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Supercritical fluid extraction of flavonoids from Scutellariae Radix[1]

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Abstract

An optimal condition of supercritical fluid extraction (SFE) for flavonoids of *Scutellaria baicalensis* was developed. In this study, various temperatures, pressures and modifiers were studied. The conventional extraction methods were conducted in parallel for comparison. The crude extracts were qualitatively compared by TLC and GC–MS, and the contents of flavonoids were determined by HPLC. The amounts of baicalin, baicalein and wogonin in the Scutellariae Radix obtained by supercritical fluid extraction and a conventional sonic shaking method were 137.6 mg/g, 8.6 mg/g and 2.2 mg/g, 113.5 mg/g, 5.7 mg/g and 2.3 mg/g, respectively. Application of SFE for extraction of the flavonoids from Scutellariae Radix was preferable. The optimal conditions of SFE was as follows: supercritical carbon dioxide–MeOH–water (20:2.1:0.9), 50°C and 200 bar.

Keywords: *Scutellaria baicalensis*; Extraction methods; Flavonoids; Baicalin; Baicalein; Wogonin

1. Introduction

The roots of *Scutellaria baicalensis* Georgi (Labiatae) have been used in the Chinese medicine for the treatment of inflammation, fever and headache [1]. Recent studies had shown that baicalin and baicalein showed a detoxifying effect and could inhibit peripheral capillary permeability, and wogonin was found to inhibit the proliferation of tumor cells [2]. Tadato, Song, Tsuyoshi and Shuzo et al. [3–6] have documented the constituents of the plant. The three constituents mentioned above were isolated from Scutellariae Radix by extraction with organic solvent, followed by column separation and purification, to obtain pure compounds. The conventional extraction consumed large amounts of solvents, required lots of time, and polluted the environment. Supercritical fluid extraction [7] was developed in 1960. In this technique, supercritical carbon dioxide is widely used as an extraction solvent. Carbon dioxide has the following advantages: chemically inert, low toxicity, no pollution problem, and shorter concentration time. These advantages have attracted increasing interest from researchers. Nowadays, the application of supercritical fluid extraction (SFE) includes the industries of food, pharmacy and environmental engineering etc. [8–21]. For the extraction of polar or ionic compounds, organic solvents have been added as modifiers or the compounds were first derivatized to decrease their polarity [14,20].

Recently, Pan et al. applied SFE to Scutellariae Radix to extract essential oil [22]. In this study, we

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focused on the extraction of flavonoids by the same technique. Since flavonoids are more polar than essential oils, especially baicalin can not be extracted by supercritical carbon dioxide alone. According to the strategies illustrated by Luque de Castro and Tena [23], the pressure and temperature of the supercritical fluid were varied, and polar modifiers were used to improve the extraction yields of flavonoids from Scutellariae Radix.

2. Experimental

2.1. Material

The root of *Scutellaria baicalensis* was purchased from a natural drug retail outlet in Taipei, dried to constant weight and pulverized.

2.2. Chemicals and solvents

Carbon dioxide (SFE grade) was purchased from Air Products and Chemicals (PA, USA). Baicalin and wogonin were obtained from Nacalai Tesque (Osaka, Japan). Baicalein and ferric chloride were purchased from Matsuura Yakuga (Kyoto, Japan). Caffeine was purchased from Sigma (St. Louis, MO, USA). Acetonitrile, methanol, ethyl acetate, acetone and *n*-butanol of LC grade were purchased from Labscan (Dublin, Ireland). Phosphoric acid and glacial acetic acid were purchased from E. Merck (Darmstadt, Germany). Tetrachloromethane was purchased from Riedel-de Haen (Seeize, Germany). Ethanol (95%) was produced by Taiwan Tobacco and Wine Monopoly Bureau (Taipei, Taiwan).

