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# Simultaneous analysis of coumarins and secoiridoids in Cortex Fraxini by high-performance liquid chromatography–diode array detection–electrospray ionization tandem mass spectrometry

Lei Zhou, Jie Kang, Li Fan, Xiao-Chi Ma, Hai-Yu Zhao, Jian Han, Bao-rong Wang, De-An Guo *

*The State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, No. 38 Xueyuan Road, Beijing 100083, PR China*

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**Abstract**

A high-performance liquid chromatography–diode array UV detection–electrospray ionization tandem mass spectrometry (HPLC–DAD–ESI-MS) method was developed and validated for the simultaneous analysis of seven major constituents in Cortex Fraxini, including esculin, esculetin, fraxetin, fraxin, escuside, oleuropein and ligustroside. The contents of the seven constituents were determined by using HPLC–DAD, and the chemical structures of these constituents were identified by using HPLC–DAD–ESI-MS method. The separation was performed on an Agilent Zorbax SB-$C_{18}$ column (250 mm × 4.6 mm i.d., 5 μm) with gradient elution of acetonitrile and 0.3% aqueous acetic acid within 40 min. Detection was performed at 254 nm. The calibration curves showed good linearity ($r^2 > 0.9992$). The limits of detection (LOD) ranged from 1.07 to 3.19 ng/ml and limits of quantification (LOQ) ranged from 2.79 to 12.75 ng/ml, respectively. The intra- and inter-day precision was less than 5% and the accuracy was ranging from 96.49% to 103.55%. The recovery of the assay was in the range of 97.61–104.36%. The method was successfully applied to the quantification of the seven constituents in different samples of Cortex Fraxini. The results indicated that the developed method could be considered to be a simple, rapid and reliable method for the quality evaluation of Cortex Fraxini.


*Keywords:* HPLC–DAD–ESI-MS; Cortex Fraxini; Coumarins; Secoiridoids; Quantification; Quality evaluation

## 1. Introduction

Cortex Fraxini is the dried bark of *Fraxinus rhynchophylla* Hance, *Fraxinus chinensis* Roxb., *Fraxinus chinensis* Roxb. var. *acuminate* Lingelsh. and *Fraxinus stylosa* Lingelsh., and called "Qin pi " in Chinese. It is a commonly used Chinese herb belonging to the 'heat-clearing' category according to the classification of traditional Chinese medicine (TCM) and has the efficacy of clearing heat and eliminating dampness, improving acuity of vision, etc. [1].

The drug is mainly used as an antibacterial, analgesic and anti-inflammatory agent for the treatment of diarrhea, bacillary dysentery, arthritis and hyperuricemia [2–5]. Other pharmacological studies showed that it also had diuretic, anticoagulant, antiallergic [6] and antioxidant effects [7–9].

The characteristic constituents in Cortex Fraxini are coumarins including esculin, esculetin, fraxetin fraxin and escuside, etc. [2,10,11]. Besides, other kind of compounds such as lignans, secoiridoid glycosides, saponins and other phenolic compounds also existed in the plants [12–15]. Coumarins are proven to be the active constituents, which were reported to have activities such as anti-inflammation, antivirus, antiarthritis and anticancer [16–21]. Other constituents, like ligustroside and oleuropein, have antioxidant effect [22–24]. Therefore, quantification of multiple bioactive constituents in Cortex Fraxini would be of great significance for the quality evaluation of the herb. However, previous studies only aimed at the quantitative analysis of esculin and esculetin in herbal samples or the preparation of Cortex Fraxini by TLC [25–27], fluorimetry [28] and double artificial neural network (DANN)-UV [29].

* Corresponding author. Tel.: +86 10 82802024; fax: +86 10 82802700.
*E-mail address:* gda@bjmu.edu.cn (D.-A. Guo).

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Other methods including CE [30,31], CZE [32] and MEKC [33] were developed for the determination of fraxin, esculin, and esculetin. High performance liquid chromatography (HPLC) methods were applied in the simultaneous quantification of esculin, esculetin, fraxetin and fraxin [34–39]. Therefore, a simple and accurate HPLC–DAD–ESI-MS method was developed in this study for simultaneous quantification and identification of the seven main constituents in Cortex Fraxini, including esculin, esculetin, fraxetin, fraxin, escuside, oleuropein and ligustroside, to evaluate the quality of this Chinese herbal medicine.

## 2. Experimental

### 2.1. Materials and reagents

Cortex Fraxini samples were collected from local drugstores in different regions. Esculin, esculetin, fraxetin, fraxin, escuside, ligustroside and oleuropein were isolated by the author from Cortex Fraxini. These were identified by comparison of their $^{1}H$ NMR, $^{13}C$ NMR and MS data with the literature data [10,40–43]. Their purity was not less than 98% according to HPLC. HPLC grade acetonitrile was purchased from Caledon Laboratories Ltd. (Georgetown, Ont., Canada). Analytical grade acetic acid was purchased from Beijing Chemical Factory (Beijing, China). The deionized water was prepared using Millipore purification system (Millipore, Milford, MA, USA) and filtered with 0.45 μm membranes.

