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Sensitive determination of phenolic compounds using high-performance liquid chromatography with cerium(IV)-rhodamine 6G-phenolic compound chemiluminescence detection

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Abstract

A simple, selective and sensitive determination method of 20 phenolic compounds has been developed using high-performance liquid chromatography (HPLC) with chemiluminescence detection. The method is based on the chemiluminescent enhancement by phenolic compound of the cerium(IV)-rhodamine 6G system in sulfuric acid medium. Twenty phenolic compounds were separated on a XDB-C_8 column with a gradient elution using a mixture of methanol and 1.0% acetic acid as a mobile phase. Under the optimized conditions, a linear working range extends 2 orders of magnitude with the relative standard deviations of intra- and inter-day precision below 4.0%, and the detection limits (S/N = 3) were in the range of 1.5–82.1 ng/ml. The chemiluminescence reaction was compatible with the mobile phase of high-performance liquid chromatography. The proposed method has been successfully applied to the assay of phenolic compounds in red wine without any pretreatment.

Keywords: High-performance liquid chromatography; Chemiluminescence; Phenolic compound; Cerium(IV); Rhodamine 6G; Wine

1. Introduction

Phenolic compounds are usually referred to as a diverse group of naturally occurring compounds, which possess an aromatic ring bearing one or more hydroxy substituents and contain single or multiple phenolic functionalities [1]. These compounds are commonly found in higher plants and thus are part of the human diet [2]. Phenolic compounds have strong in vitro and in vivo antioxidant activities associated with their ability to scavenge free radicals, break radical chain reactions and chelate metals. During the past decade, phenolic compounds have been the object of increasing interest because of their biological properties, namely anti-inflammatory, anti-histaminic and anti-tumor activities, and as free radical scavengers and protection against cardiovascular diseases [3–6]. Because these compounds are present in the normal diet of humans, it is important to develop a better understanding of the role of these compounds and the adequate levels of intake for good health. Moreover, there are numerous phenolic compounds in our living environments. Most of them are generated artificially and found in wastewaters of chemical plants, exhaust gases of incinerators, sidestream smoke of cigarettes, etc. [7]. Most phenolic compounds are toxic and harmful to our health [7,8]. Thus, developing a highly sensitive analytical method to the simultaneous determination of phenolic compounds in different types of sample matrices such as foods and environmental samples is a key aspect of analytical field.

The determination of phenolic compounds using chromatographic methods includes gas chromatography (GC) [9], capillary electrophoresis (CE) [10,11] and high-performance liquid chromatography (HPLC) [12–17]. HPLC techniques have proved to be the most appropriate ones due to the structural similarity and diversity of phenolic compounds, allowing the analysis with sufficient precision, selectivity and within a reasonable time. Most hitherto published studies dealing with the determination of phenolic compounds employed by conventional ultraviolet–visible absorbance detection. Therefore, the application of HPLC to the determination of trace levels of phenolic

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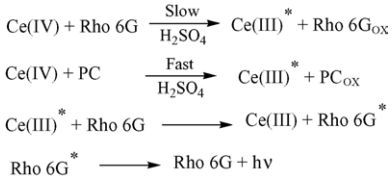
compounds was limited by the inadequate sensitivity of detector, though many pre-concentration procedures were carried out, such as solid-phase purification extract (SPE) [16], supercritical fluid extraction (SFE) [18,19] and microwave-assisted extraction (MAE) [20,21].

Chemiluminescence (CL) detection is very sensitive because the absence of a light source reduces noise and eliminates Rayleigh and Raman scattering. The cerium(IV)-involved CL reactions with sensitizers have been studied and used for the detection of a number of compounds [22–24]. For example, the CL reaction between cerium(IV) and tiopronin in sulfuric acid medium sensitized by quinine has been reported for the determination of tiopronin [25]. The cerium(IV)-rhodamine CL system has been used for the determination of N- or S-containing compounds [26–30], but not for the detection of numerous phenolic compounds. In our previous work, it was found that the reaction between cerium(IV) and rhodamine 6G in the strong sulfuric acid medium underwent weak CL, which could be greatly enhanced by 32 phenolic compounds. Phenolic hydroxyls were the main active groups for the generation of CL, and the magnitude of CL was related to the type and position of substituents in the benzene ring. There was no CL signal for the compounds without phenolic hydroxyl, such as oxygenated aromatic compounds [31]. The possible mechanism of the cerium(IV)-rhodamine 6G-phenolic compound CL reaction, as shown in Scheme 1, has been proposed due to that rhodamine 6G and phenolic compound are oxidized by cerium(IV) in sulfuric acid medium to form the excited-state cerium(III). The reaction rate between cerium(IV) and phenolic compound is faster than that of cerium(IV) with rhodamine 6G. Thus, the presence of phenolic compound can accelerate the generation of the excited-state cerium(III), and then energy is transferred from cerium(III)* to rhodamine 6G to form the excited-state rhodamine 6G, which emits its characteristic radiation at 555 nm. In Scheme 1 Rho 6G, Rho $6G_{OX}$, PC and PC_{OX} are rhodamine 6G, the oxidized form of rhodamine 6G, phenolic compound and the oxidized form of phenolic compound.

