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Ionic liquids-lithium salts based microwave pretreatment followed by ultrasonic-assisted extraction for syringin and oleuropein from Syringa reticulata var. mandshurica branch bark by dual response surface methodology

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Rapid and efficient solvent extraction of syringin and oleuropein from *Syringa reticulata var. mandshurica* branch bark was achieved using microwave-assisted ionic liquid-lithium salt pretreatment, followed by ultrasonic breakdown of the plant cell walls. The conditions for this novel sample treatment method, including microwave pretreatment time and power, ultrasound irradiation extraction time and power, types and concentrations of ionic liquid and lithium salt, and the liquid-solid ratio, were optimized using the dual response surface methodology. The proposed approach under optimal conditions was compared with conventional extraction methods. No degradation of syringin and oleuropein was observed in stability studies performed with a reference standard. The experimental results indicate that the proposed approach is a simple and efficient sample preparation technique that has strong potential for application in the field of sample analysis.

Introduction

Syringa reticulata var. mandshurica is a small deciduous tree or shrub in the Oleaceae family. China has traditionally been the distribution center for this plant and it has been used extensively as an ornamental plant in northeast China, Russian Amurskaya, the Korean peninsula and Japan because of its beautiful flowers and strong fragrance.[1,2] In addition to its wide use in landscape gardening, *S. reticulata var. mandshurica* has been extensively used in China as a folk medicine for treating chronic bronchitis and asthma.[3] Cough granules and tablets produced from the bark and branch extracts are very popular.

Extensive studies have shown that the main active components in the bark are syringin and oleuropein. Syringin is a phenyl propanoid glucoside (Fig. 1),[4] with extensive accumulation restricted to some medicinal plants.[5] It has attracted attention from medical professionals, pharmaceutical producers and researchers around the world for its wide spectrum of pharmacological activities, including anti-oxidant,[6] immunomodulatory,[7] anti-inflammatory,[8] antinociceptive,[8] anti-diabetic,[9,10] anti-hyperglycemic,[11] anti-hyperglycemic activities,[11] and it also protects cell viability,[12] and is an anti-depressant.[13] Oleuropein (Fig. 1) is a secoiridoid compound, a heterosidic ester of elenolic acid and 3,4-dihydroxy-phenylethanol.[14] Many in vivo and in vitro studies have indicated that oleuropein exhibits a wide variety of biological and pharmacological activities, including anti-thrombotic,[15] antioxidant,[16] hypolipidemic,[16] anti-ischemic,[16] immunoregulatory,[17] anti-tumor,[18-20] anti-atherogenic,[20] anti-diabetic,[21] anti-microbial,[22] anti-inflammatory,[23] and neuroprotective activities.[24] Because these two components are highly valued, their efficient extraction and analysis are very important.

Plant cell walls are dense structures, composed of cellulose, hemicellulose, pectin and other substances. Ultrasound, microwaves, and enzymatic hydrolysis methods have been used in attempts to destroy the cell wall structure, resulting in partial collapse and expansion that reduces the solvent extraction resistance between the cell wall and cytoplasm, accelerating the dissolution rate of the active ingredients, improving the extraction efficiency, and shortening the extraction time. Enzymes are macromolecular substance with catalytic function, but they have high molecular weight and are bulky, so steric effects lead to poor permeability, limiting their effectiveness. For extraction of syringin and oleuropein, glycosides are used to degrade the cellulose in cell walls and

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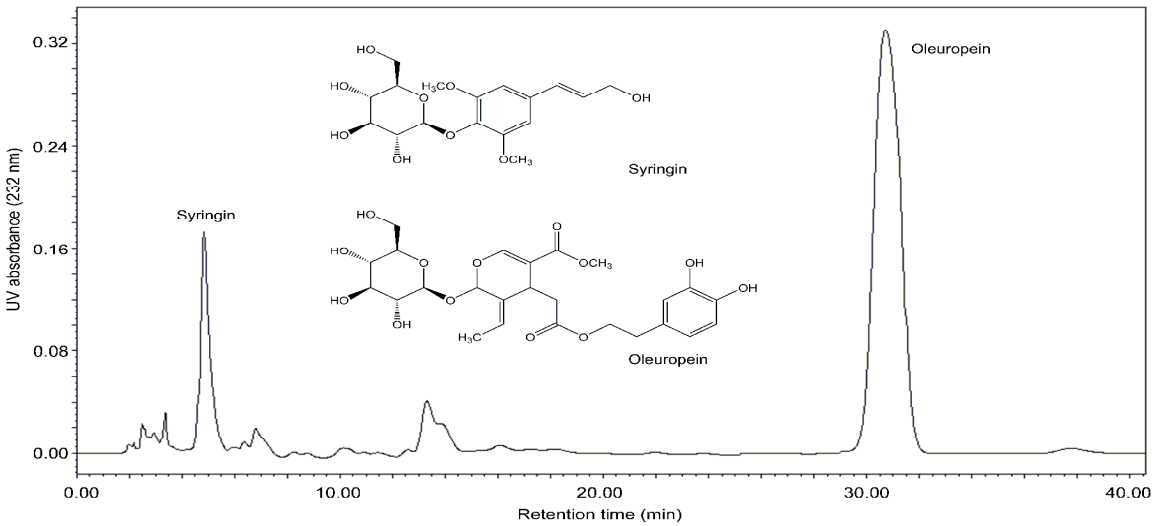


Fig. 1. HPLC profile of target analytes in an extract obtained using a 0.6 M C4mimBr-LiCl aqueous solution as extraction solvent. Insert: molecular structures of syringin and oleuropein.

hemicellulose through deglycosylation. Although microwave and ultrasonic methods have been used widely to analyze samples during the processing of plant ingredients, the outcomes are generally not ideal, often requiring multiple extraction.

Ultrasound in the field of extraction has proven to be a green technology: extraction intensification in aqueous media, low energy consumption and enhanced extract quality when compared to conventional maceration.[25] The advantages of microwave include reduction of solvent consumption, extraction duration, faster energy transfer, high reproducibility and general applicability to a wide range of plant matrices.[25] This article is a combination technology of ultrasound and microwave.

Ionic liquids are already applied in green extraction of natural products for their solvent power, high chemical and thermal stability, and as a non-flammable and non-VOC solvent.[26] Ionic liquids are compounds that comprise at least one organic ion and at least one delocalized charge. Ionic liquids lack a stable lattice structure, resulting in them being liquid below 100 °C. Ionic liquids have been used widely in recent years, notably in the extraction of active ingredients such as glycosides,[27,28] flavonoids,[29] triterpenoids,[29] coumarins,[30] and lignans.[31,32] Recently, Ionic liquids have been used as efficient solvents for the dissolution of various types of bio-macromolecules such as cellulose and hemicellulose,[33,34] starch,[35] chitin[36] and protein fiber[37] with high efficiency. It has been reported that ionic liquids can efficiently dissolve cell walls, which are composed of cellulose and lignin,[38] allowing active components to be separated from the cells. Since the earliest reports, many further experiments have been carried out in this field.

