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Study and Application of HRP-like Catalyst Copper 2-Hydroxy-1-naphthaldehyde-2-aminothiazole [Cu^{II}-$(HNATS)_2$]

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2-Hydroxy-1-naphthaldehyde-2-aminothiazole (HNATS) and its copper complex [Cu^{II}-$(HNATS)_2$] had been synthesized. The complex of [Cu^{II}-$(HNATS)_2$] was used to mimic the active group of horseradish peroxidase (HRP). The catalytic characteristics of this HRP-like catalyst in the H_2O_2-phenol- 4-AAP redox coupling reaction was studied, and the catalytic activity of [Cu^{II}-$(HNATS)_2$] was compared with those of HRP and other Schiff base metal complexes. It was found that [Cu^{II}-$(HNATS)_2$] exhibits a good catalytic activity and could be used as a novel catalyst in the determination of hydrogen peroxide (H_2O_2). The reaction mechanism, optimal experimental conditions and interferences of coexisting substances were discussed. Under the experimental conditions established, the linear relationship between the absorbance at 504 nm (A_{504}) and H_2O_2 concentration was in the range of 5.6×10^{-6}~1.1×10^{-4} mol/L, with a correlation coefficient (r) of 0.9990. The linear regression equation was $A_{504} = 2540\times C$ (mol/L) – 3.0×10^{-3} with a detection limit of 1.7×10^{-6} mol/L. In this paper, the complex [Cu^{II}-$(HNATS)_2$] was applied successfully with catalytic spectrophotometric method to the determination of (–O-O-H) in polyethylene glycol.

Key words: Cu^{II}-$(HNATS)_2$, HRP-like catalyst, catalytic spectrophotometric method, hydrogen peroxide

Enzymes have been widely used in medicine and analytical biochemistry because of their rapidity and high selectivity [1,2,3], but many natural enzymes are expensive and their solutions are not very stable [4,5,6]. We hope to substitute these natural enzymes with some common stable chemicals. Horseradish peroxide (HRP) can catalyze reactions of substrates with hydrogen peroxide (H_2O_2) [4,7,8,9], and by coupling this catalytic reaction with the catalytic reaction of glucose oxidase, glucose can be determined indirectly [10,11,12]. Metalloporphyrin system has been widely used to mimic HRP in the determination of H_2O_2, ascorbic acid and glucose [4,13,14,15]. But it is comparatively difficult to synthesize metalloporphyrins, most of which are slightly soluble in water [16,17]. In contrast to the metalloporphyrins, Schiff base metal complexes dissolve easily and their solutions are stable. It is easy to synthesize Schiff base metal complexes and introduce some conjugate groups, *e.g.* thiazole ring, naphthalene ring and benzene ring to them, which can provide some atoms (N, O, S)

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that can be easily coordinated [5,18]. In this paper, we found that the complex [Cu^{II}-$(HNATS)_2$] had similar catalytic activity as that of HRP. It can also be applied to the determinations of glucose, superoxide dismutase (SOD) and superoxide anion radical ($O_2^{-\bullet}$) besides H_2O_2.

EXPERIMENTAL

Apparatus: All absorbance measurements were carried out on a UV-265 spectrophotometer (Shimadzu, Japan). All pH measurements were made with a pH-3 digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China). The elemental analysis was carried out on a MT-3 model CHN elemental analyzer. The infrared spectrum was measured by employing a PE-983 spectroscopic analyzer (KBr discs, Perkin-Elmer).

Reagents: 2-Hydroxy-1-naphthaldehyde-2-aminothiazole (HNATS) and its copper complex [Cu^{II}-$(HNATS)_2$] were synthesized by ourself. Copper salicylaldehyde-2-amino-4-phenylthiazole [Cu^{II}-$(SAPTS)_2$], copper salicylaldehyde-2-aminothiazole [Cu^{II}-$(SATS)_2$], copper 2-hydroxy-1-naphthaldehyde-2-amino-4-phenylthiazole [Cu^{II}-$(HNAPTS)_2$], copper 2-hydroxy-1-naphthaldehyde salicyloylhydrazone [Cu^{II}-HNSH], copper 2-hydroxy-1-naphthaldehyde phenyloylhydrazone [Cu^{II}-HNPH], copper bis-(2-hydroxy-1-naphthaldehyde) thiocarbohydrazone [Cu^{II}-BHNTZ] and copper bis-(2-hydroxy-1-naphthaldehyde) oxocarbohydrazone [Cu^{II}-BHNOZ] were prepared according to the methods in the literature [8,19].

