Simultaneous extraction and purification of alkaloids from *Sophora flavescens* Ait. by microwave-assisted aqueous two-phase extraction with ethanol/ammonia sulfate system

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**Abstract**

A rapid and effective method of integrating extraction and purification for alkaloids from *Sophora flavescens* Ait. was developed by microwave-assisted aqueous two-phase extraction (MAATPE) based on the high efficiency of microwave-assisted extraction (MAE) and the demixing effect of aqueous two-phase extraction (ATPE). The aqueous two-phase system (ATPS), ethanol/ammonia sulfate was chosen from seven combinations of ethanol/salt systems, and its extraction properties were investigated in detail. Key factors, namely, the compositions of ATPS, solvent-to-materials ratio, and the extraction temperature were selected for optimization of the experimental conditions using response surface methodology (RSM) on the basis of the results of the single-factor experiment. The final optimized conditions were, the compositions of ATPS: ethanol 28% (w/w) and \((\text{NH}_4\text{)}_2\text{SO}_4\) 18% (w/w), solvent-to-material ratio 60:1, temperature 90°C, extraction time 5 min, and microwave power 780 W. MAATPE was superior to MAE, the latter using a single solvent, not only in extraction yield but also in impurity content. Moreover, compared with the combination of MAE and ATPE in the two-step mode, MAATPE demonstrated fewer impurities, a better yield (63.78 ± 0.45 mg/g) and a higher recovery (92.09 ± 0.14%) in the extraction and purification of alkaloids. A continuous multiphase-extraction model of MAATPE was proposed to explicate the extraction mechanism. MAATPE revealed that the interaction between microwave and ATPS cannot only cause plant cell rupture but also accelerate demixing, improving mass-transfer from solid–liquid extraction to liquid–liquid purification. MAATPE simplified procedures also contributed to the lower loss occurrence, better extraction efficiency, and reduced impurity to target constituents.

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1. Introduction

*Sophora flavescens* Ait. also named as Kushen, belongs to leguminous plant of *Sophora*. As a typical traditional Chinese medicine, it has been commonly used for the treatment of viral hepatitis, cancer, viral myocarditis, gastrointestinal hemorrhage, and skin diseases such as eczema, colitis, and psoriasis [1]. Alkaloids and flavonoids are reportedly the major active constituents of this plant; alkaloids, in particular, have attracted increasing attention to their high pharmacological activities, which exhibit sedative, analgesic, and other central nervous system inhibition effects as well as anti-typeric, anti-tumor, and anti-myocardial actions [1–16]. So far, more than 20 alkaloids have been isolated from the root, leaves and flowers of *S. flavescens* Ait. [2–6,17–20]. Oxy-matrine and matrine are known as the main alkaloids in the extracts, and have been widely used as primary ingredients in pharmaceutical preparations in various forms, such as suppositories, capsules, tablets and creams [1,21–24]. Moreover, alkaloids have also been used as green pesticides in agriculture due to insecticidal effects [25,26]. The huge demands for alkaloids in the market drive further investigations aimed at the improvement of methods for extraction and purification.

Alkaloids in *S. flavescens* Ait. are usually extracted through conventional methods, such as solvent soaking extraction, heat reflux extraction, and soxhlet extraction [1,27], which are subject to remarkable shortcomings, including the lengthy process, the high cost of organic solvents, low recovery, and toxic solvent residuals in the products, etc. Various approaches, including ultrasonic, microwave, supercritical fluid and ionic liquid sorbent have been...
developed to reduce the extraction time, minimize solvent consumption, increase the extraction yield, and improve the quality of extracts [19,28–30]. Microwave-assisted extraction (MAE) becomes increasingly popular in traditional Chinese medicine for extracting the active constituents from organisms and plants due to its beneficial characteristics of quick heating, low quantity solvent used, decreased energy consumption and pollution [31–34]. It is a unique technique, in which molecules and polar bonds in the extraction medium can be agitated by microwave [35–37]. Via the interactions of microwave, the weak bonds of the target constituents to the matrix are disrupted and the solvent-to-matrix material penetration is accelerated, leading to a fast release of constituents from matrix materials.