2.3. Equipment

The SFE was equipped with an Isco SFX 2-10 extractor, a Model 100DX Syringe Pump, and a 10-ml stainless steel sample cartridge, with a 1/16 in. stainless tube (Lincoln, USA) (1 in.=2.54 cm). The high-performance liquid chromatography system was composed of a Waters 600 pump and a controller, a 486 tunable absorbance detector (Millipore, Boston, MA, USA), a Hitachi L-7200 autosampler (Hitachi, Tokyo, Japan), and a Shiunn Haw Computing integrator (Scientific Information Service). The reduced pressure evaporation was carried out with a Rotavapor EL 130, a Recirculation Chiller Buchi 700, a Vacuum/Distillation Controller 168 (Buchi, Flawil, Switzerland) and an Aspirator EYELA A-3S (Rikakikai, Tokyo, Japan). The GC–MS system was composed of an HP-6890 GC system, an HP-5973 mass selective detector, an HP 6890 Series Injector, and an HP MSD Chemstation (Hewlett-Packard, Palo Alto, CA, USA).

2.4. High-performance liquid chromatography

Cosmosil 5C_{18}-AR (5 and 15 cm×4.6 mm I.D.) reversed-phase columns (Nacalai Tesque, Kyoto, Japan) were used. The mobile phase was composed of 0.1% phosphoric acid (A) and acetonitrile (B). The gradient elution was as follows: time 0 min A–B (90:10); time 30 min A–B (35:65); time 35 min A–B (90:10). The flow-rate was 1 ml/min and the detection wavelength was 277 nm.

2.5. Gas chromatography and mass spectrometry

A 30 m×0.25 mm HP-5MS (crosslinked 5% diphenyl–95% dimethylpolysiloxane) capillary column was used (0.25 μm film thickness). The operation conditions were as follows: splitless injection; 50.0 ml/min purge flow; oven, with initial temperature at 100°C for 2 min, ramping 10°C/min to 280°C, then holding at 280°C for 15 min; injection temperature 250°C; ionization source temperature 230°C; 70 eV electron impact mode; solvent delay 4 min; injection volume 1 μl; using helium as carrier gas at 1 ml/min.

2.6. Thin-layer chromatography

TLC plates were silica gel Merck 60 F_{254} 20×20 cm (E. Merck). The two mobile phases were *n*-butanol–water–glacial acetic acid (7:2:1, v/v/v) and tetrachloromethane–acetone (5:3, v/v). Ferric chloride spray was used as a chromogenic reagent. Observation was achieved at 254 nm.

2.7. Extraction

Pulverized Scutellariae Radix (1 g) was accurately weighed, then extracted by the following methods.

Table 1
The amount of crude extract from Scutellariae Radix (1 g) using conventional extraction methods ($n=5$)

Extraction method	Extraction solvent	Yield (mean±S.D.; mg/g)	R.S.D.(%)
Percolation overnight	MeOH	160.2±2.19	1.37
Ultrasonic shaking	MeOH–water (70:30)	396.9±4.42	1.11
	MeOH	201.1±2.95	1.47
	EtOH	161.2±1.50	0.93
	Me_2CO	47.5±0.75	1.58
	EtOAc	31.3±1.08	3.45

2.7.1. SFE method

The pulverized sample was packed into a 10-ml sample cartridge. Methanol or 70% methanol (1 ml, 2 ml and 3 ml, respectively) was added, and 20 ml liquid carbon dioxide was used as extraction solvent. The extraction temperature was set at 40, 50, 60 and 70°C, respectively. Liquid carbon dioxide at high pressure (200, 300 and 400 bar) was then allowed to flow into the sample cartridge. When the pressure reached 200, 300, 400 bar, the vent valve of the extractor was opened immediately and carefully, so that the soluble fraction was collected through tubing to a test tube filled with 10 ml methanol. The extraction process was run for 10–15 min, and was repeated three times. The crude extract was obtained by reduced pressure evaporation.

