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Ultrasound-assisted Supercritical Carbon Dioxide Extraction of Ursolic Acid from *Rabdosia rubescens*

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Abstract. Ursolic acid (UA) exhibits an antineoplastic activity on several kinds of cancer. In this study, the temperature of 32–64 °C and pressure of 11.0–32.0 MPa were used to extract UA from *Rabdosia rubescens* using ultrasound-assisted supercritical carbon dioxide (USC–CO_2) with ethanol solution as a cosolvent. The optimized condition for maximal extraction yield of UA using USC–CO_2 process was at a pressure of 28.5 MPa and temperature of 55 °C with 11.6% of ethanol (80%, v/v) as cosolvent, leading to the extraction yield of 2.386 mg/g dry plant weight. Comparing with other conventional methods, USC–CO_2 extraction revealed superior extraction yield and efficiency. In conclusion, USC–CO_2 procedure can be considered as a promising green alternative to extract UA from *R. rubescens* for food, cosmetic and pharmaceutical industries with benefits of no chemical waste, rich in active compounds and short extraction time.

keywords: *Rabdosia rubescens*, ursolic acid, ultrasound-assisted supercritical CO_2 extraction, cosolvent.

1 Introduction

The entire dried *Rabdosia rubescens* (Hemsl.) Hara plant (known as Dong-ling-cao in China) is the most popular healthy teas throughout Asia for health care and diseases prevention worldwide [1]. Traditionally, *R. rubescens* is used for the treatment of stomachache, pharyngitis, sore throat, cough, wrestling injuries and tumors in Chinese folk medicine. Recently, this herb has gained attention to its anticancer activates [2]. Ursolic acid (UA, 3β-hydroxy-urs-12-en-28-oic acid) (Fig. 1) is one important constituent of *R. rubescens* and is a common constituent of medical herbs and edible

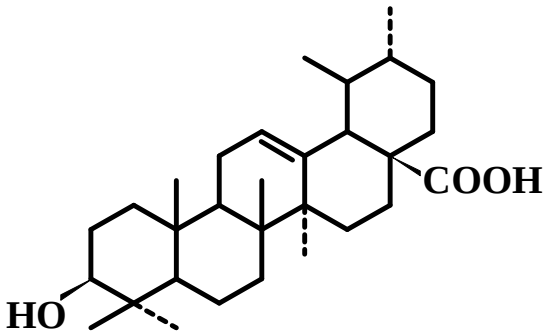

Fig. 1 Chemical structure of ursolic acid (UA).

plants, which widely applied in medicine with many beneficial functions to human health. In recent years, the literature furnishes numerous data on UA induce apoptosis in a wide variety of cancer cells, including hepatocellular carcinoma, prostate carcinoma, colorectal cancer, acute myelogenous leukemia, skin tumorigenesis, cervical carcinoma and lung carcinoma [3]. Therefore, UA is an important anticancer constituent and contribute to the pharmacological efficacy of the herb.

Early works have reported the extraction of UA in other natural materials using supercritical carbon dioxide (SC–CO_2) [4,5] and traditional solvent extraction method [6]. However, the traditional solvent extraction methods are usually characterized by the consumption of large volumes of solvent and energy, low extraction yields, lengthy extraction procedures and the potentially deleterious degradation of labile compounds. SC–CO_2 extraction has capabilities to overcome the limitations [7]. CO_2 has been recognized as an ideal non-polar extraction solvent when CO_2 is beyond its critical point (31.1 °C and 7.38 MPa). More importantly, CO_2 is nontoxic, and can be readily recovered after extraction. CO_2 is considered to be safe for humans and SC–CO_2 extraction process is an acceptable environmentally friendly alternative. Furthermore, the extracts obtained by using SC–CO_2 extraction are of superior quality as compared with those obtained by using the conventional organic solvent extraction methods. SC–CO2 extraction has become an important separation technique in the field of food, pharmaceutical and cosmetic industries.

Because polar analyte is insoluble in SC–CO2 due to its lack of polarity, pure SC–CO2 fails to extract organics from sample matrix. The solubility of polar compounds in SC–CO2 can be enhanced by cosolvents (or modifiers) was reported by Wei and Yang [8] in the extraction of oleanolic and ursolic acid (OA and UA) from Hedyotis diffusa using ultrasound-assisted SC–CO2 extraction under temperature of 30–63 °C and pressure of 10.4–30 MPa. Domingues et al. [4] issued the similar results in the extraction of triterpenic acids from Eucalyptus globulus bark using 5% aqueous ethanol as a cosolvent. Yang et al. [9] also used 11.9% ethanol solution (80%, v/v) as a cosolvent to extract OA and UA from Scutellaria barbata D. Don using ultrasound-assisted SC–CO2 extraction.

