

# 离子液体-微波辅助提取黄芩饮片中四种黄酮类成分

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**摘要:** 采用 HPLC 法建立黄芩饮片中黄芩苷、汉黄芩苷、黄芩素、汉黄芩素四种黄酮类化合物同时测定的方法; 并且优选离子液体-微波辅助提取 (IL-MAE) 黄芩饮片中四种黄酮类成分的最佳工艺。优选的色谱分离条件为: Agilent TC-C<sub>18</sub> (250 × 4.6 mm, 5.0 μm) 色谱柱, 流动相为 0.4% 磷酸水 (A) 和甲醇 (B), 梯度洗脱程序为 0 ~ 10 min, 40% ~ 50% B; 10 ~ 20 min, 50% B; 20 ~ 30 min, 50% ~ 60% B; 30 ~ 40 min, 60% ~ 80% B; 50 min, 80% B, 检测波长 280 nm。优选的最佳的黄芩饮片 IL-MAE 条件为: 以溴化-1-丁基-3-甲基咪唑盐为溶剂, 固-液比为 1: 100, 提取时间为 3 min, 微波功率为 264 W; 将该工艺所得四种成分的含量与采用中国药典 (2010 版) 所记载的“回流提取法”所得含量进行对比, IL-MAE 显著地提高了黄芩饮片中四种黄酮类成分的含量并且缩短了提取时间。研究表明, 所建立的 HPLC 分析方法灵敏、准确, 可用于黄芩饮片的质量控制; 所优选的黄芩饮片的 IL-MAE 简单, 高效, 快速, 无污染。

**关键词:** 黄芩饮片; 离子液体; 微波辅助提取; 高效液相色谱法; 黄酮

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## Ionic Liquid-based Microwave-Assisted Extraction of Four Flavonoids from *Scutellariae Radix* Slices

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**Abstract:** A rapid reversed-phase high performance liquid chromatography method was developed for the simultaneous determination of 4 flavonoids, namely baicalin, wogonoside, baicalein and wogonin. The chromatographic separation was performed on an Agilent TC-C<sub>18</sub> column (250 × 4.6 mm, 5.0 μm) with 0.4% phosphoric acid (A) and methanol (B) as mobile phases. The gradient elution program was as follows: 0-10 min, 40% -50% B; 10-20 min, 50% B; 20-30 min, 50% -60% B; 30-40 min, 60% -80% B; 40-50 min, 80% B. The detection wavelength was set at 280 nm. Under the developed HPLC method, the ionic liquid-based microwave-assisted extraction (IL-MAE) of 4 flavonoids from *Scutellariae Radix* slices was optimized. The optimal IL-MAE conditions were determined as follows: 1.0 M 1-butyl-3-Methylimidazolium bromide ([C<sub>4</sub>min]Br) employed as extractant, the ratio of solid to liquid was 1: 100, extracting for 3 min, the microwave power was 264 W. Compared with traditional solvent reflux extraction (SRE) recorded in Chinese pharmacopoeia (2010 edition), IL-MAE significantly improved the extraction yields of 4 flavonoids and shortened the extraction duration (from 3 h to 3 min). In conclusion, the developed HPLC method was sensitive, accurate, specific, and applicable for the quality control of *Scutellariae Radix* slices, and the optimized IL-MAE method was a simple, efficient, rapid, and green extraction technique.

**Key words:** *Scutellariae Radix* slices; ionic liquid; microwave-assisted extraction; high performance liquid chromatography; flavonoids

*Scutellariae Radix*, the dried roots of *Scutellaria ba-*

*icalensis* Georgi, has been widely used for centuries for the treatment of pyrexia, jaundice and diarrhea and is officially listed in Chinese pharmacopoeia. Modern researches indicated that flavonoids including baicalin, wogonoside, baicalein and wogonin were the major bioactive constituents of *Scutellariae Radix* with many

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pharmacological properties, such as anti-oxidation, anti-sepsis, antiviral, and anti-inflammatory. Hence, flavonoids were regarded as the indexes in the quality evaluation of *Scutellariae Radix* in medicinal industry.

An appropriate extraction process is important for ensuring medicinal value of *Scutellariae Radix*. The solvent reflux extraction (SRE)<sup>[1,2]</sup> is the most widely used extraction method. The main drawbacks of SRE were time-consuming and high energy consumption. Nowadays, ultrasonic-assisted extraction (UAE)<sup>[3]</sup> and microwave-assisted extraction (MAE)<sup>[4]</sup> have been employed in the extraction of *Scutellariae Radix*. Although these two methods greatly shortened the extraction duration and showed high efficiency, they do not satisfy the demand of green chemistry because of using harmful solvents during the extraction process.

