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IONIC LIQUID-MICROWAVE-BASED EXTRACTION OF AMENTOFLAVONE FROM *SELAGINELLA TRACHYPHYLLA*

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Abstract

Selaginella trachyphylla is widely used as folk medicine in south China. Amentoflavone (AME) is the main active ingredient of *S. trachyphylla* because of its significantly physiological effects. Therefore, a rapid, effective and environmental-friendly extraction approach of AME from *S. trachyphylla* is an urgent need to implement. In this study, ionic liquid (IL) as a green solvent combined with microwave-assisted extraction (IL-MAE) for AME from *S. trachyphylla* was proposed. After screening different ILs, the important extraction parameters, including IL concentration, liquid-solid ratio, extraction temperature, irradiation time and extraction power, were investigated in detail. Results indicated that n-butylpyridinium tetrafluoroborate ([Bpy]BF4) exhibited the highest extraction capacity among different ILs and was chosen as the optimal extractant. Moreover, the best extraction parameters were 0.8 mmol/l of [Bpy]BF_4, 55°C of irradiation temperature, 9.6 : 1 ml/g of liquid-solid ratio, 400 W of microwave power and 40 min of irradiation time, which showed the AME yields from *S. trachyphylla* was 1.63 mg/g. Compared to extraction by percolation and maceration, IL-MAE not only shortened extraction time (only 40 min) but also reduced the solvent consumption with sample preparation, which indicated AME of IL-MAE from *S. trachyphylla* is an efficient, fast and environment-friendly method.

Introduction

Selaginella trachyphylla Spring, a dry herb, being distributed in the southwest of China, including Guizhou, Guangdong, Guangxi, Hunan and Hainan provinces, has a very long history of uses in traditional Chinese medicines (Schmidt *et al.* 2019). In addition, *S. trachyphylla* has a variety of pharmacological effects such as anti-oxidation, anti-allergic, disease-resistant, anti-inflammatory and immune regulation (Li *et al.* 2017). The main active constituents of *S. trachyphylla* include biflavonoids, organic acids and triterpenoids (Sui *et al.* 2016).

Amentoflavone (AME) (Fig. 1), known as a biflavonoid, is the main active ingredient of *S. trachyphylla* (Kim *et al.* 2020). AME has wide range of bioactivities including anticancer, free radicals scavenging, antibacterial, anti-inflammatory, anti-viral, increasing white blood cells, protecting liver and gallbladder, lowering blood pressure, blood fat, blood sugar and exciting central nervous system (Wang *et al.* 2015).

Ionic liquids (ILs) as green solvents are ionic compounds composed entirely of organic cations and inorganic or organic anions at room temperature. Compared to conventional organic solvents, the most remarkable feature of ILs is its "designability". By changing the composition of different anions and cations, it is possible to synthesize ionic liquids of different structure and regulate their physicochemical properties over a wide range (Xu *et al.* 2019). ILs also have unique and incomparable advantages: such as almost negligible vapor pressure, non-volatility, non-flammablity, good chemical stability, non-toxic and non-polluting (Qian *et al.* 2016). At present, the widespread use of IL coupled with microwave-assisted extraction (MAE) of natural medicines has been reported in recent years (Krishnan *et al.* 2020).

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Ahmad *et al.* (2017) reported the extraction of polyphenolic compounds from *Peperomia pellucida* (L.) by MAE method using IL solution as solvent. The results showed that the proposed method can extract polyphenolic compounds more effectively compared to traditional extraction methods. The efficient extraction of phenolic alkaloids from lotus root using the MAE method based on 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF4]) was reported by Arrahman *et al.* (2020). Compared to the traditional MAE and HRE method, the IL-based MAE method showed higher extraction efficiency (from 0.9 to 43.7%) and shorter extraction time (from 120 to 2 min), which indicated that IL-MAE was an efficient, fast and simple sample pretreatment technique.