Recently, the simultaneous application of SC–CO2 and ultrasound to improve the efficiency of traditional SC–CO2 extraction process has been proposed [10]. Ultrasound-induced micro-stirring and solvent cavitation had some physical consequences,

including the cracking and damage of plant cell walls, solvent diffusion, interfacial turbulence and a reduction of external resistance to mass transfer. Consequently, the mass transfer of the solvents into the raw materials and the soluble constituents into the solvents will be improved. USC–CO2 extraction technique significantly decrease the extraction time and increase the extraction yields in natural materials while utilizing less severe operating parameters, such as temperature, pressure, CO2 flow rate. However, there are almost no previous reports regarding the USC–CO2 extraction process of UA from R. rubescens.

The objective of this study was to apply an USC–CO_2 extraction process for the preparation of UA-enriched extracts from *R. rubescens* and to compare these extracts with those obtained from a conventional solvent extraction technique. The novel method consists of static extraction (with ultrasound-assisted), followed by SC–CO_2 dynamic extraction (with ultrasound-assisted), which is the first time that this method has been investigated. The ultrasound-assisted SC–CO_2 extraction of UA from *R. rubescens* was performed at several temperatures (32, 39, 47, 55, and 64 °C) and pressures (11.0, 18.5, 23.5, 28.5 and 32.0 MPa) with ethanol solution as a cosolvent.

2 Materials and methods

2.1 Plant materials

Five samples of *R. rubescens* (Sample RR1 to RR5) were purchased from different local Chinese medicinal shops (Taiwan). All the samples were sorted and identified by the Department and Graduate Institute of Pharmacology, Kaohsiung Medical University (Kaohsiung, Taiwan) and the quality of the samples complied with the Chinese Pharmacopoeia.

2.2 Solvents and reagents

CO_2 was purchased in the liquid form from Yun-Shan Gas Co. Ltd. (Tainan, Taiwan). UA was procured as HPLC reference standards from the Sigma Chemical Co. (St. Louis, MO, USA). Methanol, ethanol, acetone, acetonitrile, ethyl acetate, *n*-hexane and 85% phosphoric acid were bought from Merck Co. (Darmstadt, Germany).

2.3 Ultrasound-assisted supercritical carbon dioxide (USC–CO_2) extraction

All experiments were conducted using ultrasound-assisted extraction (static extraction) with a 15 min duration, followed by SC–CO_2 extraction (with ultrasound-assisted). The static stage lasted 15 min for all experiments, while the dynamic stage varied from 10 to 150 min (with ultrasound-assisted). More details about the equipment and its operation have recently been described elsewhere [11]. For each experiment, a mixture of dried and ground herb (20 g, 0.36 mm) and glass beads (1 mm diameter) was placed in a 152 mL stainless steel extraction vessel (SS304, i.d. of 2.2 cm and length of 40.0 cm).

2.4 Other extraction methods

Various extraction methods [12], including heat-reflux extraction (HRE), and conventional SC–CO_2 extraction, were also investigated and compared to the USC–CO_2 extraction process.

2.5 HPLC analysis

HPLC analysis of UA was carried out on a Jasco HPLC system with a LiChrospher® C-18 analytical column (250 mm × 4 mm i.d., 5-μm particle size). The mobile phase was composed of acetonitrile (A) and 0.1% phosphoric acid (B). More details about the equipment and its separation conditions can be found in the previous work [13].

2.6 Statistical Analysis

All yields were calculated based on a moisture-free basis. The mean and standard error of the mean were calculated from six experiments. The results are expressed as the mean ± SD. Analysis of variance (ANOVA) was carried out using Tukey's method with a significance level of P <0.05 using 2010 Microsoft Office Excel (Microsoft Co., USA) and Origin version 6.1 software (Origin Lab Co., Northampton, MA, USA).

