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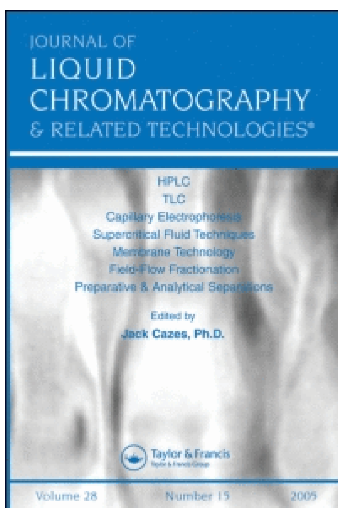

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Analysis of Nine Bioactive Compounds in *Eucommia ulmoides* Oliv. and Their Preparation by HPLC-Photodiode Array Detection and Mass Spectrometry

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ABSTRACT

A rapid, specific, and reproducible high-performance liquid chromatography (HPLC)-photodiode array (DAD)-electrospray ionization mass spectrometry (ESIMS) method has been developed for determination of nine bioactive compounds from *Eucommia ulmoides* Oliv. (*E. ulmoides*). The samples analyzed contained cortex, leaves, extracts, mixed preparation of traditional Chinese patent medicines, and beverages of *E. ulmoides* Oliv. Identification of the nine compounds, namely, aucubin, geniposidic

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acid, chlorogenic acid, caffeic acid, geniposide, (+)-pinoresinol-di-*o*-β-D-glucopyranoside, rutin, quercetin, and kaempferol, was based on retention time, UV, and mass spectra by comparison with standards. Peak area in the selected ion monitoring (SIM) chromatogram was used for quantification of nine compounds in samples. This developed method can control the quality of medicinal materials of *E. ulmoides* Oliv. and its preparation more effectively.

Key Words: *Eucommia ulmoides* Oliv.; Traditional chinese patent medicines; Plant materials.

INTRODUCTION

Eucommia ulmoides Oliv. (*E. ulmoides*) has been widely used as a medicinal herb in China, Japan, and Korea. It was recorded in the first-ever written documentation of traditional Chinese medicine; "*Shen nung ben tsao jing*" (*The Holy Farmer's Material Medica*) ca. 25 A.D., as a top grade medicinal herb because of its nontoxicity and for enhancing the vital energies etc.[1] In the Chinese Pharmacopoeia,[2] the medicinal part is roasted cortex of *E. ulmoides* Oliv. Modern pharmacological researches show that cortex and leaves of the plant has anti-hypertensive, suppressing on mutagenicity et al., effects. Its cortex and leaves have been used in plant medicines, traditional Chinese patent medicinal preparations, and food supplements more and more often.

Eucommia ulmoides Oliv. contains various types of compounds in chemical classes such as iridoids, lignans, phenylpropanoids, and flavonoids, etc. These compounds own some specific bioactivities. Figure 1 depicts the chemical structures of nine compounds that were found in the plant. Research showed various pharmacological properties of *E. ulmoides* are mainly attributed to lignans and iridoid glycoside.[3] Aucubin has been shown to inhibit NF-κB activation in mast cells and protect CCl_4-induced liver damage.[4,5] Geniposidic acid exhibited the effect against aging, promoted the collagen synthesis, and improved the turnover rate of the stratum corneum in false age model rats.[6,7] Geniposide showed an antithrombotic effect in vivo.[8] (+)-Pinoresinol-di-*o*-β-D-glucopyranoside has antihypertensive activity.[9] Chlorogenic acid is the most specific T1 inhibitor. T1 is a Glc-6-P-specific translocase that mediates penetration of the hexose phosphate through the membrane.[10] Flavonoids, including quercetin and kaempferol etc., are strong antioxidants that occur naturally in food and can inhibit carcinogenesis in rodents.[11,12]

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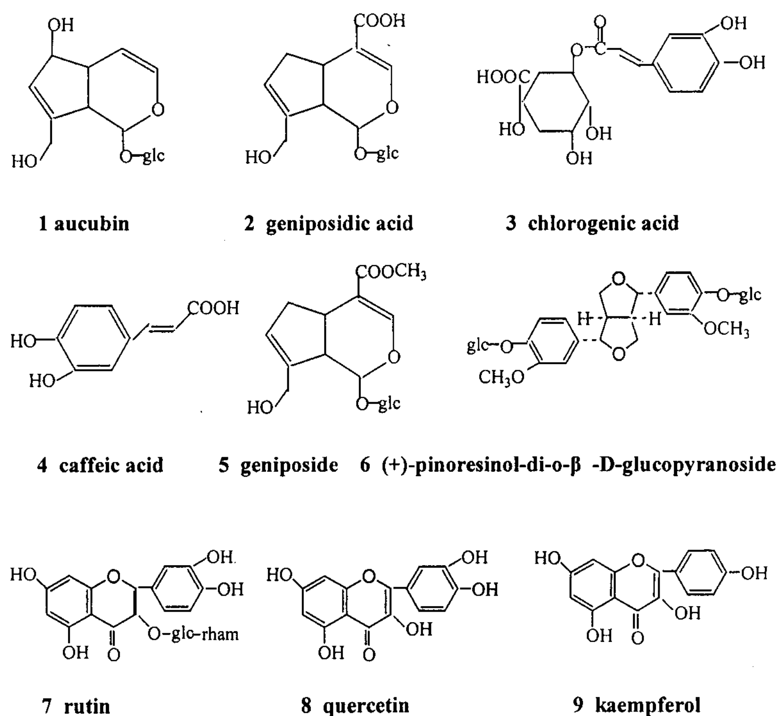

Figure 1. Structures of iridoids, lignans, phenylpropanoids, and flavonoids in *E. ulmoides*.