Table 2
The amount of crude extract from Scutellariae Radix (1 g) using supercritical fluid extraction ($n=3$)

T(°C)	P(bar)	Amount(mean±S.D.; mg/g), (R.S.D.%)					
		MeOH–CO_2			70% MeOH–CO_2		
		1:20	2:20	3:20	1:20	2:20	3:20
40	200	–	–	115.0±2.90 (2.52)	–	–	366.3±5.18 (1.41)
	300	–	–	117.1±10.0 (8.54)	–	–	369.2±8.22 (2.23)
	400	–	–	124.9±6.40 (5.12)	–	–	370.9±7.77 (2.09)
50	200	66.5±1.85 (2.78)	128.5±5.20 (4.05)	155.7±9.50 (6.10)	211.0±3.15 (1.49)	353.7±8.60 (2.43)	412.5±4.50 (1.09)
	300	72.6±5.95 (8.20)	118.2±6.45 (5.46)	156.4±8.15 (5.21)	222.5±3.05 (1.37)	348.4±7.45 (2.14)	410.9±6.53 (1.59)
	400	64.0±4.70 (7.34)	108.0±7.95 (7.36)	194.6±8.45 (4.34)	193.0±.70 (1.40)	329.4±5.95 (1.81)	404.6±8.59 (2.12)
60	200	68.3±6.00 (8.78%)	150.3±3.65 (2.43)	157.8±2.50 (1.58%)	–	–	389.9±5.03 (1.29)
	300	78.0±5.50 (7.05)	148.6±1.35 (0.91)	168.6±2.50 (1.48)	–	–	381.5±10.22 (2.68)
	400	65.5±2.85 (4.35)	137.2±7.15 (5.21)	194.3±6.25 (3.22)	–	–	412.8±7.25 (1.76)
70	200	94.3±1.95 (2.07)	126.0±4.75 (3.77)	178.1±2.20 (1.24)	–	–	401.5±11.20 (2.79)
	300	80.2±7.20 (8.98)	146.5±3.80 (2.59)	150.3±5.65 (3.76)	–	–	378.6±16.01 (4.23)
	400	77.7±3.10 (3.99)	144.0±3.75 (2.60)	199.1±5.75 (2.89)	–	–	401.5±6.54 (1.63)

2.7.2. Conventional extraction methods

The pulverized sample was extracted three times with 20 ml of solvent by ultrasonic shaking for 30 min. Various extraction solvents were used: 70% methanol, methanol, ethanol, acetone, and ethyl acetate. In a second extraction method, the pulverized sample was percolated overnight with methanol. The crude extracts were obtained by reduced pressure evaporation.

2.8. Comparison of crude extract from SFE and conventional extraction methods

2.8.1. Comparison of crude extracts by TLC and GC–MS

Crude extract, baicalin, baicalein and wogonin standards were accurately weighed, dissolved in methanol to afford the same concentration (1 mg/ml), and analyzed by TLC and GC–MS.

2.8.2. Quantitative analysis of crude extract by HPLC

The calibration curve was established using standard solutions of baicalin, baicalein, and wogonin. The concentrations of calibrators are 1, 2, 4, 6, 8, 20, 30 μg/ml for baicalin and baicalein and 0.5, 1, 2, 4, 6, 8, 20 μg/ml for wogonin. Caffeine was used as the internal standard.

Each crude extract was accurately weighed, and dissolved in 70% methanol. After filtration, caffeine was added to a final concentration of 20 μg/ml. The soluble fraction was filtered (0.45 μm Millipore) before the injection.

3. Results and discussion

3.1. Quantitative comparison of the extracts

The amounts extracted by conventional methods were listed in Table 1. Ethyl acetate and acetone

