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On-line high-performance liquid chromatography–diode array detection–electrospray ionization–mass spectrometry–chemiluminescence assay of radical scavengers in *Epimedium*

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ABSTRACT

An on-line analysis method for the simultaneous detection and identification of radical scavenging compounds in plant extracts was developed by combining HPLC with hydrogen dioxide radical scavenging and HPLC–DAD–MS–CL system. The structural identification and activity characteristics of various constituents could be rapidly achieved by the on-line assay of UV, MS and CL in one run. In 4 species of *Epimedium* studied 32 compounds, including phenolic acids, 8-isopentenyl-flavonoid glycosides and flavonoid glycosides containing a ortho-hydroxyl group, were identified by comparison with authentic standards and published mass data. Among these compounds, phenolic acids and flavonoid glycosides containing an ortho-hydroxyl group could obviously inhibit CL, which suggested their strong radical scavenging activity. These four species each exhibited different active properties, which might correlate to their respective quality. The results indicated that the on-line HPLC–DAD–MS–CL system would be a potential method to rapidly and sensitively screen radical scavengers in herbal medicines, and could display an integrated fingerprint based on different detectors.

1. Introduction

Epimedium, a well-known Chinese herbal medicine, is widely distributed in China with about 30 species. In Chinese Pharmacopoeia, the aerial parts of five species of this plant are designated as the official source for using Herba Epimedii, including *Epimedium brevicornum* Maxim., *E. sagittatum* Maxim., *E. pubescens* Maxim., *E. wushanense* T.S. Ying and *E. koreanum* Nakai [1,2]. Flavonoids are considered as the main active components in *Epimedium*, and possess favorable antioxidant, antiosteoporosis and antitumor activities [3–5]. Some analysis methods like HPLC, HPLC–MS/MS and capillary zone electrophoresis (CZE) have been applied to determine a few flavonoids in *Epimedium* [6–8]. It is well known that the integrated contributions of multiple chemical components are responsible for their therapeutic effects. Although many compounds in *Epimedium* have been identified by HPLC–MS [1], their active properties yet unclear. Therefore, the on-line analysis by chromatographic separation combined with different detectors is gaining rapidly popularity to simultaneously obtain the active properties and structural information of various ingredients in complex matrices. To some extent, this method will also reveal the comprehensive quality of herbal medicines according to their multiple active constituents.

Reactive oxygen species (ROS) play an important role in damaging a variety of surrounding tissues and organs and in many pathological processes such as aging, atherosclerosis and chronic inflammation [9–11]. Plant ROS scavengers are considered to be relatively safe and favorably effective. Many studies have focused on the analysis of natural ROS scavengers, including high-performance liquid chromatography–diode array detector–mass spectrometry (HPLC–DAD–MS) [12], HPLC–MS [13], gas chromatography–mass spectrometry (GC–MS) [14], capillary electrophoresis-chemiluminescence (CE–CL) [15] and CE–DAD [16]. These methods are very sensitive and useful for the qualitative or the quantitative analysis of multiple ingredients in herbal medicines but for the lack of activity assessment.

In order to search for bioactive compounds in plants, tedious preparative isolation and purification processes are necessary to obtain a single compound for evaluation [16]. Efficient approaches for seeking new active compounds from complex

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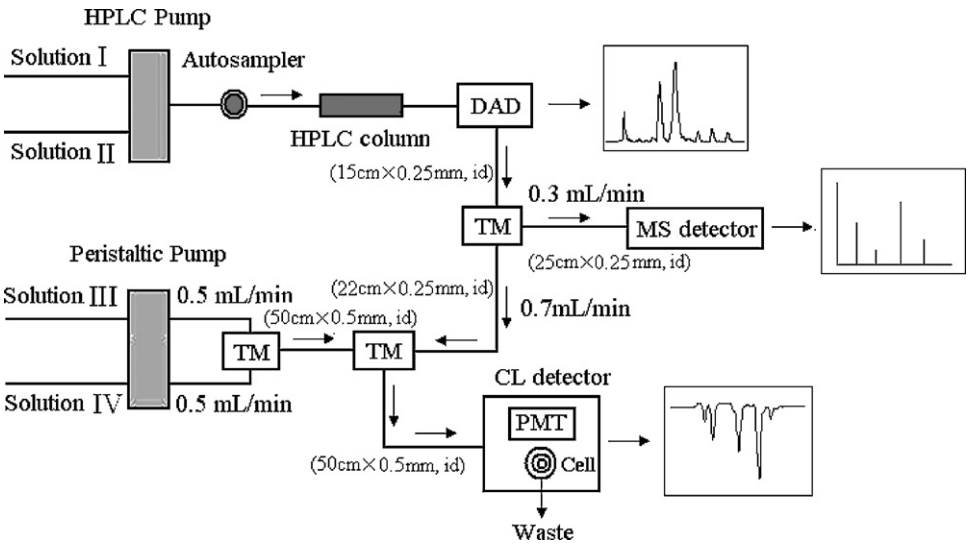


Fig. 1. HPLC–DAD–MS–CL detection apparatus. TM, Mixing Tee; Cell, glass coil (80 μL); PMT, photomultiplier.