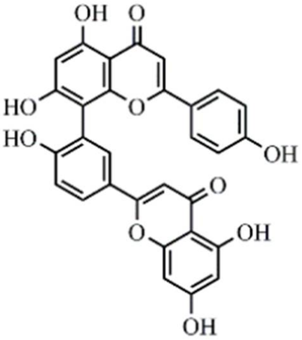


Fig. 1. Chemical structures of amentoflavone.

On the basis of the above background, different ILs were used and compared for the extraction of AME from *S. trachyphylla* in this work. Then important extraction conditions (IL concentration, microwave power, liquid-solid ratio, radiative temperature and extraction time) were evaluated on the extraction efficiency of target ingredients. The recycling of ILs was also studied. Moreover, the established IL-MAE approach was compared with several traditional extraction methods, including ethanol-MAE, maceration and percolation, which is expected to provide new meaningful reference for amentoflavone from *S. trachyphylla.*

Materials and Methods

Selaginella trachyphylla was collected from Simian Mountain (Zunyi, China). Amentoflavone (AME) standard (98% purity) was purchased from Dida Technology Co., Ltd. (Guiyang, China). All ILs were purchased from Chengjie Chemical Co. Ltd. (Shanghai, China). Chromatographic grade and analytical grade chemicals were purchased from Hengxin Chemical company (Zunyi China).

IL-MAE method was carried out in a microwave extraction system (Xianghu, China) with irradiation frequency of 250 MHz. In short, the operation process was as follows: 10.0 g of *S. trachyphylla* sample and different concentration ILs were placed in a round bottom flask. After the samples were extracted, the mixture was filtrated, concentrated and dried. Finally, *S. trachyphylla* extract was obtained which was dissolved for HPLC analysis.

The liquid phase chromatographic system (Agilent, USA) consisted of G1329B autosampler, G1311C pump, C_{18} column (250 × 4.6 mm, 5 μm, Phenomenex, USA) and G1314F DAD detector. For HPLC analysis, the mobile phase composed of 0.1% formic acid (A) and acetonitrile (B); the gradient elution procedure was as follows: 0 - 8 min, 42 - 50% B; 9 - 18 min, 50 - 56% B; 19 - 40 min, 57 - 70% B. Other optimized chromatographic conditions were as follows: flow rate was 0.8

ml/min, the injection volume was 10 µl and the detection wavelength was 330 nm. AME of the extract was quantified by a DAD detector following above HPLC separation condition. The chromatographic peak of the AME was verified by comparing the retention time with the reference standard of AME (Fig. 2). Based on the external standard method, quantification of the target molecules was carried out and their corresponding calibration curves were as follows: CA: Y = 27.95X -35.257 (R = 0.9997).

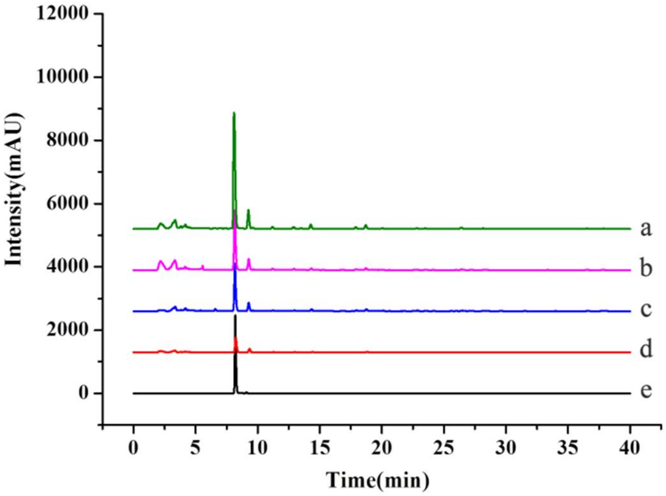
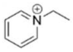
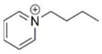
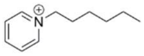
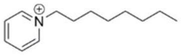
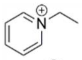
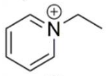
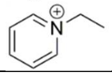


Fig. 2. The HPLC chromatographic profile of *S. trachyphylla.* (a) MAE-IL, (b) MAE-ethanol, (c) percolation extraction, (d) maceration extraction, (e) amentoflavone standard.