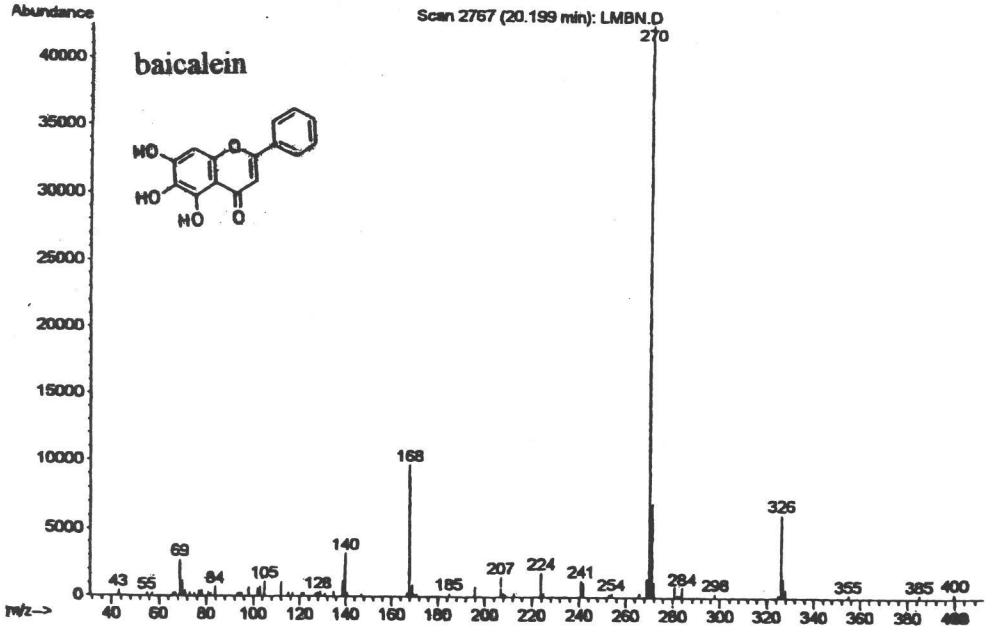

Fig. 1. EI mass spectra of baicalein. The experimental conditions are described in the text.

gave low yields. The highest extraction yield was found with extraction by 70% methanol under ultrasonic shaking, suggesting that mechanical shaking was superior to percolation. Table 1 showed that the yield of crude extract was proportional to the polarity of solvent.

Because the yield extracted by pure supercritical carbon dioxide was not satisfactory, and changes in pressure and temperature negligibly improved the yield, it was thought that increasing the polarity of the extraction solvent might overcome the low yield. Methanol (70%) and methanol were chosen as modifiers in this study. The amounts of crude extracts by SFE under various conditions are listed in Table 2. The concentrations of modifier used were 5, 10 and 15% (v/v), (in liquid carbon dioxide).

Under certain temperature and pressure, the higher the percentages of modifiers, the higher was the extraction yield achieved. By using the same percentages of modifiers, the amounts extracted with 70% methanol were higher than those with methanol (as shown in Table 2). This could be explained by the fact that polar constituents in the plant would be easier to extract with a more polar solvent.

Table 2 shows that at a certain pressure, with 70% methanol as a modifier, when the temperature was raised from 40 to 50°C, the extraction yield increased. The increment rates were 12.6%, 11.3% and 9.1% at 200 bar, 300 bar and 400 bar, respectively. When the temperature was increased from 50 to 60°C, the extraction yield decreased somewhat. As the temperature changed from 60 to 70°C, at 200 bar, the yield increased, while at a pressure of 300 or 400 bar, the yield decreased. These results suggested that 50°C was the optimal temperature for extraction.

At 40°C and 50°C, pressure changes had no significant influence on the extraction yield. At 60 or 70°C, as the pressure changed from 200 to 300 bar, the yield decreased; as the pressure changed from 300 to 400 bar, the yield increased. As a result, 200 bar was the critical pressure.