All Schiff base metal complexes (5.0×10^{-5} mol/L) were dissolved in dimethylformamide (DMF); 4-aminoantipyrine (4-AAP) solution (5.0×10^{-3} mol/L) was diluted with deionized water; HRP (purchased from Sino – American Biotechnology company, Beijing) was applied at 2.5 μg/mL and hydrogen peroxide (H_2O_2) solution (0.003%, w/w) was prepared by dilution of a 30% (w/w) solution with deionized water (standardized by titration with potassium permanganate). Tris (hydroxy-methyl) aminomethane-HCl (Tris-HCl) buffer (0.2 mol/L, pH = 6.4) was used.

The following surfactant solutions were used: sodium dodecyl sulfate (SDS) solution (1%, w/w), β-cyclodextrin (β-CD) solution (1.0 mol/L), polyvinyl alcohol (PVA) solution (5%, w/w), Triton X-100 solution (1%, w/w), Tween 80 solution (1%, w/w) and cetyl trimethyl ammonium bromide (CTMAB) solution (1%, w/w).

The chemicals below were used as reducing substrate: phenol; o-nitrophenol; p-dihydroxybenzene; gallic acid; pyro-gallic acid; p-nitrophenol; o-dihydroxybenzene; m-dihydroxybenzene; methylnaphthylamine; 8-hydroxyquinoline; oxaldehyde-bis(second-hydroxy)aniline; mandelic acid; reducing phenolphthalein; 1-nitroso-3-naphthol; o-aminophenol; 1,3,5-trihydroxybenzene and 2,6-dinitrophenol. Their aqueous solutions (2.0×10^{-2} mol/L) were prepared. All chemicals used were of analytical or higher grades.

Synthesis and properties of HNATS and [Cu^{II}-$(HNATS)_2$]:

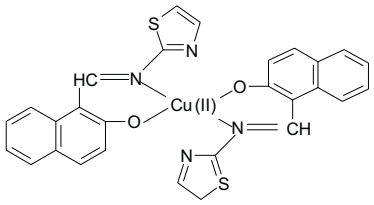
Synthesis and properties of HNATS: 0.69 g (0.004 mol) of 2-hydroxy-1-naphthaldehyde was dissolved in 10 mL of 95% (v/v) ethanol. Then it was added dropwise into a solution, which was prepared by dissolving 0.40 g (0.04 mol) of 2-aminothiazole in 15 mL of 95% (v/v) ethanol at room temperature by agitating. The mixture was refluxed in 80°C water bath for 2 hours, then it was cooled. After recrystallization from 95% (v/v) ethanol for 2~3 times, the orange yellow needle shaped crystals were obtained with a yield of 40%.

The melting point of HNATS is 157–158°C. Elemental analysis gave a composition of C 66.18, H 3.90, N 11.14%, which was in good agreement with the theoretical composition of HNATS, C 66.14, H 3.94, and N 11.02%. The infrared spectrum of HNATS (KBr discs) was obtained and the bands were assigned as follows: 2800–3150 (O–H···N); 1625 (CH=N); 1320 (C–N); 1225 (Ar–O) cm^{-1}.

Synthesis and properties of [Cu^{II}-$(HNATS)_2$]: 1.01 g (0.004 mol) of HNATS was dissolved in 95% (v/v) ethanol, and it was slowly mixed with a 95% (v/v) ethanol solution containing a 0.40 g (0.002 mol) amount of $Cu(Ac)_2{\cdot}H_2O$. The mixture was refluxed at 80°C for 4 hours, filtered while hot, washed with 95% (v/v) ethanol and deionized water in turns for three times, then dried in vacuum. The brown powder

was obtained with a yield of 61%. The melting point of [Cu^{II}-$(HNATS)_2$] is over 330°C. Elemental analysis gave a good agreement with the theoretical composition.

The infrared spectrum of [Cu^{II}-$(HNATS)_2$] (KBr discs) was obtained and the bands were assigned as follows: 3050 (Ar–H); 1620 (CH=N); 1370 (C–N); 1305 (Ar–O) cm^{-1}. The stoichiometry of the complex was studied by molar ratio and continuous variation method. The two methods showed that the composition of the complex was 1:2 (Cu^{II}:HNATS). The proposed structure of [Cu^{II}-$(HNATS)_2$] is shown in Scheme 1.