Ionic liquid (IL), one kind of fused salt, is in liquid state at room temperature with a variety of advantages such as environmental friendly, high thermal stability, low vapor pressure, nonflammability and high solubility in water and other organic solvents<sup>[5]</sup>. Therefore, it is widely used in sample pretreatment fields, such as liquid-liquid extraction and membrane separation<sup>[6]</sup>. In recent years, many progresses in extraction and separation of natural products using ionic liquid had been made. Zhang *et al.*<sup>[7]</sup> reported the extraction of chlorogenic acid from *Lonicera japonica* Thunb. by ionic liquid-based ultrasonic-assisted (IL-UAE) with 1-butyl-3-methylimidazolium tetrafluoroborate ( $[C_4min] BF_4$ ) aqueous solution as solvent. Lu *et al.*<sup>[8]</sup> reported that phenolic alkaloids in *Nelumbo nucifera* Gaertn. were extracted by ionic liquid-based microwave-assisted (IL-MAE). Ionic liquid-based microwave-assisted extraction (IL-MAE) has received considerable attention as an alternative extraction technique. The technique has been demonstrated to be time- and energy-saving and highly efficient.

In the present study, a new HPLC method was developed for the simultaneous determination of four flavonoids from *Scutellariae Radix* slices. In conjunction with the IL-MAE, the HPLC method was applied for the determination of flavonoids, including baicalin, wogonoside, baicalein and wogonin in *Scutellariae Radix*. The

results were compared with that of traditional solvent reflux extraction (SRE).

## Material and Methods

### Material

Baicalin, wogonoside, baicalein and wogonin (purity > 99%) were purchased from Yuanye Biotechnology Co. Ltd. (Shanghai, China); 1-butyl-3-methylimidazolium chloride ( $[C_4min] Cl$ , 99.0%), 1-butyl-3-methylimidazolium bromide ( $[C_4min] Br$ , 99.0%), 1-butyl-3-methylimidazolium tetrafluoroborate ( $[C_4min] BF_4$ , 99.0%) were purchased from Shanghai Chengjie Chemical Co. (Shanghai, China); HPLC-grade methanol was obtained from Tianjin Shield Specialty Chemical Ltd. Co. (Tianjin, China); Other reagents were at least of analytical grade and water was prepared by automatic double-purified water distilling apparatus (Shanghai Yarong Biochemical Factory, China).

*Scutellariae Radix* slices (SRS) were purchased from Dongfang drug store (Jinan, China). The species was identified by Professor Fengqin Zhou (Shandong University of Traditional Chinese Medicine, Jinan, China).

### Instruments

An Agilent 1200 HPLC system (Agilent Co., USA) was equipped with a G1311A quaternary pump, a G1314B VWD detector and a G1329B autosampler. IL-MAE was performed on a MM823LA6-NS Midea microwave oven (Midea group Co. Ltd., China).

### Extraction of flavonoids from SRS

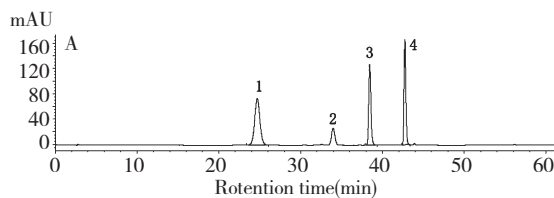
Dried SRS were pulverized into fine powder, sieved through a No. 60 mesh and subjected to SRE and IL-MAE. (1) SRE: According to Chinese Pharmacopoeia (2010 edition), the SRS powder (0.1 g) was accurately weighed and extracted with 20 mL of 70% ethanol for 3 h; (2) IL-MAE: according to the methods of Ma<sup>[9]</sup>, 0.1 g of SRS sample powder was accurately weighed and extracted with 20 mL of ionic liquid solution. Previous study had shown that the category of anion greatly affected water miscibility of IL. Hence, the most commonly employed 1-butyl-3-methylimidazolium with various hydrophilic anions ( $BF_4^-$ ,  $Br^-$ ,  $Cl^-$ ) were tested in this work. The additional extraction conditions, including IL concentration, solid-liquid ratio, microwave power, and

extraction time were investigated in this paper.

The extracts were cooled to room temperature, compensated the lost weight and then filtered. 1.0 mL of extracts were accurately measured and put in 10 mL brown volumetric flask, and topped up to the volume with extraction solvent, respectively. All the final extracts were filtered through a 0.45  $\mu\text{m}$  membrane before HPLC analysis.