In recent years, HPLC with CL detection becomes more and more attractive for the determination of the analytes at trace level in complex matrices because of its high sensitivity, high selectivity and wide dynamic range. Zhang and Danielson [32] developed an on-line quinine-sensitized photo-oxidation with quenched CL detection method for phenols using liquid chromatography. In our lab, several HPLC-CL methods have been developed for the simultaneous determination of phenolic compounds [33,34]. However, these HPLC-CL methods only respond the limited phenolic compounds and are not suitable for the simultaneous detection of numerous phenolic compounds. In this paper, we developed a highly selective and sensitive method for the simultaneous determination of 20 phenolic compounds with good CL signals by coupling HPLC with the cerium(IV)-rhodamine 6G CL reaction. It was found that the CL reaction was compatible with the tested mobile phase of HPLC. The conditions for the good separation and the maximal CL intensities of phenolic compounds were optimized. The proposed HPLC-CL method has been successfully used for the simultaneous detection of some phenolic compounds in the wine samples.



Scheme 1.

2. Experimental

2.1. Chemicals and solutions

Methanol was of HPLC grade. All other chemicals were of analytical grade and used without further purification. Redistilled water was used throughout. Cerium(IV) sulphate tetrahydrate was obtained from Shanghai Yaolong Metal Company (Shanghai, China). Rhodamine 6G was obtained from Merck (Darmstadt, Germany). Tyrosine was obtained from Kangda Amino Acid Company (Shanghai, China). Phloroglucinol, pyrogallol, phenol, resorcinol and gallic acid were obtained from China Medicine Group Beijing Regents Company (Beijing, China). 3,5-Dihydroxybenzoic acid and *m*-hydroxybenzoic acid were purchased from Fluka Chemie (Bucks, Switzerland). 2,3-Dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid were purchased from Acros Organics (Fairlawn, New Jersey, USA). Salicylic acid, *p*-hydroxybenzoic acid, 2,4-dihydroxybenzoic acid, *m*-aminophenol and 1-naphthol were purchased from Shanghai Reagents (Shanghai, China). *trans*-Resveratrol was purchased from Oais. Biotech. Inc. (Xi'an, China). Puerarin, quercetin, kaempferol and isorhamnetin were obtained from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

The stock solutions of 1.0×10^{-4} g/ml phenolic compounds were prepared by dissolving appropriate amount of compounds in redistilled water or methanol and remained stable during three months if stored in refrigerator at 4 °C. Standard solutions of phenolic compounds were prepared by further dilution of the stock solutions with redistilled water. The stock solution of 1.0×10^{-3} M rhodamine 6G was prepared with redistilled water and kept at ambient temperature in the dark. The working cerium(IV) solutions were prepared daily in sulfuric acid prior to use.

The HPLC mobile phases were fresh daily prepared, filtered through a 0.22 μm membrane filter (Xinya, Shanghai), and then degassed before injecting into the column.

2.2. Instrumentation

HPLC-CL detection system consisted of HPLC system and CL detection system, as shown in Fig. 1. The HPLC system was Agilent 1100 series (Agilent Technologies, USA), including a quaternary pump, a vacuum degasser, a thermostat column compartment, a diode array and multiple wavelength detection (DAD) system, a manual sample valve injector with a 100 μl loop, and an analytical column (Zorbax Eclipse XDB-