3 Results and discussions

3.1 USC–CO_2 extraction of UA from *Rabdosia rubescens*

Because SC–CO_2 is a non-polar substance, SC–CO_2 often fails to extract polar organic compounds from raw matrices. The addition of polar cosolvent was necessary to overcome the solubility limitations of pure SC–CO_2, which allowed the extraction of more polar substances from target samples. Yang and Wei [14] found that the addition of 11.6% (v/v) aqueous ethanol (ethanol/water = 80/20, v/v) was optimum to obtain the highest OA and UA yield from *H. diffusa*. The result supported previous findings that ethanol/water was the best cosolvent for the extraction of triterpene acids from *S. barbata* D. Don in SC–CO_2 [9].

One important effect is that cosolvent improves the swelling of matrix, which may affect the cell structure, improves the diffusion of target compounds from matrix and enhances the dissolution into the SC–CO_2. At the initial stages of extraction, the interaction of cosolvent and matrix is anticipated to be greater. Then it should be stabilized so that the concentration of cosolvent in the system is constant for the rest period. The complex interactions among the target matrix, target compounds and SC–CO_2 lead to the higher amounts of OA and UA being extracted. Therefore, the subsequent experiments were carried out with 11.6% (v/v) aqueous ethanol (ethanol/water = 80/20, v/v) as cosolvent for extraction of UA from *R. rubescens*.

For SC–CO_2 extraction, the extraction pressure and temperature are two main parameters that influence the extraction efficiency and selectivity. In this study, the effects

of extraction pressure and temperature on the extraction yield of UA were performed for 75 min dynamic stage (with ultrasound-assisted) at a mean particle size of 0.36 mm, a static extraction time of 15 min (with ultrasound-assisted), a SC–CO_2 flow rate of 2.1 mL/min (STP) and a cosolvent (80% aqueous ethanol) percentage of 11.6% (v/v). The ultrasound for static and dynamic stages was fixed at an ultrasonic frequency of 40 kHz, a power of 185 W and an ultrasound cycle of 75% (intermittent sonication). Fig. 2 shows the effect of various temperatures (32, 39, 47, 55 and 64 °C) and pressures (11.0, 18.5, 23.5, 28.5 and 32.0 MPa) on the extraction yield of UA form *R. rubescens* with ethanol:water (80:20, v/v) as cosolvent. The extraction yield of UA enhanced significantly with increase of pressure (11.0–28.5 MPa) at the operating temperatures.

These results were predictable because raising the extraction pressure, at constant temperature, leads to a higher density of SC–CO_2, which increases the solubility of UA. A similar outcome was reported by Patinha et al. [5], who found the triterpenic acids can significantly extracted at 20 MPa and 60 °C using SC–CO_2. The results were also consistent with those reported by de Melo et al. [15] and Domingues et al. [4] using SC–CO_2 to extract triterpenic acids from *Eucalyptus globulus* bark. However, as the pressure increases from 28.5 to 32.0 MPa at various temperatures, the increase of pressure did not have any significant effect on the extraction yield of UA. This result might be explained by the fact that the decrease in the diffusivity at higher pressure leads to a reduction in the interaction between SC–CO_2 and solute contained within matrix. This makes no effective changes in extraction yield of UA at28.5–32.0 MPa, which implied an optimum pressure for practical purposes. The best extraction pressure for the USC–CO_2 extraction of UA from *R. rubescens* with aqueous ethanol as cosolvent was accomplished at 28.5 MPa in this study. Topal et al. [16] also found the similar findings that the increasing pressure from 20 to 40 MPa resulted in a gradual increase in lycopene yield, while further increases in pressure from 40 to 50 MPa did not improve the yield of lycopene.

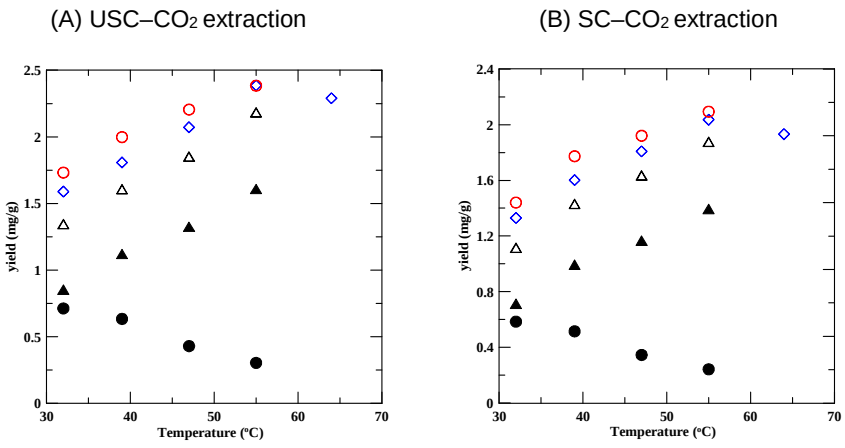

Fig. 2. Effects of extraction pressure and temperature on the extraction yield of OA using (A) USC–CO_2 and (B) conventional SC–CO_2 extractions.