### Preparation of standard solutions

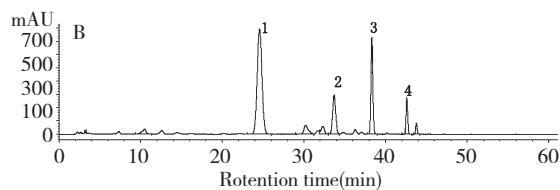
Standard stock solutions of 4 flavonoids were prepared by respectively dissolving them in ethanol. Then, proper amounts of 4 standard stock solutions were accurately measured and put in 50 mL brown volumetric flask and topped up to the volume with 70% ethanol. The final concentrations of 4 reference compounds were baicalin (46.4  $\mu\text{g}/\text{mL}$ ), wogonoside (9.92  $\mu\text{g}/\text{mL}$ ), baicalein (17.2  $\mu\text{g}/\text{mL}$ ) and wogonin (5.76  $\mu\text{g}/\text{mL}$ ). The



standard solution was stored in dark place at 4 °C.

### HPLC analysis

An Agilent TC-C<sub>18</sub> column (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ ) was used for chromatographic separation. The mobile phases were: 0.4% phosphoric acid (A) and methanol (B). The gradient elution program was: 0-10 min, 40% -50% B; 10-20 min, 50% B; 20-30 min, 50% -60% B; 30-40 min, 60% -80% B; 40-50 min, 80% B; the flow rate was 1.0 mL/min; the detection wavelength was set at 280 nm. Under these conditions, all the analytes were separated in less than 45 min. All chromatographic measurements were operated at room temperature. Quantification of the analytes was based on the peak area using the external calibration method. The chromatograms of standard solution and SRS were shown in Fig. 1.



Peaks 1-baicalin, 2-wogonoside, 3-baicalein, 4-wogonin

Fig. 1 Typical HPLC chromatograms of mixed standards solution (A) and SRS sample (B)

### Validation of the HPLC method

*Linearity, limits of quantification (LOQ) and detection (LOD)*

Linearity of the curves was established by injecting standard solutions with six different volumes (1, 5, 10, 15, 20, 30  $\mu\text{L}$ ). The calibration curves were established by plotting the peak area (Y) versus amount (X). Linear regression was used to calculate the coefficient of determination ( $R^2$ ).

The standard solution was diluted with 70% ethanol to a series of appropriate concentrations for the determination of limits of quantification (LOQ) and detection (LOD). LOD and LOQ under the chromatographic conditions were separately determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively.

*Precision, repeatability, accuracy and stability*

The precision were evaluated by relative standard deviation (RSD). The accuracy were investigated by injecting

6 repeated of the same sample solutions on the same day (intra-day) and over three consecutive days (inter-day), respectively.

Repeatability was performed by weighing 6 pieces of SRS powder (0.1 g) independently, and the samples were extracted and prepared according to the IL-MAE above.

Recovery studies were carried out by adding accurate amounts of the 4 standards to approximate 0.1 g of SRS powder, and then the samples were extracted and analyzed according to the method (IL-MAE) described above. The average percentage recoveries were evaluated by calculating the ratios of detected amount versus added amount.

Stability was assessed by detecting the same sample solution at 6 different times within 48 h at room temperature.

### Optimization of IL-MAE conditions

The SRS sample(0.1 g) was placed into a round-bottom flask(100 mL) and 20 mL 1-butyl-3-methylimidazolium with various hydrophilic anions either (i)  $\text{BF}_4^-$ , (ii)  $\text{Br}^-$  or (iii)  $\text{Cl}^-$  solvent was added, and the concentration of  $[\text{C}_4\text{MIN}]\text{Br}$  from 0.50 to 2.00 M, the solid-liquid ratios (1:5, 1:10, 1:20, 1:50, 1:100, 1:200, and 1:400). The power was set at 136,264,440,616 or 800 W. The suspensions were irradiated with microwave as follows:30-720 s. It was allowed to cool for about 15 min to room temperature, compensated the lost weight and then filtered. 1.0 mL of extracts was transferred to a 10 mL volumetric flask(10 mL) and made up to the mark using the appropriate solvent. The mixture was filtered using nylon syringe filter(0.45  $\mu\text{m}$ ) before HPLC

analysis.

## Results and Discussion

### Validation of the HPLC method

*Linearity, limits of quantification (LOQ) and detection (LOD)*

Linear calibration curves were obtained by plotting the peak area against the amount of the respective standard and were found to be linear over the range(Table 1). All the analytes showed good linearity with correlation coefficient( $R^2$ ) ranging from 0.9996 to 1.0000 for the 4 standards. The range of LOD for 4 compounds was from 0.06 to 0.13  $\mu\text{g}/\text{mL}$ , and the LOQ was from 0.21 to 0.42  $\mu\text{g}/\text{mL}$ (Table 1).