### 2.2. HPLC-UV analysis

An Agilent 1100 liquid chromatography system (Agilent, Waldbronn, Germany) equipped with a quaternary solvent delivery system, an autosampler and a diode array detector was used. The separations were carried out on an Agilent Zorbax SB-$C_{18}$ column (250 mm × 4.6 mm i.d., 5 μm). The mobile phase consisted of solvent A (acetonitrile) and solvent B (0.3% aqueous acetic acid, v/v). Gradient elution was as follows: initial 0–14 min, linear change from A–B (12:88, v/v) to A–B (16:84, v/v), 14.5–22 min, the elution mobile phase composition was kept at A–B (21:79, v/v), 22.5–30 min, linear change from A–B (26.5–73.5, v/v) to A–B (30–70, v/v), 30–40 min, linear change from A–B (30–70, v/v) to A–B (35–65, v/v). The detection wavelength was 254 nm. The column temperature was set at 30 °C. The flow rate was 1.0 ml/min and sample injection volume was 5 μl. The chromatographic data were recorded and processed with an Agilent chemstation workstation.

### 2.3. HPLC–DAD–ESI-$MS^n$ analysis

As to HPLC–DAD–ESI-MS analysis, a Finnigan LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) was connected to an Agilent 1100 HPLC instrument coupled with a quaternary solvent delivery system, an autosampler and a diode array detector via an ESI interface. The chromatographic conditions were as described above. For full scan ESI-MS analysis, the spectra were recorded in the range of *m/z* 80–900 in negative ion mode. Ultrahigh pure helium (He) was used as the collision gas and high purity nitrogen ($N_2$) as the nebulizing gas. The optimized parameters were as follows: ion spray voltage, 4.5 kV; sheath gas, 50 arbitrary units; auxiliary gas, 10 arbitrary units; capillary temperature, 300 °C; capillary voltage, 12 V; tube lens offset voltage, 40 V. $MS^n$ data were acquired in the automatic data-dependent mode.

### 2.4. Extraction and isolation

The dried barks (4.5 kg) were pulverized and extracted with hot 95% ethanol three times, for 2 h each time. After filtration, the solutions were combined and concentrated using rotary evaporator under reduced pressure. The ethanol extract was suspended in water and extracted successively with petroleum ether, $CHCl_3$, EtOAC, and *n*-BuOH [44–46]. The $CHCl_3$ extracts (20 g) were separated on a glass column using silica gel (65 cm × 5 cm i.d.), eluting with petroleum ether–EtOAC gradient solvent system. The fraction, which was eluted by petroleum ether–EtOAC (20:1, v/v), was further purified by column chromatography and semipreparative HPLC to give fraxin (12 mg). The EtOAC extracts (120 g) were chromatographed over a glass column on silica gel (90 cm × 10 cm i.d.), eluting with gradients $CHCl_3$–MeOH (100:1 to 1:1, v/v). Fraction 1, eluted by $CHCl_3$–MeOH (50:1, v/v), gave esculetin (700 mg) after repeated recrystallization. Fraction 2 was separated using column chromatography on silica gel eluting with a gradient solvent of cyclohexane–EtOAC and cyclohexane–acetone to give fraxin (185 mg). Fraction 3 was separated using column chromatography on Sephadex LH-20 eluting with a gradient solvent of MeOH–$H_2O$ and semipreparative HPLC eluting with gradients acetonitrile–$H_2O$ to give ligustroside (280 mg), escuside (8 mg) and oleuropein (15 mg). Fraction 4, eluted by $CHCl_3$–MeOH (1:1, v/v), was subjected to repeated column chromatography on silica gel eluted with a gradient solvent of $CHCl_3$–MeOH and then by ODS column chromatography finally eluting with gradients of MeOH–$H_2O$ to give esculin (65 mg). Structures of the seven compounds are shown in Fig. 1.

### 2.5. Sample preparations

The dried powder of the bark (0.5 g) was accurately weighed and extracted by sonication with 15 ml of 80% methanol for 45 min. The extracted solution was adjusted to the original weight, and then the aliquot of the supernatant was filtered through 0.45 μm nylon membrane (Whatman, UK) before HPLC injection.

## 3. Results and discussion

### 3.1. Optimization of extraction method

In this study, the extraction method, extraction solvent and extraction time were investigated in order to obtain reasonable experimental results and satisfactory extraction efficiency.