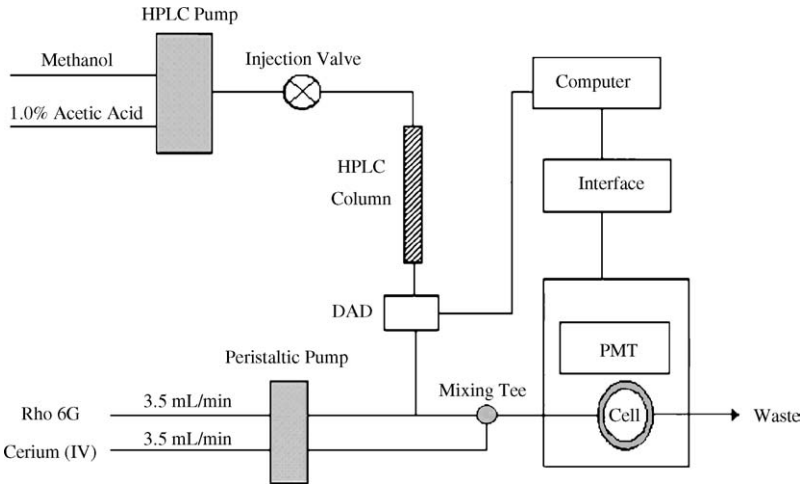


Fig. 1. Schematic diagram of HPLC-CL system.

C_8, 150 mm × 4.6 mm i.d., 5 μm; Agilent Technologies, USA). The CL detection was conducted on a flow injection CL system (Remax, China) consisted of a model IFFM-D peristaltic pump, a mixing tee and a model IFFS-A CL detector equipped with a flat glass coil (used as reaction coil and detection cell, 188 mm × 1.5 mm i.d.) and a photomultiplier. The data from the CL detector was acquired by Agilent Interface 35900E and processed by Chemstation A.08.03 run on a DELL smartpc 100 personal computer.

2.3. Procedure

Phenolic compounds were separated by XDB-C_8 column at 25 °C with a gradient elution program at a flow rate of 1.0 ml/min. The mobile phase consisted of methanol (A) and 1.0% acetic acid (B). 1.0% acetic acid (pH 2.75) was prepared by the ratio of glacial acetic acid and redistilled water (v:v = 1:99). The gradient elution program was: 90–85% B (0–6 min), 85–80% B (6–20 min), 80–70% B (20–21 min), 70–65% B (21–30 min), 65–60% B (30–31 min), 60–55% B (31–50 min), 55–45% B (50–55 min). This was followed by a 10 min equilibrium period with initial conditions prior to the injection of next sample. The UV spectra were recorded between 250 and 400 nm for the identification of phenolic compounds and for the test of peak purity. The column effluent from DAD was first mixed with 5.0×10^{-5} M rhodamine 6G solution via a PEEK tube, then combined with 8.0×10^{-3} M cerium(IV) containing 0.2 M sulfuric acid solution at a mixing tee. Solutions of cerium(IV) and rhodamine 6G were delivered by the same peristaltic pump at a flow rate of 3.5 mL/min, respectively. The light emission was monitored by the photomultiplier tube.

2.4. Compounds identification and quantification

Identification of the phenolic compounds was carried out by comparing their retention times and spectra to those of available standards. Identified peaks were then confirmed by spiking samples with standard mixtures. A diode array detector recording at 254, 270, 290, 306, and 365 nm was used to detect phenolic compounds. Quantification was performed according to an external standard method. The quantitative determination was based on the relative CL intensity $\Delta I = I_S - I_0$, where I_S is the CL intensity of phenolic compound and I_0 the intensity of blank signal.

2.5. Method validation

Method linearity was evaluated by the correlation coefficients (r) of the calibration curves generated with more than 10 standard mixtures. For each single phenolic compound, the limit of detection (LOD) was fixed as three times the signal-to-noise ratio (S/N = 3). Repeatability of peak heights (ΔI) was calculated by the RSD of 11 injections carried out on the same day, whereas, for method reproducibility, seven injections were randomly executed in a 20-day period. Accuracy data are calculated as (mean analyzed concentration)/(nominal concentration) × 100. To evaluate method recovery, actual sample was spiked with mixtures of phenolic compounds and the percent ratios between the recovered and expected concentrations were calculated.