**Table 1 Calibration equations, LOD and LOQ for the 4 compounds**

Compound	Calibration equation $Y = a + bX^a$	Linear range ( $\mu\text{g}$ )	Correlation coefficient ( $R^2$ )	LOD <sup>b</sup> ( $\mu\text{g}/\text{mL}$ )	LOQ <sup>b</sup> ( $\mu\text{g}/\text{mL}$ )
Baicalin	$Y = 3590.67X - 15.86$	0.0464 ~ 1.3920	1.0000	0.13	0.42
Wogonoside	$Y = 4015.18X - 5.96$	0.00992 ~ 0.2976	0.9996	0.11	0.38
Baicalein	$Y = 6922.45X + 13.51$	0.0172 ~ 0.5160	0.9999	0.06	0.21
Wogonin	$Y = 6955.36X - 13.63$	0.00576 ~ 0.1728	0.9997	0.07	0.22

<sup>a</sup>Y and X were the peak areas and amount( $\mu\text{g}$ ) of the compound, respectively.

<sup>b</sup>The LOD was defined as the concentration for which the signal-to-noise ratio was 3; the LOQ was defined as the concentration for which the signal-to-noise ratio was 10.

### Precision, repeatability, accuracy and stability

As shown in Table 2, the intra-day and inter-day variations were less than 3%; the average content of 4 compounds was baicalin(90.01 mg/g), wogonoside(20.19 mg/g), baicalein(16.69 mg/g), wogonin(5.36 mg/g) with RSD less than 3% respectively; the percentage recoveries were in the range of 97.9% -102.8% with

RSD less than 3%; Stability was assessed by detecting the same sample solution at 6 different times within 48 h at room temperature. The RSD values of the peak areas were all lower than 3%, indicating that it was accurate to analyze the samples within 2 days by this method.

**Table 2 Precision, recoveries and stabilities of the 4 compounds( $n = 6$ )**

Compound	Precision		Repeatability		Recovery		Stability RSD(%)
	Intra-day RSD(%)	Inter-day RSD(%)	Content (mg/g)	RSD	Recovery mean(%)	RSD (%)	
Baicalin	0.65	1.22	90.01	0.96	102.8	2.34	1.13
Wogonoside	0.98	1.83	20.19	1.48	99.84	2.56	1.56
Baicalein	1.01	1.98	16.69	1.96	102.67	1.78	2.17
Wogonin	1.13	2.26	5.36	2.37	97.9	1.26	2.45

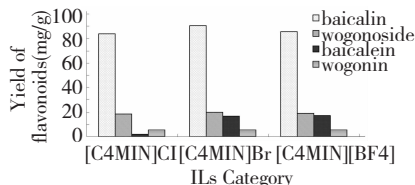
### Optimization of IL-MAE conditions

#### Selection of IL

As shown in Fig. 2 the extraction yields of baicalin(90.

6 mg/g) and baicalein(16.6 mg/g) were higher by IL of  $[\text{C}_4\text{min}]\text{Br}$  than those extracted by the other two ILs, and these 3 ILs have exhibited the similar effects

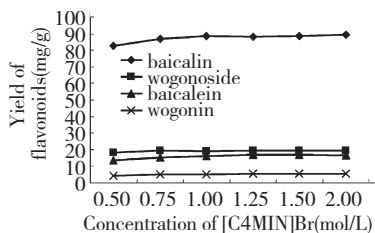
on the extraction yields of wogonoside and wogonin. Hence, IL of [C<sub>4</sub>min]Br was selected for the extraction of flavonoids in SRS.



**Fig. 2 Comparison of the extraction yield of flavonoids by 3 ILs and the effect of ILs category on the extraction efficiency of flavonoids of SRS**

#### [C<sub>4</sub>min]Br concentration

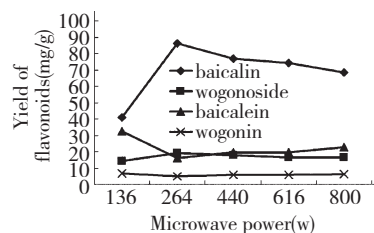
The effect of [C<sub>4</sub>min]Br concentration on the extraction yields of flavonoids from SRS was illustrated in Fig. 3. The increase of [C<sub>4</sub>min]Br concentration ranging between 0 and 1.00 mol/L led to a small increase of the extraction efficiency, whereas the increase of [C<sub>4</sub>min]Br concentration ranging between 1.0 and 2.0 mol/L showed no further variation of the extraction yields. This suggested that the 1.00 mol/L [C<sub>4</sub>min]Br was the appropriate concentration in this study.