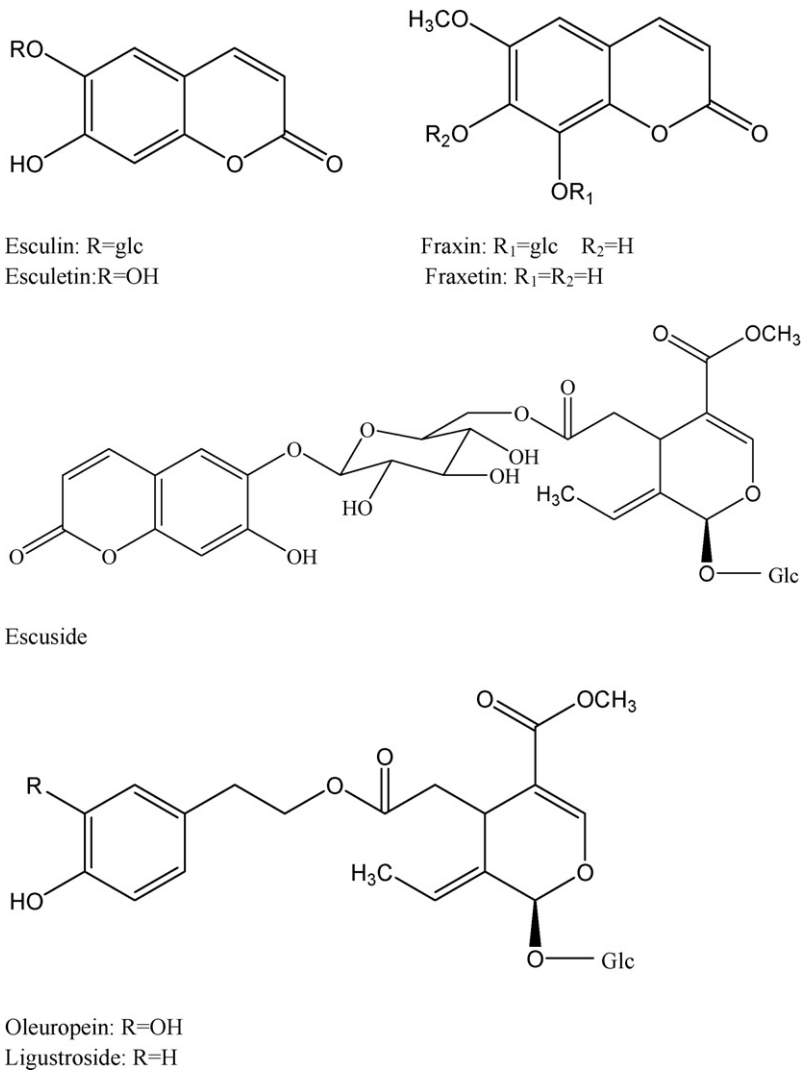


Fig. 1. Structures of seven compounds in Cortex Fraxini.

The results showed that ultrasonic extraction was similar to refluxing, but ultrasonic extraction was more convenient, so ultrasonic extraction was applied in the experiments. The samples were extracted using 30%, 50%, 80%, 100% methanol and 95% ethanol, respectively, to investigate the effects of different solvents. The results showed that 80% methanol was the most effective solvent (Fig. 2). Consequently, the samples were extracted with 80% methanol by ultrasonic extraction for 15, 30, 45 and 60 min to screen optimal extraction time. The results showed that the compounds could be entirely extracted within 45 and 60 min (Fig. 3). Therefore, 45 min was selected as an appropriate extraction time.

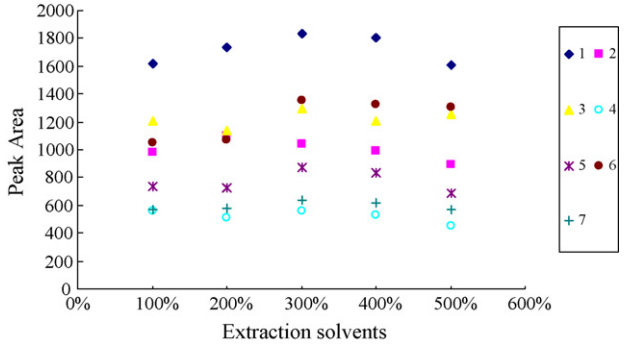


Fig. 2. Extraction efficiency of different extraction solvents: (1) esculin; (2) fraxin; (3) esculetin; (4) fraxetin; (5) escuside; (6) oleuropein; (7) ligustroside.

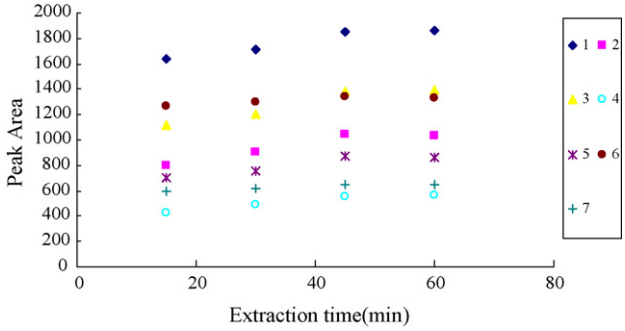


Fig. 3. Extraction efficiency of different extraction time: (1) esculin; (2) fraxin; (3) esculetin; (4) fraxetin; (5) escuside; (6) oleuropein; (7) ligustroside.