However, the capability of pure ionic liquids to dissolve cell walls is limited, and is strongly affected by the presence of moisture in the ionic liquid-plant tissue reaction system. Therefore, the use of additives and removal of moisture have attracted attention. Recently, the most notable additive has been lithium salts,[39,40] which can improve the solubility of the cell wall in some ionic liquids.

The aim of the work reported in this paper was to develop a rapid, efficient ionic liquid-lithium salt microwave pretreatment under dehydrating conditions to improve the solubility of cell walls, followed by ultrasonic-assisted extraction (UAE), to extract syringin and oleuropein from *S. reticulata var. mandshurica* branch bark. A range of parameters, including pretreatment method, types and concentrations of ionic liquids and lithium salts, pH, liquid-solid ratio, ultrasound irradiation power and time, were systematically optimized. The structure features of *S. reticulata var. mandshurica* branch bark before and after extraction were observed using an scanning electron microscope (SEM).

Experimental

Chemicals and plant materials

Syringin and oleuropein standards (98% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and phosphoric acid of chromatographic grade was purchased from Thermo Fisher Scientific Inc. (Shanghai, China). All ionic liquids, including 1-butyl-3-methylimidazolium bromide [C4mim]Br, 1-butyl-3-methylimidazolium chloride [C4mim]Cl and 1-benzyl-3-methylimidazolium bromide [Bzmim]Br were bought from Shanghai Cheng Jie Chemical Co. Ltd (Shanghai, China). Other solvents and chemicals used in this study were of analytical grade and purchased from Aladdin Industrial Inc. (Shanghai, China). Deionized water purified using a Milli-Q water purification system from Millipore (Bedford, MA, USA) was used throughout. All solutions and samples prepared for chromatographic analysis were filtered through a 0.45- μm nylon membrane (Tianjin Fuji Science and Technology Co., Ltd. Tianjin, China) before injection into a high performance liquid chromatography (HPLC) system (Waters Corp., Milford, MA, USA).

S. reticulata var. mandshurica branch bark was hand-harvested in October 2014 from the Botanical Garden of Northeast Forestry University, Harbin, Heilongjiang Province, China. Dried branch bark was further ground to 60–80 mesh fine powder prior to use.

Apparatus

The microwave extractor used in experiments was a MAS-II (2450 MHz) microwave accelerated reaction system, manufactured by Shanghai Sineo Microwave Products Co. (Shanghai, China), which incorporates a time controller, an infrared temperature sensor, an electromagnetic stirrer and a circulating water-cooling system. During experiments, time, temperature, irradiation power, and stirring speed were controlled through an electronic control panel. Temperature was monitored by the infrared temperature sensor and controlled by feedback to a microwave power regulator. The reaction flask (100 mL) was placed into the microwave resonance cavity and connected to a Clevenger apparatus through a hole located at the top of the microwave extractor. The distillate was continuously condensed in a cooling system outside the microwave cavity.

For the ultrasonic-assisted extraction experiments, an ultrasound bath was used as an ultrasonic source. The bath (KQ-250DB, Kunshan Ultrasonic Co. Ltd., China) was a rectangular container (23.5 × 13.3 × 10.2 cm), to which 50 kHz transducers were annealed at the bottom. The bath power rating was 250 W, with a control range of 0–100% and the temperature was controlled by water recirculation.

HPLC analysis and quantification

Chromatographic analyses were performed using a Waters HPLC system consisting of a pump (Model 1525), an auto-sampler (Model 717 plus), UV detector (Waters 2487 Dual k absorbance detector) and automatic column temperature control box (Model717). A Kromasil-C18 column (5 μm, 4.6 × 250 mm, Akzol Nobel, Sweden) was used, with a methanol–water–phosphoric acid (40:60:0.5, v/v/v) mobile phase at a flow rate of 1.0 mL/min, 10-μL sample injection volume, 25 °C column temperature, and detection wave-length 232 nm. The elution time for each sample was 40 min, using isocratic elution, and the retention times of syringin and oleuropein were about 5 and 31 min, respectively (Fig. 1). For a standard sample solution, various amounts of syringin and oleuropein were dissolved in methanol to yield stock solutions. Corresponding calibration curves for each compound were fitted by the linear equations $Y_{Syringin} = 8720630x - 4976$ (with regression coefficient $r = 0.9998$, for sample number n = 6) and $Y_{Oleuropein} = 6399391x + 100936$ ($r = 0.9992$, n = 6).

Microwave-assisted ionic liquids-lithium salt treatment followed by ultrasound extraction procedures

Extraction of the target compounds comprised two continuous processes: microwave-assisted ionic liquid-lithium salt pretreatment, followed by ultrasonic–assisted extraction. In the first stage, an accurately weighed amount of various ionic liquids and lithium salts were fully dissolved in acetonitrile. One gram of dried sample powder was mixed with 10 mL of these mixtures in a 100 mL flask and thoroughly stirred. The acetonitrile was then rapidly distilled off at about 70 °C using a rotary evaporator under reduced pressure. The purpose of this approach was to use a small amount of acetonitrile to reduce the viscosity of the ionic liquid–lithium salt system, making it easier to mix with the sample powder. The flask was then placed centrally within the microwave resonance cavity and the sample powder microwaved under a range of conditions. In the second step, deionized water was added to the dark slurry produced in the reaction flask during the first step and the flask then partially immersed in the ultrasonic bath containing 2.5 L of water. The types and amounts of lithium salts and ionic liquids, the heating temperature and time, ultrasound power, ultrasound irradiation time, and ratio of solid to liquid were optimized.

Optimization of the microwave pretreatment stage

Table 1. Experimental designs of microwave pretreatment stage and results.