**Fig. 3 Effect of [C<sub>4</sub>min]Br concentration on the extraction efficiency of flavonoids of SRS**

#### Microwave power

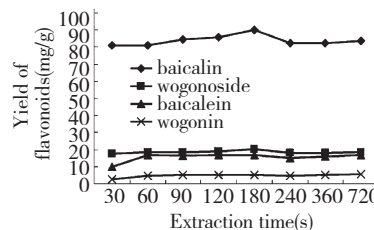
The level of microwave power can affect the extraction yields of active constituents by influencing the temperature in extraction system. The results were shown in Fig. 4. The yields of baicalin and wogonoside increased with the increasing of the microwave power from 136 W to 264 W, and in this range, the yield of baicalein decreased slightly. There was little effect on the obtained yield of wogonin no matter what microwave power was used. Therefore, the microwave power of 264 W was selected as the extraction power.



**Fig. 4 Effect of microwave power on the extraction efficiency of flavonoids of SRS**

#### Extraction duration

To some degree, the extraction duration is in direct proportion to the extraction efficiency under the given microwave power. Fig. 5 showed the changes of yields of 4 flavonoids with the increasing of extraction duration up to 180 s. When the extraction duration was longer than 180 s, the yields of 4 flavonoids decreased. The main reason may be that the excessive extraction would result in decomposition of flavonoids in SRS. Hence, 180 s was thought to be the appropriate extraction duration.



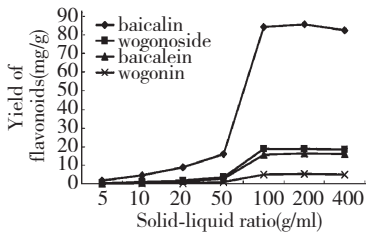
**Fig. 5 Effect of extraction duration on the extraction efficiency of flavonoids of SRS**

#### Solid to liquid ratio

A series of extractions with different solid-liquid ratios (1:5, 1:10, 1:20, 1:50, 1:100, 1:200 and 1:400) were performed for evaluating the effect of solvent volume on the yields of 4 flavonoids. As shown in Fig. 6, the yields of 4 flavonoids increased with the increasing of solvent volume before the solid to liquid ratio reached 1:100; After that the yields of the 4 flavonoids were not significantly increased with the further increasing of the solvent volume. Therefore, a solid-liquid ratio of 1:100 (g/mL) was used in the IL-MAE.

Hence, according to the above experiments, the optimal extraction conditions were as follows: the IL solvent was 1.0 M 1-butyl-3-Methylimidazolium bromide ([C<sub>4</sub>min]Br), the ratio of solid/liquid (g/mL) was 1:100, the

extraction duration was 180 s, and microwave power was 264 W.



**Fig. 6 Effect of solid/liquid ratio on the extraction efficiency of flavonoids of SRS**

### Comparison of IL-MAE and SRE

The yields of 4 flavonoids obtained by IL-MAE were compared with those obtained by SRE recorded in Chinese Pharmacopoeia (2010 edition). As shown in Table 3, comparing with the traditional SRE, the IL-MAE significantly shortened the extraction duration (from 3 h to 3 min) and improved the yields. For instance, the yields of baicalin, wogonoside, baicalein and wogonin were enhanced by 8.3%, 11.8%, 5.0% and 2.8%, respectively.

**Table 3 Comparison of flavonoids yields obtained by IL-MAE and SRE ( $n = 3$ )**

Compound	IL-MAE (mg/g)	SRE (mg/g)
Baicalin	90.01	83.13
Wogonoside	20.19	18.07
Baicalein	16.69	15.90
Wogonin	5.36	5.22

### Conclusion

In this study, IL-MAE conditions were optimized for the extraction of 4 flavonoids (baicalin, wogonoside, baicalein and wogonin) in SRS. The optimal IL-MAE conditions were as follows: the IL solvent was 1.0 M 1-butyl-3-Methylimidazolium bromide ( $[C_4\text{min}]Br$ ), the ratio

of solid/liquid (g/mL) was 1:100, the extraction duration was 180 s, and microwave power was 264 W. In addition, a HPLC method was developed and validated for the simultaneous determination of 4 flavonoids in SRS. The established HPLC method was found to be simple, stable and accurate, and it can be used for the simultaneous determination of the 4 flavonoids in SRS.

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