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Microwave-assisted aqueous two-phase extraction of alkaloids from *Radix Sophorae Tonkinensis* with an ethanol/Na_2HPO_4 system: Process optimization, composition identification and quantification analysis

Shiyu Zhou[a], Xuehao Wu[b], Yuanxiang Huang[b], Xiujuan Xie[b], Yuyang Lin[b], Huajun Fan[b,*], Laicheng Luo[a], Wei Zhang[a,*], James Z. Tang[c]

[a] School of Basic Courses, Guangdong Pharmaceutical University, Guangzhou, 510006, China
[b] School of Pharmacy, Guangdong Pharmaceutical University, Guangzhou, 510006, China
[c] School of Pharmacy, Faculty of Science and Engineering, University of Wolverhampton, Wolverhampton, WV1 1LY, United Kingdom

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ABSTRACT

A rapid method for simultaneous extraction and separation of multiple alkaloids from *Radix Sophorae Tonkinensis* (RST) was developed by microwave-assisted aqueous two-phase extraction (MAATPE) using the aqueous two-phase extraction system (ATPS) of ethanol/Na_2HPO_4 as the extraction solvent. The effects of key factors on extraction yield were investigated by utilizing single-factor experiment coupled to response surface methodology (RSM). The regression model by RSM was significant ($p < 0.0001$) and adequate for prediction of process efficacy, the optimized conditions were successfully validated by the parallel experiments with the yield very close to the predicted value. The optimum conditions were summarized as follows: the phase ratio of 2.60 for the ATPS, the particle size of 100 mesh, the liquid-to-material ratio of 75:1, the extraction temperature of 90 °C and the extraction time of 5 min, respectively. In MAATPE process, alkaloids were extracted preferentially from RST in the top phase with a higher yield and shorter extraction time than those of heating reflux extraction (HRE) and ultrasonic-assisted extraction (UAE). Nine alkaloids extracted were identified and quantified by high-resolution ultra-performance liquid chromatography-quadrupole-orbitrap mass spectrometry (UPLC-Q-Orbitrap/MS) and HPLC with UV detection. The contents of matrine, sophocarpine, oxymatrin, sophoranol, oxysophocarpine, 5α-hydroxysophocarpine, sophoridine, cytisine and *N*-methylcytisine in RST were quantified in range of 0.493–10.284 mg/g with recoveries of 90.26–106.3% and RSD's of 0.8–2.1%, respectively. Moreover, the MAATPE mechanism was explored using the different extraction systems in combination of scanning electron microscopy (SEM) morphological studies. Significant differences in extraction yield and cell rupture exhibited that the addition of the salt in the ethanol-water mixture not only improved the thermal and demixing effects, but also accelerated the mass transfer and biphasic extraction processes. MAATPE integrated the advantages of microwave-assisted extraction (MAE) and aqueous two-phase extraction (ATPE) was proved as a green, efficient and promising alternative to extraction of alkaloids from RST.

1. Introduction

Radix Sophorae tonkinensis (RST) is the dried roots and rhizomes of *Sophora tonkinensis* Gapnep of the family Leguminosae. It is also named Shandougen in Chinese and has been commonly used as a traditional Chinese medicine for the treatment of detoxification and alleviation of pain (Chinese Pharmacopoeia Commission, 2015a; Chen et al., 2017). Quinolizidine alkaloids as the main beneficial components in RST have been demonstrated the pharmacological effects such as anti-inflammation, anti-tumor, and antiviral activities, etc. (Chen et al., 2017; Han et al., 2016; Pan et al., 2015; Tang et al., 2013). In recent decades, more and more alkaloids including matrine-type and cytisine-type have been extracted from RST in succession for further development of biological activities (Ding et al., 2006; Zhang et al., 2016b). Some matrine-type alkaloids have exhibited insecticidal effects for green pesticides in agriculture (Ma et al., 2018; Villaverde et al., 2016; Xiong et al., 2016; Zanardi et al., 2015). Also, cytisine-type alkaloids were reported to have shown multi-pharmacological effects of the neuron protection and antidepressant property, etc. (Li et al., 2013; Rouden et al., 2014). Recently, toxic side effects of the alkaloids have attracted

* Corresponding authors.
E-mail addresses: junhuafan@126.com (H. Fan), gzhangw@163.com (W. Zhang).