2.6. Sample solution preparation

The tested Chinese red wine (Grand Dragon) produced by Yantai Weilong Grape Wine Co., Ltd. (Shandong, China) was natural variety and obtained at local markets. Wine was stored in the dark at 4 °C and analyzed immediately after bottle opening. The wine sample was filtered through 0.22 μm Millipore membrane (Xinya, Shanghai, China). The filtrated solution was injected directly for the DAD and CL detection.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The effectiveness of HPLC separation was tested using standard solutions containing 20 phenolic compounds. The

tested phenolic compounds include *m*-aminophenol, tyrosine, phloroglucinol, gallic acid, pyrogallol, 3,5-dihydroxybenzoic acid, resorcinol, 2,5-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid, *m*-hydroxybenzoic acid, phenol, puerarin, salicylic acid, *trans*-resveratrol, quercetin, 1-naphthol, kaempferol and isorhamnetin. The gradient elution profile was optimized to obtain the highest resolution of phenolic compounds and the shortest time of analyses. The results indicated that chromatographic gradient systems composed of methanol and water, when adding acetic acid sharpen peak shapes and improve analytical sensitivity and resolution for the HPLC analysis of phenolic compounds [16,35]. Therefore, methanol and water containing acetic acid were used as the mobile phase in this study. Under the condition of 0.5% (v/v) acetic acid, the effects of methanol concentration of the mobile phase on phenolic compound separation were studied. Isocratic elution at 10% (v/v) methanol concentration resulted in poor chromatographic separation of *m*-aminophenol, tyrosine, phloroglucinol, gallic acid and pyrogallol, and 1-naphthol, kaempferol and isorhamnetin were eluted after 80 min. As methanol concentration was increased from 10 to 15%, the co-elution of five phenolic compounds was also obtained. Therefore, an optimal gradient was needed. However, the optimized gradient was affected by the poor separation among 2,4-dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid, *m*-hydroxybenzoic acid, phenol and puerarin, and then the effects of the acetic acid concentration on the resolution were studied in the range of 0.2–1.5% (v/v). It was found that a moderate addition 1.0% of acetic acid yielded a good separation. As a result, the best resolution of all peaks was obtained using a gradient of the mobile phase consisting of methanol and 1.0% acetic acid within 55 min (the profile see Section 2.3). The retention times of phenolic compounds were described as shown in Table 1.

As for HPLC-CL detection, the mobile phase of HPLC is not only suitable for the separation of analytes but also compatible with the CL reaction. Fortunately, in this work, the resulting mobile phase of methanol–1.0% acetic acid was found to be suitable for the separation of phenolic compounds and compatible with the cerium(IV)-rhodamine 6G CL system in sulfuric acid medium.

3.2. Optimization of CL system

To obtain the maximal relative CL intensity, the effects of sulfuric acid, cerium(IV), rhodamine 6G concentration, and flow rate on relative CL intensity were investigated. The phenolic compound concentration used for the optimization experiments was 8.0×10^{-7} g/ml.

Cerium(IV) is not readily soluble in water, but becomes stable when dissolved in sulfuric acid. The effects of sulfuric acid concentration on the relative CL intensities of phenolic compounds were studied over the range 0.05–0.4 M. The relative CL intensities for *m*-aminophenol, pyrogallol and resorcinol were increased with increasing sulfuric acid concentration up to 0.1 M, above which the relative CL intensity decreased. For *p*-hydroxybenzoic acid and 2,4-dihydroxybenzoic acid, the relative CL intensities were increased with increasing sulfuric acid concentration. On the contrary, the relative CL intensities for 2,5-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid were decreased with increasing sulfuric acid concentration. The other 13 phenolic compounds were almost found to generate the maximum emission when sulfuric acid concentration was 0.2 M. As a compromise, 0.2 M sulfuric acid was chosen for the further studies.

The effects of cerium(IV) concentration on the relative CL intensities of phenolic compounds were investigated in the range 3.0×10^{-3}–2.0×10^{-2} M in 0.2 M sulfuric acid medium.

Table 1
Retention time, linear range and regression equation of phenolic compounds by HPLC-CL method