matrices continue to be one of the major challenges in the field of analytical chemistry. On-line methods such as HPLC–DPPH/ABTS–UV [17,18], HPLC–DPPH–mass spectrometry (MS) [19], HPLC–DPPH/ABTS–UV–MS [20,21], HPLC–DPPH–SPE one-dimensional and two-dimensional nuclear magnetic resonance (NMR) [22] and HPLC–DPPH/ABTS–UV–NMR [23] have been used to evaluate [1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) scavenging activities of antioxidants in plant extracts. In addition, an on-line HPLC-biochemical detection (HPLC–BCD)–MS method has been developed for angiotensin-converting enzyme (ACE) inhibitors in complex mixtures [24]. Liquid chromatography coupled with high resolution mass spectrometry and an online continuous-flow AChE bioassay has been developed for rapid assessment of chemical and biological degradation products of tacrine and their bioactivity for acetylcholinesterase (AChE) [25]. Chemiluminescence (CL) is a sensitive detection method and is widely accepted in various fields, especially in the determination of ROS scavenging activity. Currently, HPLC–CL on-line detection using flow injection analysis meets the requirement of multiple component detection in complex samples [26], and has been developed to screen antioxidants in herbal medicines [27,28]. H_2O_2, a ubiquitous free radical generated from many cell types, can activate signaling processes and induce cytotoxicity and plays an important role in biological systems. Therefore, the aim of this study was to develop an on-line HPLC–DAD–MS–CL method to rapidly identify H_2O_2 scavengers in 4 species of *Epimedium*. The chemical characteristic, active properties and structural elucidation of H_2O_2 scavengers are simultaneously displayed.

2. Experimental

2.1. Materials and reagents

The aerial parts of *E. sagittatum* Maxim (E.S), *E. pubescens* Maxim (E.P), *E. wushanense* T.S. Ying (E.W), and *E. koreanum* Nakai (E.K) were provided by the Shanxi Institute of Biochemical Drug Control (Xi'an, China). Standard substance epimedin A, epimedin B, epimedin C, and icarrin were purchased from Jiangsu Provincial Academy of Traditional Chinese Medicine (Nanjing, China). All chemicals used were of analytical reagent grade. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) from Fluka (Buchs, Switzerland), hydrogen peroxide (30% H_2O_2 in water), Na_2CO_3 and $NaHCO_3$ from Nanjing Chemical Reagent Corporation (Nanjing, China), and EDTA from Shanghai Chemical Reagent Corporation (Shanghai, China), were used for CL detection. Acetonitrile of chromatographic grade (Dikma, USA) and formic acid of analytical grade (Nanjing Chemical Plant, China) were used for the preparation of

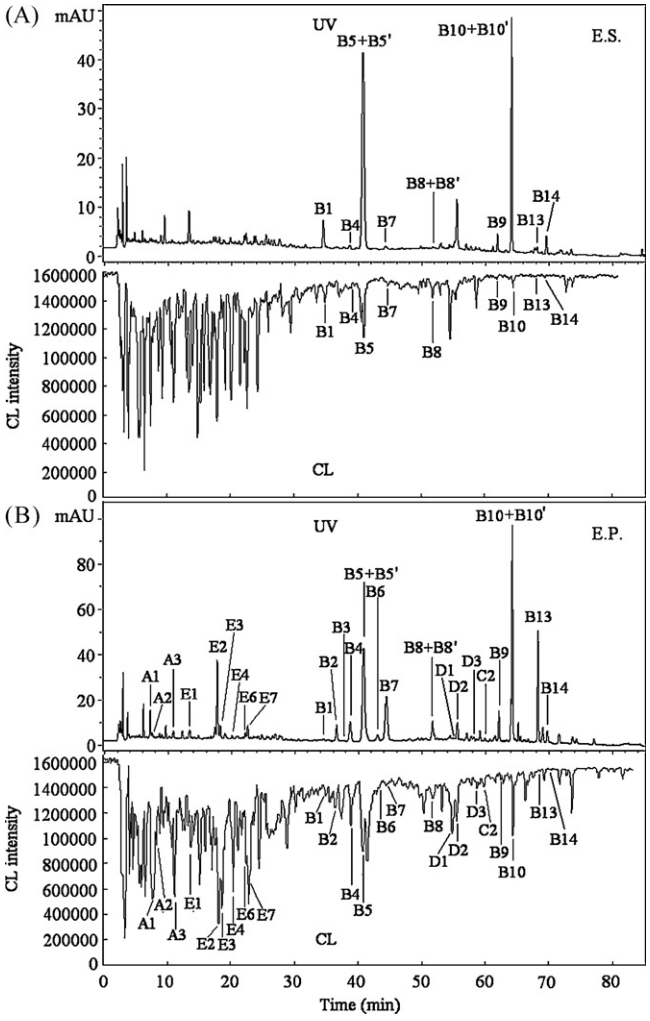


Fig. 2. Chromatograms and active profiles of E.S and E.P by on-line HPLC–DAD–MS–CL detection. (A) E.S and (B) E.P.