Table 1
Negative ESI-MS data of the seven constituents

| Peak no. | $t_R$(min) | MS (*m/z*) | $MS^n$ fragments (*m/z*) | Identification |
|---|---|---|---|---|
| 1 | 5.89 | 339 $[M-H]^-$ | 177 $[M-H–glc]^-$; 133 $[M-H–glc–CO_2]^-$ | Esculin |
| 2 | 8.69 | 369 $[M-H]^-$ | 207 $[M$-H–glc$]^-$; 192 $[M$-H–glc–$CH_3]^-$; 164 $[M$-H–glc–$CH_3$–CO$]^-$ | Fraxin |
| 3 | 10.34 | 177 $[M-H]^-$ | 133 $[M-H–CO_2]^-$ | Esculetin |
| 4 | 13.25 | 207 $[M-H]^-$ | 192 $[M-H–CH_3]^-$; 164 $[M-H–CH_3–CO]^-$ | Fraxetin |
| 5 | 21.52 | 725 $[M-H]^-$ | 563 $[M-H$-glc$]^-$; 493 $[M-H–glc–C_4H_6O]^-$; 461 $[M-H–glc–C_4H_6O–CH_3OH]^-$; 339 $[M-H–glc–C_4H_6O–CH_3OH–C_6O_3H_2]^-$; 177 $[M-H–glc–C_4H_6O–CH_3OH–C_6O_3H_2–glc]^-$ | Escuside |
| 6 | 27.01 | 539 $[M-H]^-$ | 377 $[M-H–glc]^-$; 307 $[M-H–glc–C_4H_6O]^-$; 275 $[M-H–glc–C_4H_6O–CH_3OH]^-$; 139 $[M-H–glc–C_4H_6O–CH_3OH$–hydroxytyrosol$]^-$ | Oleuropein |
| 7 | 30.22 | 523 $[M-H]^-$ | 361 $[M-H–glc]^-$; 291 $[M-H–glc–C_4H_6O]^-$; 259 $[M-H–glc–C_4H_6O–CH_3OH]^-$; 139 $[M-H–glc–C_4H_6O–CH_3OH$–tyrosol$]^-$ | Ligustroside |

### 3.2. Optimization of chromatographic conditions

Different types of chromatographic columns were compared to optimize the separation. The coumarin compounds could not be resolved and no sharp peaks were obtained on Phenomenex Luna column (250 mm × 4.6 mm i.d., 5 μm), while escuside could hardly be separated from adjacent peaks on Waters Spherisorb ODS2 column (250 mm × 4.6 mm i.d., 5 μm) and Alltech Platinum 100A column (250 mm × 4.6 mm i.d., 5 μm). Because Agilent Zorbax SB-$C_{18}$ column (250 mm × 4.6 mm i.d., 5 μm) was more applicative for acidic mobile phase and the baseline was smoother in this separation than using Agilent Zorbax Extend-$C_{18}$ column (250 mm × 4.6 mm i.d., 5 μm), Zorbax SB-$C_{18}$ column was selected.

Different composition of mobile phases, such as acetonitrile–water, methanol–water and acetonitrile–aqueous acetic acid, were investigated. The results showed that coumarin compounds could not be well separated when using acetonitrile–water system. In the methanol–water system, the separation of the compounds was satisfactory but the baseline was unsmooth. Acetonitrile–aqueous acetic acid system was chosen as mobile phase after several trials, which showed to be beneficial to the improvement of separation and peak shape.

The UV wavelength was set at 254 nm, where all the marker compounds had adequate absorption. Besides, it was found that the separation was better when the column temperature was kept at 30 °C than 20, 25 and 35 °C.

### 3.3. Identification of seven constituents in Cortex Fraxini

The MS data of the seven major constituents and their total ion chromatograms (TIC) were acquired under the conditions described above. In MS spectra, four coumarins: esculin, esculetin, fraxetin and fraxin exhibited quasi-molecular ions $[M-H]^-$ at *m/z* 339, 369, 177 and 207, respectively. In $MS^n$ spectra, the fragment ions of lacking glucose, $CH_3$, CO or $CO_2$ were detected as neutral fragments. The MS spectrum of oleuropein showed a pseudomolecular ion at *m/z* 539 $[M-H]^-$ and the fragments were consistent with the reported fragmentation pattern: the ion at *m/z* 337 arose from the loss of glucose [47]; the ion at *m/z* 307 was characteristic of the loss of a $C_4H_6O$ fragment [48]; the fragment at *m/z* 275 might derive from the loss of $CH_3OH$ from the elenolic fragment of the molecule; the ion at *m/z* 139 indicated the loss of hydroxytyrosol fragment. The MS spectrum of ligustroside showed the pseudomolecular ion at *m/z* 523 $[M-H]^-$, the fragments at *m/z* 361, 291, 259 and 139 were observed in $MS^n$ spectra. As reported for oleuropein, these fragments corresponded to the loss of glucose, $C_4H_6O$, $CH_3OH$ and tyrosol, respectively. Escuside, consisting of one coumarin glucoside unit linked to a secoiridoid moiety, showed the pseudomolecular ion at *m/z* 725 $[M-H]^-$ in MS spectra. Fragments at *m/z* 563, 493, 461, 339, and 177 were detected in the $MS^n$ spectra; the presence of these fragments was consistent with that of the constituents described above. The retention time ($t_R$), MS and $MS^n$ fragment ions data of each constituent are listed in Table 1. The MS fragmentation patterns and UV spectra of the seven constituents fitted well with their chemical structures. On the basis of omparison of the retention times and the spectral data with those of standards, the seven constituents in Cortex Fraxini were identified using the HPLC–DAD–ESI-MS method.