A number of methods have been reported for analyses of *E. ulmoides*. Most of the methods involve chromatography, such as thin-layer,[13] high-performance liquid chromatographic techniques,[14–18] micellar electrokinetic capillary chromatography,[19] and capillary electrophoresis.[20] But these methods had seldom simultaneously determined iridoids, phenylpropanoids, lignans, and flavonoids in *E. ulmoides* in the analytical preparation of mixed traditional Chinese patent medicines. This is probably due, at least, in part to a lack of sensitivity and selectivity of previously published methods. In the Chinese Pharmacopoeia, the quality control index of medicinal materials of *E. ulmoides* is the content of (+)-pinoresinol-di-*o*-*β*-D-glucopyranoside. And the analytical method is also the HPLC method.[2] But the control method is limited and cannot reflect the whole quality of the medicinal materials.

In this paper, an HPLC-DAD-ESI-MS method for the rigorous qualitative and quantitative analysis of nine bioactive ingredients of *E. ulmoides* as a new

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means of quality control was developed. The nine bioactive compounds were quantified through selected ion monitoring (SIM). A good linear relationship between response and concentration was observed. The method developed was successfully applied to the analysis of cortex, leaves and extracts of *E. ulmoides* and its mixed preparation of traditional Chinese patent medicine, such as Duzhong tea and Tianma Duzhong Jiaonang.

EXPERIMENTAL

Reagents and Materials

Aucubin, geniposidic acid, and geniposide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chlorogenic acid, (−)-epigallocatechin gallate (EGCG), and caffeine were obtained from Sigma Chemicals (St. Louis, MO). Caffeic acid, rutin, quercetin, and kaempferol were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

(+)-Pinoresinol-di-*o*-β-D-glucopyranoside was kindly provided by Doctor Deyama Takeshi of Yomeishu Seizo Ltd. (Nagano, Japan).

HPLC-grade methanol was obtained from Tedia Company, Inc. (Fairfield, OH). Deionized water was obtained using an Ultra water system from Millipore (Milford, MA). Cortex and leaves of *E. ulmoides* were picked from Zhangjiajie national forestry park of China. The plant material identification was made by Professor Li Jian-Zhong, Department of Botany, Hunan Normal University. The Chinese traditional patent medicine containing *E. ulmoides* (Tianma duzhong jiaonang) and duzhong tea were purchased from drugstores and supermarkets (Changsha, China). Extracts of *E. ulmoides* were kindly donated by Doctor Peng Mijun of Central South University (Changsha, China).

Standard Solutions

A mixed standard solution containing aucubin (0.11 mg/mL), geniposidic acid (0.24 mg/mL), geniposide (0.31 mg/mL), chlorogenic acid (0.22 mg/mL), caffeic acid (0.20 mg/mL), rutin (0.26 mg/mL), quercetin (0.20 mg/mL), kaempferol (0.20 mg/mL), and pinoresinoldiglucopyranoside (0.03 mg/mL) was prepared with methanol and used for methods development.

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Treatment of *Eucommia ulmoides* Samples

Pulverized *E. ulmoides* powder and extracts was weighed (0.5 g), except that Duzhong tea and Tianma duzhong jiaonang were weighed (1.0 g), placed in 10 mL of 50% methanol aqueous solution in an ultrasonic device for 30 min for extraction. The supernatants were then separated after centrifugation at 10,000 rpm for 5 min. The residue was once again extracted in the same conditions. The combined supernatant was placed in a 25 mL volumetric flask and diluted to volume with 50% methanol aqueous solution. Sample solution, 2-mL, was filtered through a 0.45 μm PTFE filter into an HPLC vial for future HPLC analysis.

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Liquid Chromatography/Electrospray Ionization-Mass Spectrometry System

Analyses of the standard and sample solutions were carried out using a Waters Alliance 2695 liquid chromatographic system (Milford, MA), interfaced to a 996 DAD detector and a Micromass ZQ 2000 electrospray mass spectrometer (Manchester, UK). LC separations were made on a 250 × 4.6 mm spherigel analytical column (Johnsson Dalian, China) at 30°C. The mobile phase consisted of (A) water containing 0.1% formic acid and (B) methanol. The gradient elution had the following profile: 0–5 min, 15% B; 5–18 min, 15–30% B; 18–23 min, 30% B; 23–24 min, 30–35% B; 24–35 min, 35–50% B; 35–45 min, 50–90% B; 45–45.5 min, 90–15% B; 45.5–50 min, 15% B. The flow rate was 1 mL/min. The injection volume was 10 μL. UV spectra recorded was in the range of 195–400 nm.