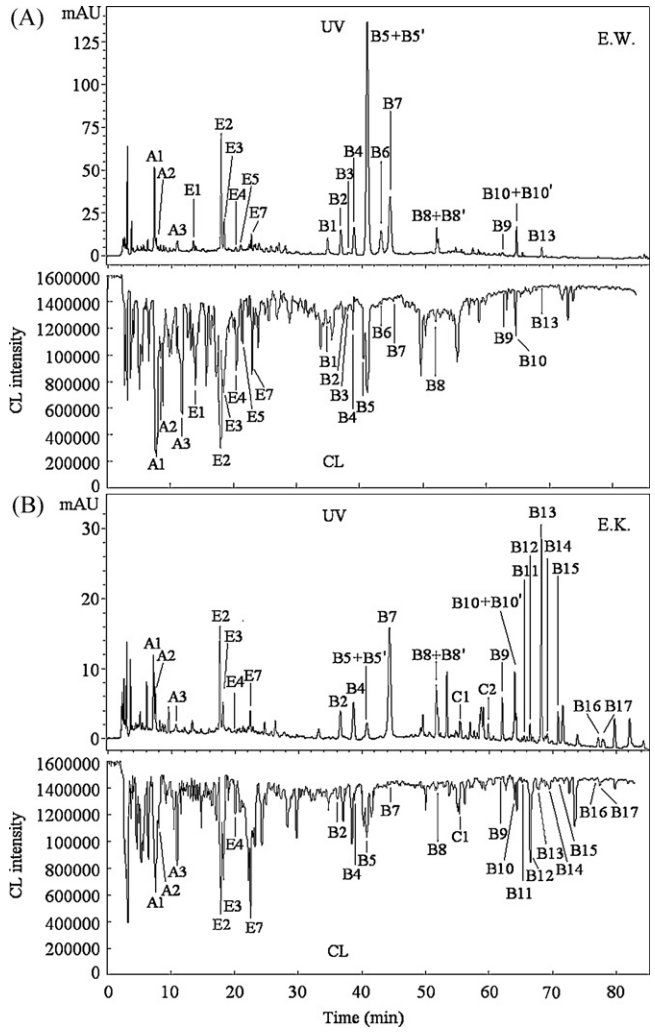


Fig. 3. Chromatograms and active profiles of E.W and E.K by on-line HPLC–DAD–MS–CL detection. (A) E.W and (B) E.K.

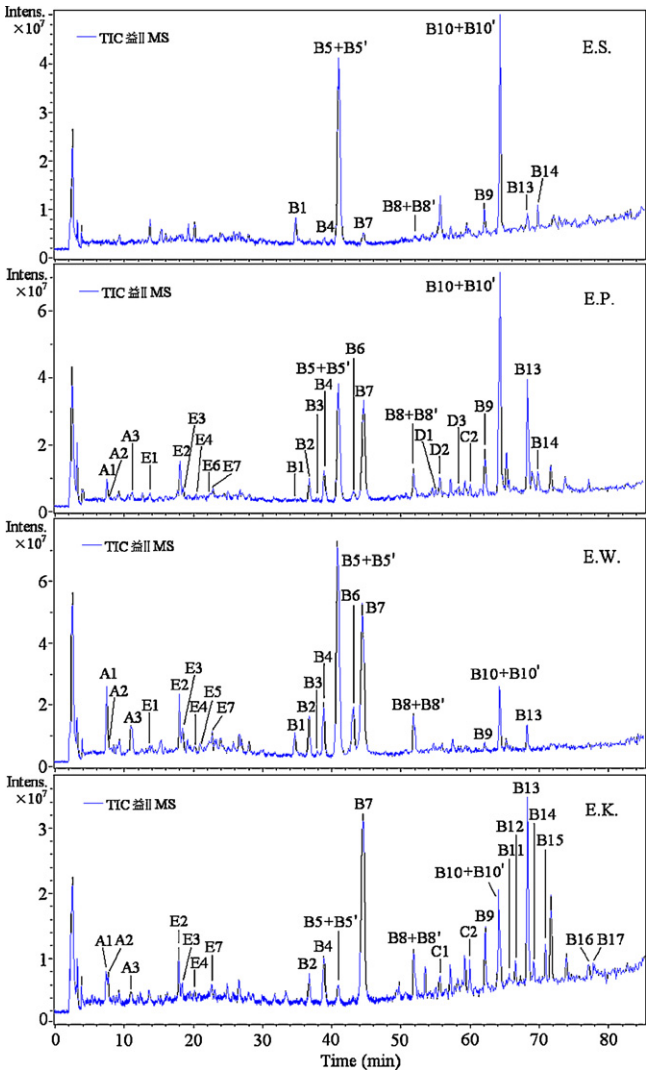


Fig. 4. The total ion chromatograms of 4 species of *Epimedium*.

mobile phase. The water used was purified by a Millipore water purification system (Milford, MA, USA).

2.2. CL reagent solutions

A 1.82×10^{-2} M stock solution of luminol was prepared in 0.1 M Na_2CO_3 solution and stored in a refrigerator for at least 3 days before dilution. Luminol solutions were prepared by diluting the stock solution in 0.1 M carbonate buffer (pH 11.0) containing 6.3×10^{-3} M EDTA. H_2O_2 solution was immediately prepared before use by diluting 30% H_2O_2 solution in water. All solutions were protected from light throughout the process of CL detection.

2.3. Sample preparation

Aerial parts of Herba Epimedii from 4 species were milled to 60 mesh powder, and dried at 60 °C for 6 h. 0.25 g samples accurately weighed were extracted with 10 mL of 70% methanol for 1 h in an ultrasonic bath. The solutions were filtered through 0.45 μm membranes prior to use, and an aliquot of 5 μL was injected into HPLC for analysis.

2.4. HPLC–DAD–MS–CL instrumentation

The equipment used was an Agilent 1100 series HPLC system consisting of a binary pump, autosampler, thermostated column compartment, and a photodiode array detector (DAD). A LiChrospher C_{18} column (250 mm × 4.6 mm, particle size 5 μm) (Hanbon Sci & Tech, Nanjing, China) was used for all chromatography separations. The mobile phase consisted of aqueous formic acid (0.1%, v/v) (A) and acetonitrile (B) using a gradient program of 15–25% (B) in 0–20 min, 25–27% (B) in 20–30 min, 27% (B) in 30–40 min, 27–40% (B) in 40–55 min, 40–50% (B) in 55–65 min, 50–60% (B) in 65–80 min and 60–100% (B) in 80–85 min. The flow rate was 1.0 mL min^{-1} and column temperature was maintained at 30 °C. The detection wavelength was set at 270 nm.

The above HPLC system was interfaced with an Agilent 1100 LC/MSD Trap XCT ESI (Agilent Technologies, MA, USA). The ESI–MS^3 spectra were acquired in negative ionization modes recorded over a mass range of *m*/*z* 100–900. Capillary voltage was 3200 V. Drying gas temperature was set at 350 °C with a flow rate of 9.0 L min^{-1} and a nebulizing pressure of 40 psi. Data were processed by LC/MSD Trap Software 4.2 and Data Analysis 2.2.