### 3.4. Validation of the assay

#### 3.4.1. Linearity, limit of detection and limit of quantification

All seven reference standards were dissolved in 80% methanol to final concentration of 189.70 μg/ml for esculetin, 152.20 μg/ml for fraxin, 180.84 μg/ml for esculetin, 25.38 μg/ml for fraxetin, 229.50 μg/ml for escuside, 119.70 μg/ml for oleuropein and 50.16 μg/ml for ligustroside, respectively. The solution was diluted to proper concentration to establish calibration curves for mixed standards. Each calibration curve consisted of six different concentrations from low to high and was set up with the peak areas of the standards determined under the HPLC-UV conditions mentioned above versus their injected quantity. All calibration curves showed good linearity ($r^2 > 0.9992$). The results are presented in Table 2. The limits of detection (LOD) ranged from 1.07 to 3.19 ng/ml and limits of quantification (LOQ) ranged from 2.79 to 12.75 ng/ml for all the reference standards, respectively.

Table 2
Calibration curves of the seven constituents in Cortex Fraxini

| Compound | Regression equation | $r^2$ | Linear range ($\times 10^3$ ng) | LOD (ng ml$^{-1}$) | LOQ (ng ml$^{-1}$) |
|---|---|---|---|---|---|
| 1 | $y = 2.1963x - 136.0562$ | 0.9993 | 0.190–7.78 | 1.17 | 3.51 |
| 2 | $y = 3.0669x - 106.2706$ | 0.9992 | 0.151–6.20 | 2.32 | 8.40 |
| 3 | $y = 1.3699x + 76.9199$ | 0.9995 | 0.181–7.41 | 1.42 | 5.02 |
| 4 | $y = 1.3681x + 8.4917$ | 0.9998 | 0.0240–0.978 | 1.41 | 4.23 |
| 5 | $y = 4.7553x - 43.9795$ | 0.9997 | 0.230–9.41 | 3.19 | 12.75 |
| 6 | $y = 1.7454x + 34.4806$ | 0.9998 | 0.120–4.91 | 1.07 | 3.33 |
| 7 | $y = 1.6869x + 14.1617$ | 0.9998 | 0.502–2.06 | 1.39 | 2.79 |

### 3.4.2. Precision

Intra-day precision was examined by analyzing the standard solution within 1 day, and inter-day precision was determined for 3 independent days. Both assays were determined by performing three different concentration levels (high, medium and low) of the reference standards. The results are shown in Table 3. The R.S.D. values (%) were from 0.20–2.75 to 0.07–2.83, respectively.

### 3.4.3. Accuracy

The recovery test was performed to evaluate the accuracy of the method by spiked known quantities of the mixed standards to the samples with known contents of each standard. Then the resultants were extracted and analyzed immediately. The added standards were prepared in three different concentration levels (high, medium and low) and each concentration was analyzed in triplicates. The content of each standard was determined by the corresponding calibration curve. The recovery of the method was calculated by using the ratio of detected amounts to added amounts. The results of average recovery are given in Table 4 and were in the range of 97.61–104.36%.

### 3.4.4. Repeatability and stability

Six samples collected from the same source were extracted and analyzed using the above-mentioned HPLC condition. The repeatability of the method was estimated by calculating the R.S.D. values of seven compounds in these samples. In order to investigate the stability of the samples, the sample solution was injected and determined by HPLC at different time points: 0, 2, 4, 6, 8, 12, 16 and 24 h, respectively. The results are listed in Table 5, and showed high repeatability of the method and good stability of the samples.

## 3.5. Sample analysis

The established analytical method was applied to determine the contents of the seven constituents in 22 Cortex Fraxini

