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A novel HPLC-UV/nano-TiO_2-chemiluminescence system for the determination of selenocystine and selenomethionine

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ABSTRACT

Active oxygen species from the photocatalytic reaction in aqueous solution react with luminol to emit strong chemiluminescence (CL), and this can be inhibited by the UV decomposed-products of selenocystine (SeCys) or selenomethionine (SeMet). Based on this phenomenon, a novel hyphenated technique, HPLC-UV/nano-TiO_2-CL, was established for the determination of SeCys and SeMet. The effects of pH, the UV irradiation time, the TiO_2 coated on the inner surface of the reaction tubing, and the Co^{2+} catalyst concentration on the CL intensity and/or chromatographic resolution were systematically investigated. Under these optimized conditions, the inhibited CL intensity has a good linear relationship with the concentration of SeCys in the range of 0.04–10.6 $\mu g\,mL^{-1}$ or SeMet in the range of 0.05–12.4 $\mu g\,mL^{-1}$, with a limit of detection (S/N = 3) of 6.4 $\mu g\,L^{-1}$ for SeCys or 12 $\mu g\,L^{-1}$ for SeMet. As an example, the method was preliminarily applied to the determination of the selenoamino acids in garlic and rabbit serum, with a recovery of 88–104%.

1. Introduction

Selenium is present in the biosphere in chemical forms analogous to those of sulfur. It has been found that selenocystine (SeCys) or selenomethionine (SeMet) are the predominant forms of the Se-containing proteins [1]. In the mammalian body, selenium mainly exists as SeCys, an essential component of glutathione peroxidase, which inhibits the oxidation role of peroxides and hydroperoxides, thereby protecting immunocompetent cells and slowing down ageing process. Supplemental selenium usually takes the form of SeMet, which has been proved to be the dominant form in plants. A certain percentage of SeMet is known being incorporated non-specifically into various proteins in place of methionine, which could introduce certain health risks when supplementing diets of infants [2,3]. Although these two selenoamino acids, SeCys and SeMet, play different roles in the biological body, they often constantly transform to each other. For example, SeMet is generally metabolized in the human body into SeCys [4]. As the metabolic behavior, the bioavailability and the effects of Se are thus dependent to a large extent on its chemical form. Therefore, there is a demand for the determination of selenoamino acids, especially SeCys and SeMet, in food supplementation and body fluid in many fields of Se research such as environmental chemistry, nutrition, and biomedical and life sciences.

A large number of techniques for the determination of SeCys and SeMet in food supplementation and body fluid have been used, including liquid chromatography (LC)–inductively coupled plasma-mass spectrometry (ICP-MS) [3,5,6]; gas chromatography (GC)–ICP-MS [4]; GC-atomic plasma emission spectroscopy (GC-AED) [7]; LC-microwave decomposition-hydride-generation-atomic fluorescence spectrometry (AFS) or atomic absorption spectrometry (AAS) [8,9]; liquid chromatography-UV irradiation-hydride generation-quartz cell atomic absorption spectrometry (LC-UV irradiation-HG-QCAAS) [10] or HPLC-UV-HG-AFS [11–13], and high-performance anion-exchange chromatography with integrated pulsed amperometric detection [14].

Chemiluminescence (CL) has been widely used as a sensitive detector for HPLC since the 1980s [15,16]. Due to the simple optical structure and especially no optical interference from any light source, CL offers obvious advantages over many conventional detection techniques in terms of instrumental simplicity, cost-effectiveness and compactness [15]. Besides, its good selectivity, high sensitivity, wide linear working range and rapid analysis render it a highly promising tool in the determination of organic compounds, e.g., phosphatidylcholine hydroperoxides [17], amikacin [18], quinolones [19], propylthiouracil and methylthiouracil [20]. Up to now, to the best of our knowledge, LC-CL has not been used for the determination of selenoamino acids since they could not

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produce CL through their direct reaction with any luminescence reagents. Therefore, the aim of the current study is to evaluate the feasibility of HPLC combined with CL for the determination of these selenoamino acids. In this work, SeCys and SeMet separated by HPLC were decomposed by UV/nano-TiO_2 and the products were used to inhibit the chemiluminescence reaction of luminol with active oxygen species produced by H_2O photocatalysis.