(●:11.0 MPa, ▲: 18.5 MPa, △: 23.5 MPa, ◇:28.5 MPa, ○: 32.0 MPa)

[1] UA yield (mg/g) = weight of the extracted UA/ weight of feeding material.

[2] USC–CO_2 extraction was processed under the static extraction time of 15 min, dynamic extraction time of 80 min, mean plant particle size of 0.36 mm, CO_2 flow rate of 2.1 mL/min with 11.6% aqueous ethanol (80% ethanol) as cosolvent and an ultrasonic frequency of 40 kHz, a power of 185 W and an ultrasound cycle of 75% (intermittent sonication).

[3] SC–CO_2 extraction was processed under the static extraction time of 30 min, dynamic extraction time of 180 min, mean plant particle size of 0.36 mm, CO_2 flow rate of 2.4 mL/min with 11.6% aqueous ethanol (80% ethanol) as cosolvent.

Because the increase in temperature could have either a positive or a negative effect on the yield of interest compounds, the influence of temperature on SC–CO_2 extraction was more difficult to predict than that of pressure. It is well-known that increasing temperature reduces the density of SC–CO_2, which is not favorable to the dissolution of solute. On the other hand, the saturated vapor pressure of solute increases in according with the temperature, which enables solutes to dissolve in SC–CO_2 more easily. Moreover, the higher temperature the more intense heat motion of solute in aqueous solution. This enables solutes to overcome the adsorbing energy of solution and to desorb more rapidly from active sites of sample matrix by SC–CO_2 at higher temperatures. Consequently, the solubility of solute was likely decreased, kept constant, or increased with rising temperatures at constant pressure, which depended on the balance between these three types of influences competed with each other. Therefore, this temperature-dependent behavior is not quite usual in extraction systems employing SC–CO_2 as the solvent.

By looking at the effect of temperature on the yield of UA as plotted in Fig. 2, a retrograde solubility behavior exists in the supercritical state for the interest compound. At lower pressure of 11 MPa, the extraction yield decreased with increasing temperature, but at the other four higher pressures (18.5, 23.5, 28.5 and 32.0 MPa), the inverse trend was observed. Similar phenomena have been documented for various solid solute in SC–CO_2 system. For example, it is in accord with supercritical carbon dioxide extraction of microalgae lipid [17] and extraction of rice bran oil by supercritical carbon dioxide [18]. At lower pressure (11 MPa), SC–CO_2 density can be largely decreased by increasing temperature, the resultant yield decrease is mainly due to the more dominant effect of the decrease in density. In contrast, the extraction yield increases with the increasing temperature (32–55 °C) at higher constant pressure (18.5–32.0 MPa) because the density of SC–CO_2 is less dependent on temperature at higher pressures, when compared with the decrease of density, increase of vapor pressure and desorb energy. However, with further increase of temperature from 55 to 64 °C, the yield of UA starts to decrease with increasing temperature. In fact, the extraction at this temperature (64 °C) seemed to deteriorate UA where the extracted yield slightly went down to 2.290 mg/g dry plant. Similar findings were evidenced by Patinha et al. [5] for the SC–CO_2 extraction of triterpenic acids from *Eucalyptus globulus* bark, and by Yang et al. [9] for the USC–CO_2 extraction of OA and UA from *S. barbata* D. Don.

To evaluate the efficiency of USC–CO_2 extraction process, the conventional SC–CO_2 extraction (a static extraction time of 30 min followed by a dynamic extraction of 150 min) was also performed, which are reported in Fig. 2. The process efficiency of USC–CO_2 and conventional SC–CO_2 extraction methods were quantitatively related to extraction yield. It is apparent that the yield extracted by SC–CO_2 extraction with ultrasound-assisted using less severe conditions was higher than that extracted by SC–CO_2 extraction without ultrasound-assisted. The increase in extraction efficiencies and yields were attributed to the intensification of mass transport due to physical effects on the surface of the particles through disruption of the cell structure of the sample, enhancing the intraparticle diffusion. Another possible mechanism responsible to the extraction efficiencies of USC–CO_2 procedure is agitation because there are no feasible mechanical stirrers to be used for SC–CO_2 procedure.