Aqueous two-phase extraction (ATPE) was first introduced by Albertson in the separation of biomolecules. It offers an alternative to the conventional liquid–liquid extraction due to its properties, such as the high yield, the environment-friendly features, the easiness to scale-up as well as lower costs and the diminished damage to the biological activity of molecules [38]. ATPE has been widely applied in the recovery and purification of biomolecules, including proteins, enzymes, and antibiotics [39–43]. The success of ATPE is largely dependent on the selection of the aqueous two-phase system (ATPS), which is usually composed of two or more phase-forming substances in water (e.g., two different polymers, a polymer and a salt, two or more different surfactants). However, most phase-forming polymers and surfactants are too viscous to process, and difficult to form transparent solutions. Recent investigations have shifted the paradigm from fragile biomolecules to small molecules in natural products. The ATPS is moderated by a short-chain mer and a salt, two or more different surfactants). However, most phase-forming polymers and surfactants are too viscous to process, and difficult to form transparent solutions. Recent investigations have shifted the paradigm from fragile biomolecules to small molecules in natural products. The ATPS is moderated by a short-chain alcohol and salt solution. It offers the advantages of low viscosity, easy demixing, solvent recycling, a more environment-friendly process, and the gained larger popularity in extracting the active constituents from medicinal plants [44–50].

The combination of microwave with extraction has advantages in achieving high yields in the reactions, in which polar solvents such as methanol, ethanol and water were used [51,28]. However, MAE recovers also more impurities, resulting in a more complicated sample pretreatment for qualitative and quantitative analyses. In our recent investigation, ATPE was used for purification after MAE [52]. The recovery of the alkaloids was in the range from 91.03% to 94.46%. ATPSs had a high electric constant, and could be formed when the mixture showed two phase separation at the phase detection point. The ATPS system and MAATPE extraction process. ATPSs were prepared according to the phase diagram plotted by Xiaojin Liu et al. [52]. Each of the salts tested (ammonium sulfate, dipotassium hydrogen phosphate, sodium carbonate, sodium sulfate, calcium chloride, potassium dihydrogen phosphate or sodium chloride) was dissolved in deionized water, respectively. Ethanol was subsequently added dropwise into each tube until the solution became turbid. The point at which the solution first became turbid was designated as the turbid point and the quantity of added ethanol was recorded. The phase diagram was plotted according to different ethanol concentrations versus ammonium sulfate concentrations at different turbid points.

2.2. Instruments and apparatus

All extraction experiments were performed on an EXCEL microwave extraction system (PreeKem Scientific Instruments Co., Ltd., China) equipped with a digital timer, power and a temperature controller. HPLC analysis was carried out using Agilent 1200 Infinity chromatograph (Agilent Technologies Co., Ltd. USA).

2.3. Plotting phase diagram of ATPS

The phase diagram was determined by turbidity titration method. A certain amount of salt (NH₄)₂SO₄ or K₂HPO₄ was added into a dozens of tubes containing deionized water, respectively. Ethanol was subsequently added dropwise into each tube until the solution became turbid. The point at which the solution first became turbid was designated as the turbid point and the quantity of added ethanol was recorded. The phase diagram was plotted according to different ethanol concentrations versus ammonium sulfate concentrations at different turbid points.

2.4. The preparation of ATPS

ATPSs were prepared according to the phase diagram plotted by Xiaojin Liu et al. [52]. Each of the salts tested (ammonium sulfate, dipotassium hydrogen phosphate, sodium carbonate, sodium sulfate, calcium chloride, potassium dihydrogen phosphate or sodium chloride) was dissolved in deionized water, respectively. The salt solution was mixed with ethanol by a vortex stirrer. ATPS was formed when the mixture showed two phase separation at the cloud point. The phase ratio (x) was calculated by measuring the volumes of top (V_top) and bottom (V_bottom) phases.

\[ x = \frac{V_{\text{top}}}{V_{\text{bottom}}} \]