At 50°C, with the modifier of 10% v/v, when the

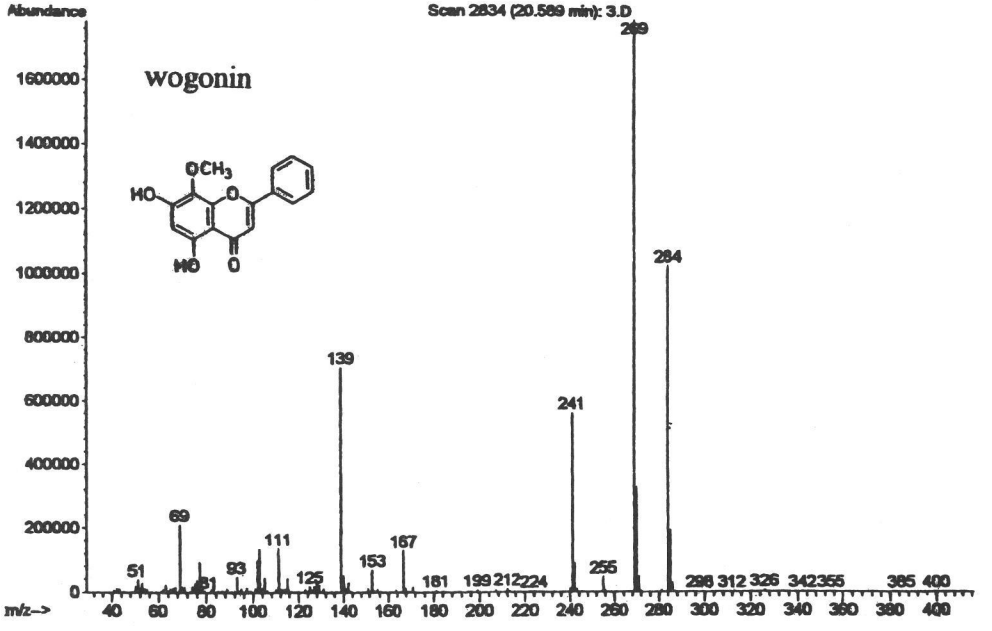

Fig. 2. EI mass spectra of wogonin. The experimental conditions are reported in the text.

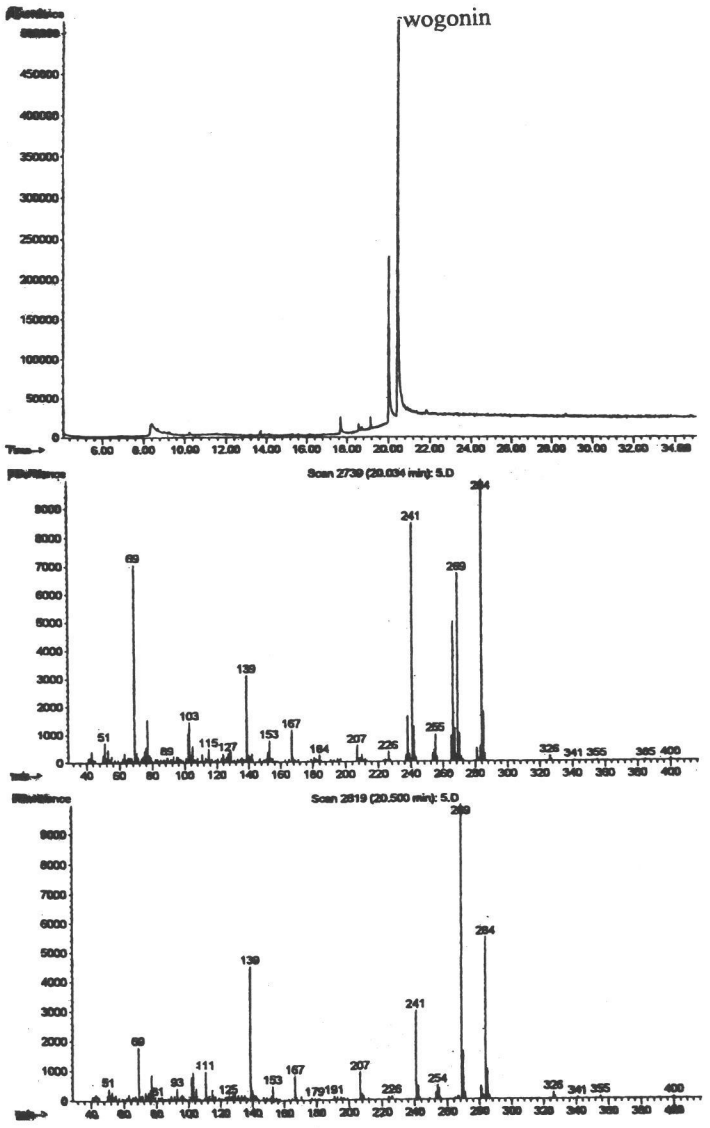

Fig. 3. GC–MS chromatogram and mass spectra of the two major peaks of the crude extract from SFE with MeOH–water (70:30) as a modifier. The experimental conditions are provided in the text.