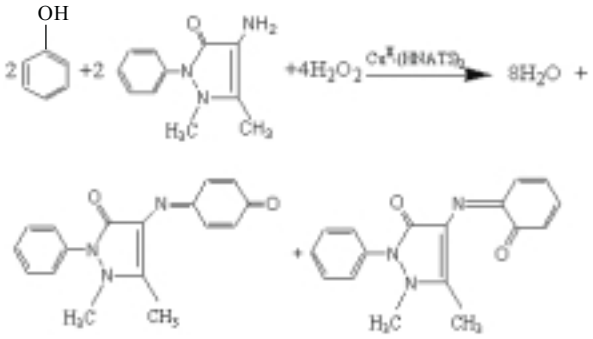


Scheme 1. The proposed structure of the Schiff base copper complex [Cu^{II}-$(HNATS)_2$].

Procedure: In a 10 mL colorimetric tube, 2 mL of Tris-HCl buffer (pH 6.4), 0.50 mL of phenol (2.0×10^{-2} mol/L), 0.50 mL of 4-AAP (5.0×10^{-3} mol/L), 0.20 mL of Schiff base metal complex solution (5.0×10^{-5} mol/L), 3 mL of sodium dodecyl sulfate (SDS) solution (1%, w/w) and 0.30 mL of hydrogen peroxide (H_2O_2) solution (0.003%, v/v) were added. The tube was then diluted to the mark with distilled deionized water. The solution was put in a 1.0 cm quartz cell. The absorbance at 504 nm (A_{504}) was recorded after 15 min.

RESULTS AND DISCUSSION

Mechanism of the H_2O_2-phenol-4-AAP coupling reaction catalyzed by [Cu^{II}-$(HNATS)_2$]: The products of the redox coupling reaction under the catalysis of HRP had a maximum absorbance at 505 nm, and the maximum absorbance wavelength of the products of the reaction catalyzed by [Cu^{II}-$(HNATS)_2$] was at 504 nm. They had the same wavelength of maximum absorbance approximately. So both of them were quinolone dyestuffs. The possible mechanism of the reaction is shown in Scheme 2.



Scheme 2. The mechanism of the H_2O_2-phenol-4-AAP coupling reaction catalyzed by Cu^{II}-$(HNATS)_2$.

Comparison of catalytic activities of HRP and HRP-like catalysts: The redox coupling reaction under the catalysis of HRP and HRP-like catalysts was studied. Their catalytic activities were compared and the results are shown in Fig. 1. It was found that Cu^{2+}, HNATS, CuBHNTZ and CuBHNOZ had no catalytic activities. The order of catalytic activities of other species is following: HRP > $[Cu^{II}$-$(HNATS)_2]$ > Cu^{II}-$(HNAPTS)_2$ > Cu^{II}-$(SAPTS)_2$. We found that the catalytic activity of HRP was higher than those of all Schiff base copper complexes with only mimic prosthetic-group structure, for HRP has not only prosthetic-group but also special cubic protein structures, which make that HRP have higher catalytic activities. $[Cu^{II}$-$(HNATS)_2]$ had higher catalytic activity than Cu^{II}-$(HNAPTS)_2$, which demonstrated that their spatial structures affected their catalytic activities. The delocalized behavior of electron cloud in naphthalene ring was higher than that in benzene ring, and the catalytic activity of Cu^{II}-$(HNAPTS)_2$, whose ligand had naphthalene ring was higher than that of Cu^{II}-$(SAPTS)_2$, whose ligand had benzene ring. The fact demonstrated that delocalized behavior of electron cloud in ligands was one of the factors that determined the catalytic activity. From the results it was clear that Schiff base copper complexes having thiazole ring had catalytic activity, but those with no thiazole ring exhibited no catalytic activity. It showed that thiazole ring was the key structure group in the catalytic reaction. We found that $[Cu^{II}$-$(HNATS)_2]$ had highest catalytic activity in these CuLn HRP-like catalysts. So $[Cu^{II}$-$(HNATS)_2]$ was selected as catalyst to catalyze the H_2O_2-phenol-4-AAP redox coupling reaction.