2.5. MAATPE procedure

Each sample (0.5 g sieved by 80-mesh) and ATPS (30 mL) were put into an extraction vessel, which was then sealed and placed in the microwave extraction system. The extraction was conducted at
90 °C under 780 W for 5 min. After cooling in the oven, the extract was filtered to remove the solid residue. The filtrate was further separated into two phases. The top and bottom phases were collected separately. The residues were obtained after removal of the solvent by a controlled evaporation. For HPLC analysis, the obtained residues were dissolved in methanol and diluted up to 50 mL. The extraction was assessed by the yield (Y) and recovery (R) of alkaloids, which were calculated as follows:

\[
Y = \frac{m_t}{m} \quad (1)
\]

\[
R = \frac{m_t}{m_T} \times 100\% \quad (2)
\]

where \(m_t\) is the total quantity of alkaloids extracted from the plant material (\(m\)); \(m_T\) is the quantity of alkaloids extracted in the top phase, and \(m_b\) is the quantity of alkaloids in the bottom phase.

2.6. HPLC analysis

The concentrations of alkaloids in the extracts were determined by HPLC with a UV detector at 220.0 nm using a Phenomenex Gemini C18 Column (5 μm, 250 mm × 4.6 mm) as the stationary phase. The mobile phase was made of methanol (A), acetonitrile (B) and 0.1% (w/v) ammonia solution (pH = 10.3) (C). Alkaloids were eluted at 30 °C in the following gradient mode: 0–6 min: 8.5–9.0% A, 9.5–9.0% B and 82% C; 6–11 min: 9.0–16.0% A, 9.0–16.0% B and 82.0–68.0% C; 11–45 min: 16.0% A, 16.0% B, and 68.0% C. The injection volume was 20 μL. The flow rate was 1.0 mL/min.

2.7. Experimental design and statistical analysis

A four-variable, three-level central composite design (CCD) was applied to determine the best combination of the extraction variables for the yield and recovery of alkaloids from S. flavescens Ait. The range and center point values of four independent variables (Table 4) were based on the results of single-factor experiments. The response to the design experiment is given in Table 5. All data were determined by HPLC in triplicate, and the results were averaged. The Design-Expert software version 8.0 was employed for the regression analysis and the optimization.

Experimental data were fitted to a quadratic polynomial model and the model was explained by the following quadratic equation:

\[
Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j \quad (3)
\]

where \(X_i\), \(X_j\) are the input variables, which influence the response function \(Y\); \(\beta_0\) is the intercept; \(\beta_i\), \(\beta_{ij}\) are the coefficients of the linear, quadratic, and the interaction term, respectively.

Table 1

The formation and property of ATPSs [52].

<table>
<thead>
<tr>
<th>Ethanol</th>
<th>Phase composition</th>
<th>Phase demixing</th>
<th>Formation feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄</td>
<td>Quick</td>
<td>Easy, stable, and transparent</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Slow</td>
<td>Easy, stable, and transparent</td>
<td></td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>Quick</td>
<td>Stable, turbid in top phase</td>
<td></td>
</tr>
<tr>
<td>Na₂SO₃</td>
<td>No</td>
<td>Unstable, precipitation in bottom phase</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>No</td>
<td>Difficult</td>
<td></td>
</tr>
<tr>
<td>KH₃PO₄</td>
<td>No</td>
<td>Difficult</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td>Difficult</td>
<td></td>
</tr>
</tbody>
</table>

Table 2

The effect of the concentration of ethanol on extraction of alkaloids.

<table>
<thead>
<tr>
<th>C_{Ethanol} (%)</th>
<th>C_{Solvent} (20°C) (%)</th>
<th>Δκ (10⁸) (s/cm)</th>
<th>Demixing time (s)</th>
<th>Phase ratio (x)</th>
<th>Yield (mg/g)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>20</td>
<td>48.44</td>
<td>117</td>
<td>0.48</td>
<td>3.17</td>
<td>14.17</td>
</tr>
<tr>
<td>22</td>
<td>20</td>
<td>54.64</td>
<td>66</td>
<td>0.72</td>
<td>14.50</td>
<td>67.09</td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>60.16</td>
<td>40</td>
<td>0.94</td>
<td>20.47</td>
<td>85.18</td>
</tr>
<tr>
<td>28</td>
<td>20</td>
<td>64.14</td>
<td>23</td>
<td>1.16</td>
<td>18.47</td>
<td>87.11</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>85.48</td>
<td>15</td>
<td>1.38</td>
<td>17.35</td>
<td>91.17</td>
</tr>
</tbody>
</table>

Notes:
- \(Δκ\) here is the conductivity difference between the top and bottom phases.
- \(X_1\) is ammonium sulfate (%).
- \(X_2\) is solvent-to-material ratio (mL: g).
- \(X_3\) is extraction temperature (°C).

