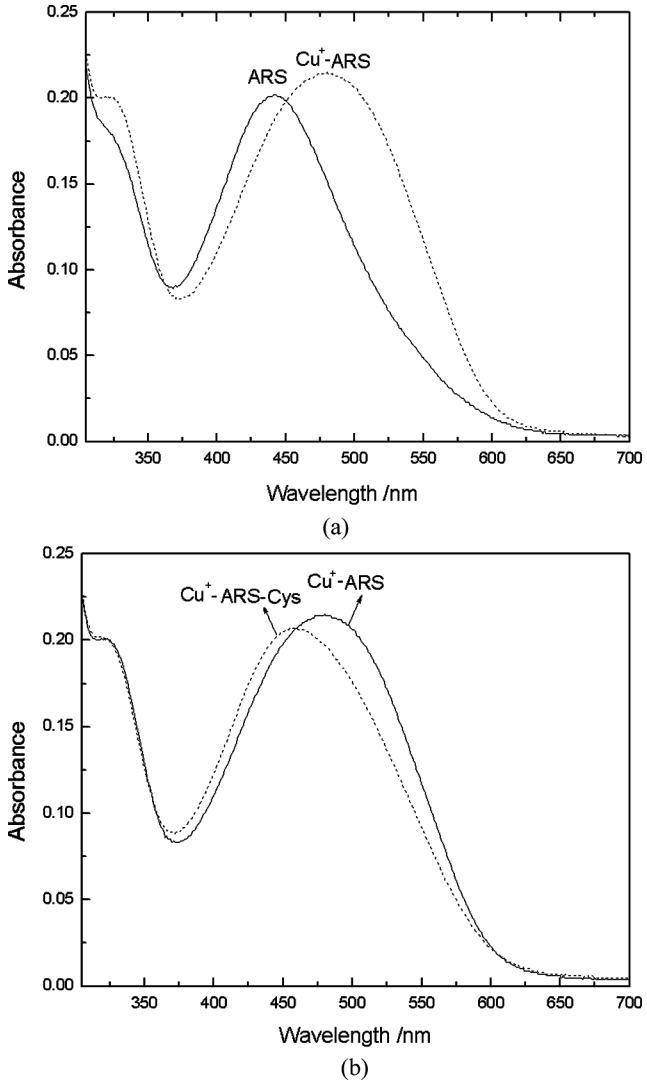


FIGURE 3 **(a) Absorption spectra of ARS and Cu^{+}–ARS in pH 5.2 B-R buffer. (b) The absorption spectra of Cu^{+}–ARS and the mixture of Cu^{+}–ARS and Cys in pH 5.2 B-R buffer. [ARS] = 5.0×10^{-5} mol L^{-1}, [Cu^{+}] = 2.5×10^{-5} mol L^{-1}, [Cys] = 1.5×10^{-5} mol L^{-1}.**

the mixture of Cu^{+}–ARS and Cys, respectively. The maximum wavelength of complex Cu^{+}–ARS centered at 487 nm, which blue shifted 14 nm compared with that of Cu^{2+}–ARS. It also exhibited a stable complex formation between ARS and Cu^{+}. However, the addition of Cys into the solution of Cu^{+}–ARS led the absorption spectra blue shift along with absorbance decrease at 487 nm. It was obvious that Cu^{+}–ARS complex decomposed and Cu^{+}–Cys formed, which exhibited that Cu^{+}–Cys complex was more stable than Cu^{+}–ARS. Thus the mechanism of Cu^{2+}–ARS responding to Cys could be expressed as follows:

$$4\mathrm{RSH} + 2\mathrm{Cu}^{2+} - (\mathrm{ARS})_2 \rightarrow 2\mathrm{Cu}^{+} - (\mathrm{RS}) + \mathrm{RSSR} + 4\mathrm{ARS} + 4\mathrm{H}^{+}$$

where RSH is cysteine, Cu^{2+}–$(ARS)_2$ is the complex between Cu^{2+} and ARS, RSSR is cystine, Cu^{+}–(RS) is the complex between cysteine and Cu^{+}, and ARS is Alizarin Red S.

It was concluded that Cu^{2+}–$(ARS)_2$ selectively and sensitively responded to Cys, which was ascribed to the deoxidation property at Cys and competitive binding action between Cys and ARS for Cu^{+}.

Analytical Application

Optimal Experimental Condition

In order to optimize experimental condition, the effect of acidity and reaction time on the determination of Cys was investigated. The fluorescent