The CL detector was equipped with a flat glass coil of 80 μL as a detection cell and a photomultiplier operated at −800 V. All other parts of the HPLC–DAD–MS–CL detection system were

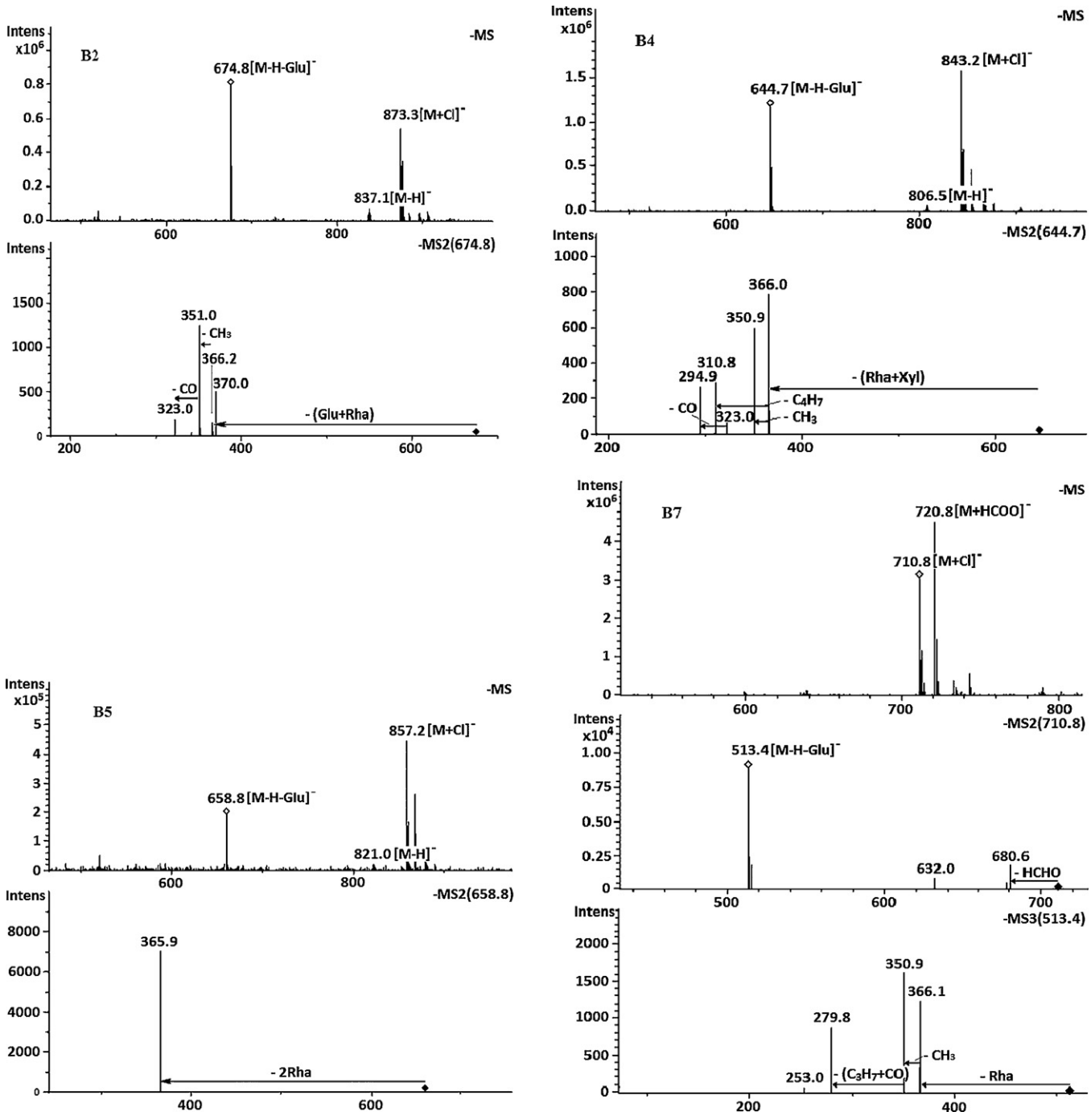


Fig. 5. Negative MS spectra of compound B2, B4, B5 and B7.

interconnected with 0.25- and 0.5-mm i.d. polyether ether ketone (PEEK) tubes. The solution passing through the DAD detector was split by a T-tube connecting two PEEK tubes with the same inner i.d. and different length for the simultaneous detection of MS and CL (split ratio, 0.3:0.7). The split ratio was measured according to the flow rate of outlet of ESI capillary in MS detector. Luminol and H_2O_2 solutions were delivered by a dual pathway peristaltic pump and mixed immediately in the tube. The mixed solution was then combined with the split solution and finally arrived at the CL detector (Fig. 1). The flow rates of luminol and H_2O_2 solutions were optimized by determination of the flow rate of the solution from the outlet of the ESI capillary in order to keep the split ratio (0.3:0.7) in the HPLC–DAD–MS–CL system.

3. Results and discussion

3.1. Design of HPLC–DAD–MS–CL method

CL is often used to determine the ROS scavenging activity of free radicals such as H_2O_2, $^{\bullet}OH$ and $O_2^{\bullet-}$ [29–31]. Luminol is usually chosen as CL reagent because of its quick reaction