Table 3

The effect of the concentration of (NH₄)₂SO₄ on extraction of alkaloids.

<table>
<thead>
<tr>
<th>C_{(NH₄)₂SO₄} (%)</th>
<th>C_{Ethanol} (%)</th>
<th>Δκ (10⁸) (s/cm)</th>
<th>Demixing time (s)</th>
<th>Phase ratio (x)</th>
<th>Yield (mg/g)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>25</td>
<td>58.15</td>
<td>64</td>
<td>1.05</td>
<td>13.47</td>
<td>68.52</td>
</tr>
<tr>
<td>20</td>
<td>25</td>
<td>59.42</td>
<td>41</td>
<td>0.95</td>
<td>17.81</td>
<td>80.63</td>
</tr>
<tr>
<td>22</td>
<td>25</td>
<td>61.18</td>
<td>33</td>
<td>0.82</td>
<td>16.98</td>
<td>80.82</td>
</tr>
<tr>
<td>24</td>
<td>25</td>
<td>63.08</td>
<td>32</td>
<td>0.63</td>
<td>16.31</td>
<td>80.07</td>
</tr>
</tbody>
</table>

Notes:
- \(Δκ\) here is the conductivity difference between the top and bottom phases.
- \(X_1\) is ammonium sulfate (%).
were easy to and ethanol/(NH$_4$)$_2$SO$_4$ or ethanol/K$_2$HPO$_4$ were the equilibrium concentrations of alkaloids and ethanol/(NH$_4$)$_2$SO$_4$ presented a smaller phase zone. In the comparison, shown in Fig. 1, ATPS from ethanol/(NH$_4$)$_2$SO$_4$ had better performance in extraction. Results showed that ATPS from ethanol/(NH$_4$)$_2$SO$_4$ had better recovery ($R$) than that from ethanol/K$_2$HPO$_4$. Partition coefficients ($K$) of both oxymatrine and matrine in ATPS from ethanol/(NH$_4$)$_2$SO$_4$ were higher than those from ethanol/K$_2$HPO$_4$. Moreover, ATPS from ethanol/(NH$_4$)$_2$SO$_4$ made of ethanol/K$_2$HPO$_4$ presented a smaller phase ratio $a$, which was beneficial for the further extraction and enrichment of alkaloids by MAATPE mentioned below. Similar findings were reported in extraction of lignans [50].

The partition coefficient ($K$) of the target compound (oxymatrine and matrine) was calculated using the following equation:

$$K = \frac{C_T}{C_B}$$  

where $C_T$ and $C_B$ were the equilibrium concentrations of alkaloids extracted in the top phase and bottom phase, respectively.

### 3.4. Study of the formation of aqueous two-phase region

The study of the formation of the aqueous two-phase region could provide information for further optimization of the ATPS compositions for MAATPE. A moderated phase diagram of the two ATPSs made of ethanol/(NH$_4$)$_2$SO$_4$ and ethanol/K$_2$HPO$_4$ systems at room temperature is shown in Fig. 2.

![Phase diagrams of ethanol/(NH$_4$)$_2$SO$_4$ and ethanol/K$_2$HPO$_4$ systems](image)