Table 1. Chemical structures of ionic liquids.

Ionic liquids	Cations	Anions
[Epy]BF_4 (N-ethylpyridinium terafluorobroate)		BF_4^-
[Bpy]BF_4 (N-butylpyridinium terafluoroborate)		BF_4^-
[Hpy]BF_4 (N-hexylpyridinium terafluoroborate)		BF_4^-
[Opy] BF_4 (N-octylpyridinium terafluoroborate)		BF_4^-
[Epy]HSO_4 (N-octylpyridinium hydrogen sulfate)		HSO_4^-
[Epy]Br (N-ethylpyridinium bromide)		Br^-
[Epy]PF_6 (N-ethylpyridinium hexafluorophosphate)		PF_6^-

According to the results of single factor test, a Box-Behnken central combination design was employed to optimize the IL-MAE process. The microwave temperature (X1), liquid/solid ratio (X2) and IL concentration (X3) were taken as the independent variables (Table 2). AME content of the extract was taken as the response value Y. The experiments including a total of 17 runs were provided by the factorial design (Table 3). Next each run was carried out 3 times, the value of AME yields was the mean values. The relationships between the three determined variables and the responses of AME content were established by the quadratic equation:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3$$

where, Y is the determined response of AME (mg/g); β_0, β_i, β_{ii}, and β_{ij} represent the coefficients of regression; and X1, X2 and X3 represent the three variables.

Table 2. Levels and codes of factors chosen for experiment.

	Factors		
Level	Extraction temperature (°C)	IL concentration (mmol/l)	Soild-liquid ratio (g/ml)
-1	50	0.6	1 : 8
0	60	0.8	1 : 10
1	70	1.0	1 : 12

Table 3. The results of experiment design.

RUN	X_1	X_2	X_3	AME
	Extraction temp. /°C	Solid-liquid ratio /g/ml	ILs conc. /mmol/l	mg/g
1	70	10	0.6	1.14
2	70	8	0.8	1.18
3	70	12	0.8	0.96
4	60	10	0.8	1.57
5	60	10	0.8	1.57
6	60	12	0.6	1.26
7	50	1	1.0	1.41
8	50	8	0.8	1.38
9	60	10	0.8	1.58
10	60	12	1.0	1.22
11	60	10	0.8	1.58
12	60	8	1.0	1.31
13	60	8	0.6	1.37
14	50	10	0.6	1.43
15	70	10	1.0	1.02
16	60	10	0.8	1.60
17	50	12	0.8	1.33

Different extraction methods on the yields of AME were compared in detail, inclulding MAE-ethanol, maceration extraction (ME) and percolation extraction (PE). On the basis of the extraction methods proposed by Jiang *et al.* (2018), the experimental operation was carried out according to the best conditions of each method. In brief, 10.0 g of herb powders was extracted with 95% ethanol, and then the extraction liquid was filtrated, concentrated (5000 rpm, 5°C) and dried in the oven. The extracts of several methods were dissolved with methanol and filtered for further HPLC analysis.

After the optimization of IL-MAE, the recovery of AME and IL from *S. trachyphylla* extract was explored as follows: The *S. trachyphylla* extract was dissolved in hot water (1 : 20, g : V).

Then IL and AME were extracted using ethyl acetate (Vethyl acetate : Vextract = 3 : 1, V/V). The aqueous layer, containing IL, was reused in the microwave extraction. As mentioned earlier, IL and AME were extracted again with ethyl acetate. Based on the above operation method, the stability of [Bpy]BF_4 was evaluated in 6 different cycles. The recovery of AME was measured by the HPLC-DAD detection method.