3.2 Identification and quantification

HPLC analysis was carried out for the identification and quantification of UA in crude extract.

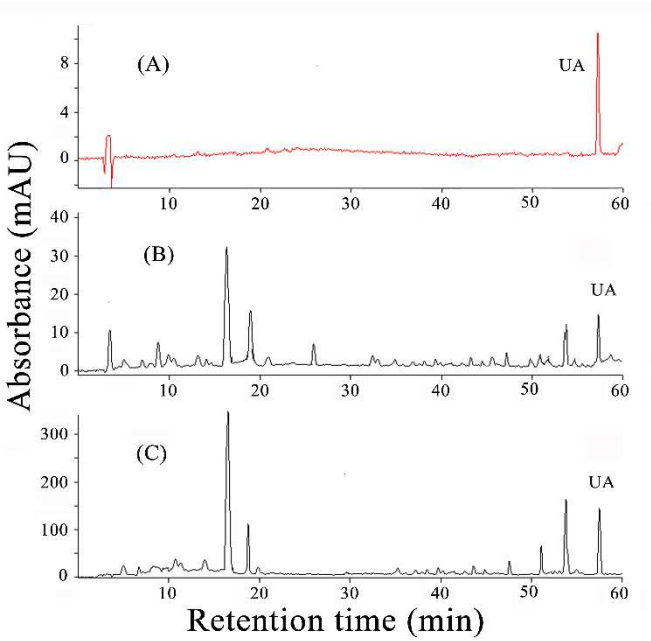

Fig. 3. HPLC chromatograms of (A) the reference compound and *Rabdosia rubescens* extracts obtained using (B) HRE and (C) USC–CO_2 extractions.

UA standard was used for the identification of peaks in extracts extracted using HRE and USC–CO_2. The chromatogram of the standard shows that UA is eluted at retention time of 57.17 min (Fig. 3A). Figs. 3B and C represented the chromatograms

of selected extracts that exhibit similar patterns with the predominant peak at the retention time of 57.17 min, belonging to UA. Consequently, the UA yields are expressed as milligrams of UA /g of dried weight plant (mg/g DW).

3.3 Compared USC–CO_2 extraction and other classical extraction procedures

In order to evaluate the yield of UA in *R. rubescens* crude extract, heat-reflux extraction (HRE) with aqueous ethanol as solvent was performed resulting a maximum UA yield of 1.65 mg on a dry weight basis (mg/g of dry *R. rubescens*). The extraction condition of HRE was shown in Table 1.

Table 1. Comparison of the extraction conditions and extraction yields obtained using various extraction methods.

Extraction parameters	Extraction mode		
	HRE	SC–CO_2	USC–CO_2
Herbal sample	RR1	RR1	RR1
Mean particle size (mm)	0.36	0.36	0.36
Plant weight (g)	20	20	20
Stirring rate (rpm)	300	—	—
Static extraction time (min)	—	30	15
Dynamic time (min)	—	150	100
Extraction time (min)	60 ×4 (4 cycles)	180	115
Extraction temperature (°C)	Boiling point	55	55
Extraction pressure (MPa)	—	28.5	28.5
Liquid/solid ratio (mL/g)	16	72.0	48.0
CO_2 flow rate (mL/min)	—	2.4	2.1
Extraction cycles	4	—	—
Duty cycle of ultrasound exposure (%)	—	—	75
Percentage of cosolvent (80% ethanol, v/v) in SC−CO_2 (%, v/v)	—	11.6	11.6
UA Yield (mg/g)[a]	1.65 ± 0.06	2.09 ± 0.08	2.39 ±0.09
Ethanol (%, v/v)[b]	80%	—	—

[a] Values are written as the mean ± SD of six replications and are calculated based on plant dry weight basis (RR1).
[b] Ethanol concentration in water (%, v/v).

4 Conclusions

The results from this study indicate that the extraction of UA from *Rabdosia rubescens* using ultrasound-assisted SC–CO_2 extraction process is feasible. The optimal extraction conditions utilize 28.5 MPa, 55 °C, a 15 min static extraction time, a 100 min dynamic extraction time, a 0.36 mm mean plant particle size, 2.1 mL/min CO_2 and

a 40 kHz ultrasonic frequency at 185 W with a 75% cycle (intermittent sonication). Under the optimized conditions, the extraction yield of the UA reached 2.39 mg/g. The results obtained are helpful for full utilization the novel process to extract UA from *R. rubescens*.

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