Run	X_1	X_2	X_3	X_4	Y
1	-1	1	-1	1	5.45
2	2	0	0	0	5.60
3	1	-1	-1	1	5.96
4	0	0	0	0	5.50
5	0	-2	0	0	5.38
6	1	1	-1	1	5.53
7	-1	1	1	1	5.80
8	-1	-1	1	1	5.40
9	0	0	2	0	4.62
10	1	1	1	1	5.46
11	0	0	0	0	5.49
12	-1	-1	-1	-1	5.26
13	-1	-1	1	-1	4.45
14	0	0	0	0	5.48
15	-1	1	-1	-1	5.41
16	0	0	0	0	5.47
17	0	0	-2	0	5.13
18	1	-1	1	-1	4.93
19	0	0	0	0	5.66
20	0	2	0	0	5.54
21	1	1	1	-1	4.77

22	1	-1	1	1	5.06
23	0	0	0	0	5.21
24	-2	0	0	0	5.41
25	0	0	0	-2	5.28
26	1	-1	-1	-1	5.39
27	1	1	-1	-1	5.59
28	-1	1	1	-1	5.15
29	0	0	0	2	5.51
30	-1	-1	-1	1	5.51

[a]The results were obtained with Design Expert 8.0 software; [b] X_1 is the amount of lithium chloride (mg), the coded levels of 2, 1, 0,-1 and -2 are 44, 55, 66, 77 and 88 mg, respectively; X_2 is the pretreatment temperature (°C), the coded levels of 2, 1, 0,-1 and -2 are 90, 95, 100, 105 and 110 °C, respectively; X_3 is the microwave irradiation time (min), the coded levels of 2, 1, 0,-1 and -2 are 1, 1.5, 2, 2.5 and 3 min, respectively; X_4 is the amount of [C4mim]Br (mmol) the coded levels of 2, 1, 0,-1 and -2 are 6, 8, 10, 12 and 14 mmol, respectively; Y is total yield of syringin and oleuropein (mg/g).

To investigate the relationships between each of the factors, the operating conditions were optimized by response surface methodology (RSM) using a central composite rotatable design (CCRD) software package for data processing. CCRD with three factors was applied using the Design-Expert 8.0 (Minneapolis, MN, USA) package without blocking. The range of pretreatment conditions tested were microwave temperature 80–120 °C, microwave time 1–3 min, amount of [C4mim]Br 0–0.018 mol and of LiCl 0–66 mg. Specific protocols for each experimental factor are shown in Table 1.

Optimization of the ultrasonic-assisted extraction stage

To investigate the relationships between each of the factors, the operating conditions were again optimized by RSM, using the CCRD software for data processing. The ranges of ultrasonic-assisted extraction factors were ultrasonic power 100–250 W, ultrasonic time 10–60 min, and ratio of liquid–solid 10–30 mL/g. Specific protocols for each experimental factor are shown in Table 2.

Table 2. Experimental designs of ultrasonic-assisted extraction stage using CCRD and results.

Run	X_1	X_2	X_3	Y
1	0	0	0	6.15
2	0	0	0	5.95
3	0	0	0	5.84
4	-1	1	-1	4.90
5	0	0	0	5.98
6	0	1.68	0	5.96
7	-1	1	1	6.01
8	0	-1.68	0	5.10
9	0	0	0	6.11
10	-1.68	0	0	4.89
11	1	1	-1	5.54
12	0	0	0	5.89
13	0	0	1.68	5.84
14	-1	-1	-1	4.48
15	1	-1	-1	5.78
16	1	-1	1	5.46
17	-1	-1	1	5.44
18	1	1	1	6.11
19	1.68	0	0	6.05
20	0	0	-1.68	5.55

[a]The results were obtained with Design Expert 8.0 software; [b] X_1 is the liquid-solid ratio (mL/g), the coded levels of 1.68, 1, 0,-1 and -1.68 are 15, 17, 20, 23 and 25 mL/g, respectively; X_2 is the ultrasonic irradiation power (W), the coded levels of 1.68, 1, 0,-1 and -1.68 are 150, 170, 200, 230 and 250 W, respectively; X_3 is the ultrasonic irradiation time (min), the coded levels of 1.68, 1, 0,-1 and -1.68 are 5, 7, 10, 13 and 15 min, respectively; Y is total yield of syringin and oleuropein (mg/g).

Comparison of approach with the reference and conventional methods

The main technical parameters of reference methods for the extraction of syringin and oleuropein from *S. reticulata var. mandshurica* branch bark are listed in Table 3. For Sample 1 and 2, 10 mmol [C4mim]Br and 77 mg inorganic additive were diluted and suspended in a small amount of acetonitrile (10 mL) to reduce solvent viscosity, and then thoroughly mixed with 1.0 g air-dry sample,. Acetonitrile and moisture were then removed by a rotary evaporator at 95 °C and −0.09 MPa vacuum. The samples were then microwaved for 1.6 min at 385 W, 20 mL of water was added to obtain a [C4mim]Br concentration of 0.6 M, and the suspension was finally extracted for 13 min by ultrasound irradiation at 230 W. For Sample 3, 12 mmol [C4mim]Br and 77 mg lithium chloride were mixed, dissolved and diluted with 20 mL of water to decrease system viscosity, and then thoroughly mixed with a 1.0-g air-dried sample, the suspension was extracted for 13 min with 230 W ultrasound irradiation. For Samples 4 and sample 5, 77 mg inorganic additive was dissolved with a small amount of acetonitrile (10 mL), and then thoroughly mixed with a 1.0 g air-dry sample. Acetonitrile and moisture were then removed by a rotary evaporator at 95 °C and −0.09 MPa vacuum. The samples were then microwaved for 1.6 min at 385 W, 20 mL of water was added to obtain a [C4mim]Br concentration of 0.6 M, and the suspension was finally extracted for 13 min by ultrasound irradiation at 230 W. For Sample 3, 12 mmol [C4mim]Br and 77 mg lithium chloride were mixed, dissolved and diluted with 20 mL of water to decrease system viscosity, and then thoroughly mixed with a 1.0-g air-dried sample, the suspension was extracted for 13 min with 230 W ultrasound irradiation. For Samples 4 and sample 5, 77 mg inorganic additive was dissolved with a small amount of acetonitrile (10 mL), and then thoroughly mixed

Table 3. Comparison with other extraction methods under the optimal conditions, mean ± S.D (n = 3).

No.	Solvent	Inorganic additive	Whether microwave treatment	Yield (mg/g)		
				Syringin	Oleuropein	Total
1	0.6 M [C4mim]Br[a]	77 mg LiCl	Yes	0.75 ± 0.05	5.46 ± 0.22	6.21 ± 0.27
2	0.6 M [C4mim]Br[a]	77 mg NaCl	Yes	0.56 ± 0.02	4.47 ± 0.21	5.03 ± 0.23
3	0.6 M [C4mim]Br[b]	77 mg LiCl	No	0.37 ± 0.02	3.46 ± 0.17	3.83 ± 0.19
4	Pure water[c]	77 mg LiCl	Yes	0.19 ± 0.01	1.78 ± 0.08	1.97 ± 0.09
5	Pure water[c]	77 mg NaCl	Yes	0.19 ± 0.01	1.72 ± 0.07	1.91 ± 0.08

[a] 12 mmol [C4mim]Br and 77 mg inorganic additive were mixed, dissolved and diluted with a small amount of acetonitrile (10 mL) to decrease system viscosity, and then uniformly and fastly mixed with 1.0 g air-dry sample. After that the acetonitrile and moisture were removed by rotary evaporator at 70 °C and -0.09 MPa vacuum and microwave irradiation for 1.6 min under 385 W as pretreatment. 20 mL of water was added, bringing the concentration of [C4mim]Br to 0.6 M, and then the suspension was extracted for 13 min under 230 W ultrasonic irradiation.

