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Simultaneous Quantitation of Nine Constituents of *Fraxinus rhynchophylla* using High Performance Liquid Chromatography - Diode Array Detector

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Abstract – A high-performance liquid chromatography-diode array detector (HPLC-DAD) method was established for quantitative evaluation of nine constituents of *Fraxinus rhynchophylla* such as four coumarins, esculin (**1**), fraxin (**2**), esculetin (**3**), fraxetin (**4**), three lignans, syringaresinol 4,4'-*O*-β-diglucoside (**5**), pinoresinol 4-*O*-β-glucoside (**6**), pinoresinol (**9**), one secoiridoid, oleuropein (**7**), and one coumarinolignan, cleomiscosin C (**8**). The preferred chromatographic condition was obtained on Phenomenex Gemini-NX (3 μm, C18 110A, 150 × 4.60 mm) and the mobile phase was composed of water and acetonitrile using a gradient elution. The wavelength was set at 220 nm. Extraction condition of these constituents in *F. rhynchophylla* was also optimized through extraction time, extraction solvent and extraction method using established method. From this study, extraction at 70 °C with the mixture of ethanol and water for more than 12 h was suggested to be good extraction condition for these constituents. Quantitation of nine constituents in different *F. rhynchophylla* samples was also successfully accomplished with the newly established method.
Keywords – *Fraxinus rhynchophylla*, Qualitative evaluation, HPLC-DAD

Introduction

Fraxinus species, which widely grow in Asia and Europe, have been used for the treatment of inflammation and infectious diseases in traditional medicines (Bae, 1999). *Fraxinus* species produce various coumarins, secoiridoids, lignans and coumarinolignans, which responsible for pharmacological effects (Kim *et al*., 1999; Jiang *et al*., 2008; Peng *et al*., 2010; Kostova and Iossifova, 2007).

Recently, patented extract of *Fraxinus* species named Fraxipure™ is developed people with diabetes or obesity (Ibarra and Sang, 2011). It reduced fasting blood glucose and insulin levels in clinical and in vivo study (Visen *et al*., 2009). It also effectively reduced body weight and fat accumulation in high-fat diet obese mice (Ibarra and Sang, 2011). Related to active constituents, secoiridoids reduced fat accumulation in adipocytes by PPAR-γ activation and reduced fat absorption by the inhibition of the pancreatic lipase activity (Bai *et al*., 2010; Choi *et al*., 2011; Ahn *et al*., 2013). Coumarins and lignans from *Fraxinus* species also have been reported to have anti-obesity, by reducing fat accumulation and fat absorption, respectively (Shin *et al*., 2010; Ahn *et al*., 2012). Therefore, coumarins, secoiridoids and lignans, the major bioactive constituents of *Fraxinus* species, are suggested to exert its anti-obesity activity through multiple actions.

To data, analytical method only for phenolic compounds such as lignans and phenylethanoids has been reported (Sanz *et al*., 2012). To ensure the pharmacological activity of *F. rhynchophylla* for anti-obesity, qualitative and quantitative analysis of multiple bioactive constituents is needed. High performance liquid chromatography coupled with diode array detector (HPLC-DAD) method was developed for simultaneous quantitation of four different classes of constituents of *F. rhynchophylla* such as coumarins, secoiroids, lignans and coumarinolignans. Nine compounds, such as four coumarins, esculin (**1**), fraxin (**2**), esculetin (**3**), fraxetin (**4**), three lignans, syringaresinol-4,4'-*O*-β-diglucoside (**5**), pinoresinol-4-*O*-β-glucoside (**6**), pinoresinol (**9**), one secoiridoid, oleuropein (**7**) and one coumarinolignan, cleomiscosin C (**8**) were used as the marker for analysis for *F. rhynchophylla* (Fig. 1A). The contents of these constituents in samples from different extraction conditions and from different cultivation were analyzed using developed method.

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Experimental

General experimental procedures – Analysis was performed using a Waters HPLC system equipped with Waters 600 Q-pumps, a 996 photodiode array detector, and Waters Empower software using Phenomenex Gemini-NX 3 μm C18 110A (150 × 4.60 mm) for quantitation.