Results and Discussion

Several ethanol solutions of ILs were investigated to infer their ability for extraction of AME from *S. trachyphylla*. For this initial evaluation, the same operational conditions were maintained in all experiments, namely solid-liquid ratio of 1 : 10 g/ml, microwave power of 400 W and extraction time of 40 min at 60°C. The physical and chemical properties of different ILs are influenced by their structures combined by different anions and cations. Thus, the extraction efficiencies of target compounds also vary between different ILs (Boli *et al.* 2020). Fig. 3A shows that n-butypyridinium ($[Bpy]^+$) ILs with BF_4^- obviously affected the extraction yields of AME ($p <$ 0.05), which may be attributed to the acidity of the [Epy]BF_4 ethanol solution, which is higher than that of other anions. For cations, four ILs including [Epy]BF_4, [Bpy]BF_4, [Hpy]BF_4 and [Opy][BF_4] were evaluated. As shown in Fig. 3B, the extraction efficiency ($p < 0.05$) was significantly improved with increasing the alkyl chain length from ethyl to butyl. The results might be related to the improvement of lipotropism and hydrophobic effects, and hydrogen bonding of [Bpy]BF_4 with AME (Li *et al.* 2019). However, when the carbon chain length exceeded C-4, the AME yields decreased significantly. It might be due to hydrophobic effect gradually decreased and steric effect between IL and AME gradually increased by inch. As the above results showed, [Bpy]BF_4 was the best IL and used for subsequent study.

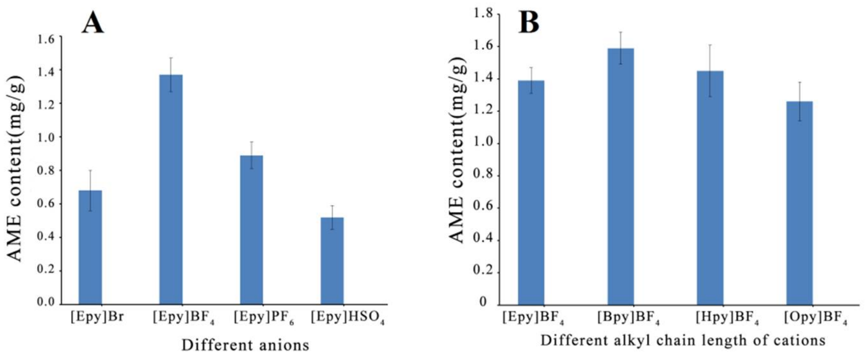


Fig. 3. Effect of the (A) IL anions and (B) IL cations for amentoflavone from *S. trachyphylla.*

The effects of IL concentrations of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mmol/l on AME yields from *S. trachyphylla* were investigated. Other extraction conditions for MAE were as follows: extraction power of 400 W, solid-liquid ratio of 1 : 10 g/ml, extraction time of 40 min, radiative temperature of 60°C. It could be seen from Fig. 4A that with the increase of IL concentration from 0.2 to 0.8 mmol/l, the yield of AME also increased from 1.11 to 1.58 mg/g. It can be attributed to the improvement of the absorption and mass transfer ability of [Bpy]BF_4 at high concentrations (Mai *et al.* 2020). Conversely, the extraction efficiency decreased gradually when [Bpy]BF_4 concentration increased from 0.8 to 1.2 mmol/l because of the increase of solution viscosity and the decline of mass transfer ability. Based on the results, [Bpy]BF_4 solution of 0.6, 0.8 and 1.0 mmol/l were evaluated for the further RSM analysis.

The liquid-solid ratio is also an important parameter that affects the extraction rate of the active ingredients of natural products (Seitkalieva *et al.* 2018). In order to evaluate the effects of solvent to solid ratio for the extraction of the target analytes, different liquid-solid ratios (6 : 1, 8 : 1, 10 : 1, 12 : 1, 14 : 1 and 16 : 1 ml/g) were investigated and analyzed in detail. As could be seen from the results in Fig. 4B, when the liquid-solid ratio changed from 6 : 1 ~ 10 : 1 ml/g, the extraction efficiency of AME increased notably. Nevertheless, as the solvent to solid ratio was above 10 : 1 ml/g, the yields of AME gradually decreased. This may be a suitable solvent to solid ratio sufficient for extraction, and if the amount of solvent is too large, a large amount of impurities will be generated, which is disadvantageous to MAE. Thus, the solvent to solid ratio of 8 : 1 to 12 : 1 ml/g were selected to optimize by RSM for sample preparation.