[b] 12 mmol [C4mim]Br and 77 mg inorganic additive were mixed, dissolved and diluted with 20 mL of water to decrease system viscosity, and then uniformly mixed with 1.0 g air-dry sample, the suspension was extracted for 13 min with 230 W ultrasonic irradiation.

[c] 77 mg inorganic additive was dissolved with a small amount of acetonitrile (10 mL), and then uniformly and fastly mixed with 1.0 g air-dry sample. After that the acetonitrile and moisture were removed by rotary evaporator at 110 °C and -0.09 MPa vacuum and microwave irradiation for 1.6 min under 385 W as pretreatment, 20 mL of water was added, and then the suspension was extracted for 13 min under 230 W ultrasonic irradiation.

with a 1.0-g air-dried sample. The acetonitrile and moisture were then removed by rotary evaporator at 110 °C and −0.09 MPa vacuum and the samples microwaved for 1.6 min at 385 W as a pretreatment, 20 mL of water was added, and then the suspension was extracted by ultrasound irradiation for 13 min at 230 W. The suspension was filtered through a 0.45-μm filter for HPLC analysis.

Statistical analysis

A one way ANOVA test was used to indicate the significance of any differences in extraction yields of target analytes, syringin and oleuropein. The results of HPLC analysis were expressed as the mean of extraction yield.

Results and discussion

Microwave pretreatment stage

Screening of the ionic liquids Ionic liquids possess unconventional characteristics that make them suitable for a wide range of uses. The structure of ionic liquids determines their characteristics and different combinations of anions and cations influence the extraction efficiency of target components.[41] The results demonstrate that pretreatment of *S. reticulate var. mandshurica* branch bark by ionic liquids is indeed beneficial for releasing target components embedded in the plant matrix. As shown in Fig. 2a, the syringin and oleuropein yields decreased in the order [C4mim]Br > [Bzmim]Br > [C4mim]Cl > Control. Clearly, the three imidazolium ionic liquids expressed different effects on the yields of syringin and oleuropein. Cellulose can be dissolved in ionic liquids because of their ability to break the extensive network of hydrogen bonds (including both inter-and intra-

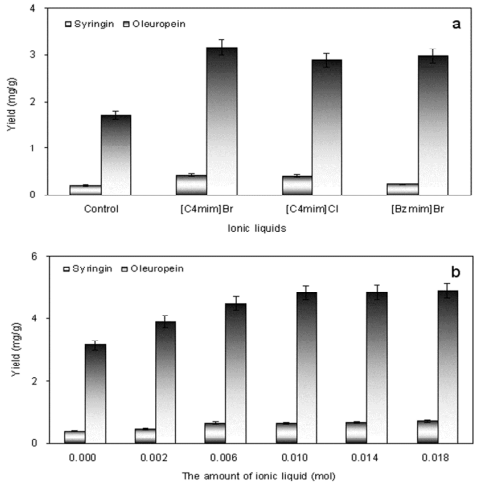


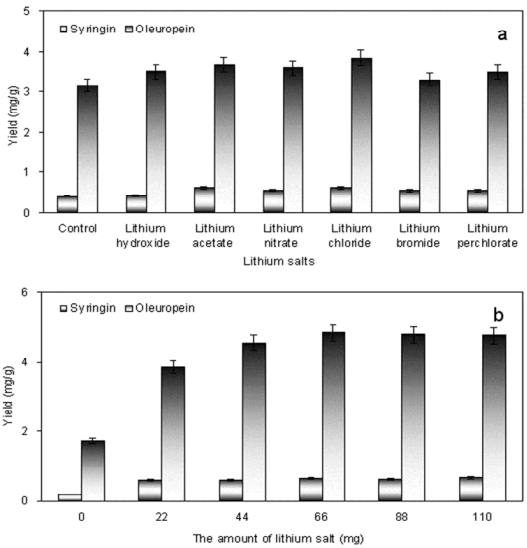
Fig. 2. Effects of the type (a) and amount (b) of ionic liquid. A certain amount of ionic liquid and LiCl were mixed, dissolved and diluted with a small amount of acetonitrile (10 mL) to decrease system viscosity, and then uniformly and rapidly mixed with 1.0 g of air-dried sample powder. The acetonitrile and moisture were then removed by rotary evaporator at 70 °C and −0.09 MPa vacuum and the sample then microwaved for 2 min at 385 W as a pretreatment. A suitable amount of water was added and then the suspension was extracted for 15 min at 250 W ultrasonic irradiation power

molecular hydrogen bonds) existing in cellulose and to form new hydrogen bonds between the carbohydrate hydroxyl protons and the ionic liquid anions.[42,43] [C4mim]Br showed significantly higher efficiency for the extraction of syringin and oleuropein, compared with other ionic liquids. From the above results, the [C4mim]Br was thus identified as the most effective extraction solvent. Different concentrations of ionic liquid can affect the yield of target analytes. Use of the optimal ionic liquid concentration is important in the extraction of target analytes, so extraction was performed in solutions containing different amounts of ionic liquid (2 to 18 mmol). In light of the results shown in Fig. 2b, it can be concluded that the extraction yields of syringin and oleuropein significantly increased when the concentration of ionic liquid was in the range of 2 to 10 mmol. However, a further increase in the ionic liquid concentration did not significantly increase the extraction yield. We believe that the high viscosity of the solvent at high concentration leads to a reduction in molecular velocity, resulting in decreased extraction yield. Similar results were reported for the extraction of alkaloids from *Catharanthus roseus*.[41] Hence, the range 6 to 14 mmol was chosen for further optimization experiments.

Effect of type and concentration of the lithium salts

As mentioned above, pure ionic liquids have limited solvent capacity for the cytoderm (cell wall), and lithium salts have recently attracted significant attention as an effective additive for improving the solvent capacity of ionic liquids.[37] Therefore, to improve the extraction yield, it was necessary to investigate the effects of the types and amounts of lithium salts used on the solubility of cellulose.