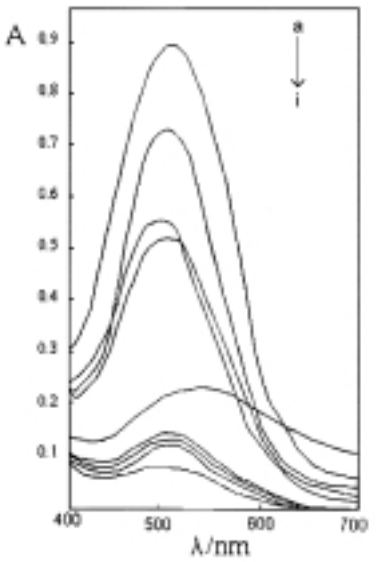


Figure 1. Comparison of catalytic activities of (a) 0.125 μg/mL HRP; (b) 1.0×10^{-5} mol/L Cu^{II}-$(HNATS)_2$; (c) 1.0×10^{-5} mol/L $Cu(HNAPTS)_2$; (d) 1.0×10^{-5} mol/L $Cu(SAPTS)_2$; (e) 1.0×10^{-3} mol/L Cu^{2+}; (f) 1.0×10^{-5} mol/L CuBHNOZ; (g) 1.0×10^{-5} mol/L CuBHNTZ; (h) 1.0×10^{-5} mol/L $Cu(SATS)_2$ and (i) 1.0×10^{-5} mol/L HNATS in the redox coupling reaction.

Selection of the reducing substrate: Phenol, aniline and their derivatives were used as substrate in the redox coupling reaction. The experimental results are shown in Table 1, which demonstrated that phenol could be well used as reducing substrate in this reaction. According to the structure, we could draw a conclusion that compounds, whose benzene ring had an electron-contributing group opposite to the hydroxy, were easy to be oxidized for its high delocalization of electron clouds. In the subsequent experiments, phenol was adopted as a reducing substrate.

Table 1. The absorbance at 504 nm (A_{504}) using different compounds as reducing substrate in the coupling reaction.

Compound (2.0×10^{-2} mol/L	A_{504}
phenol	0.198
o-nitrophenol	0.125
p-dihydroxybenzene	0.175
gallic acid	0.051
pyro-gallic acid	0.166
p-nitrophenol	0.040
o-dihydroxybenzene	0.194
m-dihydroxybenzene	0.167
methylnaphthylamine	0.069
8-hydroxyquinoline	0.032
oxaldehyde-bis(second-hydroxy)aniline	0.018
mandelic acid	0.008
reducing phenolphthalein	0.011
1-nitroso-3-naphthol	0.090
o-aminophenol	0.103
1,3,5-trihydroxybenzene	0.020
2,6-dinitrophenol	0.042

Optimization of experimental conditions.

Effect of pH: The pH of the medium had a great effect on the coupling reaction (Fig. 2). The results showed that the absorbance was high over the pH range 5.0–7.0 and was highest at pH 6.4, which was chosen as the optimum experimental pH.

Effect of the 4-AAP concentration: The effect of the 4-AAP concentration on the catalytic coupling reaction was studied. The results are shown in Fig. 3, which demonstrates that the absorbance value remained constant with increasing 4-AAP concentrations from 1.0×10^{-4} to 4.0×10^{-4} mol/L. In this paper, 2.5×10^{-4} mol/L was adopted in the subsequent experiments.

Effects of the concentration of [Cu^{II}-$(HNATS)_2$] and the reaction time: The kinetics of enzymatic reaction has been studied (Fig. 4). The reaction rate was low at a small concentration of [Cu^{II}-$(HNATS)_2$] and increased with increasing the concentration of [Cu^{II}-$(HNATS)_2$] up to 1.0×10^{-6} mol/L. The reaction was almost completed after 15 min when [Cu^{II}-$(HNATS)_2$] was applied at 1.0×10^{-6} mol/L. So 15 min and 1.0×10^{-6} mol/L of [Cu^{II}-$(HNATS)_2$] were adopted in the subsequent experiments.

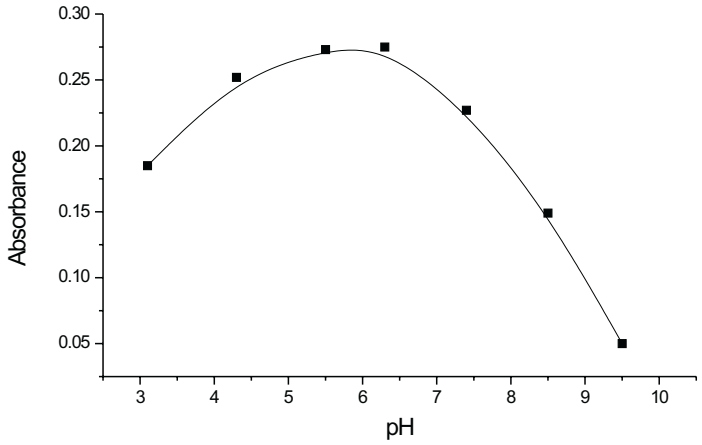


Figure 2. Effect of pH on A_{504}. Phenol, 1.0×10^{-3} mol/L; 4-AAP, 2.5×10^{-4} mol/L; H_2O_2, 9×10^{-5} %; Cu^{II}-$(HNATS)_2$, 1.0×10^{-6} mol/L.