pressure increased from 200, 300 to 400 bar, the yield decreased. The decreased amounts were 14.3%, 15.2% and 18.6%, respectively. When 5% of modifier was used, as the pressure was increasing from 200, 300 to 400 bar, the decreased amounts were 48.8%, 45.9% and 52.3%, respectively.

From Tables 1 and 2, similar results were obtained between the conventional extraction method and SFE when methanol was used at 50–70°C. However, when 70% methanol was used at 400 bar, the yield of SFE was higher than that of the conventional method.

3.2. Comparison of crude extract by TLC and GC–MS

3.2.1. TLC

Baicalin, baicalein and wogonin were detectable in each extract with TLC. The R_f value of baicalin was 0.32 in *n*-butanol–water–glacial acetic acid (7:2:1, v/v/v); R_f values of baicalein and wogonin were 0.49, 0.74 in tetrachloromethane–acetone (5:3, v/v), respectively.

The content of baicalin in ethyl acetate and acetone extracts was low, but other extracts showed higher content. The results indicated that the extraction of the polar constituent baicalin was related to solvent polarity. Less baicalin was extracted by a less polar solvent. The oil-like appearance and higher R_f value for the less polar extract confirmed that this was the case.

Using carbon dioxide alone in SFE, baicalin could not be detected; the bands of wogonin and baicalein were shown on TLC plates. Using 70% methanol or methanol as a modifier, the TLC patterns indicated the presence of baicalin and the concentration of the zone was proportional to the concentration of the modifier.

3.2.2. Identification by GC–MS

The identification of crude extract was by GC–MS. The GC–MS spectra for standard baicalein and wogonin are shown in Figs. 1 and 2, respectively. Retention time for wogonin was 20.59 min, the molecular ion peak was at *m/z* 284, *m/z* 269 was for the fragment $[M-CH_3]^+$. The retention time for baicalein was 20.20 min and its molecular ion peak was at *m/z* 270. As to baicalin, the retention time of baicalin was 20.15 min, but its molecular ion peak could not be observed at *m/z* 446.

In this study, all crude extracts showed the similar patterns of total ion chromatogram (Fig. 3). By comparison of MS fragments with library search software, wogonin was identified. But at the retention time 20.03 min, fragments of *m/z* 270 had a relatively low intensity compared to those of *m/z* 284, 269, 139 and 241. By comparison with the peak intensities of the library database, these peaks may be shown to represent the combinations of many constituents, such as baicalin, baicalein and oroxylin A. Therefore, optimal conditions for separation of the coexisting baicalein and baicalin by GC–MS requires further investigation.

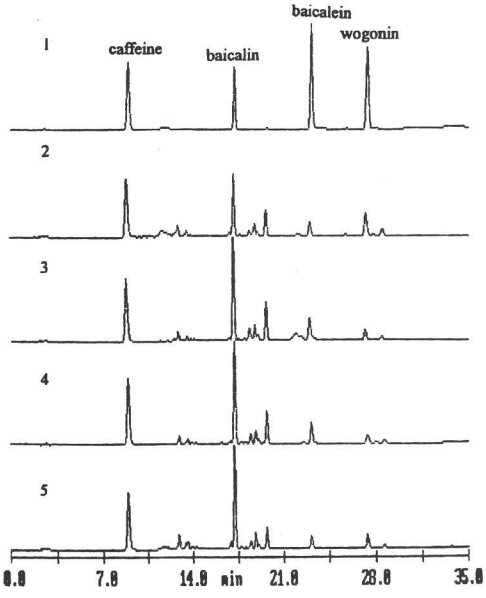

Fig. 4. HPLC chromatograms of a mixture of standard solution and various crude extracts: (1) standard solution of baicalin, baicalein and wogonin with internal standard caffeine, (2) SFE extract from supercritical CO_2 with MeOH–water (70:30) as a modifier, (3) SFE extract from supercritical CO_2 with MeOH as a modifier (4) extract from ultrasonic shaking with MeOH–water (70:30), (5) extract from ultrasonic shaking with MeOH.