Several parameters of the ESI interface were optimized through direct injection analysis of standard solution. The sensitivity of detection of chemicals (except for compound 3, 4, 6, and 7, for the numbering of the compounds see Fig. 1) in the positive ion ESI mode was found higher than in the negative ion ESI. Therefore, instrumental parameters were selected on a positive/negative alternant scan mode. Nitrogen was used as desolvation gas at flow rates of 250 L/h; the cone gas was set 50 L/h. The desolvation temperature was 250°C; the source temperature: 105°C. Capillary voltage and cone voltage was 2500 and 40 V, respectively, for ESI(+) and ESI(−). Mass values of the range of 100–800 u were measured. The eluent was split at the HPLC column end to allow 20% eluent to flow into the mass spectrometer.

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RESULTS AND DISCUSSION

ESI Mass Spectra of Standards

In order to investigate mass spectrometric-based information of each compound, direct injection experiments of the nine compounds standards were performed. $[M + Na]^+$, $[M + K]^+$, $[2M + Na]^+$, $[M - H]^-$, and $[2M - H]^-$ ions were observed under mass spectrometric conditions described in the Experimental Section. The mass spectra of phenylpropanoids and flavonoids had been reported.[21–25] Figure 2 shows the mass spectra of compound 1, 2, 5, and 6. These compounds showed the abundant positive molecular cations at m/z 369, 397, 411, and 705 in the positive ionization mode, respectively. The signals at m/z 385, 413, 427, and 721 were $[M + K]^+$ ions of these compounds. The signals at m/z 715, and 771 correspond to dimer positive ions of compounds 1 and 2. At the same time, these compounds showed deprotonated anions in negative ionization mode at m/z 345, 373, 387, and 681, respectively. Compound 1 exhibited abundant signal of $[M + HCOO]^-$ ion at m/z 391, $[2M - H]^-$ ion at m/z 691, $[2M + HCOO]^-$ion at m/z 737. Compound 2 displayed $[M + 2H_2O—H]^-$ ion at m/z 409, $[2M - H]^-$ ion at m/z 747, and fragment ions at m/z 211 arising from loss of a hexose unit. Compound 5 exhibited abundant $[M + HCOO]^-$ ion at m/z 433, $[M + 2H_2O - H]^-$ ion at m/z 423, and fragment ions at m/z 225 due to loss to glucose. Compound 6 displayed fragment ions at m/z 519 and 357 due to the loss of two hexose units.

Because the SIM mode had excellent sensitivity with high specificity, it can give more specific and exact analytical results. But in the authentic sample analysis, it is necessary to select proper qualifier ions for the detection of compounds. The simultaneous detection of both molecular ions and selected qualifier ions of nine compounds are shown in Figs. 3 and 4. The ions selected for SIM are seen in Table 1, with regard to maximum abundance of the ions of the analytes in positive and negative mode.

In summary, the present ESI-MS method provided a reliable means to distinguish and determine the nine compounds. Our results also suggested that both a positive and negative ESI-MS approach could be applied to identify the nine compounds, even if their authentic standards are not available.

LC/MS Analysis of a Mixture of Standards

Figures 3 and 4 show HPLC-ESI-MS-TIC and HPLC-MS-SIM chromatograms for the analysis of standard solution mixtures. As shown in Figs. 3 and 4, the nine components showed a good response in positive and negative