Fig. 3. Effects of the type (a) and amount (b) of lithium salt. 10 mmol of ionic liquid and a certain amount of lithium salt were mixed, dissolved and diluted with a small amount of acetonitrile (10 mL) to decrease system viscosity, and then uniformly and rapidly mixed with a 1.0-g air-dried sample powder. The acetonitrile and moisture were then removed by rotary evaporator at 70 °C and −0.09 MPa vacuum and microwaved for 2 min at 385 W as a pretreatment. 20 mL of water was added, bringing the concentration of [C4mim]Br to 0.5 M, and then the suspension was extracted for 15 min at 250 W ultrasonic irradiation power.



From Fig. 3a, it can be seen that the yields of syringin and oleuropein were enhanced significantly when a small amount of lithium salt was added into [C4mim]Br. As reported in the literature,[44] some lithium salt hydrates can dissolve cellulose through coordination of the hydroxyl groups of the cellulose with Li^+. The disruption of the intermolecular hydrogen bonds would therefore further dissolve the cell wall and release target analytes. Therefore, higher syringin and oleuropein yields were observed in [C4mim]Br-lithium salt systems than in [C4mim]Br alone. From the results shown in Fig. 3a, LiCl was thus chosen for further optimization experiments. Fig. 3b shows that syringin and oleuropein extraction yields significant increased when the amount of lithium salts added was within the range 22–66 mg, but a further increase was not obvious above 66 mg. Therefore, the range 44–88 mg was chosen for further optimization experiments.

Effects of microwave pretreatment temperature and time Optimization of the microwave pretreatment temperature is important for the efficient extraction of target analytes. Microwave extraction allows mass and heat transfer from inside the plant cell to the outside,[45] however, microwave heating conditions are harsh, so plant samples must be carefully handled to prevent pyrolysis.[46] To make it easier to control microwave heating, we chose constant gentle heating at a microwave irradiation power of 385 W, avoiding excessive power, which would lead to excessive heating and carbonized material. Fig. 4a shows the five levels of microwave pretreatment temperature selected: 80, 90, 100, 110 and 120 °C, with other parameters such as the amount of sample (1.0 g),

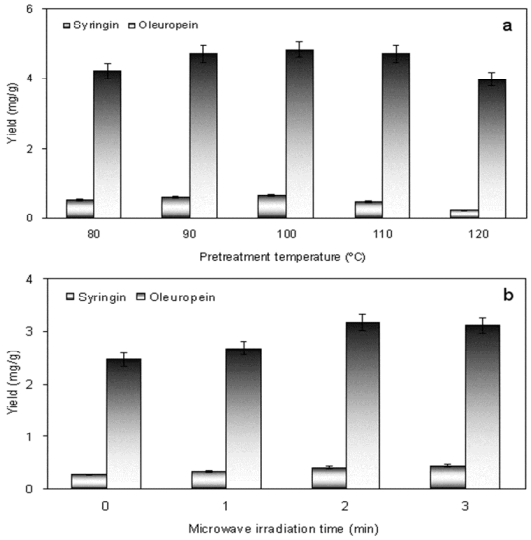


Fig. 4. Effects of microwave pretreatment temperature (a) and microwave irradiation time (b). 10 mmol of ionic liquid and 77 mg LiCl were mixed, dissolved and diluted with a small amount of acetonitrile (10 mL) to decrease system viscosity, and then uniformly and rapidly mixed with a 1.0 g air-dried sample powder. The acetonitrile and moisture were then removed by rotary evaporator at 70 °C and −0.09 MPa vacuum and microwaved for a certain time, maintaining a constant temperature at 385 W as pretreatment. 20 mL of water was added, bringing the concentration of [C4mim]Br to 0.5 M, and then the suspension was extracted for 15 min at 250 W ultrasonic irradiation power.

microwave irradiation time (2 min), kept constant. The results indicate that yields of syringin and oleuropein increased gradually when the pretreatment temperature was in the range 80–100 °C, but decreased slightly when the temperature exceeded 100 °C. This is most likely caused by the higher temperature more effectively removing moisture from the system, thus more readily dissolving the cell walls. However, at too high a temperature, while the cell walls can be dissolved, syringin and oleuropein may have been degraded, resulting in decreased yields. Therefore, the range 90–110 °C was chosen for further optimization experiments.

The effect of microwave irradiation time on syringin and oleuropein yield was bi-modal. It can be observed in Fig. 4b that an increase in microwave irradiation time resulted in an increase in syringin and oleuropein yields, reaching a maximum at 2 min. However, the yields decreased with prolonged microwave irradiation time. Therefore, the range 1 – 3 min was chosen for further optimization experiments.

Optimization of microwave pretreatment methods by RSM To better investigate the relationships between variables, we used RSM to screen conditions and used CCRD to process the data. A preliminary study of each single factor became the guiding principle for determining the scope of application of factors in the experiments. The range of lithium chloride amounts, was 44–88 mg, microwave pretreatment

Table 4. Regression coefficients and analysis of variance of the CCRD model for total yield of syringin and oleuropein with microwave pretreatment.

Source	Sum of squares	Degree of freedom	Mean square	*F*	*p*	Significant
Model	2.56	14	0.18	5.26	0.0014	**
X_1	0.02	1	0.02	0.49	0.4941	NS
X_2	0.10	1	0.10	2.77	0.1167	NS
X_3	0.70	1	0.70	20.16	0.0004	***
X_4	0.56	1	0.56	16.24	0.0011	**
X_1X_2	0.09	1	0.09	2.51	0.1343	NS
X_1X_3	0.13	1	0.13	3.63	0.0762	NS
X_1X_4	0.02	1	0.02	0.56	0.4642	NS
X_2X_3	0.14	1	0.14	3.94	0.0657	NS
X_2X_4	0.02	1	0.02	0.61	0.4487	NS
X_3X_4	0.16	1	0.16	4.72	0.0462	*
X_1^2	0.00	1	0.00	0.10	0.7606	NS
X_2^2	0.00	1	0.00	0.00	0.9954	NS
X_3^2	0.59	1	0.59	16.94	0.0009	***
X_4^2	0.01	1	0.01	0.21	0.6504	NS
Residual	0.52	15	0.03			
Lack of fit	0.42	10	0.04	1.98	0.2335	NS
Standard deviation	0.19	Coefficient of variation		3.4860		
R^2	0.83	Adequacy precision		9.2410		

* $p < 0.05$, significant; ** $p < 0.01$, highly significant; *** $p < 0.001$, extremely significant; NS, not significant.

temperature 90–110 °C, microwave irradiation time 1–3 min, and [C4mim]Br amount 6–14 mmol. The matrix for the CCRD optimization experiment is summarized in Table 1, while Table 4 shows the regression coefficients and analysis of variance of the CCRD model, with an F-value of 5.26 indicating a meaningful model, whereby there is only a 0.14% chance that this could arise from noise. Values of "Prob > F" below 0.05 indicate an important contribution of terms to the model. Therefore, X_3, X_4, X_3X_4, and X_3^2 were terms of great significance. A "Lack of fit F-value" of 1.98 implies no accuracy and results are meaningless compared with pure error. There is a 23.35% chance that a "Lack of fit F-value" takes place because of a large amount of noise. "Adeq precision" is the measure of signal-to-noise ratio. A ratio of 9.2410, greater than 4, is appropriate and indicates an adequate signal. The total yield of syringin and oleuropein (Y) was given by $Y = -4.71 + 0.17X_1 + 0.07X_2 - 1.65X_3 + 0.42X_4 - 0.02X_1X_3 + 0.04X_2X_3 + 0.10X_3X_4 - 0.59\ X_3^2$.