Table 3
The content of baicalin, baicalein and wogonin extracted from Scutellariae Radix by SFE and conventional extraction methods ($n=3$)

Extraction mode		Content (mean±S.D.; mg/g), R.S.D. (%)		
		Baicalin	Baicalein	Wogonin
Percolation overnight	MeOH	22.9±0.69 (3.01)	6.6±0.33 (5.00)	2.8±0.07 (2.50)
Ultrasonic shaking	MeOH–water (70:30)	113.5±2.17 (1.91)	5.7±0.24 (4.21)	2.3±0.10 (4.35)
	MeOH	31.5±1.43 (4.54)	5.1±0.33 (6.47)	2.8±0.04 (1.43)
	EtOH	16.7±0.99 (5.93)	4.1±0.21 (5.12)	2.7±0.10 (3.70)
SFE	MeOH–water (70:30)	137.6±5.13 (3.73)	8.6±0.54 (6.28)	2.2±0.09 (4.09)
	MeOH	25.6±1.45 (5.66)	4.8±0.30 (6.25)	3.7±0.05 (1.35)

SFE conditions were as follows: supercritical CO_2-modifier (20:3), 50°C, 200 bar.

3.3. Assay by HPLC

The extraction yields using acetone and ethyl acetate with the conventional method were too low to perform quantitative analysis. The HPLC chromatograms of the crude extracts were similar. The chromatograms of the standard solution, crude extracts from 70% methanol or methanol as modifiers in SFE or as solvents in ultrasonic shaking method are shown in Fig. 4.

The retention times for the three standards and internal standard were 8.9, 17.2, 23.1 and 27.4 min for caffeine, baicalin, baicalein and wogonin, respectively. The calibration curves (correlation coefficients) for baicalin, baicalein, and wogonin were $y=0.05089x+0.06683$ ($r=0.9995$), $y=0.07375x-0.04091$ ($r=0.9987$) and $y=0.09058x+0.04099$ ($r=0.9994$), respectively. The contents of baicalin, baicalein and wogonin were calculated from the calibration curves and listed in Table 3.

The contents of baicalin, baicalein and wogonin in various crude extracts of Scutellariae Radix are listed in Table 4. With methanol as a solvent or a modifier in SFE, the yield of baicalin ranged from 14.3 to 16.4%, baicalein ranged from 2.5 to 4.1% and wogonin ranged from 1.4 to 2.4%. With ethanol as a solvent, the yields of the above constituents were 10.4, 2.5 and 1.7%, respectively. When 70% methanol was used, the yields of baicalin, baicalein and wogonin varied from 28.6 to 33.4%, 1.4 to 2.1%, 0.5% to 0.6%, respectively. When the same extraction method was used and the solvent was changed from methanol to 70% methanol, the increments of baicalin content were 1.8 and 2.1 times,

Table 4
The percentage of baicalin, baicalein and wogonin in various extracts (SFE conditions as in Table 3)

Extraction mode		Percentage (%)		
		Baicalin	Baicalein	Wogonin
Percolation overnight	MeOH	14.3	4.1	1.7
Ultrasonic shaking	MeOH–water (70:30)	28.6	1.4	0.6
	MeOH	15.7	2.5	1.4
	EtOH	10.4	2.5	1.7
SFE	MeOH–water (70:30)	33.4	2.1	0.5
	MeOH	16.4	3.1	2.4

respectively. These results indicated that with the addition of water, highly polar baicalin was more extractable, but showed no effect on the yield of less polar wogonin and baicalein.

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