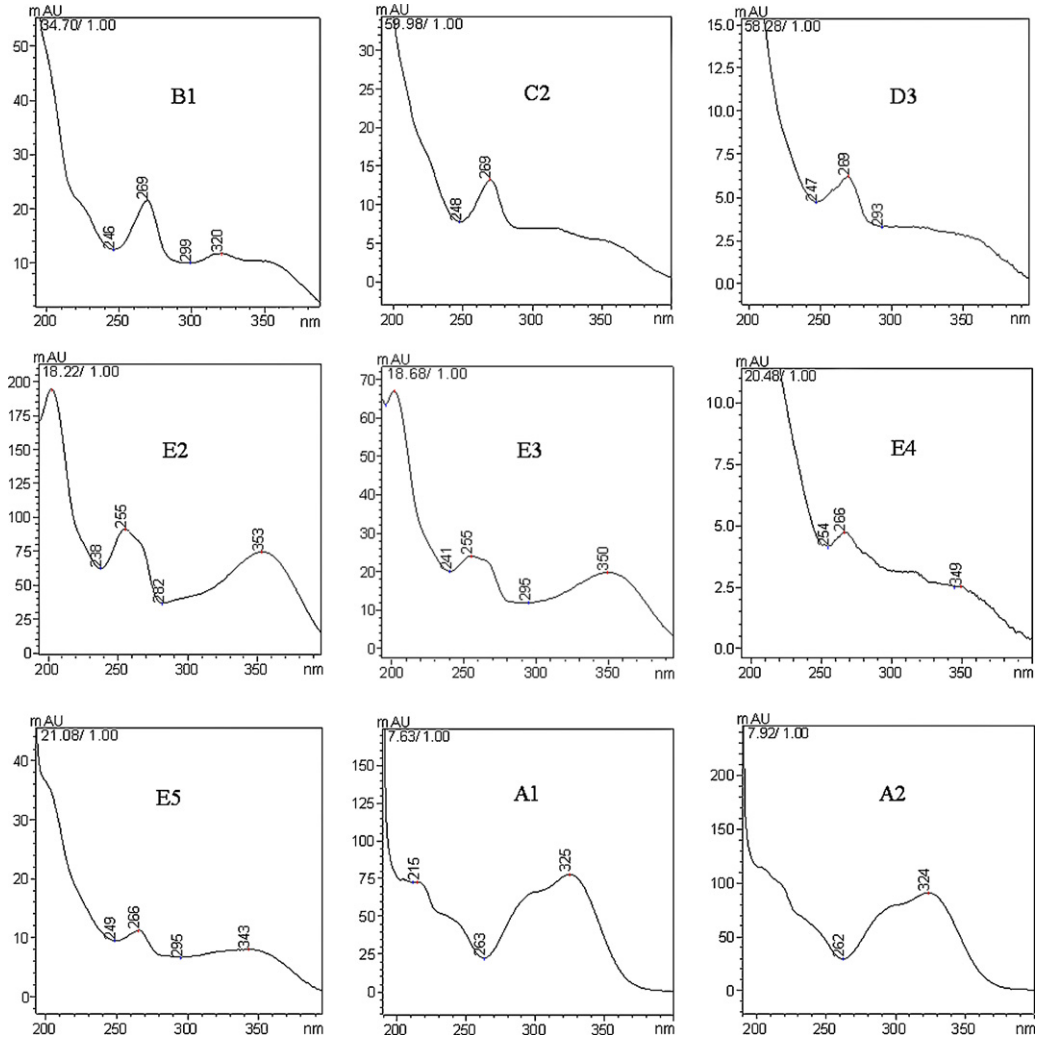


Fig. 6. UV spectra of compound A1, A2, B1, C2, D3, E2, E3, E4 and E5.

speed and excellent luminous intensity. However, the mechanism of CL detection of these three free radicals differs. H_2O_2 scavenging activity can be directly detected, while $^{\bullet}OH$ and $O_2^{\bullet-}$ require chemical reactions to be detected. Hence, the connection of multiple tubes for the on-line reactions of CL reagents is absolutely essential to produce $^{\bullet}OH$ and $O_2^{\bullet-}$ in the latter, which may significantly affect the sensitivity of radical scavenger detection and on-line combination of MS and CL. Therefore, in this study, an on-line HPLC–DAD–MS–CL method using only luminol and H_2O_2 was proposed to elucidate the activity and structural characteristics of H_2O_2 scavengers in herbal medicines.

In the present system, DAD, MS and CL detectors were simultaneously used to obtain the chemical and activity profiles and structural information of H_2O_2 scavengers in *Epimedium*. CL reaction was favored under alkaline conditions, and the radical-scavenging reaction was carried out in the buffer salt solution. These analytical conditions were not compatible with those of DAD and MS detection. To achieve the on-line combination of the three detectors, PEEK tubes with different lengths and inner diameters were selected to balance the pressure generated from the flux in different tubes. Meanwhile, the concentrations and flow rates of CL solutions had to be optimized to avoid the unacceptable baseline drift and inadequate CL intensity.

3.2. Optimization of HPLC–DAD–MS–CL detection conditions

Acids and organic reagents, which were mainly used for chromatographic separation, could induce CL baseline drift due to quenching of luminescence caused by organic reagents and the pH change of the buffer solution. To make it compatible among DAD, CL, and MS detections, the chromatographic conditions should be optimized to obtain high sensitivity and a stable baseline for CL detection, while the conditions of CL detection and chromatographic separation should be suitable for MS analysis. In our previous studies, desired results were obtained for the HPLC–DAD–CL analysis by the gradient elution using acetonitrile–0.1% phosphoric acid [27]. However, phosphoric acid was not compatible with MS detection. Therefore, the gradient elution procedures using acetonitrile–water, respectively acetonitrile–0.1% formic acid were evaluated for the analytical signal corresponding to the blank CL intensity. The results indicated that the detection of samples could not be achieved due to lower CL intensity and strong baselines drift when acetonitrile–water was used. Therefore, the gradient elution using acetonitrile–0.1% formic acid, which could enhance ionic strength and induce high CL intensity, was selected for chromatographic separation.