2. Experimental

2.1. Instrumentation

The liquid chromatography system used in this work was an LC 2130 chromatographer (Tianmei, China), with a polystyrene-divinylbenzene-based anion exchange column (Hamilton PRP X-100, 10 μm, 250 × 4.1 mm, Reno, NV, USA) with ammonium quaternary salt with methyl groups as substituents and a 7725i injector (Rheo, NV, USA) with a 100 μL sample loop.

The UV/TiO_2-CL system consisted of two peristaltic pumps (Qingpu Huxi Instrument Factory, Shanghai, China); a laboratory-made UV/nano-TiO_2 photocatalytic reaction device and a commercial computerized ultraweak luminescence analyzer (BPCL, the Institute of Biophysics, Academia Sinica, Beijing, China) for the detection of the chemiluminescence emission. The reaction device was composed of a PTFE tube (0.3 mm inner diameter) modified by TiO_2 and a Philips TUV-15 lamp (the main emission at 253.7 nm, 15 W, 44 cm long) surrounded by the PTFE tube.

2.2. Chemicals

Selenocystine (SeCys, seleno-L-cystine, 98+%, Fluka) and selenomethionine (SeMet, D,L-selenomethionine, 99+%, Acros) stock solutions with concentration of 1000 mg L^{-1} were obtained by dissolving appropriate amount of the compounds. Stock solutions were stored in the dark at 4 °C. Working solutions were prepared daily. The LC mobile phases were prepared using Na_2HPO_4 (Kelong Reagent Co., Chengdu, China) and NaH_2PO_4 (Kelong Reagent Co., Chengdu, China). Luminol was purchased from Sigma Chemical Co. (USA) and used without further purification. A 1×10^{-2} mol L^{-1} luminol stock solution was prepared by dissolving 0.4454 g of luminol in 250 mL of 0.l mol L^{-1} NaOH. Titanium dioxide (anatase, Shenzhen, China) was used as received. All other chemicals were of analytical reagent grade, and doubly distilled water (DDW) was used for the preparation of the solutions throughout this work.

2.3. TiO_2-coated PTFE tube

The preparation of nano-TiO_2 film was carried out as described by Nazeeruddin et al. [21]. The commercial TiO_2 powder (12 g) of grain size in the range of 20–50 nm was ground in a porcelain mortar with a small amount of water (4 mL) containing acetylacetone (0.4 mL) to prevent reaggregation of the particles. After the powder was dispersed by the high shear forces in the viscous paste, it was diluted by slow addition of water (16 mL) under continued grinding. Then, 0.2 mL Triton X-100 (Aldrich) was added to facilitate the spread of the colloid on the substrate. The TiO_2 colloid prepared was finally pumped through the PTFE tube to finish the wet-coating. The TiO_2-coated PTFE tube was then dried at room temperature, and calcined in a muffle furnace at 200 °C for 3.0 h. The coating and drying procedure was repeated for three times before calcination.

2.4. Sample preparation

Serum A and B were collected from rabbit A and B, respectively. And serum C was collected from rabbit A after it was immunized by several estrogens. These rabbit serum samples were stored in polyethylene bottle at 4 °C. Properly diluted samples were filtrated through a 0.2 μm nylon filter before analysis.

Garlic, a selenium food supplement, was purchased from the local market. It was washed with tap water and DDW to remove any contamination on the surface. Then it was cut into small pieces and ground in a porcelain mortar for complete homogenization of the sample. After eliminating the moisture content by freeze-drying, the freeze-dried sample weight was about 69% of that of the original sample. The extraction was carried out according to a previously reported method [22]. Briefly, 0.05 g of the freeze-dried garlic sample was accurately weighed into a plastic beaker, to which 3 mL of sodium hydroxide solution (0.1 mol L^{-1}) was then added. After extracted in an ultrasonic bath for 15 min, the mixture was filtered with a 0.2 μm nylon filter and appropriately diluted before analysis.