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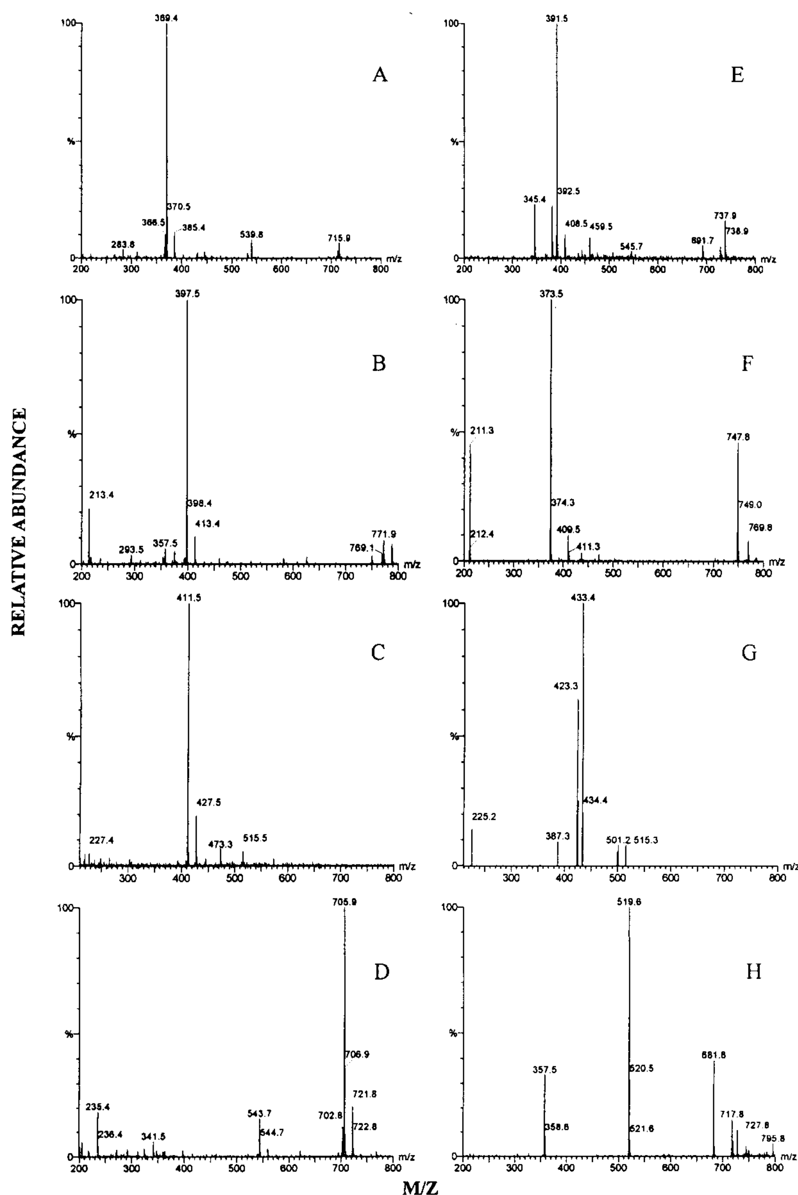

Figure 2. Positive-ion and negative-ion ESI-MS spectra: (A) is positive-ion spectra of compound 1; (B) is positive-ion spectra of compound 2; (C) is positive-ion spectra of compound 5; (D) is positive-ion spectra of compound 6; (E) is negative-ion spectra of compound 1; (F) is negative-ion spectra of compound 2; (G) is negative-ion spectra of compound 5; and (H) is negative-ion spectra of compound 6.

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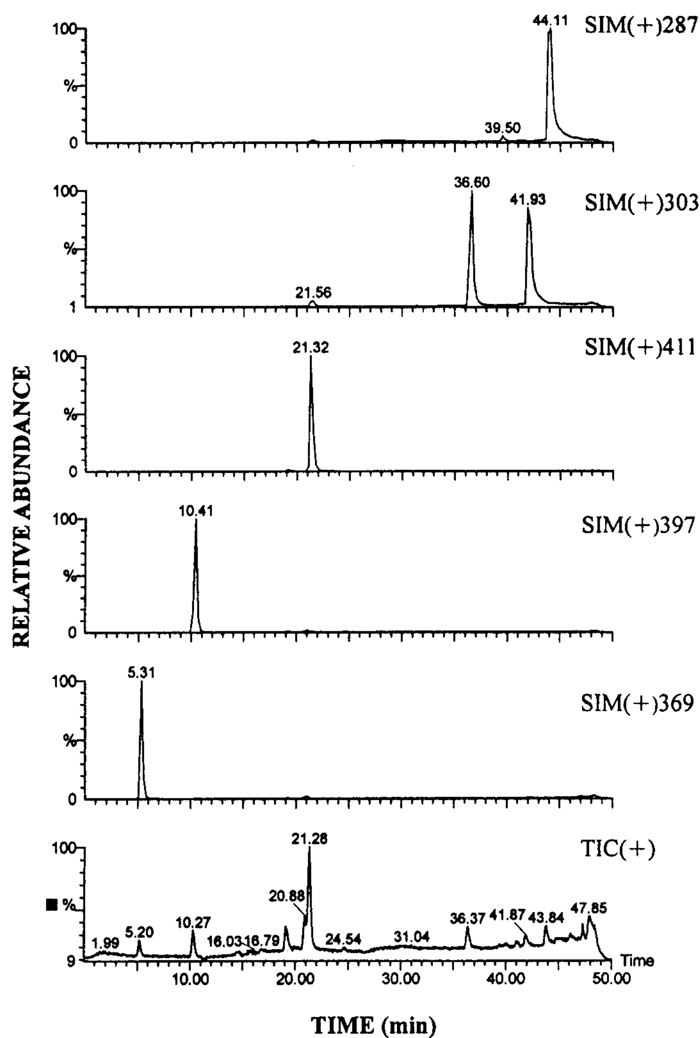

Figure 3. HPLC-ESI(+)-MS-TIC and HPLC-ESI(+)-MS-SIM chromatogram for the analysis of standard solution mixtures with a 1 : 5 post-column stream splitting. Chromatographic conditions are described in section 2.4. The peak identifications are given in Table 1.

