

Sustainable Chemistry

Subcritical Water Extraction of Sesquiterpene Lactones from *Inula racemose*Xiaofeng Chi,^[a, b] Guoying Zhang,^[c] and Shilong Chen^{*,[a, b]}

Inula racemose, a traditional Tibetan medicine with strong antifungal and anti-inflammatory activities, contains large amounts of sesquiterpene lactones. In the current study, subcritical water extraction was employed for the efficient extraction sesquiterpene lactones from *I. racemose*. Various parameters, such as extraction time (23.2–56.8 min), temperature (129.5–230.5 °C) and SW flow rate (1.3–4.7 mL/min) was investigated for optimizing the recovery of the targeted compounds. The optimal conditions for SWE were determined

as follows: extraction time of 45 min, extraction temperature of 190 °C, and SW flow rate of 3.0 mL/min, and a recovery of 71.97% was achieved. Four sesquiterpene lactone isomers were separated by *prep*-HPLC from the SWE extract and identified as igalan, isoalantolactone, alantolactone, and alloalantolactone. A comparison of SWE with traditional extraction technologies showed that subcritical water could be a green and efficient substitution for the extraction of sesquiterpene lactones from *I. racemose*.

1. Introduction

Approximately 100 species of the genus *Inula* (family: Asteraceae) are widely distributed in Asia, Africa, and Europe and used as important traditional herbal medicines.^[1] Among them, the radix of *I. racemose* has long been used in China, India, and Pakistan for the treatment of inflammation, bacterial infections, chronic gastritis, and neurodynia.^[2–5] Phytochemical investigation of the genus *Inula* revealed the presence of monoterpenes, sesquiterpenes, diterpenes, flavonoids, phenolic acids, and the sesquiterpene lactones, such as alantolactone (AL) and isoalantolactone (IAL) as the chief chemical constituents.^[2,5–6]

Sesquiterpene lactones extracted from *Inula* have gained considerable interest for their diverse pharmacological activities, such as anti-inflammatory, anti-bacterial, anti-fungal, anti-proliferative effects.^[2,4–9] Interestingly, sesquiterpene lactones were confirmed anticancer properties in a variety of cancer cells, including esophagus,^[10] breast,^[11] stomach,^[12] pancreatic^[13] and liver^[14] in recent years. Sesquiterpene lactones exhibit anticancer activity mainly by inducing the programmed cell death and inhibiting migration and invasion.^[10–14] However, limited studies have focused on sesquiterpene lactones green

and efficient extraction. Thus suitable processes for the production of high-quality extracts need to be developed.

Extraction is an important stage for obtaining target compounds from medicinal plants, which is affected by many factors and warrants judicious selection of appropriate extraction technologies and extraction processes.^[15] Recently, literatures have reported several techniques for extracting sesquiterpene lactones from *I. racemose*, including soxhlet extraction,^[16] ultrasound extraction^[17–18] and microwave-assisted extraction.^[19] However, the applicability of these methods was compromised due to the poor recovery, sluggishness, high solvent wastage, and the presence of toxic residual solvent in the extract. Supercritical fluid extraction (SFE), an environment-friendly extraction process, is preferred for extracting sesquiterpene lactones due to several process-related advantages including low extraction temperature, short operating time, excellent selectivity and no residual solvents, though high capital investment for the equipment has limited the application of this technique.^[20]

Subcritical water extraction (SWE) was first reported for the extraction of less-polar organic pollutants from the soil by Hawthorne et al. in 1994.^[21] In SWE, as the temperature is elevated from 25 °C to 250 °C at constant pressure (5 MPa), the dielectric constant of water decreases from 80 to 27.^[22] The dielectric constant of water at 250 °C ($\epsilon = 27$) lies between that of ethanol ($\epsilon = 24$) and methanol ($\epsilon = 33$) at 25 °C. As this process allows gradual changes in the polarity of water with temperature, compounds with low polarity can be extracted efficiently. Compared to the conventional methods, SWE exhibited many advantages, such as higher extraction quality, lower solvent costs, and especially no organic solvents use and no toxic solvents residues.^[22–24] The obtained extracts can be directly used as food or pharmaceutical products.^[15] Thus, SWE is a popular 'green' extraction method, widely used in the production of environmental,^[21] herbal^[23] and food samples.^[25–27] However, till to now there was rarely literature

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Table 1. CCD matrix and response values for the recovery of AL&IAL.