**Table 5**

Analysis of variance (ANOVA) for response surface model of the extraction yield of total alkaloids.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>$df$</th>
<th>Mean square</th>
<th>$F$ value</th>
<th>$P$-value</th>
<th>Significant$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>4157.60</td>
<td>15</td>
<td>277.17</td>
<td>214.43</td>
<td>&lt;0.0001</td>
<td>+++</td>
</tr>
<tr>
<td>$X_1$</td>
<td>278.50</td>
<td>1</td>
<td>278.50</td>
<td>215.45</td>
<td>&lt;0.0001</td>
<td>+++</td>
</tr>
<tr>
<td>$X_2$</td>
<td>24.15</td>
<td>1</td>
<td>24.15</td>
<td>18.68</td>
<td>0.0010</td>
<td>++</td>
</tr>
<tr>
<td>$X_3$</td>
<td>4.06</td>
<td>1</td>
<td>4.06</td>
<td>3.14</td>
<td>0.1018</td>
<td>--</td>
</tr>
<tr>
<td>$X_4$</td>
<td>47.63</td>
<td>1</td>
<td>47.63</td>
<td>36.85</td>
<td>&lt;0.0001</td>
<td>+++</td>
</tr>
<tr>
<td>$X_5$</td>
<td>777.54</td>
<td>1</td>
<td>777.54</td>
<td>598.43</td>
<td>&lt;0.0001</td>
<td>+++</td>
</tr>
<tr>
<td>$X_6$</td>
<td>2.71</td>
<td>1</td>
<td>2.71</td>
<td>2.09</td>
<td>0.1736</td>
<td>--</td>
</tr>
<tr>
<td>$X_7$</td>
<td>16.87</td>
<td>1</td>
<td>16.87</td>
<td>13.05</td>
<td>0.0036</td>
<td>++</td>
</tr>
<tr>
<td>$X_8$</td>
<td>1.02</td>
<td>1</td>
<td>1.02</td>
<td>0.78</td>
<td>0.9780</td>
<td>--</td>
</tr>
<tr>
<td>$X_9$</td>
<td>1.44</td>
<td>1</td>
<td>1.44</td>
<td>0.96</td>
<td>0.3422</td>
<td>--</td>
</tr>
<tr>
<td>$X_{10}$</td>
<td>2.04</td>
<td>1</td>
<td>2.04</td>
<td>1.21</td>
<td>0.2895</td>
<td>--</td>
</tr>
<tr>
<td>$X_1^2$</td>
<td>99.22</td>
<td>1</td>
<td>99.22</td>
<td>76.76</td>
<td>&lt;0.0001</td>
<td>+++</td>
</tr>
<tr>
<td>$X_2^2$</td>
<td>57.63</td>
<td>1</td>
<td>57.63</td>
<td>44.58</td>
<td>&lt;0.0001</td>
<td>+++</td>
</tr>
<tr>
<td>$X_3^2$</td>
<td>23.46</td>
<td>1</td>
<td>23.46</td>
<td>18.29</td>
<td>0.0012</td>
<td>++</td>
</tr>
<tr>
<td>$X_4^2$</td>
<td>49.84</td>
<td>1</td>
<td>49.84</td>
<td>38.21</td>
<td>&lt;0.0001</td>
<td>+++</td>
</tr>
<tr>
<td>Residual</td>
<td>15.51</td>
<td>12</td>
<td>1.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td>12.86</td>
<td>7</td>
<td>1.84</td>
<td>3.47</td>
<td>0.0950</td>
<td>--</td>
</tr>
<tr>
<td>Pure error</td>
<td>2.65</td>
<td>5</td>
<td>0.53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor total</td>
<td>4173.11</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ +++ most significant ($P<0.001$); ++, more significant ($P<0.01$); +, significant ($P<0.05$); --, less significant ($P>0.05$).
ethanol/(NH₄)₂SO₄ was depicted (Fig. 3a), based on the cloud and saturation curves. In this phase diagram, a clear aqueous two-phase region was illustrated in comparison to other two regions, namely, the single-phase region and the saturation region. The turning point between the single-phase and the aqueous two-phase region was determined at the cloud point. The turning point from the aqueous two-phase to the saturation region was recorded when salt precipitation was observed. The collective data indicated that ethanol concentrations from 11.4% (w/w) to 54.1% (w/w) and (NH₄)₂SO₄ concentrations from 2.1% (w/w) to 30.6% (w/w) were suitable for extraction.