ESI-MS modes. Their respective mass spectra are similar to those obtained by direct injection analysis. No unexpected adduct ions from the column materials were observed. The results demonstrated baseline separation of the nine-component mixture, except for compounds 4 and 5, within 50 min

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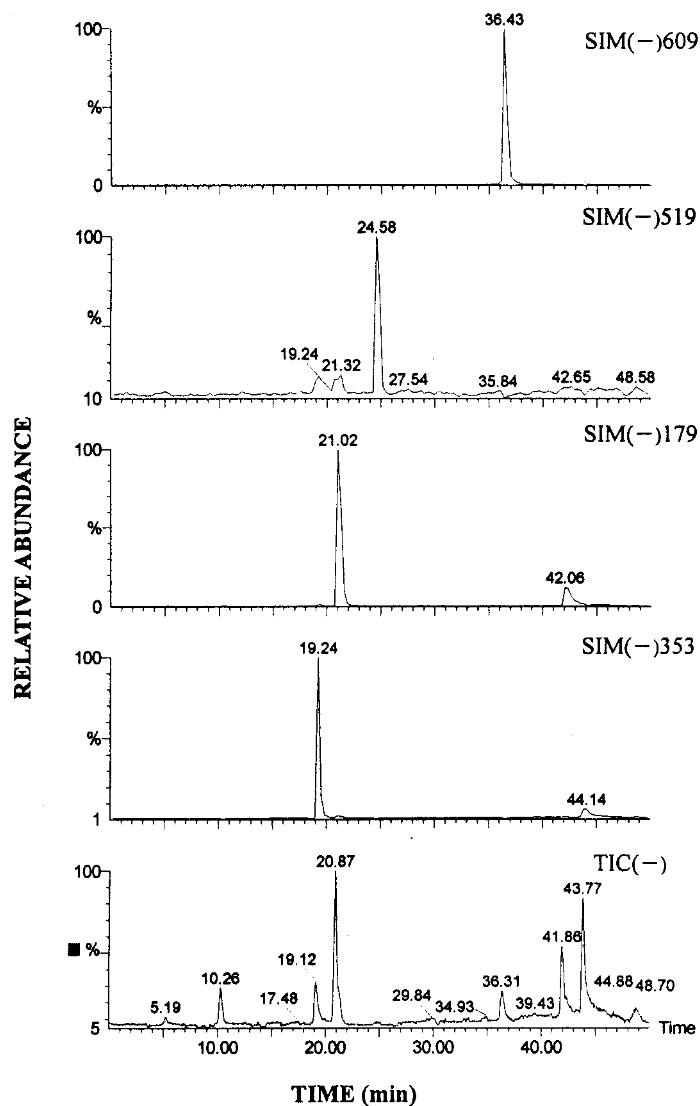

Figure 4. HPLC-ESI(−)-MS-TIC and HPLC-ESI(−)-MS-SIM chromatogram for the analysis of standard solution mixtures with a 1 : 5 post-column stream splitting. Other conditions are the same as in Fig. 3.

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Table 1. Molecular weight and qualifier ions of nine compounds selected by SIM using HPLC-ESI-MS.

Compound	Retention time (min)	Molecular weight	Qualifier ions, m/z
1	5.31	346	369(+)
2	10.41	374	397(+)
3	19.24	354	353(−)
4	21.02	180	179(−)
5	21.32	388	411(+)
6	24.58	682	519(−)
7	36.43	610	609(−)
8	41.93	302	303(+)
9	44.11	286	287(+)

Note: Where + shows positive ionization mode, − shows negative ionization mode.

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utilizing LC elution conditions, described in the Experimental Section. The baseline separation of compounds 4 and 5 can be achieved through the gentle change of mobile phase composition, but the analytical time is longer. The utility of the SIM chromatograms of m/z 179 and 411 in Fig. 4, compounds 4 and 5, obtained preferable separation without interference on the mass axis.

Figure 5 shows total wavelength detection and four selected wavelengths chromatograms. The four selected wavelengths are in maximum absorbance range of iridoids, lignans, phenylpropanoids, and flavonoids. The utility of UV detection peak profiles of different wavelengths also can quantitatively analyze the bioactive ingredients of *E. ulmoides* medicinal materials.

To check the performance of the method, the linearity, detection limit, accuracy, and precision of SIM mode for quantification of nine compounds were evaluated. The limit of detection (LOD) was calculated according to $S/N = 3$.[26] The average recoveries were calculated according to the method proposed by Cuadros et al.[27] Table 2 summarizes the results of linear range, LOD, regression coefficients (r), and recovery. The precision of the method was evaluated by carrying out eight replicate analysis of a standard solution on different days. The obtained relative standard deviation was smaller than 3% for all the compounds.