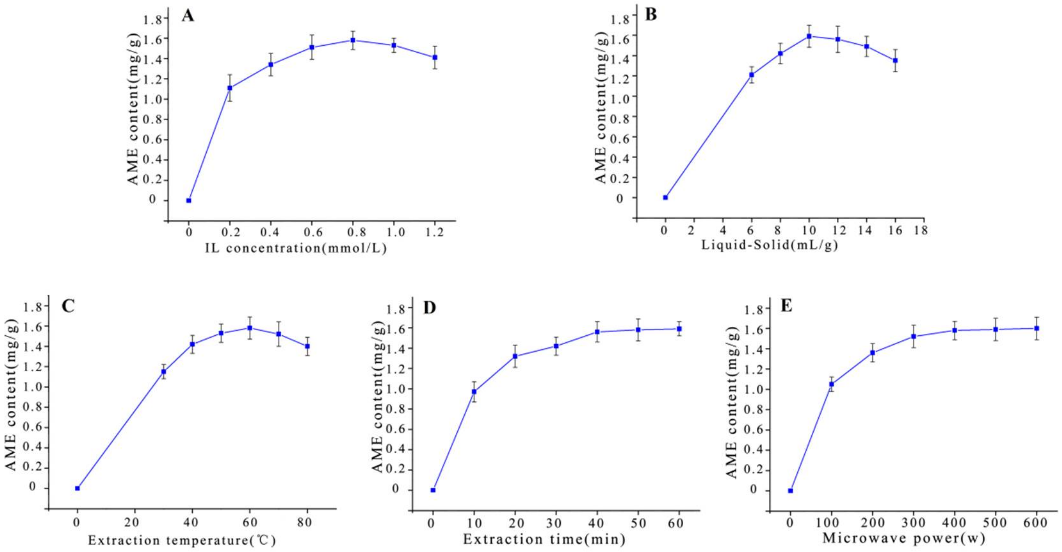


Fig. 4. Effects of extraction parameters of Amentoflavone yields of *S. trachyphylla*. (A) IL concentration; (B) Liquid-Soild ratio; (C) Extraction temperature; (D) Radiation time; (E)Microwave power.

The influences of the extraction temperature (30, 40, 50, 60, 70 and 80°C) on AME yields from *S. trachyphylla* were explored. As shown in Fig. 4C, when the temperature increased from 30 to 60°C, the results showed that AME yield changed from 1.15 to 1.58 mg/g. Conversely, the extraction yield decreased gradually when the temperature was above 60°C. Heating during the extraction can strengthen the molecular movement, soften the cell tissue, improve the targets solubility, and accelerate the solvent diffusion, thereby increasing the extraction rate of the target molecues (Mangang *et al.* 2020). However, if the temperature is too high, the extracted active ingredients may be oxidized or decomposed, resulting in a decrease of the target yields. Consequently, three levels of the extraction temperature including 50, 60 and 70°C, were studied for the further RSM optimization.

The yields for AME reached the maximum values at 40 min after extraction (Fig. 4D). This is because the extraction time is required for microwave irradiation to induce cell wall disruption and target component release into the IL solution. When the radiation time was above 40 min, the extraction yields for the analyses decreased slightly. In addition, extending the extraction time would require a lot of energy consumption and increase the cost of extraction. Hence, 40 min was selected as the optimal radiation duration for subsequent experiments.

After extraction, the output of AME reached its maximum value at 400 W (Fig. 4E). With the increase of microwave power, the internal pressure of the cell exceeds the expansion capacity of the cell wall, causing the cell wall to rupture, and the active ingredients in the cell diffuses into the extractant (Dupuy *et al.* 2011). When the radiation power was prolonged to 400 - 600 W, the extraction yields of AME were basically unchanged. Therefore, 400 W was choosed as the optimal radiation power for the next stage experiment.