Materials and chemicals – Nine standards constituents were isolated as we previously reported (Shin *et al*., 2010; Choi *et al*., 2011; Ahn *et al*., 2012, 2013). *F. rhynchophylla* samples were purchased at local market from different provinces such as Gyeongbuk, Chonbuk, Gangwon and Chungbuk and authenticated at Herbarium of College of Pharmacy at Chungbuk National University.

Sample preparation for HPLC – Stock standard solution of each compound was prepared in methanol at a concentration of 0.5 mg/ml, respectively. The appropriate amount of every standard solution was mixed and diluted with methanol as indicated.

The powdered stem barks of *F. rhynchophylla* (500 mg) were weighed accurately and extracted with 50% methanol for 24 h at room temperature. For evaluation of extraction times, powdered *F. rhynchophylla* (500 mg) was extracted with ethanol for different time periods such as 0.5, 1, 2, 4, 8, 16 and 24 h at room temperature. Effects of extraction solvent were evaluated by extraction with each mixture of ethanol-water (100%, 75%, 50%, 0% ethanol in water) for 24 h. For the optimization of extraction method, powdered *F. rhynchophylla* (500 mg) were extracted with 50% ethanol by shaking at room temperature or at 70 ºC, or by using ultrasonic apparatus. Each sample solution was filtered through 0.45 μm membrane filter before HPLC analysis.

Validation of the HPLC Methods – The linearity of calibration curves was calculated according to the International Conference on Harmonization (ICH) guidelines. Five concentrations of each compound (0.10, 0.15, 0.25, 0.30 and 0.50 mg/ml) were prepared and analyzed in triplicate. The limit of detection (LOD) and quantification (LOQ) were determined based on the method recommended by ICH (LOD = 3.3 δ/S, δ = standard deviation of the response, S = slope of the calibration curve). The precision test was carried out by the intra-day and inter-day variability for each compound. The intra-day variability was assayed at five concentrations on the same day and inter-day variability was assayed at five concentrations on three sequential days (1, 3, 5 days).

Results and discussion

Optimization of chromatographic conditions – For simultaneous determination of nine components of *F. rhynchophylla*, various mixtures of water, methanol and acetonitrile in combination with acetic acid were tested as a mobile phase. Chromatographic separation was accomplished using water containing 0.1% acetic acid (A) and acetonitrile (B) with gradient elution program as 0 - 10 min at 10% B; 10 - 15 min from 10 to 20% B; 15 - 20 min at 20% B; 20 - 25 min from 20 to 30% B; 25 - 30 min from 30 to 10% B; 30 - 40 min at 10% B. The wavelength for detection was set at 220 nm, where the nine compounds showed the maximum absorption as measured by DAD. Under this optimized analytical method, nine compounds of *F. rhynchophylla* were separated within 53 min (Fig. 1B).

Validation of developed analytical method – The specificity of peak was determined by the calculation of peak purity facilitated by DAD and ESI-MS. The absorption spectrum of a single component remained invariable at each time point in one peak. The molecular ion and fragmentation patterns of each compound were well matched with each chemical structure in its HPLC-ESI-MS spectra (Table 1). All nine standard compounds

Table 1. LC-ESI-MS^n characteristics of compounds **1 - 9**

Compound	m/z	Mode	MS^n ions m/z (relative abundance)
1	340	Negative	MS full scan : 339(100); MS^2[339]: 177(100)
2	370	Positive	MS full scan : 209(100), 393(29); MS^2[209]: 149(100), 163(65), 181(55), 194(30)
3	178	Positive	MS full scan : 150(100), 179(63)
4	208	Positive	MS full scan : 209(100); MS^2[209]: 163(69), 149(100)
5	742	Positive	MS full scan : 760(100), 765(36), 742(3); MS^2[742]: 418(100), 401(75)
6	520	Negative	MS full scan : 519(100); MS^2[519]: 357(100)
7	540	Negative	MS full scan : 539(6), 523(100); MS^2[523]: 361(100), 291(38)
8	416	Positive	MS full scan : 417(100), 433(12); MS^2[417] : 367(100), 399(55)
9	358	Positive	MS full scan : 341(4), 387(100); MS^2[387] : 337(100), 369(46)