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LC/MS Analysis of Cortex, Leaves, and Extracts of *E. ulmoides*

To evaluate the practicability of the method for the single traditional Chinese medicinal, eight different *E. ulmoides* samples, including two

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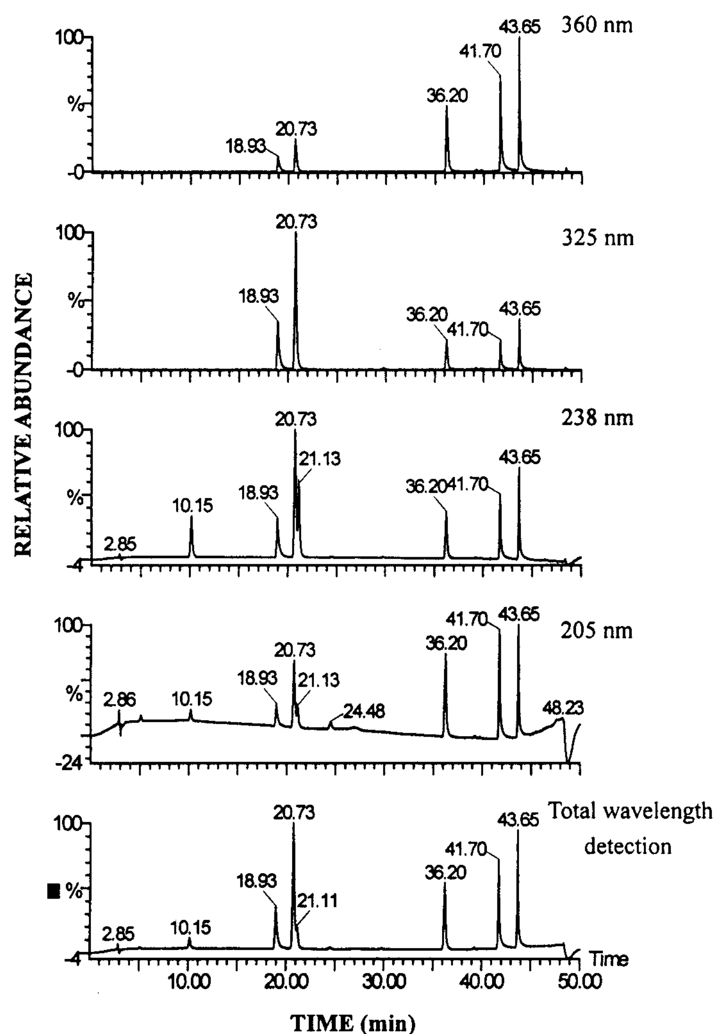

Figure 5. Total wavelength detection and four selected wavelength chromatogram for the analysis of standard solution mixtures with a 1 : 5 post-column stream splitting. Other conditions are the same as in Fig. 3.

extracts, roasted cortex, fresh cortex, dried leaves, and fresh leaves, were analyzed by the presented HPLC-UV-ESI-MS method. Figure 6 shows total wavelength detection and HPLC-MS-SIM chromatograms of the extracts. Analytical results are shown in Table 3. Their contents vary according to the

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Table 2. Linear range, regression coefficients, limits of detection (LOD), and recovery of nine compounds.

Compound	Linear range (μg)	*r*	LOD (ng)	Recovery (%)
1	0.11–1.10	0.999	0.3	98.3
2	0.24–2.40	0.999	0.4	99.5
3	0.22–4.40	0.998	0.9	98.7
4	0.20–4.00	0.996	0.8	99.4
5	0.31–4.65	0.996	0.3	95.3
6	0.03–0.45	0.992	2.0	94.5
7	0.26–5.20	0.999	0.5	99.1
8	0.20–3.00	0.999	1.6	101.1
9	0.20–4.00	0.999	1.3	97.5

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physical parts, age of the *E. ulmoides*, and geographic origin. In terms of the individual constituents, analysis results showed that roasted cortex and fresh cortex did not contain compounds 7, 8, and 9, whereas, the leaves had higher contents of compound 7, and a small amount of compounds 8 and 9. The contents of compound 1 in the fresh cortex and leaves were found to be higher than in the roasted cortex and dried leaves. Only two samples contained a few amounts of compound 4. The two extracts contained higher contents of compounds 1, 2, 5, and 6. These results show that the presented method is feasible as a means of more comprehensive quality control of *E. ulmoides* single traditional Chinese medicines.

LC/MS Analysis of Preparations of Mixed Traditional Chinese Patent Medicines Containing *E. ulmoides*

To evaluate the applicability of the method in preparation of mixed traditional Chinese patent medicine, the commercial Duzhong tea and Tianma Duzhong Jiaonang were analyzed. The interference factor was investigated carefully, as the mixed traditional Chinese medicines have multi-medicinal materials. For example, there are many catechins in Duzhong tea. The results are seen in Figs. 7 and 8. From Fig. 7, the utility of the SIM chromatogram for the nine bioactive compounds, quantitative analysis is suitable. This conclusion is supported by the following viewpoint. An SIM technique can distinguish the compounds on mass axis, even if the peaks of the compounds overlap on time axis. But, the interference of the same compounds coming from the different medicinal materials is uncontrollable. Analysis results are shown in Table 3. The concentration of compounds 2, 5, and 6 in the