Table 3
Intra- and inter-day precisions in the assay of the seven constituents

| Compound | Concentration ($\times 10^3$ ng ml$^{-1}$) | Intra-day ($n = 6$) | | | Inter-day ($n = 3$) | | |
|---|---|---|---|---|---|---|---|
| | | Found ($\times 10^3$ ng ml$^{-1}$)[a] | R.S.D. (%) | Accuracy (%) | Found ($\times 10^3$ ng ml$^{-1}$)[a] | R.S.D. (%) | Accuracy (%) |
| 1 | 0.190 | 0.190 ± 0.61 | 0.32 | 100.00 | 0.193 ± 2.71 | 1.41 | 101.58 |
| | 1.14 | 1.14 ± 0.02 | 0.17 | 100.00 | 1.14 ± 4.34 | 0.38 | 100.00 |
| | 2.09 | 2.11 ± 18.57 | 0.88 | 100.96 | 2.15 ± 60.82 | 2.83 | 102.87 |
| 2 | 0.151 | 0.151 ± 1.93 | 1.28 | 100.00 | 0.152 ± 2.52 | 1.66 | 100.00 |
| | 0.907 | 0.908 ± 2.91 | 0.32 | 100.11 | 0.906 ± 2.36 | 0.26 | 99.89 |
| | 1.66 | 1.64 ± 12.96 | 0.79 | 98.80 | 1.65 ± 27.80 | 1.69 | 99.40 |
| 3 | 0.181 | 0.180 ± 1.22 | 0.68 | 99.45 | 0.181 ± 1.23 | 0.68 | 100.00 |
| | 1.09 | 1.09 ± 2.18 | 0.20 | 100.00 | 1.09 ± 5.76 | 0.53 | 100.00 |
| | 1.99 | 1.92 ± 37.25 | 1.94 | 96.48 | 1.92 ± 9.64 | 0.50 | 96.48 |
| 4 | 0.0254 | 0.0259 ± 0.29 | 1.12 | 101.97 | 0.0263 ± 0.59 | 2.26 | 103.54 |
| | 0.152 | 0.154 ± 1.42 | 0.92 | 101.32 | 0.155 ± 1.04 | 0.67 | 101.97 |
| | 0.279 | 0.282 ± 1.75 | 0.62 | 101.08 | 0.284 ± 1.54 | 0.54 | 101.79 |
| 5 | 0.230 | 0.228 ± 1.85 | 0.81 | 99.13 | 0.231 ± 2.95 | 1.28 | 100.43 |
| | 1.38 | 1.39 ± 6.95 | 0.50 | 100.72 | 1.40 ± 16.57 | 1.19 | 101.45 |
| | 2.52 | 2.67 ± 73.43 | 2.75 | 109.12 | 2.58 ± 75.63 | 2.93 | 102.38 |
| 6 | 0.120 | 0.120 ± 0.89 | 0.74 | 100.00 | 0.122 ± 1.44 | 1.18 | 101.67 |
| | 0.720 | 0.722 ± 2.67 | 0.37 | 100.28 | 0.721 ± 0.50 | 0.07 | 100.14 |
| | 1.32 | 1.33 ± 8.38 | 0.63 | 100.77 | 1.33 ± 5.32 | 0.40 | 101.76 |
| 7 | 0.0502 | 0.0504 ± 0.53 | 1.05 | 100.40 | 0.0509 ± 0.78 | 1.54 | 101.39 |
| | 0.300 | 0.302 ± 1.27 | 0.42 | 100.67 | 0.303 ± 0.60 | 0.20 | 101.00 |
| | 0.552 | 0.0559 ± 0.50 | 0.89 | 101.27 | 0.557 ± 4.53 | 0.81 | 100.91 |

[a] Mean ± S.D.

Table 4
Recoveries of the seven constituents in Cortex Fraxini ($n = 3$)

| Compound | Added ($\times 10^3$ μg) | Detected ($\times 10^3$ μg) | R.S.D.[a] (%) | Recovery[b] (%) |
|---|---|---|---|---|
| 1 | 0.320 | 0.326 ± 5.38 | 1.66 | 101.88 |
| | 0.639 | 0.667 ± 27.62 | 4.14 | 104.38 |
| | 1.60 | 1.61 ± 17.14 | 1.07 | 100.62 |
| 2 | 0.146 | 0.142 ± 2.27 | 1.60 | 97.26 |
| | 0.292 | 0.291 ± 3.79 | 1.30 | 99.66 |
| | 0.729 | 0.716 ± 3.83 | 0.56 | 98.21 |
| 3 | 0.355 | 0.361 ± 6.77 | 1.88 | 101.69 |
| | 0.710 | 0.709 ± 6.29 | 0.89 | 99.86 |
| | 1.78 | 1.77 ± 5.17 | 0.29 | 99.43 |
| 4 | 0.0455 | 0.0446 ± 0.66 | 1.48 | 98.02 |
| | 0.0910 | 0.0896 ± 2.90 | 3.24 | 98.46 |
| | 0.228 | 0.227 ± 1.97 | 0.87 | 99.56 |
| 5 | 0.115 | 0.120 ± 2.94 | 2.45 | 104.35 |
| | 0.230 | 0.240 ± 2.78 | 1.16 | 104.35 |
| | 0.574 | 0.585 ± 7.60 | 1.30 | 101.91 |
| 6 | 0.0800 | 0.0821 ± 1.12 | 1.37 | 102.63 |
| | 0.160 | 0.162 ± 2.07 | 1.27 | 101.25 |
| | 0.400 | 0.415 ± 1.26 | 0.30 | 103.75 |
| 7 | 0.0225 | 0.0227 ± 0.58 | 2.53 | 100.89 |
| | 0.0450 | 0.0448 ± 1.32 | 2.93 | 99.56 |
| | 0.113 | 0.112 ± 0.77 | 0.69 | 99.12 |

[a] (S.D./mean) × 100.
[b] [(Mean of measured concentration − spiked concentration)/spiked concentration] × 100.

commercial samples collected from different locations. The HPLC-UV chromatograms for content determination are shown in Fig. 4. The content of each reference compound in 22 samples was quantified.