According to the single factor experiment of IL-MAE, some important parameters (IL concentration, liquid-solid ratio and irradiation temperature) that influence observably the entire extraction process were explored in detail. As shown in Table 4, the proposed models were adequate based on the significant models, non-significant "lack of fit" and satisfactory coefficients of determination (Rostamifasih *et al.* 2019). The model for AME with p-value of < 0.0001 and F values of 163.18 showed that the model was significant. The F-values of "lack of fit" were found to be 4.85 for target molecules, which indicated that the "lack of fit" was not significant. The higher values of R^2 of 0.9953 for AME, indicated that the model appeared to reasonably represent 99.53% of the experimental data variations. The following second-order polynomial equation were expressed as follows: $Y = -12.42 + 0.26\,X1 + 0.91\,X2 + 5.57\,X3 – 2.05 \times 10^{-3}X1^2 – 0.04\,X2^2 – 3.13\,X3^2 – 2.08 \times 10^{-3}X1X2 – 0.01\,X1X3 + 0.01\,X2X3$.

Table 4. ANOVA for the fitted secondary order curve for AME content.

Source	Sum of square	df	F value	p value	R^2	R^2(Adj)	Significance
Model	0.64	9	163.18	< 0.0001	0.9953	0.9892	Significant
X_1X_2	6.94×10^{-3}	1	16.04	0.0052			
X_1X_3	3.09×10^{-3}	1	7.13	0.0320			
X_2X_3	1×10^{-4}	1	0.23	0.6453			
X_1^2	0.18	1	409.4	< 0.0001			
X_2^2	0.12	1	266.24	< 0.0001			
X_3^2	0.066	1	152.4	< 0.0001			
Residual	3.03×10^{-3}	7					
Lack of fit	2.38×10^{-3}	3	4.85				Not significant
Pure error	6.53×10^{-4}	4					

Next, the interaction effect was explored between two independent variables through 3D RSM, which helped to analyze the changes of AME yields. As described in Fig. 5, all RSM figures were upward convex shaped with a maximum value of AME yields, which indicated the rationality of the prediction of 3D models. After the software optimization, the optimal process parameters for the achieved point prediction were 9.64 ml/g of liquid-solid ratio, 0.81 mmol/l of IL concentration and 54.89°C of extraction temperature, giving a predicted AME yield of 1.6258 mg/g. For easy extraction of AME, the calibration tests were suitably modified at the optimum factors of 0.80 mmol/l IL concentration, 9.6 ml/g ratio of liquid-solid and 55°C extraction temperature, which obtained AME yield of 1.63 mg/g. Therefore, the optimized extraction method may be used for sufficient extraction of AME from *S. trachyphylla*.

Fig. 6 shows the recovered results of $[Bpy]BF_4$ on the extraction of AME from *S. trachyphylla* under the optimized condition. It was found that the extraction yields of AME revealed a slight decrease with the increase of the reuse cycles. This illustrated that the extraction yields of the following 5 cycles were all greater than 90% of the satisfactory yields, which proved that [Bpy]BF_4 was the ideal solvent in MAE of the biflavonoids and could be recycled and applied for the extraction of AME more than five times.

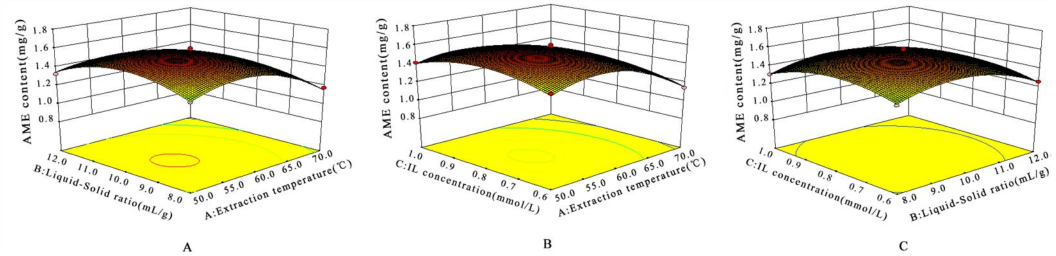


Fig. 5. Response surface plots showing interaction effects of liquid-solid ratio, extraction temperature and IL concentration on AME content.