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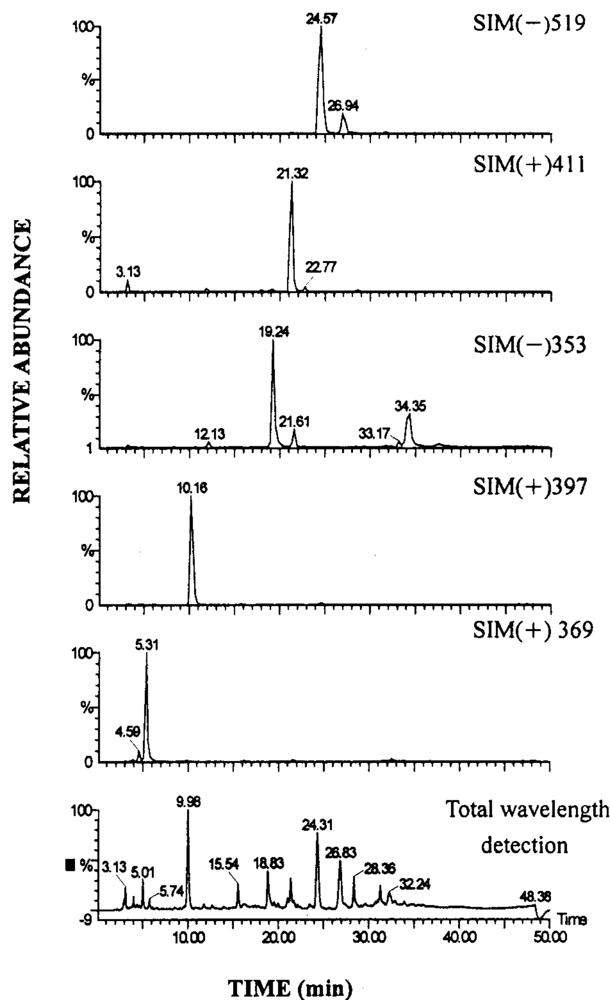

Figure 6. Total wavelength detection and SIM chromatogram for analysis of the extract of *E. ulmoides* with a 1 : 5 post-column stream splitting. Other conditions are the same as in Fig. 3.

traditional Chinese herbal medicine preparations, Tianma Duzhong Jiaonang, was less than in the herb *E. ulmoides* itself.

Figure 8 shows HPLC-UV-ESI-MS-TIC and SIM chromatograms of Duzhong tea. The constituents of Duzhong tea had good responses in both diode array total wavelength detection chromatogram and positive and

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Table 3. Quantification for *E. ulmoides* samples by HPLC-DAD-ESI-MS (%, w/w).[a]

Sample	1	2	3	4	5	6	7	8	9
Vimineous cortex (zhangjiajie)	0.005	0.022	0.017	0.001	0.008	0.079	Nd[b]	Nd	Nd
Cortex (Luguodaiyaofang)	Nd	0.021	0.023	Nd	0.008	0.054	Nd	Nd	Nd
Cortex (Liuyang)	0.011	0.421	0.025	0.001	0.090	0.388	Nd	Nd	Nd
Fresh Bark (xiangxiang)	0.020	0.117	0.010	Nd	0.005	0.037	Nd	Nd	Nd
Dried Leaves (zhangjiajie)	0.010	0.004	0.696	Nd	Nd	0.078	0.136	0.013	0.003
Fresh Leaves (xiangxiang)	0.116	0.039	0.208	Nd	Nd	0.001	0.037	0.003	0.001
Extracts (1)	1.137	1.365	0.119	Nd	0.176	0.973	Nd	Nd	Nd
Extracts (2)	2.203	1.220	Nd	Nd	0.512	0.772	Nd	Nd	Nd
Tianma duzhong jiaonang (shengyang)	Nd	0.014	0.012	Nd	0.006	0.010	Nd	0.003	0.001
Tianma duzhong jiaonang (guizhou)	Nd	0.008	0.009	Nd	0.003	0.003	Nd	0.001	Nd
Duzhong tea (supermarket)	0.403	0.266	0.248	Nd	Nd	0.004	0.115	Nd	0.008

[a]Where the results are mean of three replicate analysis, the relative standard deviation was always $<4\%$ for nine compounds.
[b]Where Nd is not detected.