Table 5
Repeatability and stability in assay

| Compound | R.S.D. (%) | |
|---|---|---|
| | Repeatability ($n = 6$) | Stability ($n = 8$) |
| 1 | 0.62 | 1.10 |
| 2 | 0.38 | 2.22 |
| 3 | 0.73 | 1.56 |
| 4 | 1.41 | 1.17 |
| 5 | 2.47 | 2.36 |
| 6 | 0.48 | 0.67 |
| 7 | 0.85 | 0.57 |

The results showed that there were remarkable variations in the contents of a single compound in different samples determined, as least 11-fold variation was found. For instance, the content of esculin was 0.324% in sample no. 12, while 3.480% in sample no. 22. In addition, even 30-fold variation was detected for fraxin between sample no. 21 and 22, 34-fold variation was detected for escuside between sample no. 10 and 15. The prominent difference of the contents might be caused by several reasons. First of all, the samples in assays originated from four plants in different species, so the types and contents of the compounds contained in the herbal samples were different. In addition, the different growth year, harvesting time and processing method of the barks might also cause the difference.

The typical coumarin compounds in Cortex Fraxini: esculetin, esculin, fraxin fraxetin and escuside were detected in all samples. The total coumarin contents in branches ranged between 2.773% and 11.667% and in stems ranged between 0.990% and 6.727%, it was obvious that the total amounts of five coumarins occupied considerable percentage in the herbal samples and they possessed a number of pharmacological activ-

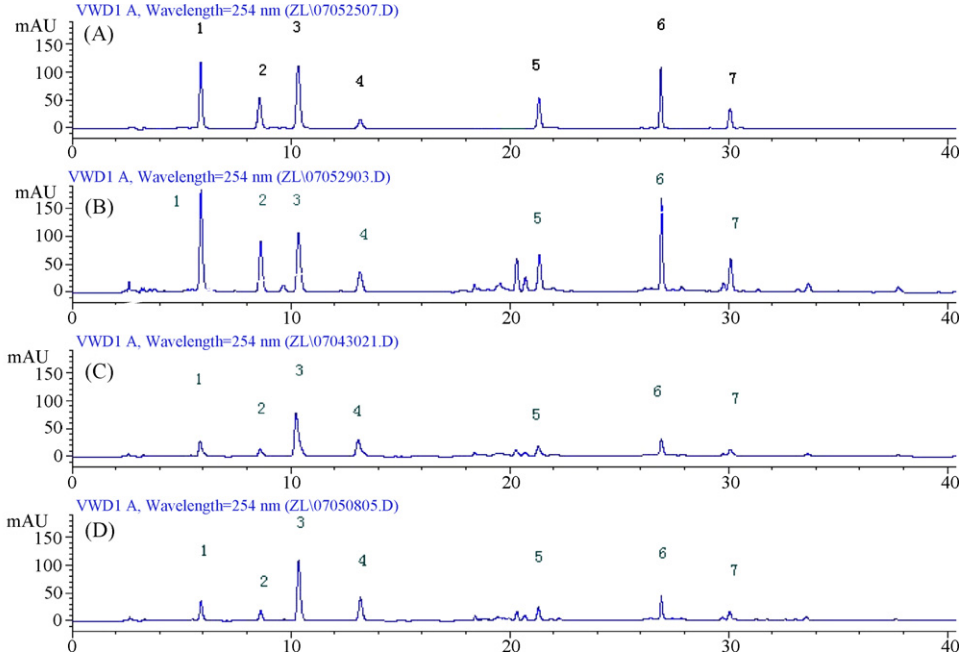


Fig. 4. HPLC-UV chromatograms (A): standard mixture; (B): Cortex Fraxini (Haerbin, Heilongjiang. China); (C): Cortex Fraxini (Dalian, Liaoning. China); (D): Cortex Fraxini (Shenzhen, Guangdong. China). (1) esculin; (2) fraxin; (3) esculetin; (4) fraxetin; (5) escuside; (6) oleuropein; (7) ligustroside.

Table 6
Contents of the seven constituents in the bark originating from branch

| Sample | Contents of the compounds (%)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Standard sample[b] | 3.133 ± 0.030 | 2.512 ± 0.017 | 1.564 ± 0.007 | 0.635 ± 0.000 | 3.083 ± 0.007 | 1.939 ± 0.010 | 0.839 ± 0.003 |
| No. 1 | 1.119 ± 0.013 | 1.018 ± 0.018 | 0.688 ± 0.010 | 0.426 ± 0.003 | 3.330 ± 0.021 | 1.261 ± 0.006 | 0.436 ± 0.009 |
| No. 4 | 3.200 ± 0.001 | 2.295 ± 0.003 | 0.177 ± 0.000 | 0.114 ± 0.000 | 3.226 ± 0.002 | 2.050 ± 0.003 | 0.929 ± 0.002 |
| No. 5 | 3.185 ± 0.001 | 2.264 ± 0.037 | 0.172 ± 0.001 | 0.111 ± 0.000 | 3.142 ± 0.030 | 1.924 ± 0.008 | 0.973 ± 0.006 |
| No. 6 | 1.673 ± 0.010 | 1.627 ± 0.010 | 0.381 ± 0.005 | 0.235 ± 0.005 | 4.372 ± 0.018 | 1.997 ± 0.016 | 0.970 ± 0.011 |
| No. 9 | 2.018 ± 0.005 | 1.616 ± 0.033 | 1.030 ± 0.020 | 0.387 ± 0.010 | 1.974 ± 0.037 | 1.280 ± 0.022 | 0.577 ± 0.010 |
| No. 14 | 3.170 ± 0.002 | 2.340 ± 0.004 | 0.122 ± 0.005 | 0.090 ± 0.001 | 3.240 ± 0.064 | 2.077 ± 0.003 | 0.955 ± 0.003 |
| No. 15 | 2.026 ± 0.023 | 1.706 ± 0.010 | 0.104 ± 0.007 | 0.131 ± 0.001 | 5.130 ± 0.012 | 2.096 ± 0.006 | 0.650 ± 0.005 |
| No. 16 | 2.016 ± 0.040 | 1.534 ± 0.007 | 0.808 ± 0.009 | 0.333 ± 0.001 | 2.489 ± 0.026 | 1.384 ± 0.013 | 0.664 ± 0.008 |
| No. 17 | 0.338 ± 0.020 | 0.287 ± 0.000 | 1.005 ± 0.000 | 0.409 ± 0.004 | 0.734 ± 0.001 | 0.404 ± 0.001 | 0.157 ± 0.001 |
| No. 22 | 3.480 ± 0.053 | 2.403 ± 0.035 | 0.786 ± 0.011 | 0.286 ± 0.005 | 4.712 ± 0.056 | 1.849 ± 0.021 | 0.764 ± 0.010 |