2.5. Procedure

Experiments were performed using the LC-UV/nano-TiO_2-CL system as depicted in Fig. 1. A 100 μL sample solution was injected into the LC system. A 40 mmol L^{-1} Na_2HPO_4–NaH_2PO_4 buffer at pH 7.0 was used as the mobile phase at a flow rate of 0.6 mL min^{-1}. In the analytical column outlet, 1 μg L^{-1} Co^{2+} was added at the same flow rate by using a peristaltic pump (P2). Then the mixture reached the laboratory-made photocatalysis reaction device (PCRD). Destruction of the organic portion of selenoamino acids was achieved in an 8 m long PTFE tube enlaced outside an UV lamp. The mixture eluted from the PCRD was propelled into the reaction cell (RC) and reacted with luminol to produce the inhibited CL. The RC was a flat-spiral glass tube with a volume of 100 μL, which was located directly in front of the window of the CR-105 photomultiplier tube (PMT) of a computerized ultraweak luminescence analyzer, BPCL. Luminol was introduced into the RC by another peristaltic pump (P1). The CL signal was detected and recorded by

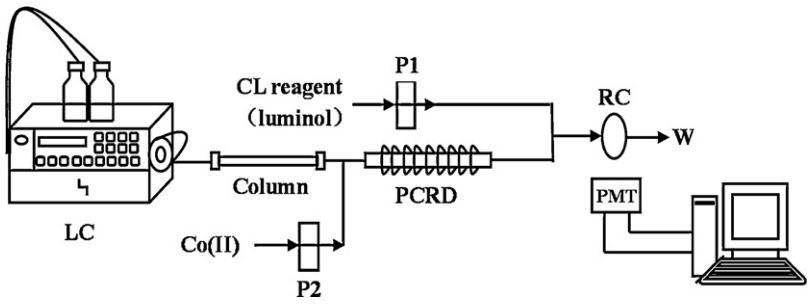

Fig. 1. Schematic diagram of the LC-UV/Nano-TiO_2-CL system. P1: peristaltic pump 1; P2: peristaltic pump 2; PCRD: photocatalysis reaction device; RC: reaction cell; PMT: photomultiplier tube; and W: waste.