As expressed through the equation, the interaction coefficients for X_1X_2, X_1X_4 and X_2X_4 were zero. Therefore, we only needed to investigate the interactions between X_1 and X_3, X_2 and X_3, X_3 and X_4 on the total yield of syringin and oleuropein. The effect of response surfaces for the independent variables on the total yield of syringin and oleuropein are shown in Fig. 5.

The conditions identified for point prediction by the software were 11.76 mol of [C4mim]Br and 77 mg lithium chloride, microwaved for 1.5 min at 95 °C. Through point prediction, the total yield of syringin and oleuropein could reach to 5.8 mg/g.

Ultrasonic-assisted extraction stage

Effects of ultrasonic irradiation power Generally, the ultrasonic irradiation power and the extraction efficiency for target analytes have a proportional relationship. To extract syringin and oleuropein from the cellular structure, the solvent must enter the cellular compartments where syringin and oleuropein are located.

Despite the use of microwave pretreatment in combination with the action of [C4mim]Br ionic liquid and a lithium salt, the macromolecular cell wall components such as cellulose and hemicellulose are dissolved only partially, which destroys part of the cell wall structure but presumably leaves part of the cell wall intact. The residual intact cell structure would prevent solvent access to syringin and oleuropein. Acoustic cavitation provides enough energy to break down the plant material releasing the cell content. In addition the mechanical action will reduce particle size allowing much more plant surface to be in contact with the extraction solvent.[47] Extractions were set to a constant time of 30 min, at 100, 150, 200, 250 W ultrasonic irradiation power. Fig. 6a indicates that the yields of syringin and oleuropein were significantly affected when the ultrasonic irradiation power was less than 250 W. Therefore, a range of 150–250 W ultrasonic irradiation power was selected for further optimization experiments.

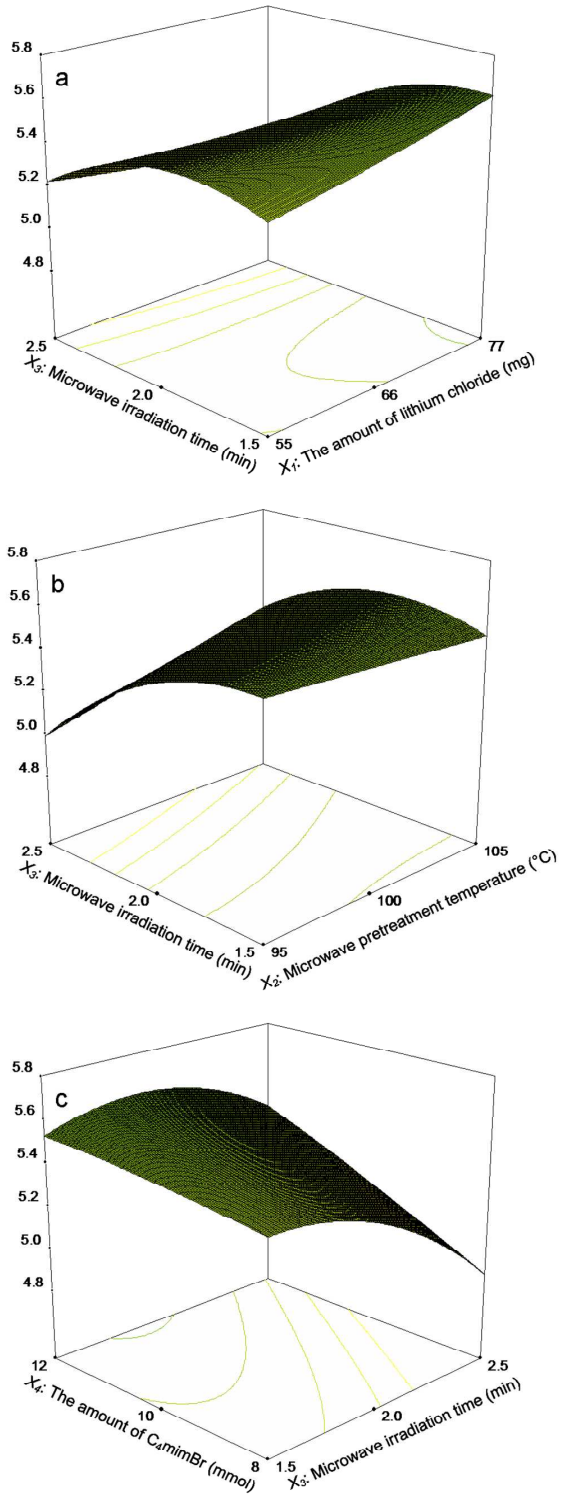


Fig. 5. The response surfaces for the effect of independent variables on total yield of syringin and oleuropein extracted: (a) the amount of lithium chloride and microwave irradiation time; (b) microwave pretreatment temperature and microwave irradiation time; (c) the amount of [C4mim]Br and microwave irradiation time.

Effect of ultrasonic treatment time To some extent, ultrasonic irradiation time is another important factor identified in this study. Extractions were therefore set at 250 W in the ultrasonic unit for 5, 10, 20, 30, 40, 50 and 60 min. The physicochemical effects of ultrasound treatment might also result in quality impairments of food products by the appearance of off-avors, modifications in physical parameters and degradation of major and minor compounds.[48] Fig. 6b illustrates that the yields of the two target analytes were increased when the ultrasonic treatment time was increased from 5 to 20 min, but increased only slightly when the treatment time was increased from 20 to 60 min, and through data analysis, no degradations of both syringin and oleuropein had been occured. Therefore, 5–15 min was chosen for further optimization experiments

Effects of liquid-solid ratio The liquid–solid ratio parameter was studied to identify values that could increase the extraction yields of the two target analytes. An excessively high liquid–solid ratio will not only cause procedural complexity but also unnecessary waste, whereas insufficient ratios would cause incomplete extraction of targets and lower extraction efficiency. Different liquid–solid ratios (10, 15, 20, 25, and 30 mL/g) were studied to determine the effect of the liquid–solid ratio on syringin and oleuropein yields. Fig. 6c indicates that the yields of syringin and oleuropein increased significantly below a liquid–solid ratio of 20 mL/g, but that yields were not significantly increased with a further increase in the amount of solvent. Therefore, a liquid–solid ratio range of 15–25 mL/g was used in the subsequent optimization study.