As important parameters influencing the CL intensity and the sensitivity of CL detection, concentrations of CL reagents and pH values of carbonate buffer were investigated. Different concentra-

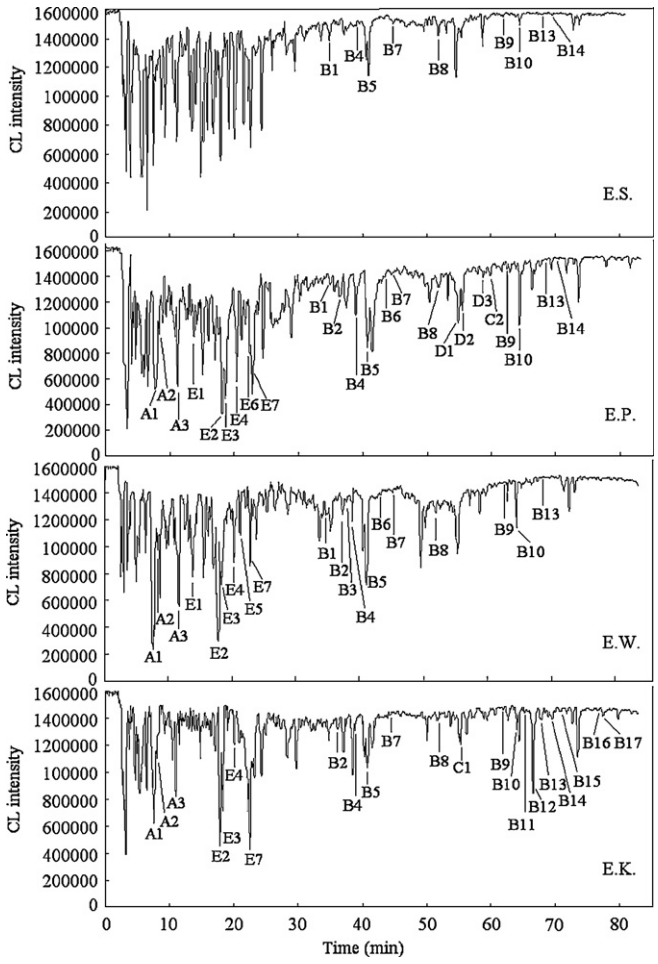


Fig. 7. CL profiles of 4 species of *Epimedium*.

tions of luminol (9.0×10^{-6}, 3.6×10^{-5}, 7.2×10^{-5}, 1.08×10^{-4} and 1.82×10^{-4} M) and H_2O_2 (1.76×10^{-4}, 8.8×10^{-4}, 1.76×10^{-3} and 3.52×10^{-3} M) were evaluated. The results indicated that higher CL intensity could be achieved by increasing the concentrations of luminol and H_2O_2. Radical scavengers in *Epimedium* could be sensitively detected when the concentrations was 1.08×10^{-4} M for luminol and 8.8×10^{-4} M for H_2O_2, respectively. Meanwhile, the concentration of H_2O_2 closely correlated with the sensitivity of CL detection. Taking the alkaline solution required into consideration for CL reaction, pH 10.0, 10.5 and 11.0 for the carbonate buffer were studied. Ultimately, pH 11.0 was selected for further studies due to the high CL intensity and the stable CL baseline in the on-line assay. Additionally, the flow rates of the CL solutions are extremely important to perform the simultaneous detection of MS and CL. Higher flow rates may cause carbonate buffer into MS detector and increase the possibility of damaging the MS detector. The different flow rates were measured disconnecting MS detector from the on-line system, and the split ratio was maintained at 7:3. Under these conditions, three flow rates (0.4, 0.5 and 0.7 mL min^{-1}) were investigated for the stable baseline and high sensitivity of CL detection. The results indicated that the flow rate of 0.5 mL min^{-1} of the CL solutions was compatible with on-line detection of CL and MS, and was used for further studies.

3.3. On-line assay of Epimedium by HPLC–DAD–MS–CL

Samples from the four *Epimedium* species, including E.S, E.P, E.W and E.K, were determined by the present HPLC–DAD–MS–CL system. Their DAD traces, CL profiles and total ion chromatograms are shown in Figs. 2–4. As shown in Figs. 2 and 3, a series of chromatographic peaks and their corresponding CL inhibition could be observed, and the degree of activity was proportional to the intensity of the negative peak. Thus, the antioxidant activities of the compounds were described by their scavenging rates (%) and calculated by the following equation:

$$\text{scavenging rate}\,(\%) = \frac{CL_0 - CL_1}{CL_0} \times 100$$

where CL_0 was the blank CL intensity (without sample) and CL_1 was the inhibited CL intensity of every compound in the samples.

According to Figs. 2–4, some minor peaks (0–30 min) in the total ion chromatograms and DAD traces were not satisfactorily separated under the present separation conditions. In CL profiles, the minor peaks showed strong CL inhibition, and exhibited noticeable advantages over some major peaks in H_2O_2 scavenging activity. In addition, some negative peaks could be observed after 30 min in Figs. 2(B) and 3(A) and (B), while only few negative peaks were found after 30 min in Fig. 2(A). Significant disparities in multiple profiles revealed different active constituents in the four species.

The minor peaks showing strong activity might be generally ignored in the chromatographic fingerprint analysis giving high weight to the major peaks. As a sensitive analysis method, the on-line HPLC–DAD–MS–CL could display integral activity of complex samples. The chemical structures and activity characteristics of various constituents in *Epimedium* could be revealed by different detectors with their respective functions.

3.4. Identification of main radical scavengers in Epimedium

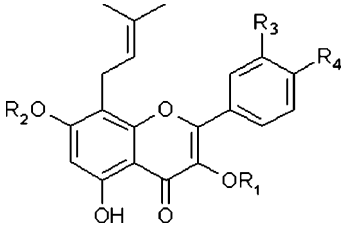
Negative ion mode was found to be more suitable for the identification of phenolic compounds than positive mode. Therefore, as many as 32 compounds in the four species were identified by on-line ESI–MS in the negative mode (Table 1). They were tentatively identified as phenolic acids (A), 8-isopentenyl-flavonoid glycosides (B, C and D), and some flavonoid glycosides containing ortho-hydroxyl group (E). Their structures were mainly confirmed by comparing their UV and mass spectra with those described in literature [1].