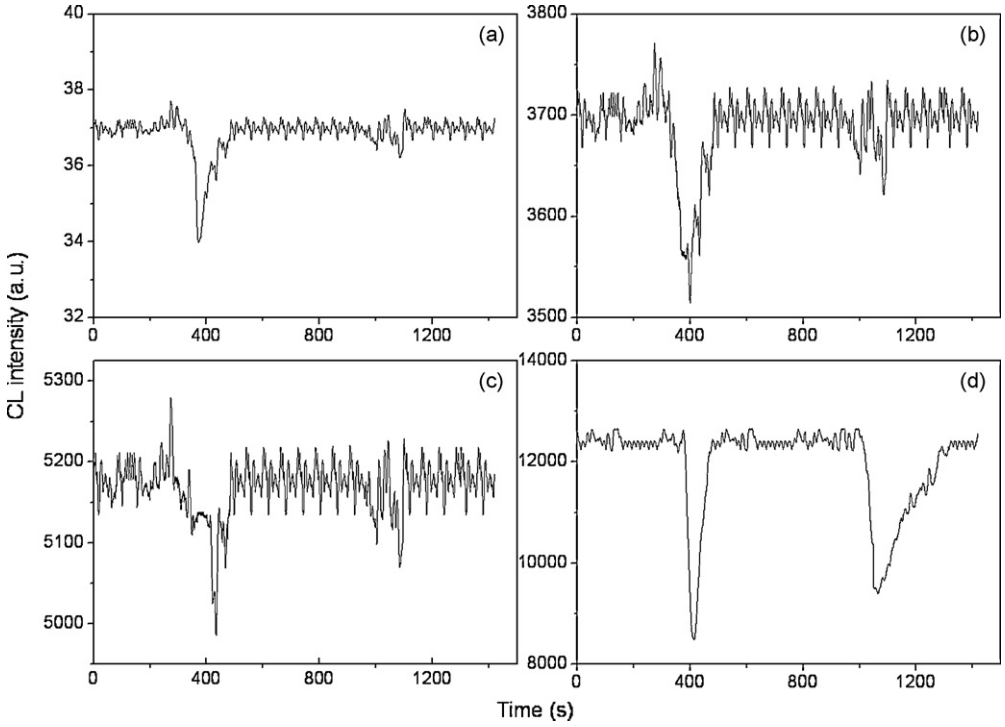

Fig. 2. Effects of UV, TiO_2 and Co^{2+} on the signals of SeCy (0.4 μg mL^{-1}) and SeMet (0.5 μg mL^{-1}), respectively. (a) TiO_2–Co^{2+}; (b) UV/TiO_2; (c) UV-Co^{2+}; and (d) UV/TiO_2–Co^{2+}. Mobile phase: 40 mmol L^{-1} phosphate buffer, pH 7.0; flow rate: 0.6 mL min^{-1}; PTFE tube: 8 m; luminol: 5×10^{-5} mol L^{-1}; and Co^{2+}: 1 μg L^{-1}.

the BPCL. Data acquisition and treatment were performed with the BPCL software running under Windows XP.

3. Results and discussion

3.1. Optimization of chromatographic separation

To obtain the optimal conditions for the separation and determination of SeCys and SeMet, the effects of the pH and the concentration of the mobile phase on the CL signal and HPLC separation were studied in detail. Different buffers including acetate buffer, phosphate buffer and carbonate buffer were used for obtaining different pH. Both SeCys and SeMet featured high retention time in alkaline medium, as reported previously [10]. In acidic medium, the CL signal decreased and the noise dramatically increased, although the retention time was shortened. At pH 7.0 (phosphate buffer), SeCys and SeMet were well separated in reasonably short time with the best S/N. The effect of the phosphate buffer at pH 7.0 was further investigated in the range of 10–80 mmol L^{-1}. In each run, 100 μL of 0.4 μg mL^{-1} SeCys and 0.5 μg mL^{-1} SeMet mixtures was injected into the LC-UV/nano-TiO_2-CL system. The retention of the selenium species was only related to the reversed-phase mechanism between the aqueous phosphate in mobile phase and the polymeric base of column. Therefore, the concentration of phosphate in the mobile phase had no obvious effect on the retention of SeCys and SeMet. It also showed a slight effect on the CL intensity. Finally, 40 mmol L^{-1} phosphate buffer was chosen for use.

3.2. Optimization of UV irradiation

Since both SeCys and SeMet absorb at 253.7 nm [13], a Philip TUV-15 Hg UV lamp with emission mainly around 253.7 nm was used. Preliminary experiments were carried out with the UV lamp on and off, respectively. As shown in Fig. 2(a), very weak chemiluminescence emission from the blank was observed with the UV lamp off. In addition, a weak CL signal for 100 μL of a mixture containing 0.4 μg mL^{-1} SeCys and 0.5 μg mL^{-1} SeMet was obtained and the CL intensity of the blank increased significantly, and a strong analytical signal was observed when the UV lamp was on, as shown in Fig. 2(d). The decomposition of selenoamino acids mainly depended on the irradiation time. The optimal irradiation time was directly determined by the length of the PTFE tube. We investigated the tube length from 2 to 10 m. The result showed that the analytical signal increased with increasing the tube length. Considering the better analytical signal and the shorter analytical time, 8 m was chosen for the rest of this work.

3.3. Effect of TiO_2

Using UV irradiation for degradation or removal of pollutants from the environment has been widely reported. However, the time for decomposition is too long for analytical use. Nano-TiO_2 is nonphotocorrosive and nontoxic, and it can shorten the UV-decomposition time of most organic compounds efficiently [23,24]. As indicated in Fig. 2(c), the analytical signal was not satisfactory when UV irradiation was used without nano-TiO_2, the use of nano-TiO_2 did improve the inhibited CL signal, as shown in Fig. 2(d).

Before and after modification with TiO_2, the inner surfaces of PTFE were observed by scanning electron microscopy (SEM), and the results are shown in Fig. 3. It can be seen that the inner surface of the PTFE tube was covered with a film of nanosized particles, with an average diameter of about 50 nm. The thickness of TiO_2 film was about 0.6 μm. Using the nano-TiO_2-coated PTFE tube, obvious analytical signal was observed, as shown in Fig. 2(d).