[a] Mean ± S.D. ($n = 3$).
[b] Control sample according to China pharmacopoeia, purchased from National Institute for the Control of Pharmaceutical and Biological Products.

Table 7
Contents of the seven constituents in the bark originating from stem

| Sample no. | Contents of the compounds (%)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 2 | 0.576 ± 0.003 | 0.192 ± 0.012 | 0.162 ± 0.012 | 0.060 ± 0.000 | – | 0.267 ± 0.008 | – |
| 3 | 1.213 ± 0.002 | 0.313 ± 0.000 | 1.140 ± 0.003 | 0.124 ± 0.006 | 0.259 ± 0.003 | 0.160 ± 0.008 | – |
| 7 | 0.580 ± 0.007 | 0.347 ± 0.010 | 1.056 ± 0.004 | 0.255 ± 0.007 | 0.313 ± 0.003 | – | – |
| 8 | 1.277 ± 0.007 | 0.472 ± 0.002 | 1.048 ± 0.004 | 0.182 ± 0.001 | 0.473 ± 0.002 | 0.234 ± 0.019 | 0.084 ± 0.001 |
| 10 | 1.299 ± 0.003 | 0.270 ± 0.001 | 0.243 ± 0.005 | 0.028 ± 0.000 | 0.151 ± 0.002 | 0.071 ± 0.004 | – |
| 11 | 1.771 ± 0.009 | 1.026 ± 0.001 | 0.628 ± 0.003 | 0.180 ± 0.001 | 0.289 ± 0.002 | 0.120 ± 0.001 | – |
| 12 | 0.324 ± 0.007 | 0.335 ± 0.000 | 0.663 ± 0.000 | 0.184 ± 0.002 | 0.108 ± 0.005 | 0.085 ± 0.000 | – |
| 13 | 2.070 ± 0.017 | 0.277 ± 0.001 | 1.337 ± 0.003 | 0.129 ± 0.000 | 0.220 ± 0.003 | 0.178 ± 0.002 | 0.069 ± 0.001 |
| 18 | 1.560 ± 0.006 | 1.284 ± 0.006 | 0.922 ± 0.001 | 0.444 ± 0.002 | 2.517 ± 0.013 | 1.267 ± 0.003 | 0.790 ± 0.005 |
| 19 | 1.012 ± 0.020 | 0.125 ± 0.002 | 1.787 ± 0.008 | 0.139 ± 0.002 | 0.385 ± 0.002 | 0.180 ± 0.004 | – |
| 20 | 2.149 ± 0.001 | 0.318 ± 0.007 | 2.289 ± 0.045 | 0.125 ± 0.004 | 0.364 ± 0.006 | 0.214 ± 0.000 | – |
| 21 | 0.147 ± 0.000 | 0.079 ± 0.002 | 0.630 ± 0.002 | 0.199 ± 0.000 | 0.455 ± 0.004 | 0.199 ± 0.009 | 0.041 ± 0.002 |

[a] Mean ± S.D. ($n = 3$).

ities. Hence, the contents of the coumarin constituents played a vital role in the quality evaluation of Cortex Fraxini.

It could also be summarized, based on the results, that the contents of the compounds in most of the barks from branch were higher than those from stem of the plants and many samples originated from stem contained no oleuropein and ligustroside or the contents were very low. The results are summarized in Tables 6 and 7. Any kind of the barks are allowed to be used in Chinese pharmacopoeia, but the barks from branch might have better curative effect on the basis of our analytical results.

## 4. Conclusion

A simple, rapid and sensitive HPLC method was developed for the determination of the constituents in Cortex Fraxini. Simultaneous quantification of a total of seven constituents was reported firstly, the previous reports on applying HPLC method to determine the samples of Cortex Fraxini only aimed at some constituents and did not include other constituents such as escuside, oleuropein, ligustroside, etc. which also occupied certain percentages in the herbal samples. The developed method could be applied as a reliable quality evaluation method for Cortex Fraxini.

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