Some isomers could be found according to the MS data in Table 1. Among them, B2, B4, B5 and B7 were identified as epimedin A, B, C and icarrin by comparison with standards. As shown in Fig. 5, the structures of epimedin A, B, C and icariin resembled each other contained the same aglycone. The analogies of structures indicated the similarities of their MS^n fragmentation behaviors. In their ESI-MS spectra, the $[M-H]^-$, $[M+Cl]^-$ and $[M+HCOO]^-$ ions were observed, while $[M-H-Glu]^-$ was exhibited as predominant ion in the MS/MS^2 spectrum. The aglycone ions at m/z 366 could be observed and their fragmentated by losing CH_3, C_3H_7, C_4H_7 and CO were also detected separately or simultaneously in the MS^2/MS^3 of these compounds. Exceptionally, the ion at m/z 366 was the only peak observed in MS^2 spectrum of epimedin C after the loss of 7-*O*-rha-(1–4)rha. The isomers of B2, B4 and B5 were tentatively assigned according to the corresponding compounds reported in a previous publication [1].

UV spectra of the compounds obtained by DAD detection are shown in Fig. 6. It is evident that these compounds were discriminated in 3 groups, which was identical to the results of MS detection. UV spectra of B, C and D were similar, but A and E showed difference. Different UV spectra of compounds in E group implied different flavonoid glycosides containing a ortho-hydroxyl group. Although $[M-H]^-$ for E2 and E4 were coincident, UV spectra between E2 and E4 were different, and the same is true for E3 and E5 as shown in Fig. 6 [32]. Similar UV spectra were obtained for A1 and A2, but different ion fragments from MS^2 and MS^3 could dis-

Table 1
Identification of phenolic acid and flavonoid glycosides in the four species.

No.	t_R (min)	$[M-H]^-$	Compounds	HPLC–ESI–MS^n m/z
A1	7.6	353	Neochlorogenic acid	MS^2[353]: 191, 179, 135. MS^3[353 → 191]: 191, 173, 155, 137, 127, 111, 109.
A2	7.9	353	Cryptochlorogenic acid	MS^2[353]: 191, 179, 173, 135. MS^3[353 → 179]: 179, 135.
A3	11.4	337	5-*p*-Coumaroylquinic acid	MS^2[337]: 191, 171, 163. MS^3[337 → 191]: 171, 127, 111.
E1	13.8	479	Myricetin-3-O-glu	MS^2[479]: 461, 316, 271.
E2	18.2	463	Quercetin-3-O-gal	MS^2 [463]: 368, 303, 302, 301, 300, 253, 254, 151. MS^3[463 → 300]: 256.
E3	18.7	447	Kaempferol-3-O-gal	MS^2[447]: 327, 285, 284, 269, 257, 178 MS^3[447 → 285]: 285.
E4	20.4	463	Quercetin-7-O-glu	MS^2[463]: 346, 301, 300, 286, 279, 266
E5	21.0	447	Kaempferol-7-O-glu	MS^2[447]: 327, 285, 284, 283, 255, 151. MS^3[447 → 284]: 256, 255, 226, 186.
E6	22.4	579	Quercetin-3-O-rha-xyl	MS^2[579]: 447, 301, 300, 271.
E7	22.7	447	Quercetin-3-O-rha	MS^2[447]: 411, 301, 300, 271, 255, 179. MS^3[447 → 301]: 257, 255.



			R_1	R_2	R_3	R_4	
B1	34.7	837	rha-(1–3)glu	glu	H	OMe	MS^1: 873, 677, 675. MS^2[675]: 368, 295.
B2	36.8	837	rha-(1–2)glu	glu	H	OMe	MS^1: 873, 837, 676, 675. MS^2[675]: 370, 367, 366, 351, 323.
B3	37.9	807	rha-(1–4)xyl	glu	H	OMe	MS^1: 843, 645. MS^2[645]: 367, 352.
B4	38.9	807	rha-(1–2)xyl	glu	H	OMe	MS^1: 843, 807, 646, 645. MS^2[645]: 367, 366, 351, 323, 311, 295.
B5	40.8	821	rha-(1–2)rha	glu	H	OMe	MS^1:857, 821, 660, 659. MS^2[659]: 367.
B5′		300					
B6	43.2	821	rha-(1–4)rha	glu	H	OMe	MS^1:857, 659, 657. MS^2[659]: 366, 351, 323.
B7	44.4	675	rha	glu	H	OMe	MS^1: 721, 711. MS^2[711]: 681, 632, 513 MS^3[711 → 513]: 366, 351, 280, 253.
B8	51.8	819	rha-(1–4)furan acid	glu	H	OMe	MS^2[819]: 657, 367.
B8′		835					
B9	62.0	675	rha-glu	H	H	OMe	MS^2[675]: 367, 366, 352. MS^3[675 → 367]: 352, 351.
B10	64.0	645	rha-(1–2)xyl	H	H	OMe	MS^2[645]: 354
B10′		659					
B11	65.5	659	rha-(1–2)rha	H	H	OMe	MS^2[659]: 495, 366, 367, 323.
B12	66.4	717	rha(OAc)-glu	H	H	OMe	MS^2[717]: 555.
B13	68.1	513	rha	H	H	OMe	MS^2[513]: 367, 366, 351, 323. MS^3[513 → 366]: 352, 351, 323.
B14	69.6	657	rha-furan acid	H	H	OMe	MS^2[657]: 513, 367.
B15	70.7	759	rha(1–4OAc)-glu(1–4OAc)	H		OMe	–
B16	76.9	801	rha(OAc)-glu(2OAc)	H	H	OMe	MS^2[801]: 759, 671.
B17	77.6	801	rha(OAc)-glu(2OAc)	H	H	OMe	MS^2[801]: 741, 367.
C1	55.6	515	H	glu	H	OH	MS^2[515]: 353. MS^3[515 → 353]: 353, 338, 310, 298, 297, 284.
C2	59.9	499	rha	H	H	OH	MS^2[499]: 353, 352. MS^3[499 → 353]: 309, 297, 291.
D1	54.9	661	rha-xyl	H	OH	OMe	MS^2[661]: 383, 382, 297, 283, 269.
D2	55.5	675	rha-rha	H	OH	OMe	MS^2[675]: 383, 297.
D3	58.2	529	rha	H	OH	OMe	MS^2[529]: 383, 382, 312, 283, 269.