TABLE 1 Interference of Foreign Substances

nterferent	Addition	Error (%)	Interferent	Addition	Error (%)
Na^+	1.0×10^{-2} mol L^{-1}	2.9	Cystine	1.0×10^{-4} mol L^{-1}	−3.2
K^+	1.0×10^{-2} mol L^{-1}	+3.5.	Tryptophan	1.0×10^{-4} mol L^{-1}	+6.0
Ag^+	1.0×10^{-3} mol L^{-1}	−5.1	Tyrosine	5.0×10^{-4} mol L^{-1}	−1.1
Zn^{2+}	5.0×10^{-4} mol L^{-1}	+4.0	Methionine	5.0×10^{-4} mol L^{-1}	+5.0
Hg^{2+}	5.0×10^{-4} mol L^{-1}	−5.0	Glycine	5.0×10^{-4} mol L^{-1}	+4.0
Mg^{2+}	1.0×10^{-3} mol L^{-1}	−8.0	Alanine	2.5×10^{-4} mol L^{-1}	+5.0
Al^{3+}	6.0×10^{-5} mol L^{-1}	−5.0	Arginine	2.0×10^{-4} mol L^{-1}	+5.0
Ca^{2+}	1.0×10^{-3} mol L^{-1}	−2.8	Aspartic acid	1.5×10^{-4} mol L^{-1}	+5.0
Ni^{2+}	1.0×10^{-4} mol L^{-1}	−4.6	Glutamine acie	5.0×10^{-4} mol L^{-1}	+5.0
Pb^{2+}	5.0×10^{-4} mol L^{-1}	+5.0	Histidine	3.5×10^{-4} mol L^{-1}	+5.0
Fe^{3+}	1.0×10^{-5} mol L^{-1}	−8.0	Hydroxyproline	2.5×10^{-4} mol L^{-1}	+5.0
Fe^{2+}	5.0×10^{-4} mol L^{-1}	−3.2	Leucine	2.0×10^{-4} mol L^{-1}	+5.0
Cd^{2+}	2.0×10^{-3} mol L^{-1}	−4.9	Phenylalanine	2.3×10^{-4} mol L^{-1}	+5.0
I^-	5.0×10^{-4} mol L^{-1}	+3.1	Lysine	2.0×10^{-4} mol L^{-1}	−3.0
$S_2O_{3^{2-}}$	5.0×10^{-4} mol L^{-1}	+5.0	Serine	2.5×10^{-4} mol L^{-1}	−4.0
ClO_{3^-}	5.0×10^{-4} mol L^{-1}	−2.0	Threonine	3.0×10^{-4} mol L^{-1}	+5.0
Br^-	1.0×10^{-2} mol L^{-1}	+5.0	Valine	2.0×10^{-4} mol L^{-1}	−1.1
S^{2-}	5.0×10^{-5} mol L^{-1}	+4.5	Cystamine	1.0×10^{-4} mol L^{-1}	+5.0
EDTA	5.0×10^{-5} mol L^{-1}	+5.0	Methionine	5×10^{-4} mol L^{-1}	4.0
BSA	2.0×10^{-5} mol L^{-1}	−4.3	GSH	1.0×10^{-4} mol L^{-1}	+5.0
HSA	2.0×10^{-5} mol L^{-1}	−4.7	Cysteamine	1.0×10^{-4} mol L^{-1}	+5.0

intensity change of the system was maximum and kept constant when pH value was from 4.5 to 6.5. We also studied the fluorescence spectra changes in different buffer. It was found that ARS did not emit fluorescence in HAc–NaAc, NaH_2PO_4–Na_2HPO_4, and HCl–hexamethylenetetramine. Only in B-R buffer solution were distinct changes observed. It was attributed that boron participated in action with ARS.[29] Thus, pH 5.2 B-R aqueous solution was selected. In this buffer solution, the reaction between Cu^{2+}–ARS and Cys was completed after 3 min at room temperature, and absorbance and fluorescence intensity of the system kept constant at least 24 h under the experimental condition. Thus, 5 min were chosen as reaction time.

Interference of Foreign Substances

Under optimal experimental condition, in the mixture of 1.0×10^{-5} mol L^{-1} Cys and 2.5×10^{-5} mol L^{-1} Cu^{2+}–ARS, the presence of the amounts of foreign substances given a Table 1 resulted in less than $\pm5\%$ error.

Sample Analysis

The feasibility of the proposed method in analyzing real samples has also been investigated. It has been used to determine cysteine in protein hydrolysate of fresh pig blood. Recovery experiments were performed, and the results are given in Table 2.

TABLE 2 Analytical Results of the Samples

Method	Added (10^{-6} mol/L)	Found (mean) (10^{-6} mol/L)	Recovery (mean) %	RSD (%) (n = 5)
Spectrophotometry	0	3.28	—	3.5
	3.0	5.55–5.94 (5.90)	88.4–94.6 (93.9)	4.7
	6.0	9.05–9.15 (9.11)	97.5–98.6 (98.2)	5.1
Spectrofluorimetry	0	3.43	—	5.0
	3.0	6.35–6.41(6.38)	98.8–99.7 (99.2)	3.1
	6.0	9.34–9.45(9.39)	99.0–100.2 (99.6)	5.5

CONCLUSIONS

In this paper, a simple, sensitive, and selective method for the determination of cysteine based on Cu^{2+}–ARS was proposed. The fluorescence and absorption spectral changes displayed a good linear response as a function of Cys concentration. The high selectivity over other amino acids was ascribed to the redox property of Cys. The proposed method has been used in analyzing Cys in protein hydrolysate of fresh pig blood with good recoveries of 88.4–100.2%.

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