The analytical stability of the nano-TiO_2 film on the inner surface of the PTFE tube was tested after the TiO_2-coated PTFE tube was used for about 300 h running time. The relative standard deviation (RSD) of the analytical signals of SeCys and SeMet was 3.4% and 1.9% ($n = 7$), respectively, without obvious change in the sensitivity compared with that of a newly prepared one.

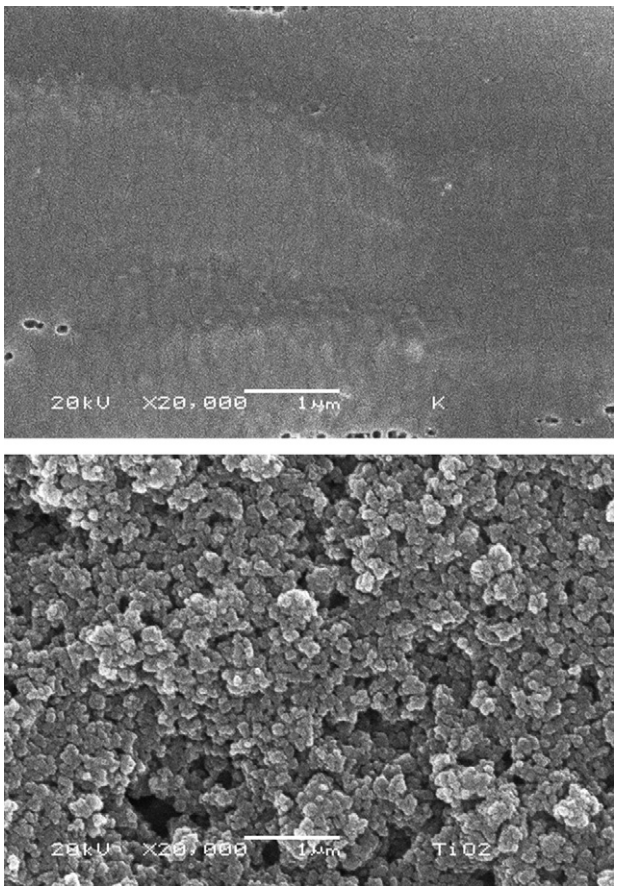

Fig. 3. SEM images of the inner surface of PTFE tube before (top) and after (bottom) nano-TiO_2-coating.

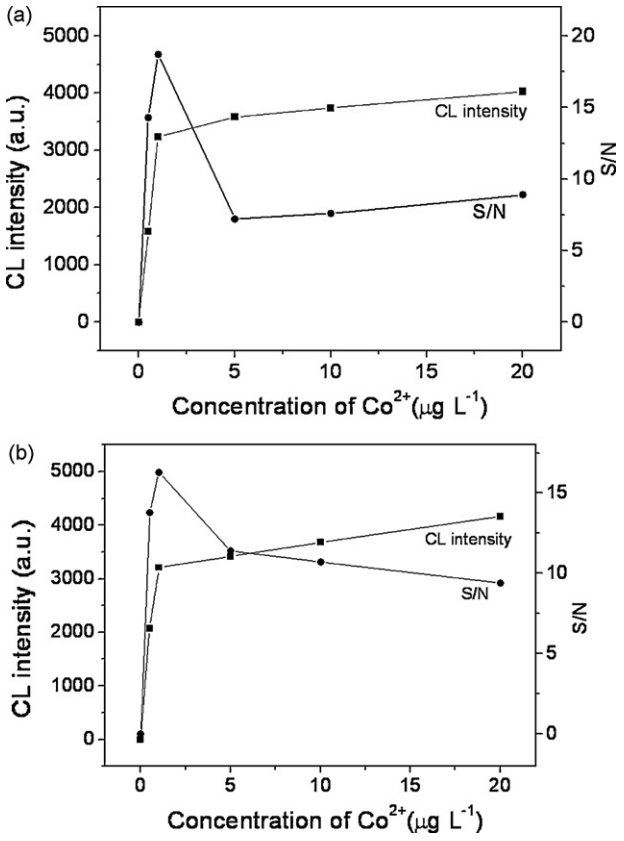

Fig. 4. Effect of Co^{2+} concentration on the signals of 0.4 μg mL^{-1} SeCys (a) and 0.5 μg mL^{-1} SeMet (b). Mobile phase: 40 mmol L^{-1} phosphate buffer, pH 7.0; flow rate: 0.6 mL min^{-1}; PTFE tube: 8 m; and luminol: 5×10^{-5} mol L^{-1}.