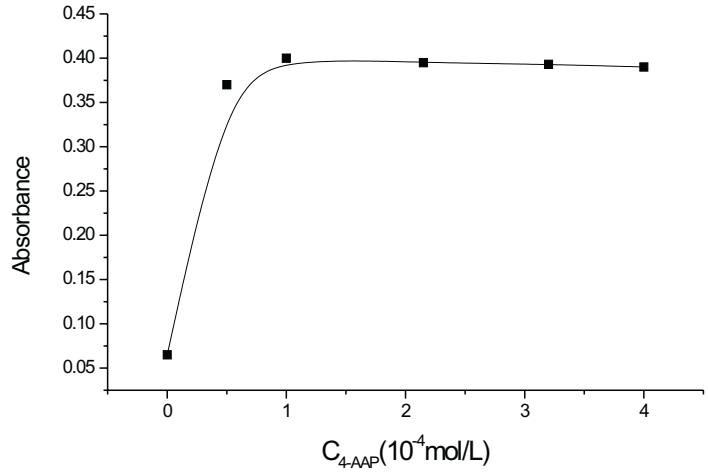


Figure 3. Influence of 4-AAP concentration on A_{504}. Phenol, 1.0×10^{-3} mol/L; H_2O_2, 9×10^{-5} %; Cu^{II}-$(HNATS)_2$, 1.0×10^{-6} mol/L.

Effect of the concentration of phenol: Under the experimental conditions established, the concentration of phenol was studied in the range of $0.5–1.5\times10^{-3}$ mol/L, and the optimum concentration was chosen as 1.0×10^{-3} mol/L.

Effects of surfactants: Sodium dodecyl sulfate (SDS), polyvinyl alcohol (PVA), β-cyclodextrin (β-CD), Tween 80, Triton X-100 and cetyl trimethyl ammonium bromide (CTMAB) were used in this reaction to improve the sensitivity. The results showed that these surfactants could improve the reaction sensitivity except Triton X-100 (Fig. 5). So SDS was selected as the optimum surfactant.

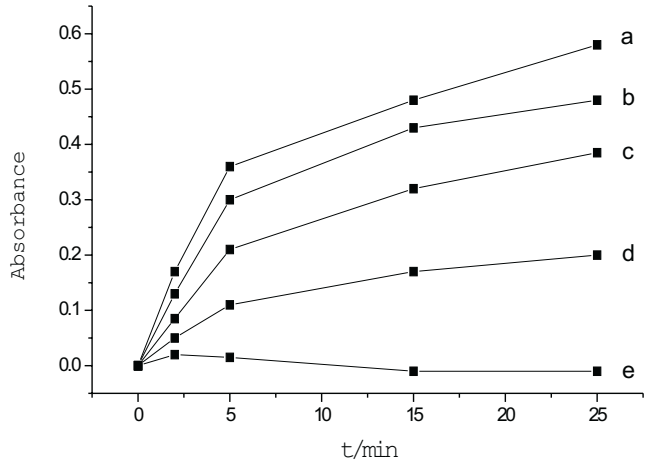


Figure 4. Effects of the concentration and the reaction time on A_{504}. Phenol, 1.0×10^{-3} mol/L; 4-AAP, 2.5×10^{-4} mol/L; H_2O_2, 9×10^{-5} %; Cu^{II}-$(HNATS)_2$: (a) 1.0×10^{-6} mol/L; (b) 8×10^{-7} mol/L; (c) 6×10^{-7} mol/L; (d) 4×10^{-7} mol/L; (e) 0.