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more and more attention due to adverse clinical cases (Chen et al., 2017; Liu et al., 2017; Tian, 2016). For pharmaceutical uses, RST is one of the most important sources of quinolizidine alkaloids (especially matrine and oxymatrine as primary ingredients in pharmaceutical preparations) besides *Sophora flavescens* Ait (Chinese Pharmacopoeia Commission, 2015b).

At present, alkaloids in RST are usually extracted by cold-mercceration, heating reflux extraction, liquid membrane extraction, ultrasonic-assisted extraction (UAE) and accelerated solvent extraction (ASE) using a single-phase solvent (Guo et al., 2016; Meng et al., 2013; Sheng and Zhou, 2008; Tang et al., 2013; Wang et al., 2017; Yang et al., 2012). However, these extraction methods are subjected to remarkable shortcomings including the lengthy process, large solvent consumption, low extraction yield with high impurities, and degradation of the unstable compound exception with UAE and ASE with shorter extraction time.

Since aqueous two-phase extraction (ATPE) was first introduced by Albertson (1986), it has been widely used in the separation of biomolecules such as proteins, enzymes, and antibiotics due to its simple operation, mild condition, friendly environment, ease of scaling-up and low costs (Glyk et al., 2015; Iqbal et al., 2016; Molino et al., 2013; Ruiz-Ruiz et al., 2012; Soares et al., 2015). An aqueous two-phase systems (ATPS) is usually consisted of two or more phase-forming substances in water (e.g., two polymers, a polymer and a salt, two or more surfactants) under a certain critical condition (Iqbal et al., 2016; Molino et al., 2013; Soares et al., 2015). Since short chain alcohols/hydrophilic solvents came into use, these ATPSs have been applied to extraction and purification of lots of compounds from natural products. It is beneficial from the moderation with efficient mass transfer, low viscosity and interfacial tension, biocompatibility and nontoxicity (Liu et al., 2013; Soares et al., 2015; Tatjana and Mirjana, 2017; Xu et al., 2017; Zhang et al., 2013). Recently, a combination technology of microwave-assisted extraction (MAE) and ATPE, known as microwave-assisted aqueous two-phase extraction (MAATPE), attracts more attentions due to advantages of quick heating, less solvent disposal, low energy consumption and controlled pollution, etc. (Chen et al., 2016; Cheng et al., 2017; Ma et al., 2013; Wang et al., 2008; Xie et al., 2017). Based on biphasic extraction capacity and microwave field intensification, MAATPE can produce greater thermal and more rapid demixing effects due to microwave enhanced interactions. As a result, MAATPE integrating extraction and purification processes into one-step procedure, can not only improve extraction efficiency, but also remove coexisted impurities. In our previous work, we observed that the herbs with roots or rhizomes in a polar medium were susceptible to microwave radiation. The interaction of microwave field with an ATPS led to cell rupture and a higher extraction yield (Cheng et al., 2017; Xie et al., 2017; Zhang et al., 2015). Thus, MAATPE as a combination of MAE and ATPE is a potential and powerful alternative to the conventional approaches for extraction alkaloids from RST. To our knowledge, there hasn't been any related report so far.