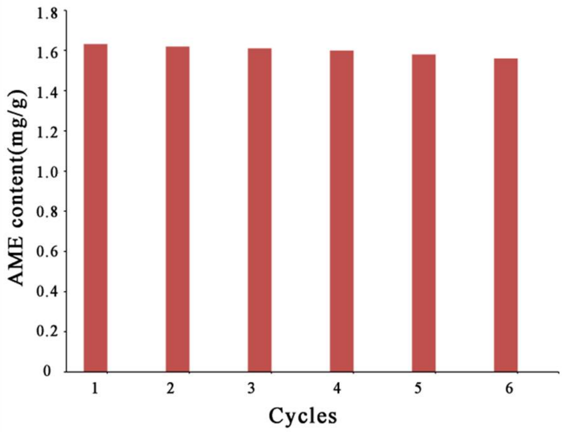


Fig. 6. Performance of recovered [Bpy]BF_4 on the yields of AME.

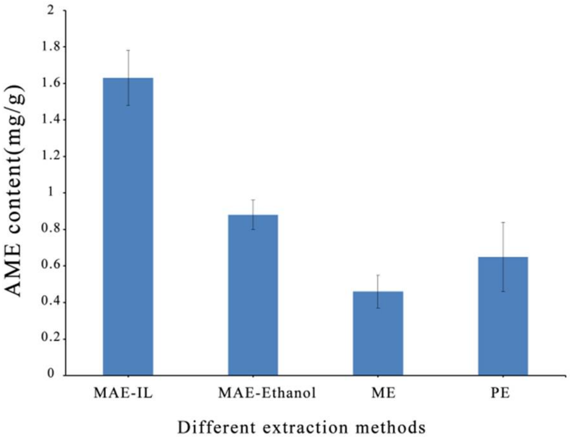


Fig. 7. Effect of four extraction methods for amentoflavone content from *S. trachyphylla*.

The characteristics of the proposed method have been compared in Fig. 7 with other methods (IL-MAE, ethanol-MAE, ME and PE) that had been used for the extraction of AME. It was clear that ME and PE required long time to extract the target compound and consumed a lot of solvent, and AME yields of the extracts was very low. However, IL-MAE not only increased the AME content about three to four times (Fig. 7), but also distinctly shortened the extraction time and reduced the solvent consumption. Additionally, extraction time of IL-MAE and MAE-ethanol was basically the same, and the AME content of IL-MAE was increased nearly two times compared to MAE-ethanol. Finally, these results revealed that the proposed method was environmental friendly, inexpensive, quick and effective technique and was suitable for the extraction of AME from *S. trachyphylla*.

In this study, an IL-MAE method was developed and applied to extract amentoflavone from *S. trachyphylla*. The effects of IL-MAE for AME yields from the herb was studied in detail. After screening seven ILs, $[Bpy]BF_4$ showed the highest extraction efficiency and was selected as the optimal IL. Based on the evaluation of single-factor test and RSM, the extraction parameters were optimized, which indicated the established IL-MAE was successfully used to extract AME from *S. trachyphylla* sample. The extraction yield of AME was 1.63 mg/g under the best process of 400 W microwave power, 0.8 mmol/l $[Bpy]BF_4$, 40 min extraction time, 1 : 9.6 g/ml solid-liquid ratio and 55°C extraction temperature. Compared to the traditional solvent extraction methods, IL-MAE showed remarkable properties of being fast, environmental friendly, solvent saving and highly efficient for the extraction of amentoflavone from *S. trachyphylla*. Thus, this study confirmed that IL-MAE was an effective method for the extraction of AME from *S. trachyphylla*.

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