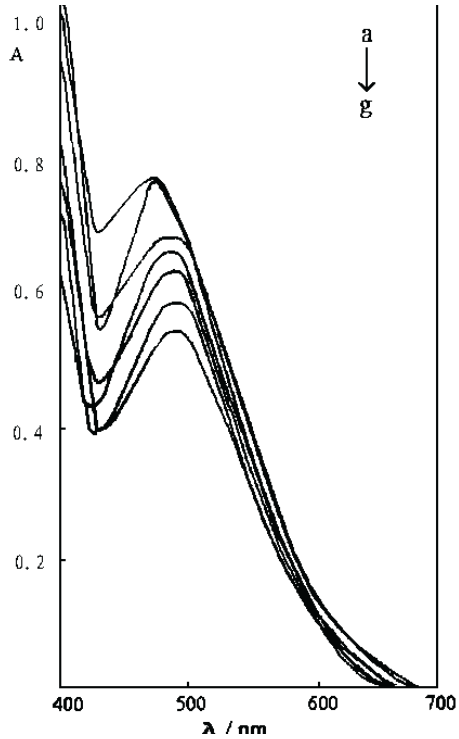


Figure 5. Effect of different surfactants on the absorbance at 500 nm (A_{500}) of a solution containing 1.0×10^{-3} mol/L phenol, 2.5×10^{-4} mol/L 4-AAP , 9×10^{-5} % H_2O_2 and 1.0×10^{-6} mol/L Cu^{II}-$(HNATS)_2$. Surfactants used: (a) 0.3% SDS; (b) 0.3% CTMAB; (c) 1.5% PVA; (d) 3.0×10^{-3} mol/L β-CD; (e) 0.3% Tween 80; (f) No surfactant; (g) 0.3% Triton X-100.

Interferences of inorganic ions and organic compounds: A systematic study of the interferences by various inorganic ions and organic compounds was carried out. The concentration of H_2O_2 was fixed at 7.0×10^{-5} mol/L and the tolerable error was fixed at a ±5% variation of the absorbance. The permitted concentrations of various interference substances were 100 times Co^{2+}, Cd^{2+}, Mn^{2+}, WO_4^{2+}, Ca^{2+}, F^-, VB_2, tyrosine, phenylalanine, tryptophan; 50 times Zn^{2+}, Ni^{2+}, Fe^{3+}; 10 times Al^{3+}; 5 times Pb^{2+}. The possible reason for interferences by inorganic ions was that cation could react with substrates or substitute Cu^{II} in Cu^{II}-$(HNATS)_2$, and anion could deprive Cu^{II} from Cu^{II}-$(HNATS)_2$. As for organic compounds, they could react with substrates under the catalysis of the HRP-like catalyst.

Analytical characteristics: Under the experimental conditions, there was a linear relationship between the absorbance at 504 nm (A_{504}) and H_2O_2 concentration in the range 5.6×10^{-6}–1.1×10^{-4} mol/L with a correlation coefficient (r) of 0.9990. The linear regression equation was $A_{504} = 2540\times C(mol/L)-3.0\times10^{-3}$. The relative standard deviation was 0.18% obtained from a series of 11 standards each containing 1.0×10^{-5} mol/L H_2O_2. The standard deviation was 0.00213 obtained from a series of 10 blank solutions. The limits of detection (k = 3) and of determination (k = 10) of the method were established according to the IUPAC definitions ($C_1 = kS_0/S$, where C_1 is the limit of detection, S_0 the standard error of blank determination, S the slope of the standard curve and k the constant related to the confidence interval) [20], and the values found were 1.7×10^{-6} mol/L and 5.6×10^{-6} mol/L, respectively.

Sample analysis: Under normal conditions, polyethylene glycol (PEGs) should have no oxidizing property, but its hydroxyl group (–OH) can be easily activated to form hydroperoxyl group (-O-O-H), which has oxidizing properties just like H_2O_2 has. So three solutions of PEGs of different polymerization degree: $PEGs_{400}$, $PEGs_{600}$ and $PEGs_{800}$ were diluted 400, 160 and 200 times, respectively. Then the content of -O-O-H in them was determined with the proposed method. The results are shown in Table 2.

Table 2. The determination of -O-O-H in the PEGs of different polymerization degree. (P = 0.95, n = 6).

Sample	H_2O_2 added (10^{-4} mol/L)	Found[a] (10^{-4} mol/L)	Recovery (%)
	0	0.465±0.001	
$PEGs_{400}$	0.250	0.720±0.002	102
	0.500	0.955±0.003	98
	0	0.325±0.001	
$PEGs_{600}$	0.250	0.573±0.003	99
	0.500	0.830±0.004	101
	0	0.220±0.001	
$PEGs_{800}$	0.250	0.465±0.002	98
	0.500	0.730±0.001	102

[a]Each sample was analyzed six times.

Acknowledgments

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