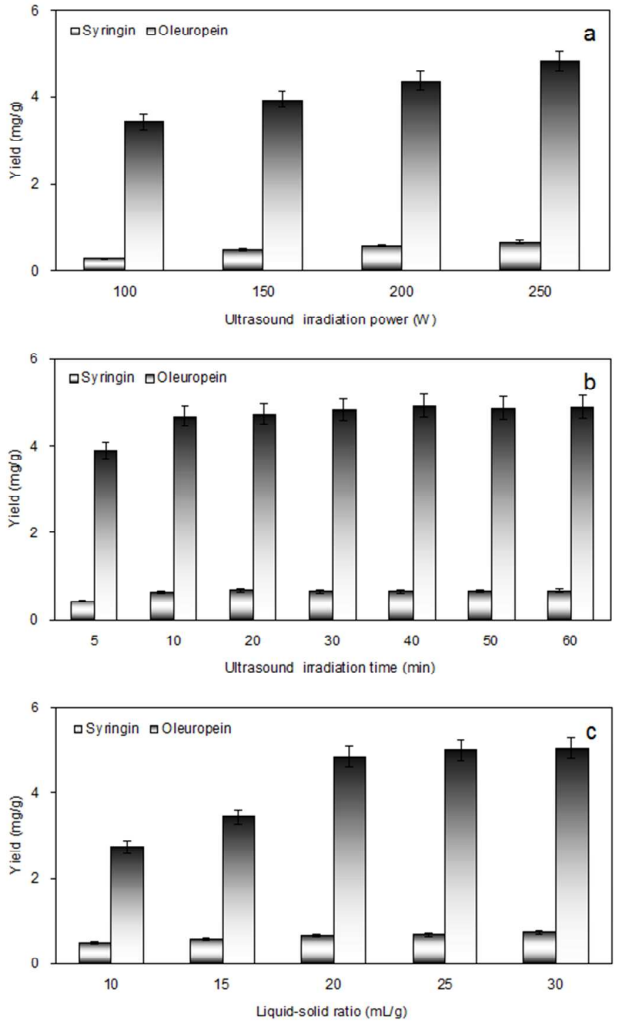


Fig. 6. Optimization of syringin and oleuropein extraction using a factorial design. Effects of ultrasonic irradiation power (a), ultrasonic irradiation time (b), and liquid-solid ratio (c) on the extraction yields of target analytes.

Optimization of ultrasonic-assisted extraction by RSM To study the relationships between all factors, we used RSM to optimize the ultrasonic-assisted extraction conditions and CCRD for data processing. CCRD with three factors at five levels was applied using Design-Expert 8.0, without blocking. A preliminary study for a single factor formed the guiding principles for scope of application of factors in the experiments. The bounds of the factors were 15–25 mL/g liquid-solid ratio, 150–250 W ultrasonic irradiation power, and 5–15 min ultrasonic irradiation time. The experimental design matrix is presented in Table 2. Twenty-one experiments were performed in triplicate, and the regression coefficients and analysis of variance of the CCRD model are shown in Table 5. The Model F-value of 12.81 indicates a significant model, whereby there is only a 0.02% chance that the value arose through noise. Values of "Prob > F" less than 0.0500 indicate that the model terms were significant, and in this case X_1, X_2, X_3, X_1X_3, X_1^2, X_2^2, and X_3^2 were identified as significant model terms. A "Lack of fit F-value" of 3.65 implies there is a 9.08% chance that it could could be attributed to noise. "Adeq Precision" is indicates the noise ratio, and a ratio of 10.877 indicates an adequate signal level. The total yield of syringin and oleuropein (Y) was given by $Y = -21.91 + 1.42X_1 + 0.09X_2 + 0.56X_3 - 0.03X_1X_3 - 0.02\ X_1^2 - 0.01X_3^2$.

As expressed through the equation, the interaction coefficients for X_1X_2, X_2X_3 and X_2^2 were zero. Therefore we only needed to study the influence on interactions between X_1 and X_3 on the total yield of syringin and oleuropein. The response surfaces for the effect of independent variables on total yield of syringin and oleuropein are shown in Fig. 7.

The conditions identified through point prediction by the software were: 228 W ultrasonic irradiation power, with a 20 mL/g liquid-solid ratio and an ultrasonic irradiation time of 13 min. Through point prediction, the total yield of syringin and oleuropein could reach 6.24 mg/g.

Verification tests

The verification tests were run in triplicate under the conditions identified through point predictions by the two response surface processes described above. The actual total yields of syringin and oleuropein totaled 6.21 mg/g with an error 0.27 mg/g (for syringin, 0.75 ± 0.05 mg/g; for oleuropein, 5.46 ± 0.22 mg/g).

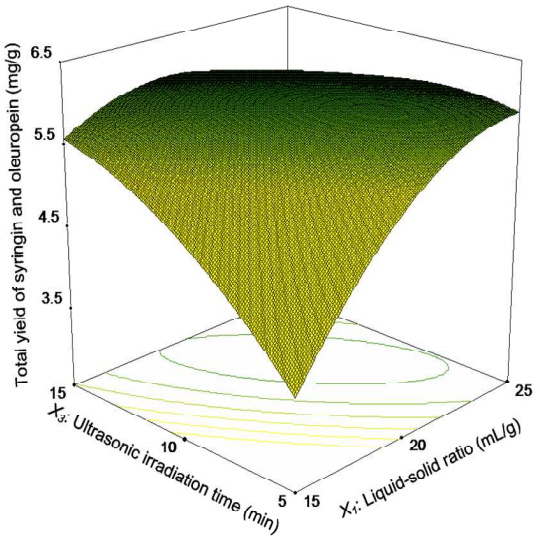


Fig. 7. Response surface plots showing the significant effects of variables ultrasonic irradiation time and liquid-solid ratio on total extraction yield of target analytes.

Comparison of approach with the reference and conventional methods

Under the optimal conditions, conventional extraction methods were compared with [C4mim]Br-based ultrasonic-assisted extraction to identify the best extraction method. were only 30%–40% of that using 0.6 M [C4mim]Br as the From Table 3, it can be seen that the yields of the two target analytes with pure water as the solvent (Samples 4 and 5) solvent (Samples 1 and 2). The results show that the ionic liquid comprised the dominant contribution towards extraction yield in the ionic liquid-water solvent extraction system. Comparing the results from microwave pretreatment (Sample 1) without pretreatment (Sample 3), we can see that microwave pretreatment greatly increased the extraction yield of syringin and oleuropein from *S. reticulata var. mandshurica* branch bark. We can therefore conclude that [C4mim]Br acted as a solvent and LiCl acted as a solubilizing additive, playing a critical role in extraction of syringin and oleuropein via the microwave pretreatment process.