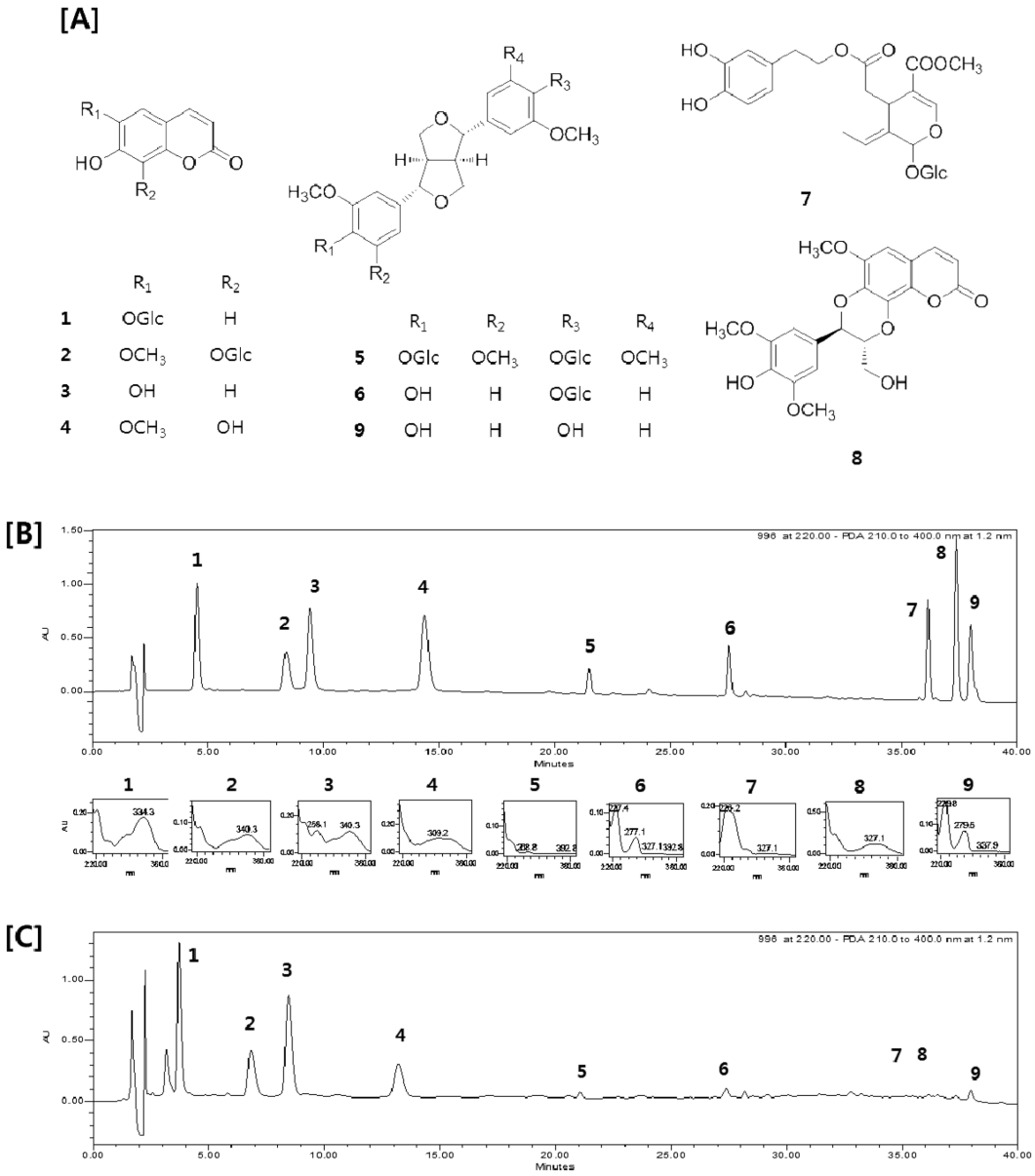

Fig. 1. (A) Structure of nine constituents of *F. rhynchophylla*, (B) HPLC chromatogram of standard compounds at 220 nm, (C) HPLC chromatogram of total extract of *F. rhynchophylla* at 220 nm.

(**1** - **9**) were clearly isolated each other in standard mixture solution (Fig. 1B) and seven compounds (**1** - **6**, **9**) were identified in *F. rhynchophylla* extract (Fig. 1C). As shown in Table 2, the calibration curves showed good linearity for all nine constituents ($r^2 > 0.9975$), and LOD and LOQ for nine compounds were less than 56.1 ng and 170.1 ng, respectively. The RSD of overall intra- and inter-day variability showed good precision of this method. The developed method had good accuracy with recovery of 93.3 - 106.7% with RSD values less than 6.68% (Table 3).