Phenolic compound	Retention time (min)	Linear range (g/ml)	Regression equation[a] $\log \Delta I = a \log C + b$
m-Aminophenol	1.8	5.0×10^{-8}–1.5×10^{-5}	$\log \Delta I = 0.606 \log C + 6.625$
Tyrosine	2.2	3.0×10^{-7}–3.0×10^{-5}	$\log \Delta I = 0.473 \log C + 5.276$
Phloroglucinol	2.4	3.0×10^{-8}–3.0×10^{-5}	$\log \Delta I = 0.738 \log C + 7.808$
Gallic acid	2.7	3.0×10^{-7}–3.0×10^{-5}	$\log \Delta I = 0.663 \log C + 6.598$
Pyrogallol	3.2	3.0×10^{-8}–1.5×10^{-5}	$\log \Delta I = 0.742 \log C + 7.679$
3,5-Dihydroxybenzoic acid	4.9	2.0×10^{-7}–2.0×10^{-5}	$\log \Delta I = 0.668 \log C + 6.716$
Resorcinol	5.4	3.0×10^{-8}–1.5×10^{-5}	$\log \Delta I = 0.665 \log C + 7.284$
2,5-Dihydroxybenzoic acid	8.8	2.0×10^{-7}–2.0×10^{-5}	$\log \Delta I = 0.705 \log C + 6.973$
p-Hydroxybenzoic acid	9.7	3.0×10^{-8}–1.0×10^{-5}	$\log \Delta I = 0.764 \log C + 7.881$
2,4-Dihydroxybenzoic acid	12.0	1.0×10^{-8}–9.0×10^{-6}	$\log \Delta I = 0.832 \log C + 8.804$
2,3-Dihydroxybenzoic acid	13.3	8.0×10^{-8}–1.0×10^{-5}	$\log \Delta I = 0.875 \log C + 8.316$
m-Hydroxybenzoic acid	14.4	2.0×10^{-7}–2.0×10^{-5}	$\log \Delta I = 0.713 \log C + 6.973$
Phenol	15.6	1.0×10^{-7}–1.5×10^{-5}	$\log \Delta I = 0.746 \log C + 7.312$
Puerarin	16.2	1.0×10^{-7}–1.5×10^{-5}	$\log \Delta I = 0.771 \log C + 7.539$
Salicylic acid	29.1	7.0×10^{-8}–1.0×10^{-5}	$\log \Delta I = 0.796 \log C + 7.859$
trans-Resveratrol	30.5	3.0×10^{-8}–1.5×10^{-5}	$\log \Delta I = 0.671 \log C + 7.184$
Quercetin	39.3	3.0×10^{-7}–3.0×10^{-5}	$\log \Delta I = 0.553 \log C + 5.903$
1-Naphthol	47.4	2.0×10^{-7}–2.5×10^{-5}	$\log \Delta I = 0.622 \log C + 6.411$
Kaempferol	49.3	8.0×10^{-9}–9.0×10^{-6}	$\log \Delta I = 0.778 \log C + 8.411$
Isorhamnetin	50.8	1.0×10^{-7}–1.0×10^{-5}	$\log \Delta I = 0.739 \log C + 7.331$

[a] ΔI: relative CL intensity; C: concentration of phenolic compound; regression coefficients: $r > 0.995$; $n \geq 10$.

For tyrosine, *m*-hydroxybenzoic acid, quercetin and 1-naphthol, the relative CL intensities were increased with increasing cerium(IV) concentration. The maximal enhanced CL intensities were all reached at the concentration of 8.0×10^{-3} M for the other 16 phenolic compounds, above which the CL intensities sharply decreased. Lower concentrations of oxidant produced lower CL emission, whereas CL emission decreased with higher cerium(IV) concentrations. Because of the increased collisional energy transfer between molecules caused by higher cerium(IV) concentration, some excited-state rhodamine 6G molecules return to the ground state by a non-radiative internal transfer process, which would decrease the chemiluminescent quantum yield. Therefore, 8.0×10^{-3} M cerium(IV) was selected for the further work.

Under the above selected conditions, the effects of rhodamine 6G concentration on the relative CL intensities of phenolic compounds were tested over the range 1.0×10^{-5}–1.0×10^{-4} M. The results showed that the optimum concentrations of rhodamine 6G for all tested 20 phenolic compounds were 5.0×10^{-5} M. Lower concentrations of rhodamine 6G gave lower CL emission because of fewer energy receptors, and higher concentrations produced a decreased CL emission due to self-absorption of radiation.

The flow rates of solutions are very important to the CL reaction and should be regulated. At the flow rates that are too slow or too high, CL is not emitted in the flow cell and hence the emitter cannot be detected. The effects of flow rate on the relative CL intensities of phenolic compounds were studied over the range 1.5–4.5 ml/min in each stream (Fig. 2). The CL intensities for phloroglucinol, resorcinol and *m*-hydroxybenzoic acid were increased with increasing flow rate up to 3.5 ml/min, above which the CL intensities decreased. For the other 17 phenolic compounds, the relative CL intensities were increased with increasing flow rate. However, higher flow rates caused more noise, higher pressure in the connection and excessive consumption of reagents. Accordingly, a flow rate of 3.5 ml/min was recommended because of greater precision of CL signals and lower consumption of reagents.