In the combination of phase ratio $\alpha$, better ATPS could be identified for MAATPE. Our recent investigation indicated that the recovery ($R$) was not the only response to the selection of ATPE [52]. When ATPS with ethanol concentration of 38% (w/w) and (NH₄)₂SO₄ concentration of 18% (w/w) was used for MAATPE, the phase ratio $\alpha$ was too high to acquire phase separation under the presence of herb material. After adjustment of the concentrations of ethanol and (NH₄)₂SO₄, ATPS, having a smaller phase ratio $\alpha$, a better extraction and enrichment could be achieved as well as a reduced usage of ethanol and operational easiness. For this reason, a 3D diagram was drawn (Fig. 3b) by adding the phase ratio $\alpha$ as the z-axis, projecting out from Fig. 3(a). Fig. 3(b) showed that the phase ratio $\alpha$ was strongly dependent on the compositions of ATPS. The findings of the extraction experiment showed that ATPS revealed a phase ratio $\alpha$ in the region of 0.7–1.2, which was easier for the subsequent operations (e.g. demixing among top, herb, and bottom phases, collection respective phases, filtration, etc.) after MAATPE.

In summary, due to its better extraction properties, such as the broader range of ATPS formation, the more suitable phase ratio $\alpha$, the higher ability of recovery, and the greater partition coefficient compared to other ATPSs, the ethanol/(NH₄)₂SO₄ system as a solvent for extraction of alkaloids is more adaptable to MAATPE application in further investigations.

### 3.5. Optimization of MAATPE conditions

#### 3.5.1. Optimization of compositions of the ATPS

In conducting a systematical study of ATPS, based on the guidance of the phase ratio $\alpha$ mentioned above, it was revealed that a broad band of ATPS compositions was found at an ethanol concentration of 20–30% (w/w) and (NH₄)₂SO₄ concentration of 18–24% (w/w). A single-factor experiment was carried out and the results
were presented in Table 2 and 3. The ethanol concentration of 25% was selected due to the high yield and relatively high recovery in the extraction of oxymatrine and total alkaloids. This selection also exhibited a phase ratio $\alpha$ in the middle region, with a relatively faster demixing time. Similarly, the $\text{(NH}_4\text{)}_2\text{SO}_4$ concentration of 20% (w/w) was selected, which is consistent with the results of the phase ratio $\alpha$ and demixing time.

3.5.2. Optimization of particle sizes and of solvent-to-material ratio

Fig. 4 shows the effect of particle sizes on the yield and recovery of oxymatrine and total alkaloids. Dry herb ground to the size of mesh number 80 was suitable for MAATPE, and the extraction displayed high yield and recovery. Fig. 5 shows the effect of solvent-to-materials ratio on the yield and recovery of oxymatrine and total alkaloids. A high yield of the extraction was found in the solvent-to-materials ratio of 50:1 and high recovery in 20–60:1, so the solvent-to-materials ratio in this study was set at 50:1.

3.5.3. The effect of microwave power and extraction temperature

Fig. 6 shows the effect of the microwave power on the yield and recovery of oxymatrine and total alkaloids. The high yield and recovery were achieved at the power of 780 W. However, the effect of the microwave power on the yield and recovery was not that significant in practical terms. As Fig. 7 illustrates, a high yield and a relatively high recovery of oxymatrine and total were found at 100 °C. Higher temperature (above 100 °C) caused a significant decrease of the yield of total alkaloids due to changes in chemical stability, highlighting the particular susceptibility of oxymatrine to high temperatures [58].

3.5.4. The effect of extraction time

Fig. 8 shows the effect of the extraction time on the yield and recovery of oxymatrine and total alkaloids. It was interesting that the high yield of total alkaloids and the high recovery of oxymatrine were found at the duration of 5 min. The yield of oxymatrine and the recovery of total alkaloids appeared comparatively stable during the extraction time window.

3.6. Optimization of MAATPE conditions with RSM

The results of the single-factor experiment could be summarized as follows: ethanol, 25% (w/w) and $\text{(NH}_4\text{)}_2\text{SO}_4$, 20% (w/w); the size of matrix materials 80 mesh; the solvent-to-materials ratio of 50:1; the microwave power, 780 W; the extraction temperature, 100 °C; and the extraction time of 5 min. Among these conditions, ATPS compositions chosen were in line with the pre-determined scales. They were variables interrelated in the extraction and needed further RSM verification. The solvent-to-materials ratio was fixed close to the top scale, leaving room for
improvement. Microwave power input, i.e. the extraction temperature caused unstable loss of ingredients in the extract [58]; hence, it deserves a further analysis. According to central composite design (CCD), shown in Table 4, the RSM was introduced to determine the best combination of extraction variables for the yield and recovery of alkaloids from *S. flavescens* Ait.