Quantitation of compounds in *F. rhynchophylla* from different sample preparation – Extraction condition greatly affects the extraction efficiency (Chen *et al*., 2012; Mulinacci *et al*., 2011). For the optimization of extraction condition, established method has been applied to the quantitation of *F. rhynchophylla* samples from different preparation using different solvent composition, extraction time and extraction method..

The effect of extraction time was first tested. As shown in Fig. 2A, the yield of compounds was increased as the extraction time increased. Especially, the yield of

Table 2. Linear regression data, LOD and LOQ of compounds

Compounds	Linear regression data		LOD (ng)	LOQ (ng)
	Equation	r^2		
1	y = 22321x + 165748	0.9983	27.8	84.1
2	y = 12753x + 8112.9	0.9995	10.5	31.7
3	y = 24503x – 89130	0.9999	56.1	170.1
4	y = 30408x – 149797	0.9994	9.9	29.9
5	y = 4541.4x + 36431	0.9999	45.1	136.6
6	y = 9099x + 103063	0.9975	29.5	89.4
7	y = 14984x + 108235	0.9976	33.6	102.0
8	y = 30639x + 295048	0.9986	35.2	106.6
9	y = 15208x + 132790	0.9987	25.6	77.6

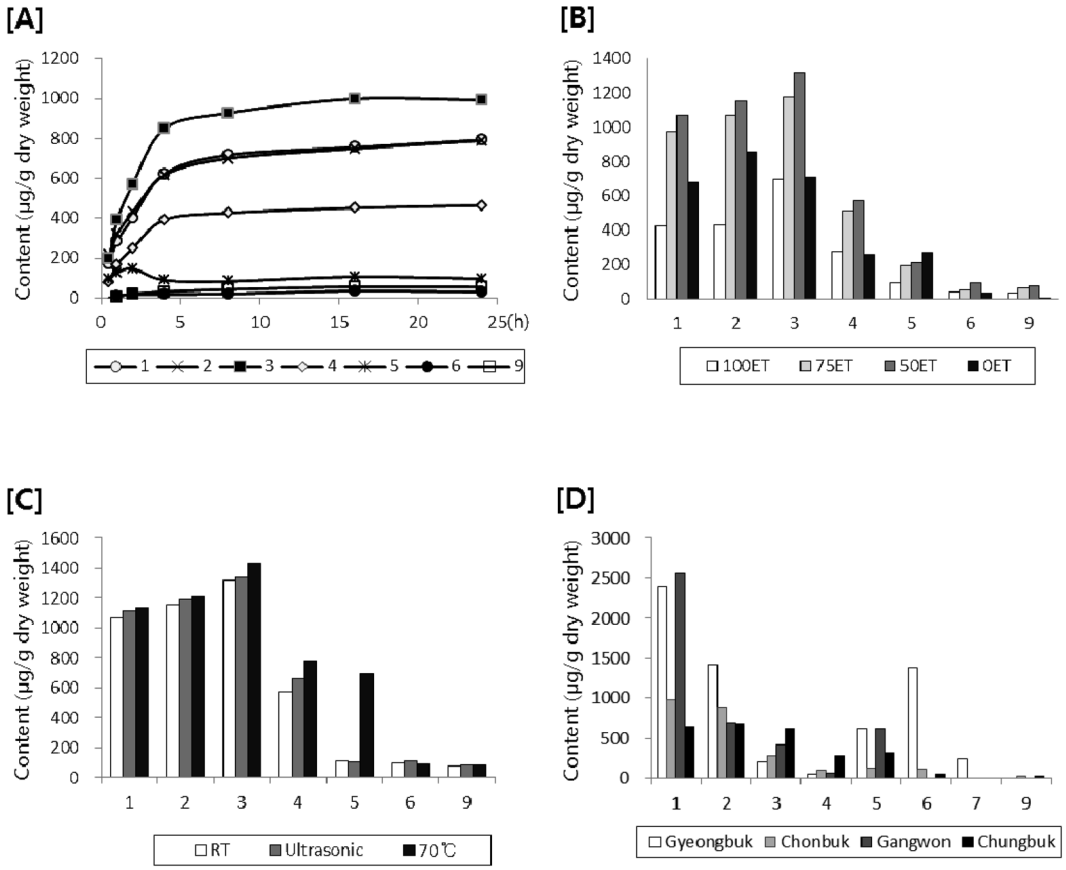

Fig. 2. Effect of extraction condition on the yield of *F. rhynchophylla*. (A) Samples were extracted with 100% ethanol for different time as indicated. (B) Samples were extracted for 24 h at room temperature with the mixture of ethanol and water (100%, 75%, 50% and 0% ethanol in water, respectively). (C) Samples were extracted for 24 h with 50% ethanol using different method as indicated. (D) Samples from different cultivation were extracted with 50% ethanol for 24 h.

coumarins was increased sharply as the extraction time increased. Next the effects of extraction solvent on extraction efficiency were evaluated. *F. rhynchophylla* was extracted with the mixture of ethanol and water (100%, 75%, 50% and 0% ethanol in water) and analyzed. As shown in Fig. 2B, the yield of compounds was 50% ethanol > 75% ethanol > 100% water ≈ 100% ethanol. Therefore, the mixture of ethanol and water is suggested to be a good solvent system for the extraction of *F. rhynchophylla*. The extraction method on extraction yield was tested by the extraction using ultrasonic apparatus or by shaking at room temperature or at 70 °C