Under the optimized conditions, a typical chromatogram of a standard mixture of 20 phenolic compounds with CL detection is shown in Fig. 3a. The degree of band broadening compared to DAD detection is in the range of 1.1–2.7%, which was calculated in peak width at half-height ($W_{h/2}$). The results demonstrate that the CL detector is good.

3.3. Method validation

Under the optimum conditions described above, the calibration curves were prepared over the range 5.0×10^{-9}–5.0×10^{-5} g/ml, and at least 10 samples covering the whole range were used for each compound. Each point of the calibration graph corresponded to the mean value from three independent peak measurements. The linearity between the logarithm of phenolic compound concentration (C) and the logarithm of CL intensity (ΔI) was good, as shown by the data in Table 1 that the regression coefficients (r) were greater than 0.995 for all the curves. For all tested phenolic compounds, the linear ranges of CL detection were more than 2 orders of magnitude. Table 2 indicates that the detection limits at a signal-to-noise ratio of three are in the range of 1.5–82.1 ng/ml.

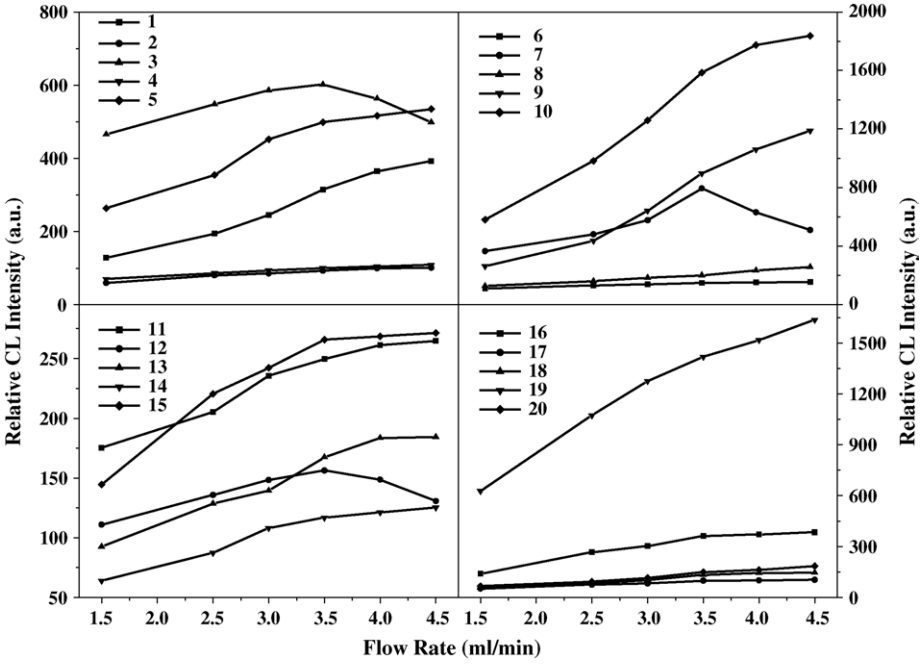


Fig. 2. Effect of flow rate on the relative CL intensities of 20 phenolic compounds at a concentration of 8.0×10^{-7} g/ml. (1) *m*-aminophenol; (2) tyrosine; (3) phloroglucinol; (4) gallic acid; (5) pyrogallol; (6) 3,5-dihydroxybenzoic acid; (7) resorcinol; (8) 2,5-dihydroxybenzoic acid; (9) *p*-hydroxybenzoic acid; (10) 2,4-dihydroxybenzoicacid; (11) 2,3-dihydroxybenzoic acid; (12) *m*-hydroxybenzoic acid; (13) phenol; (14) puerarin; (15) salicylic acid; (16) *trans*-resveratrol; (17) quercetin; (18) 1-naphthol; (19) kaempferol and (20) isorhamnetin.