All experimental data were obtained from a 30-run-experiment by HPLC analysis (as shown in Fig. 9), the yield of total alkaloids and oxymatrine were shown in Figs. 6, 7, and 8.

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**Fig. 6.** The effect of microwave power on the yield (a) and recovery (b) of oxymatrine and total alkaloids.

**Fig. 7.** The effect of extraction temperature on the yield (a) and recovery (b) of oxymatrine and total alkaloids.

**Fig. 8.** The effect of extraction time on the yield (a) and recovery (b) of oxymatrine and alkaloids.
showed the significance of $X_1$, $X_2$, and $X_3$ are the most important variables from the process described above, since both the extraction yield and recovery depend on the composition of ATPS [31,38-51,28,52-59].

Table 6

<table>
<thead>
<tr>
<th>Yield (mg/g)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Determined</td>
</tr>
<tr>
<td>64.17</td>
<td>0.71</td>
</tr>
<tr>
<td>63.66</td>
<td></td>
</tr>
<tr>
<td>64.10</td>
<td></td>
</tr>
<tr>
<td>63.01</td>
<td></td>
</tr>
<tr>
<td>63.72</td>
<td></td>
</tr>
<tr>
<td>63.31</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Mean ± standard deviation ($n = 6$).

3.7. Mechanisms of MAATPE

A schematic diagram was drawn, based on the facts and evidences observed in our study of MAATPE. First, Fig. 10(a) showed that the herb powder sample was located in between the salt-rich bottom phase and the ethanol-rich top phase, which was different from conventional extraction. Comparatively, this MAATPE experienced two processes: solid–liquid extraction of alkaloids from the herb matrix into the bottom phase, and a liquid–liquid extraction from the bottom to the top phase (Fig. 10b). In the above process, the salt played two roles in the improvement of demixing and absorbing microwave. Owing to its higher conductivity, the salt solution could facilitate the separation from the miscible ethanol. This had a key part in the bottom phase, where solid–liquid extraction occurred between the herb material and the salt-rich phase. Due to the higher conductivity of the bottom phase, microwave could cause a stronger internal heating and molecular agitation, and thus, facilitated the breakdown of the weak interplay of alkaloids with the matrix and speeded up the removal of the targeted constituents and impurities [31,35,37]. On the contrary, the ethanol top phase, having a lower conductivity, did not contribute much to the solid–liquid extraction. However, it still could act as purification during the liquid–liquid extraction. In MAATPE process, the alkaloids were extracted preferentially from the herb to the bottom phase, and migrated to the top phase for their purification. Thus, among the above multiple phases, the mass-transfer equilibriums of the alkaloids were considered one of the key factors for the improvement of the extraction yield and purification recovery. At a certain high temperature, the liquid–liquid extraction could continuously drive alkaloids from the salt-rich bottom phase into the ethanol-rich top phase; forming an alkaloids enrichment favored equilibrium, which could be further enhanced by microwave agitation.

Second, MAATPE introduced changes in the surface morphologies of the herb samples; Fig. 11 shows the effect of microwave onto the morphologies of the herb matrix. In comparison, the morphologies of herb matrix materials collected after MAATPE, two conventional extraction treatments and non-treated as provided, were observed by scanning electron microscopy (SEM). SEM micrographs illustrated that MAATPE resulted in micro-cracks on the cell surface of the matrix, whereas the conventional extractions exhibited surface shrinkage. This was because the MAAP could cause cell rupture [59]. In addition of the internal heating, microwave with higher frequency could accelerate the dipole rotation and
collision, having a synergistic effect regarding the mechanical disturbance. Additionally, MAATPE also accelerated demixing. Demixing of MAATPE (45 s) is much faster than the spontaneous demixing of ATPE (1.5 min) [60]. The fast demixing may contribute to a more efficient purification with a higher recovery, especially when the conductivity difference ($D_k$) between two phases is increased, as shown in Table 2 and 3. The demixing effect under the microwave-assisted condition could benefit remarkably sample pretreatment before the quantitative analysis.