In this study, MAATPE was utilized for extraction and separation of multiple alkaloids from RST with an ATPS of ethanol/disodium hydrogen phosphate system. Using a closely pressurized microwave system, MAATPE conditions including the screening of the ATPS, the phase ratio of the ATPS, extraction temperature and time, particle size, and liquid-to-material ratio, were investigated under single-factor tests, respectively. Subsequently, key factors were further optimized using response surface methodology (RSM) for improving the extraction efficiency. Accordingly, alkaloids' composition in the extract was qualitatively analyzed by UPLC-Q-Orbitrap/MS. Finally, multiple alkaloids in RST were quantified using high performance liquid chromatography (HPLC) with UV detection. MAATPE mechanism was explored in the different extraction medium with scanning electron microscopy observations for the study of biphasic property, extraction process and herb surface structures.

2. Materials and methods

2.1. Materials and reagents

Crude drug of RST was obtained from Guangxi province in China, and identified by a botany professor in School of Traditional Chinese Medicine at Guangdong Pharmaceutical University in comparison with monography in the pharmacopoeia (Chinese Pharmacopoeia Commission, 2015a). After being dried and ground into a fine powder, the samples were sieved (40–200 mesh) and stored in desiccators at room temperature. The moisture of RST powder was determined as 10.82 ± 0.09% (w/w) by measuring the weight difference between before and after drying a given sample according to the method in Chinese Pharmacopoeia Commission.

Oxymatrine and matrine (purities were ≥98.0%) were purchased from Xi'an Xuhuang Bio-Tech Co., Ltd. (China). Oxysophocarpine, sophocarpine and sophoridine (purities ≥ 98.0%) were bought from Shanghai Pureone Biotechnology Co., Ltd. (China). Cytisine and *N*-methylcytisine were obtained from Chengdu Herbpurify Co., Ltd. (China). Bromothymol blue (Tianxin Fine Chemical Co., Ltd. China); Acetonitrile and methanol (HPLC grade, Merck Darmstadt Ltd., Germany); All other chemicals were analytical grade (Guangzhou Chemical Reagent Factory, China).

2.2. Preparation of ATPS

Phase diagram of ATPS was first prepared by turbidity titration method at a certain temperature (Zhang et al., 2015). Certain amounts of salts (NaH_2PO_4, Na_2HPO_4, Na_3PO_4) were dissolved in deionized water, respectively. Ethanol was subsequently added dropwise into the salt solution until it became turbid. The concentrations of both ethanol and salt at every level were recorded accurately to acquire enough data. Then phase diagrams were plotted with different ethanol concentrations versus phosphate salts concentrations at different turbid points.

An ATPS of ethanol/phosphate system was further prepared according to the phase diagram plotted (See Fig. 1). A certain amount of phosphate was dissolved in deionized water by heating in a water bath, ethanol was then added, and they were mixed by a vortex stirrer. The ATPS was formed when the mixture separated into two phases at the cloud point.

2.3. MAATPE procedure

All MAATPE experiments were performed on an EXCEL microwave extraction system (PreeKem Scientific Instruments Co., Ltd., China) equipped with a digital timer, power and temperature controller. The mixture of 0.5 g of sample (100 mesh) and 30 mL of the ATPS (ethanol of 35.85% (w/w) and Na_2HPO_4 of 15.38% (w/w)) were added into an extraction vessel. The sealed vessel was placed in the microwave extraction system for extraction of alkaloids at 90°C for 5 min. The extract was immediately filtered to remove the herb residue. The top phase was collected for the following evaluation when two phases were separated.

2.4. UV–vis analysis

Total alkaloids in the extract were determined by bromothymol blue method with matrine as standard according to the following procedure. The mixture of 5 mL of phosphate buffer (KH_2PO_4-NaOH, pH = 7.0) and 5 mL of 0.12% bromothymol blue solution (w/w) were added to 1.0 mL of sample solution from top phase. After mixing well, the mixture was left standing for 3 min. Then 10 mL of chloroform was added for extraction of ion-associates produced by alkaloids, and then standing until chloroform layer appeared. After discarded aqueous phase, the extract obtained was detected at 417 nm on a 2550 ultraviolet spectrophotometer (Shimadzu, Japan) against reagent blank. Similarly, matrine standard solutions were also determined according