While obvious change in analytical signal occurred when it was used for about 400 h running time. Five TiO_2-coated PTFE tubes were prepared as that described in Section 2.3. Using these tubes respectively, the RSD of the analytical signals of SeCys and SeMet was less than 2.8% and 3.1%, respectively. This means that the batch coating procedure is reliable.

3.4. Optimization of chemiluminescence detection

The luminol CL emission is usually produced in alkaline medium, and the NaOH concentration of 0.1 mol L^{-1} was optimized for use in this work. The luminol concentration also affects the CL intensity. The CL intensity increased with the concentration of luminol in the range of 1×10^{-6}–1×10^{-4} mol L^{-1}. Considering the consumption of reagent and the sensitivity for the determination, the optimal concentration of luminol was 5×10^{-5} mol L^{-1}.

It should be noted that Co^{2+} plays a key role in the current system. As shown in Fig. 2(b), without Co^{2+}, the analytical signal was not satisfactory even when 100 μL of a mixture containing 0.4 μg mL^{-1} SeCys and 0.5 μg mL^{-1} SeMet was injected. When Co^{2+} was introduced, an obvious analytical signal was observed, as shown in Fig. 2(d). The concentration of Co^{2+} affected the background signal and the analytical signal. As shown in Fig. 4(a) and (b), the analytical signals of SeCys and SeMet increased with the concentration of Co^{2+} in the range of 0.5–20 μg L^{-1}, while the best S/N was obtained with 1 μg L^{-1} Co^{2+}. Therefore, the optimal concentration of Co^{2+} was 1 μg L^{-1}.

3.5. Analytical figures of merit

The calibration graphs were linear over a concentration range of 0.04–10.6 μg mL^{-1} and 0.05–12.4 μg mL^{-1}, with correlation coefficients of 0.9997 and 0.9999 for SeCys and SeMet, respectively. The RSD of the analytical signals of 0.4 μg mL^{-1} SeCys and 0.5 μg mL^{-1} SeMet were 3.4% and 1.9% ($n = 7$), respectively. The absolute limits of detection, evaluated as that equivalent to three times of the stan-

Table 1
Analytical results of SeCys and SeMet in garlic and rabbit serum

Samples	SeCys			SeMet		
	Found (μg L^{-1})	Spiked (μg L^{-1})	Recovery (%)	Found (μg L^{-1})	Spiked (μg L^{-1})	Recovery (%)
Garlic	ND	50[a]	95	19[a]	20[a]	90
Rabbit serum A	105	106	91	ND	200	96
Rabbit serum B	89	106	104	ND	200	88
Rabbit serum C	142	106	102	181	200	91

ND: not detected.
[a] Selenium species in freeze-dried samples (μg g^{-1}).