Comparing lithium salt with sodium salt, from Samples 1 and 2, we can see that lithium also plays an important role in the extraction syringin and oleuropein, while from Samples 4 and 5, we can also see that lithium has no effect in water extraction of syringin and oleuropein. Hence, we believe that lithium salt act synergistically, rather than alone.

Table 5. Regression coefficients and analysis of variance of the CCRD model for total yield of syringin and oleuropein with ultrasonic-assisted extraction.

Source	Sum of squares	Degree of freedom	Mean square	*F*	*p*	Significant
Model	3.97	9	0.44	12.81	0.0002	***
X_1	1.18	1	1.18	34.17	0.0002	***
X_2	0.59	1	0.59	17.21	0.0020	**
X_3	0.58	1	0.58	16.74	0.0022	**
X_1X_2	0.04	1	0.04	1.22	0.2953	NS
X_1X_3	0.41	1	0.41	12.01	0.0061	*
X_2X_3	0.14	1	0.14	3.92	0.0758	NS
X_1^2	0.57	1	0.57	16.45	0.0023	**
X_2^2	0.45	1	0.45	13.12	0.0047	**
X_3^2	0.20	1	0.20	5.90	0.0355	*
Residual	0.34	10	0.03			
Lack of fit	0.27	5	0.05	3.65	0.0908	NS
Standard deviation	0.1857	Coefficient of variation			3.2854	
R^2	0.9202	Adequacy precision			10.8767	

* $p < 0.05$, significant; ** $p < 0.01$, highly significant; *** $p < 0.001$, extremely significant; NS, not significant.

General morphology changes after extraction

Fig. 8 shows images of samples under different treatment conditions. Fig. 8a shows an image of raw material after grinding. Cellular tissue edges are clear and there are some attachments above these, which is probably tissue debris generated when the material was crushed. As we can see from Fig. 8b, after microwave-assisted ionic liquid/lithium salt pretreatment, the surface of the cellular tissue was covered with a layer of membrane material, and no crystalline lithium salt, demonstrating that the ionic liquid and lithium salt were mutually dissolved. After ultrasonic-assisted extraction, the membrane material on the surface of the cellular tissue disappeared and the edge of the cellular tissue appeared to be rounded, indicating that the cell wall was locally dissolved

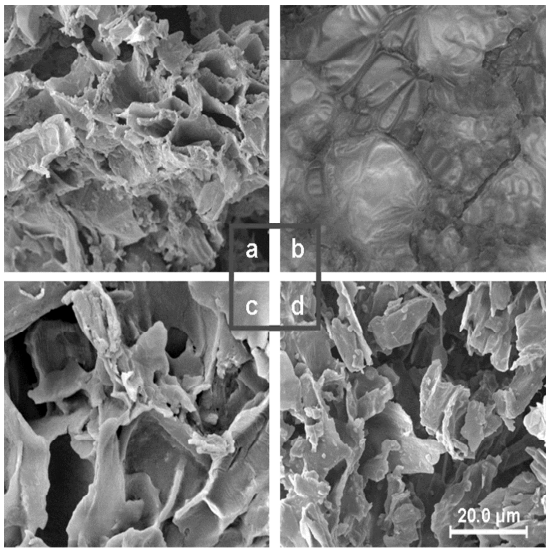


Fig. 8. Scanning electron microscope images of the bark of *Syringa reticulata var. mandshurica*: (a) untreated sample; (b) a sample after [C4mim]Br-LiCl pretreatment by microwave irradiation; (c) sample shown in b after ultrasonic-assisted extraction; (d) the sample after ultrasonic-assisted extraction with water.

by the ionic liquid/lithium salt (Fig. 8c). However, bark powder extracted with water (Fig. 8d) appeared to be only slightly affected and was very similar to the untreated sample, with attachments to the cell wall washed away by water, making the cell wall interface appear clearer. The results show that ionic liquid-lithium salt microwave pretreatment could be used to destroy the microstructure of plant tissue so that the solvent can enter the sample and extract the target analytes.

Conclusions

In this study, a novel extraction method comprising ionic liquid-lithium salt-based microwave pretreatment, followed by ultrasonic-assisted extraction, was operated and optimized by dual response surface methodology for the extraction of syringin and oleuropein from *S. reticulata var. mandshurica* branch bark. The optimal extraction conditions were identified as follows: with 77 mg lithium chloride added, microwave irradiation for 1.5 min at 95 °C; ultrasonic-assisted extraction stage, 13 min ultrasonic irradiation at 228 W and a liquid–solid ratio of 20. Under the optimal extraction conditions, the extraction yields of syringin and oleuropein were 0.75 ± 0.05 mg/g and 5.46 ± 0.22 mg/g, respectively. The present results demonstrate that the proposed process is an efficient, simple and rapid extraction method for plant sample analysis.

Acknowledgements

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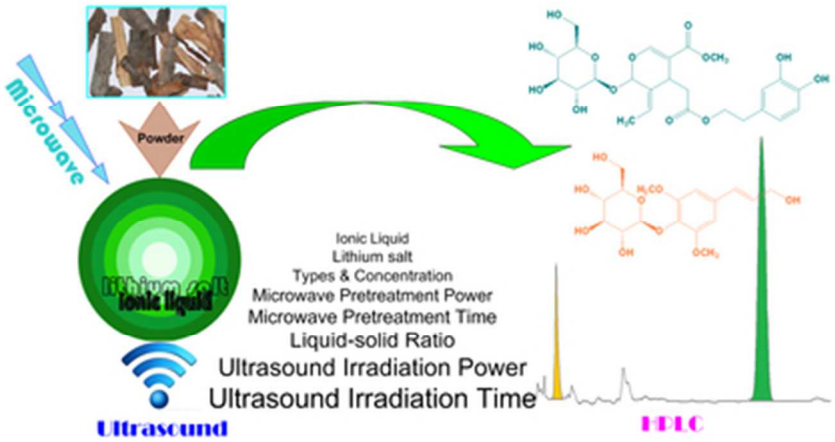

Ionic liquids/lithium salts solvent system was successfully introduced into the separation technique for the preparation of syringin and oleuropein from Syringa reticulata var. mandshurica branch bark, microwave irradiation pretreatment followed by ultrasound extraction procedure was adopted and optimized using dual response surface methodology.
17x9mm (600 x 600 DPI)