Table 3. Inter- and intra-day precision of compounds

Compound	Spiked amount (μg)	Inter-day		Intra-day	
		Content (μg)	RSD [a] (%)	Content (μg)	RSD [a] (%)
	1.0	0.943	3.79	0.970	2.25
1	2.5	2.449	4.04	2.465	4.94
	5.0	4.734	2.37	4.994	6.68
	1.0	0.979	1.82	0.977	1.55
2	2.5	2.466	3.16	2.466	2.73
	5.0	4.859	1.03	4.896	1.25
	1.0	1.044	4.40	1.028	2.55
3	2.5	2.578	3.01	2.517	1.16
	5.0	5.059	1.80	5.088	2.54
	1.0	1.039	3.74	1.034	2.53
4	2.5	2.538	3.25	2.526	1.96
	5.0	5.034	2.11	5.140	2.99
	1.0	0.941	2.56	0.955	2.30
5	2.5	2.465	6.18	2.551	4.75
	5.0	4.723	2.90	4.937	2.62
	1.0	0.933	3.67	0.913	1.31
6	2.5	2.491	3.67	2.568	2.39
	5.0	4.897	1.77	5.051	2.28
	1.0	0.985	3.51	0.972	3.21
7	2.5	2.568	4.10	2.622	1.62
	5.0	4.926	2.08	5.018	5.84
	1.0	0.963	4.25	0.960	3.49
8	2.5	2.549	3.03	2.592	1.34
	5.0	4.881	1.24	4.947	3.49
	1.0	0.916	4.25	0.910	3.73
9	2.5	2.380	3.00	2.437	1.48
	5.0	4.757	2.35	4.961	6.03

[a] RSD (%) = (SD of amount detected / mean of amount detected) × 100 (n = 5).

(Fig. 2C). Although the yield was relatively high at 70 ºC extraction, the extraction method did not exert great effect.

Quantitation of compounds in F. rhynchophylla from different cultivation – Established analytical method was applied to the quantitation of compounds in the samples from different cultivation. As shown in Fig. 3D, quantitation of nine constituents in different *F. rhynchophylla* samples was also successfully accomplished with the newly established method. The contents of esculin (**1**), pinoresinol 4-*O*-β glucoside (**6**) and oleuropein (**7**) showed considerable variation between samples.

Conclusively, an HPLD-DAD method was developed for the quantitation of nine constituents such as four coumarins; esculin, fraxin, esculetin, fraxetin, three lignans; syringaresinol 4,4'-*O*-β diglucoside, pinoresinol 4-*O*-β-glucoside, pinoresinol, one secoiridoid; oleuropein and one coumarinolignan; cleomiscosin C in *F. rhynchophylla*. Quantitation of these compounds in different *F. rhychophylla* samples showed great difference in the yield of constituents. Therefore, quantitation of these compounds using developed analytical method might provide basic requirement for pharmacological activity of *F. rhynchophylla*.

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