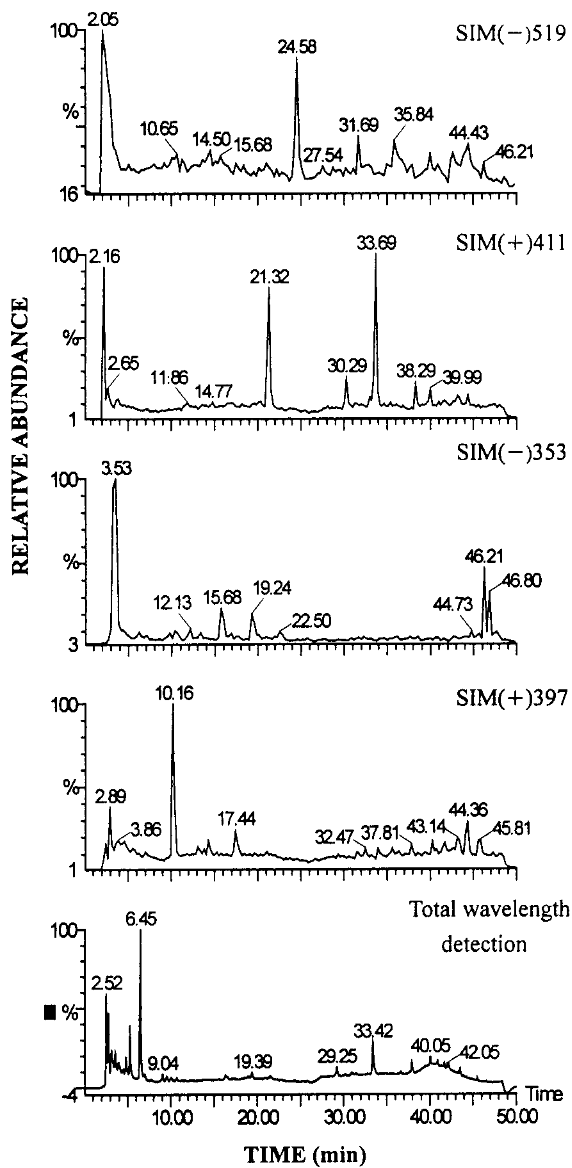

Figure 7. Total wavelength detection and SIM chromatogram for analysis of the Tianma Duzhong Jiaonang with a 1 : 5 post-column stream splitting. Other conditions are the same as in Fig. 3.

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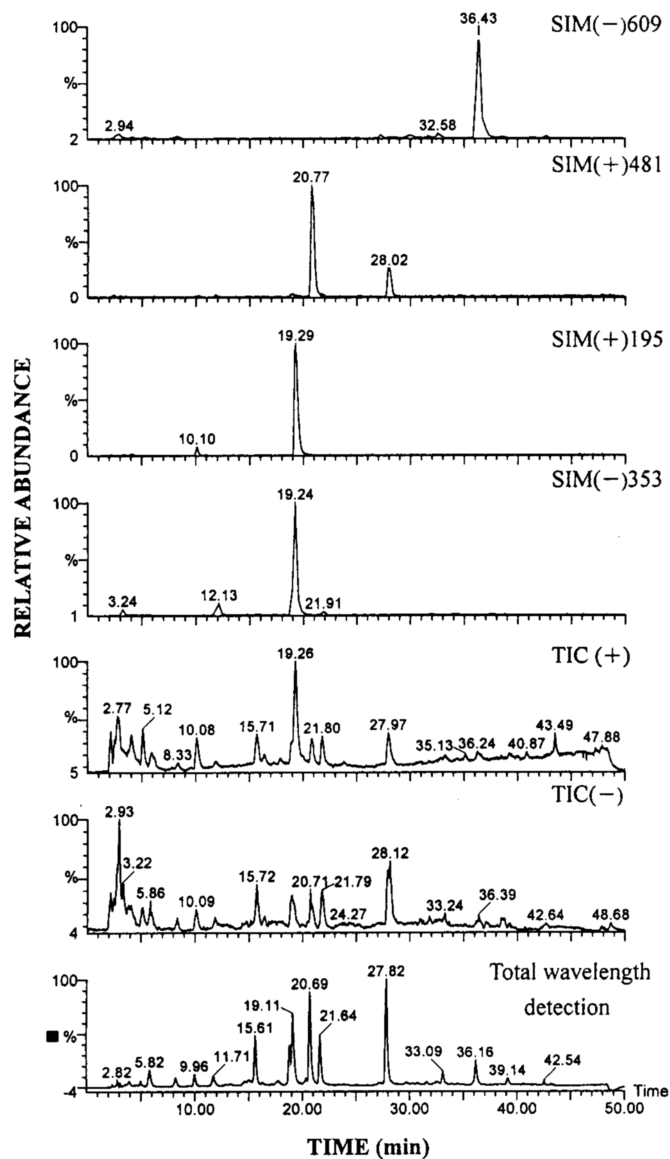

Figure 8. HPLC-UV-ESI-MS-TIC and SIM chromatogram for analysis of the Duzhong tea with a 1 : 5 post-column stream splitting. Other conditions are the same as in Fig. 3.

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negative ESI-MS modes. Chlorogenic acid and caffeine had partial overlaps. The retention time of EGCG, caffeic acid, and geniposide is 20.77, 21.02, and 21.32 (SIM time), respectively. The SIM technique successfully solves this analysis problem. Identities of EGCG and caffeine were also established from LC retention times by comparison with commercial standards. The m/z 481 and 195 are positive ions of EGCG and protonated ion of caffeine. Chromatograms of SIM 481 and 195 were shown in Fig. 8. From the results, the method is suitable for Duzhong tea quality control analysis.

CONCLUSIONS

The HPLC-UV-ESI-MS method for the measurement of nine components in cortex, leaves, extracts, preparations of mixed traditional Chinese medicines, and beverages of *E. ulmoides* has been made successfully. The nine components could be detected at the nanogram on-the column level. The method described in this report can have widespread use for quality control of *E. ulmoides* Oliv. and its preparations.

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