Table 2
Scavenging rates (%) of compounds in four species.

Peak	t_R (min)	$[M-H]^-$	Scavenging rate (%)			
			E.S	E.P	E.W	E.K
A1	7.60	353	–	58.7	85.8	56.4
A2	7.90	353	–	23.5	21.4	22.9
A3	11.4	337	–	58.4	69.3	43.2
E1	13.8	479	–	30.0	41.8	–
E2	18.2	463	–	71.9	81.5	68.7
E3	18.7	447	–	61.6	61.9	52.4
E4	20.4	463	–	55.3	43.4	12.3
E5	21.0	447	–	–	31.4	–
E6	22.4	579	–	34.2	–	–
E7	22.7	447	–	58.9	52.2	66.6
B1	34.7	837	7.90	5.85	7.78	–
B2	36.8	837	–	11.4	11.1	7.08
B3	37.9	807	–	–	3.40	–
B4	38.9	807	1.73	35.2	23.8	4.98
B5	40.8	821	26.2	47.3	44.6	27.8
B6	43.2	821	–	3.31	0.59	–
B7	44.4	675	3.25	7.53	1.11	1.61
B8	51.8	819	6.92	6.20	6.43	4.41
D1	54.9	661	–	23.7	–	–
D2	55.5	675	–	20.5	–	–
C1	55.6	515	–	–	–	15.0
D3	58.2	529	–	4.64	–	–
C2	59.9	499	–	4.32	–	–
B9	62.0	675	1.50	3.22	1.04	1.28
B10	64.0	645	5.09	32.1	22.3	10.9
B11	65.5	659	–	–	–	2.40
B12	66.4	717	–	–	–	42.2
B13	68.1	513	0.63	2.52	0.34	5.75
B14	69.6	657	0.12	5.59	–	4.92
B15	70.7	759	–	–	–	1.95
B16	76.9	801	–	–	–	0.73
B17	77.6	801	–	–	–	3.72

criminate the two compounds based on the published mass data [1].

3.5. Radical scavengers in Epimedium from different species

The scavenging rates (%) of active ingredients in the samples obtained by the on-line HPLD–DAD–MS–CL method were listed in Table 2. A and E could strongly inhibit CL, and several B, C and D compounds displayed different CL inhibition in 4 species. However, it was evident that A and E might be more effective than the other compounds in H_2O_2 scavenging activity. As predominant ingredients in *Epimedium*, 8-isopentenyl-flavonoid glycosides including B, C and D with different substitute groups displayed great differences in H_2O_2 scavenging activity. In these constituents, B2, B3, B4, B5, B10, B12, C1, D1 and D2 possessed strong activities, while the activities of B1, B6, B7, B8, B9, B11, B13, B14, B15, B16, B17, C8 and D3 were relatively weaker. Different activities showed for the compounds in 4 species mainly correlated with their predominance in respective chemical structures and contents in different species.

From the results of ESI-MS^3 detection, the significant differences of the four species in chemical constituents could be found in Figs. 2–4. In Fig. 2(A) and 4, there were few peaks in the DAD trace and total ion chromatogram, and B group showing minor activity was the predominant constituent in E.S. Some unknown ingredients in E.S exhibited strong activities, and their structures still needed to be identified. A, B, C and E were main ingredients in E.W according to Fig. 3(A), while E.P contained A, B, C, D and E (Fig. 2(B)). As shown in Fig. 3(B), more constituents could be observed in E.K than in the others, and most of B compounds were present in E.K. However, although E.K seemed to exhibit a richer chemical composition, E.W and E.P contained more active compounds.

It was obvious that chemical ingredients in the four species were significantly different, which probably led to their different bioactivities. As the main chromatographic peaks in *Epimedium*, B could effectively distinguish the four species based on UV traces and total ion chromatograms (Figs. 2–4). However, the characteristics of A and E showing stronger CL inhibition in active fingerprints might be also important for uniquely identifying and/or separating the four species (Fig. 7). Therefore, the appropriate analysis for the bioactivity evaluation of multiple ingredients in *Epimedium* was necessary. The combination assay of chemical structures and activity characteristics might effectively evaluate the quality of *Epimedium*.

In this study, only H_2O_2 scavenging activity of *Epimedium* was investigated. Some pharmacology experiments were greatly needed to validate the results. To further verify the power and the wide applicability of HPLC–DAD–MS–CL technique, more applications on analysis of herbal medicines are needed.

4. Conclusions

In the present study, an on-line HPLC–DAD–MS–CL method was applied to screen the radical scavengers in 4 species of *Epimedium*. The results indicated that this method could rapidly and sensitively identify radical scavengers in *Epimedium*. The proposed strategy could achieve the activity determination of multiple compounds in complex matrixes. However, this method is currently limited to the analysis of H_2O_2 scavenging activity, and still needs to be studied for the investigation of the other oxygen free radicals.

The on-line HPLC–DAD–MS–CL method, indicating the chemical and activity features of multiple ingredients in complex matrices, could comprehensively and properly reveal the quality characteristics of *Epimedium* from the four species described in this study. More objective conclusions in quality evaluation of herbal medicines might be obtained by accumulating more data of on-line HPLC–DAD–MS–CL approach.

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