Table 2
Detection limit, precision and accuracy of phenolic compounds

Phenolic compound	Detection limit (ng/ml)		Precision (RSD, %)		Accuracy (%)	
	CL	DAD	Intra-day	Inter-day	Intra-day	Inter-day
			1.2	2.5	99.1	101.2
m-Aminophenol	7.5	135.4[a]	1.5	2.9	96.1	100.1
Tyrosine	25.4	221.3[b]	2.8	1.9	100.4	99.4
Phloroglucinol	5.3	545.8[b]	3.0	2.3	103.7	95.0
Gallic acid	40.4	154.6[a]	1.3	2.0	96.3	96.3
Pyrogallol	8.7	399.2[a]	2.4	2.3	98.7	92.8
3,5-Dihydroxybenzoic acid	30.6	663.5[b]	2.6	2.8	95.4	98.7
Resorcinol	4.0	434.1[a]	2.2	3.1	105.0	96.4
2,5-Dihydroxybenzoic acid	32.9	852.7[b]	1.9	2.0	102.7	100.4
p-Hydroxybenzoic acid	8.2	356.2[b]	1.7	2.6	101.3	103.4
2,4-Dihydroxybenzoic acid	2.9	425.6[b]	0.9	3.9	99.8	100.4
2,3-Dihydroxybenzoic acid	27.3	500.8[b]	1.0	2.4	98.5	100.9
m-Hydroxybenzoic acid	40.3	352.1[b]	1.7	1.9	95.1	95.9
Phenol	30.1	1100.3[a]	2.1	2.1	93.9	98.6
Puerarin	26.5	208.4[b]	2.8	2.7	104.7	99.5
Salicylic acid	18.0	304.2[c]	3.5	1.4	103.5	104.3
trans-Resveratrol	6.6	84.7[d]	2.7	3.0	100.9	103.8
Quercetin	24.9	621.4[e]	2.4	3.4	101.8	100.7
1-Naphthol	26.3	987.5[c]	1.3	3.3	99.1	94.4
Kaempferol	2.3	814.2[e]	1.4	1.1	96.7	95.7
Isorhamnetin	23.9	525.8[e]	2.5	2.7	98.3	104.2

[a] Wavelength of DAD detection: 270 nm.
[b] Wavelength of DAD detection: 254 nm.
[c] Wavelength of DAD detection: 290 nm.
[d] Wavelength of DAD detection: 306 nm.
[e] Wavelength of DAD detection: 365 nm.

The detection limits of CL detection for 20 phenolic compounds were about 1–2 orders of magnitude lower than those of DAD detection. The results demonstrate that the HPLC-CL method is sensitive for the detection of all tested phenolic compounds.

The intra-day precision was tested with 11 repeated injections of phenolic compound solution at the concentration level of 8.0×10^{-7} g/ml. The inter-day precision of the proposed method was studied by analyzing 8.0×10^{-7} g/ml phenolic compound solution, with seven injections randomly executed in a 20-day period. The relative standard deviations (RSD) were below 4.0%. The intra- and inter-day accuracy was also determined by the same procedure (Table 2). The obtained data demonstrated that the proposed analytical method provided good validation.

3.4. Application

Red wines include a number of phenolic compounds, which are secondary metabolites naturally present in wine grapes and/or produced during the winemaking process, such as monomeric flavonoids, anthocyanins, phenolic acids and polymeric tannins. The characteristic spectrum of phenolic compounds in wines depends on the origin of the natural wine, the species and origin of wood, the characteristics of the barrel, and on the circumstances and duration of the winemaking process [36]. The analytical application of the proposed CL detection system was tested by determining phenolic compounds in red wine. The identification of phenolic compounds in red wines was carried out by retention time and UV spectra. The chromatographic profile of phenolic compounds in red

Table 3
Concentration and recovery of phenolic compounds in red wine determined by the HPLC-CL method

Phenolic compound	Concentration[a] (ng/ml)		Added (ng/ml)	Recovered (ng/ml)	Recovery (%)
	CL	DAD			
Gallic acid	5314.2 ± 100.7	5289.5 ± 107.8	2000.0	7225.4	95.5
p-Hydroxybenzoic acid	2871.5 ± 40.9	ND	2000.0	4863.9	99.6
2,4-Dihydroxybenzoic acid	938.6 ± 15.8	ND	500.0	1449.7	102.2
Salicylic acid	463.7 ± 11.1	ND	500.0	957.8	98.8
trans-Resveratrol	244.3 ± 5.4	ND	200.0	451.7	103.8
Kaempferol	43.9 ± 0.6	ND	20.0	63.7	99.0

ND: not detected.
[a] Concentration: mean ± SD, $n = 3$.