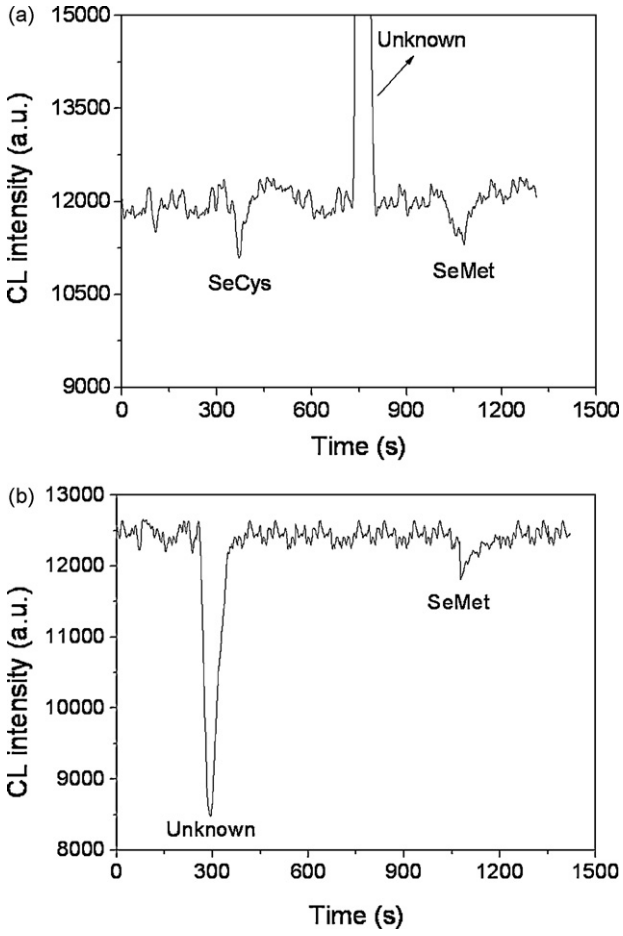

Fig. 5. Chromatograms of SeCys and SeMet in 20-fold diluted (a) rabbit serum C and (b) garlic sample solutions. Mobile phase: 40 mmol L^{-1} phosphate buffer, pH 7.0; flow rate: 0.6 mL min^{-1}; PTFE tube: 8 m; luminol: 5×10^{-5} mol L^{-1}; and Co^{2+}: 1 μg L^{-1}.

dard deviation of the background noise, were 6.4 and 12 μg L^{-1} for SeCys and SeMet, respectively (for 100 μL injection).

3.6. Analysis of real samples

The applicability of this method for the simultaneous determination of SeCys and SeMet in real samples was preliminarily examined by analyzing garlic purchased from the local market and rabbit serums. Both SeCys and SeMet were detected in rabbit serum C, while only SeCys was detected in rabbit serums A and B, and only SeMet was detected in garlic, as shown in Table 1. Selenium standard was then spiked into the four kinds of samples and the recoveries of each selenium species were from 88% to 104%. The chromatograms of samples, rabbit serum C and garlic were shown in Fig. 5(a) and (b), respectively.

3.7. Possible mechanism

Much attention has been given to the "advanced oxidation processes" in wastewater treatment. This term is used to describe an oxidation mechanism, which depends on the production of active free radicals such as hydroxyl radicals (•OH), the most important oxidative reagent [25,26]. According to the following equation [25]:

$$2H_2O \xrightarrow{(h\nu)} e_{(eq)}^- + {}^\bullet OH(aq) + H_3O^+$$

The reaction is possible since the more energetic photons (λ < 192 nm) required for this reaction can be emitted by the mercury lamp used in this work. In addition, it has also been reported that hydroxide and peroxide would be produced upon the oxidization of water [27]. Based on the phenomena found in the present work and many previous studies on the CL of luminol [28,29], we believe the background CL emission could be from the oxidation of luminol by oxygen-containing species such as •OH and peroxide generated from the oxidation of H_2O under the UV irradiation.

The catalytic activity of Co^{2+} has been widely used in CL systems. To investigate the catalytic activity of Co^{2+}, we carried out two preliminary tests. One is to introduce Co^{2+} by pump 1, the other is to introduce Co^{2+} by pump 2. When Co^{2+} was introduced by pump 1, it was directly added into the luminol CL system. Under this condition, although the background the CL intensity rapidly increased, the analytical signal was hardly observed. When Co^{2+} was introduced by pump 2, it was introduced into the photocatalytic reaction of analytes. The background CL intensity increased, while obvious analytical signal was observed. The phenomena showed that Co^{2+} acted not only as a catalyst for the luminol CL system, but also as a co-catalyst of TiO_2 for the photo-decomposition of SeCys and SeMet. The analytical signal (the inhibited CL intensity) is therefore proposed to be originated from the consumption of oxygen-containing species, which most probably participates in the decomposition of the analytes.

4. Conclusion

In the presence of Co^{2+}, selenocystine and selenomethionine can be decomposed rapidly by the UV (253.7 nm) irradiation on the surface of nano-TiO_2 immobilized on the inner surface of a PTFE tube; and then the decomposed-products inhibit the CL from the reaction of luminol with oxygen-containing species. Based on this, a novel LC-UV/nanoTiO_2-CL system was developed for the determination of selenoamino acids. Although further work on the study of the luminescence mechanism and the improvement of the detection limits is necessary, this method is simple, inexpensive and prospective for the determination of selenocystine and selenomethionine in many types of biological samples.

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