3.8. Comparison of different extraction methods

In our studies, MAATPE is a relatively new approach. It combines conventional extraction, ATPE and MAE. For validation of MAATPE, a comparative study of the one-step MAATPE with the two-step MAE-ATPE was carried out using ATPS, water/ATPS, and ethanol/ATPS, respectively. MAATPE was carried out under the RSM optimized conditions, whereas MAE-ATPE followed MAE with ethanol or water before ATPE, according to the recent protocols [51,28]. The results in Table 7 show that the one-step MAATPE significantly improved the extraction yield compared to the two-step (single solvent) MAE-ATPE, while the recovery remained the same. As illustrated in Fig. 12, there were 6 alkaloids, named oxymatrine, N-oxysophocarpine, sophoridine, N-methylcytisine (identified by LC-MS), matrine, and sophocarpine in the top phase of the extraction by MAATPE. According to the purities of the extracts, analyzed by HPLC chromatograms, MAATPE yielded an extract with more constituents in a higher content and fewer impurities, whereas MAE-ATPE approaches, using only ethanol, presented somewhat similar results, but with more impurities. MAATPE, combining the extraction and purification in one step, demonstrated the best performance among the three methods as shown in Table 7. Thus,

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**Fig. 10.** Schematic diagrams of the multiple-phase process of MAATPE: (a) visual distribution of three phases; (b) the continuous multiphase-extraction model for MAATPE extraction and purification.

**Fig. 11.** The pictures of the herb by electron scanning microscopy: MAATPE (a); heat reflux extraction (b); soaking extraction (c); non-extracted (d).
Table 7
The results of extracted alkaloids from *Sophora flavescens* Ait. by different methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Mode</th>
<th>Extracting solvent</th>
<th>Yield (mg/g)</th>
<th>Relative purity (%)</th>
<th>Recovery (%)</th>
<th>Relative purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAE-ATPE</td>
<td>1</td>
<td>MAE prior to ATPE</td>
<td>63.78</td>
<td>92.09</td>
<td>-</td>
<td>69.54</td>
</tr>
<tr>
<td>MAE-ATPE</td>
<td>2</td>
<td>MAE prior to ATPE</td>
<td>53.04</td>
<td>40.67</td>
<td>89.51</td>
<td>56.21</td>
</tr>
<tr>
<td>MAATPE</td>
<td>Simultaneously</td>
<td>ATPS</td>
<td>20.50</td>
<td>30.44</td>
<td>91.85</td>
<td>70.33</td>
</tr>
</tbody>
</table>

* The relative purity of total alkaloids is the ratio of the peak area of oxymatrine, N-oxysophocarpine, sophoridine, matrine, and sophocarpine to the total peak area except N-methylcytisine without its standard.

MAATPE simplifying experimental procedures can be applied not only in sample pretreatment prior to quantitative analysis but also in the industrial scale-up production.

4. Conclusions

In this study, a microwave-assisted aqueous two-phase extraction (MAATPE) was developed for the first time to enhance the yield and recovery of alkaloids from *S. flavescens* Ait. MAATPE, integrating MAE with ATPE into one-step procedure, provided a rapid and effective method for the simultaneous extraction and purification. ATPS of ethanol/ammonium sulfate was carefully selected as a multi-function extraction solvent, and assessed by exploring the sophisticated 3D phase diagram. The protocol for MAATPE was screened through the single-factor experiment and optimized by RSM. The optimized conditions improved significantly the yield and recovery of the alkaloids compared with those of the conventional approaches. For a better understanding of MAATPE, we depicted a continuous multiphase model of the conjugated extraction processes. In this model, MAATPE has a significant impact on the solid–liquid extraction that might be involved in the surface demixing time for phase separation, morphological changes, and effective method for the simultaneous extraction and purification of active constituents in microbial cells or plants.

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**References**


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