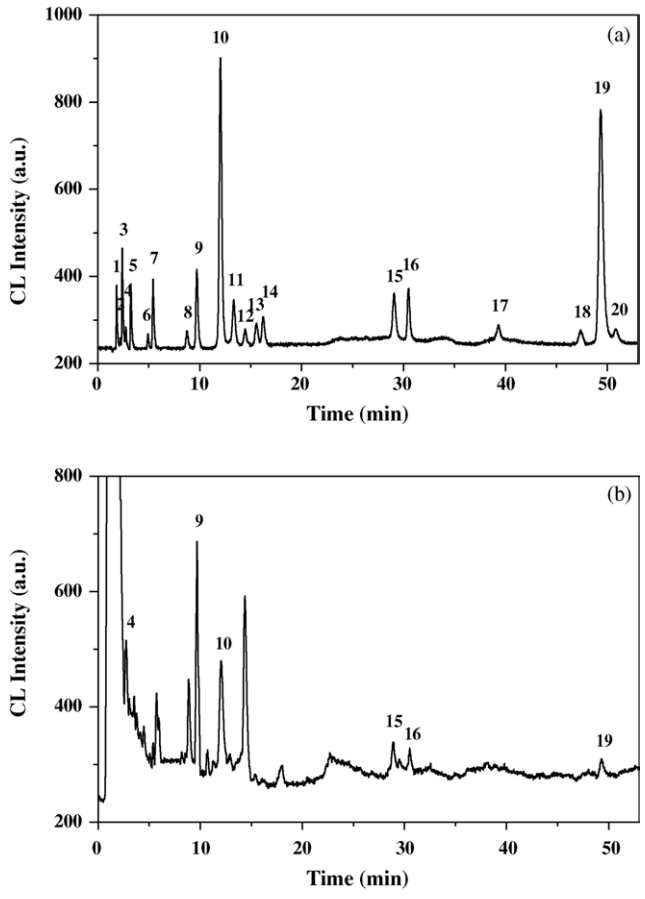


Fig. 3. CL chromatograms of (a) 20 standard phenolic compounds (1.0×10^{-6} g/ml) and (b) red wine sample. Conditions are given in Section 2.3. Peaks: (1) *m*-aminophenol; (2) tyrosine; (3) phloroglucinol; (4) gallic acid; (5) pyrogallol; (6) 3,5-dihydroxybenzoic acid; (7) resorcinol; (8) 2,5-dihydroxybenzoic acid; (9) *p*-hydroxybenzoic acid; (10) 2,4-dihydroxybenzoic acid; (11) 2,3-dihydroxybenzoic acid; (12) *m*-hydroxybenzoic acid; (13) phenol; (14) puerarin; (15) salicylic acid; (16) *trans*-resveratrol; (17) quercetin; (18) 1-naphthol; (19) kaempferol and (20) isorhamnetin.

wine by CL detection shows that there is no interference by matrix (Fig. 3b). Six phenolic compounds were identified as gallic acid, *p*-hydroxybenzoic acid, 2,4-dihydroxybenzoic acid, salicylic acid, *trans*-resveratrol and kaempferol, and the concentration of tested phenolic compounds in red wine was shown in Table 3. It is worth noting that phenolic compounds with lower content cannot be detected using DAD detection in the tested red wine, but was detectable by CL detection except gallic acid. In order to evaluate the validity of the proposed method for the determination of phenolic compounds in wine sample, a recovery experiment was carried out by adding the known amounts of phenolic compounds to sample solution, and the good recoveries varied from 95.5 to 103.8%. The good recovery results indicated that the proposed HPLC-CL method was reliable for the quantification of phenolic compounds in red wine sample.

4. Conclusions

In this paper, a new HPLC-CL detection method has been developed for the determination of phenolic compounds based on the enhancement of cerium(IV)-rhodamine 6G CL in sulfuric acid medium. The method allows for the simultaneous and sensitive detection of phenolic compounds in red wine without preconcentration or derivatization step, and offers wider linear range, lower detection limit, and shorter analysis time. Moreover, the CL reaction is compatible with the mobile phase of HPLC. Based the obtained results, the following conclusions can be drawn. First, the proposed method extends the scope of CL detection in HPLC to the determination of phenolic compounds. Second, the proposed method can be a valuable tool (simple and inexpensive) for the determination of phenolic compounds in foods and environmental samples. Third, this CL system could tolerate distinct acidic medium rather than an alkaline medium like the luminol CL system, thus the analytes stable in acidic solution could be detected safely.

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