Run	Independent variable			Recovery of AL&IAL (%)	
	X ₁	X ₂	X ₃	Experimental (n = 3)	Predicted
1	-1	-1	-1	43.56 ± 1.24	45.66
2	-1.6818	0	0	55.97 ± 0.83	57.12
3	0	0	1.6818	65.53 ± 2.12	66.30
4	1	-1	1	54.47 ± 2.45	56.89
5	0	1.6818	0	56.06 ± 1.73	58.76
6	0	0	0	71.88 ± 0.99	71.46
7	0	0	0	69.54 ± 0.65	71.46
8	0	0	0	74.31 ± 2.42	71.46
9	0	0	-1.6818	61.87 ± 1.69	60.78
10	-1	1	1	63.02 ± 2.09	61.94
11	1	1	-1	66.60 ± 0.74	67.40
12	0	-1.6818	0	43.88 ± 1.41	40.86
13	0	0	0	70.94 ± 1.63	71.46
14	1.6818	0	0	70.11 ± 1.91	68.64
15	1	-1	-1	53.56 ± 1.65	54.87
16	-1	-1	1	53.76 ± 1.13	53.19
17	-1	1	-1	59.58 ± 0.86	57.39
18	0	0	0	73.54 ± 0.93	71.46
19	1	1	1	68.32 ± 1.08	66.44
20	0	0	0	68.47 ± 1.58	71.46

reported on the sesquiterpene lactones extraction by SWE. Furthermore, the effect of extraction parameters on the quality of sesquiterpene lactones is poorly understood.

In the current study, we want to address: (1) the effect of SWE process variables on the recovery of sesquiterpene lactones extracted from *I. racemose*; (2) the pros and cons of SWE compared with traditional extraction technologies; (3) the main compounds of the SWE extract. Extraction experiments were designed using response surface methodology (RSM) with central composite design (CCD) to explore the effect of the extraction parameters (extraction time, temperature, and SW flow rate) on the recovery of the targeted compounds (AL and IAL). The applicability and significance of the model were valued by Analysis of variance (ANOVA). Furthermore, a comparison of the SWE with traditional extraction methods was conducted and the main compounds of the SWE extracts were also purified and identified.

2. Results and discussion

In the current research, the total recovery of AL and IAL (*R*) was calculated by Equation (1).

Recovery of AL & IAL (wt.%)

$$= \left(\frac{\text{Yield of AL\&IAL}}{\text{Total amount of AL \& IAL}} \right) \times 100 \quad (1)$$

The total amount of AL & IAL was obtained via the SE process mentioned in the Supporting Information. The extraction yield was calculated as $4.78 \pm 0.04\%$ w/w of the *I. racemose* roots, which was considered as the total extractable AL & IAL. The yield of AL&IAL was the actual extracted amount by different parameters of SWE or other methods (UAE, SFE).

2.1 Evaluation of RSM model

The central composite design (CCD) developed for the process optimization is presented in Table 1. The regression coefficients of the quadratic model have been shown in Table 2, and the significance of each term was determined by *p*-values. The results demonstrated that all linear and quadratic terms were significant ($p < 0.05$), while all the interactions between the terms were insignificant ($p > 0.05$). Hence, the total recovery of AL & IAL (*R*) after eliminating the insignificant terms can be represented by Equation (2).

$$R = 71.46 + 3.43X_1 + 5.32X_2 + 1.64X_3 - 3.03X_1^2 - 7.65X_2^2 - 2.80X_3^2 \quad (2)$$

The results of the ANOVA of the experimental model have been shown in Table 3. The regression model was found highly significant ($p = 0.000$), while the lack of fit was not significant ($p = 0.131$), implied the high accuracy and reliability. The regression coefficient (R^2) and the adjusted regression coef-

Table 2. Regression coefficients of predicted second-order polynomial model for AL&IAL recovery.

Term	Coefficient	SE Coefficient	t-value	p-value
β ₀	71.4558	1.0759	66.413	0
X ₁	3.4276	0.7139	4.802	0.001
X ₂	5.32	0.7139	7.452	0
X ₃	1.6421	0.7139	2.3	0.044
X ₁ ²	-3.0321	0.6949	-4.363	0.001
X ₂ ²	-7.653	0.6949	-11.013	0
X ₃ ²	-2.7987	0.6949	-4.027	0.002
X ₁ X ₂	0.2012	0.9327	0.216	0.834
X ₁ X ₃	-1.3763	0.9327	-1.476	0.171
X ₂ X ₃	-0.7438	0.9327	-0.797	0.444

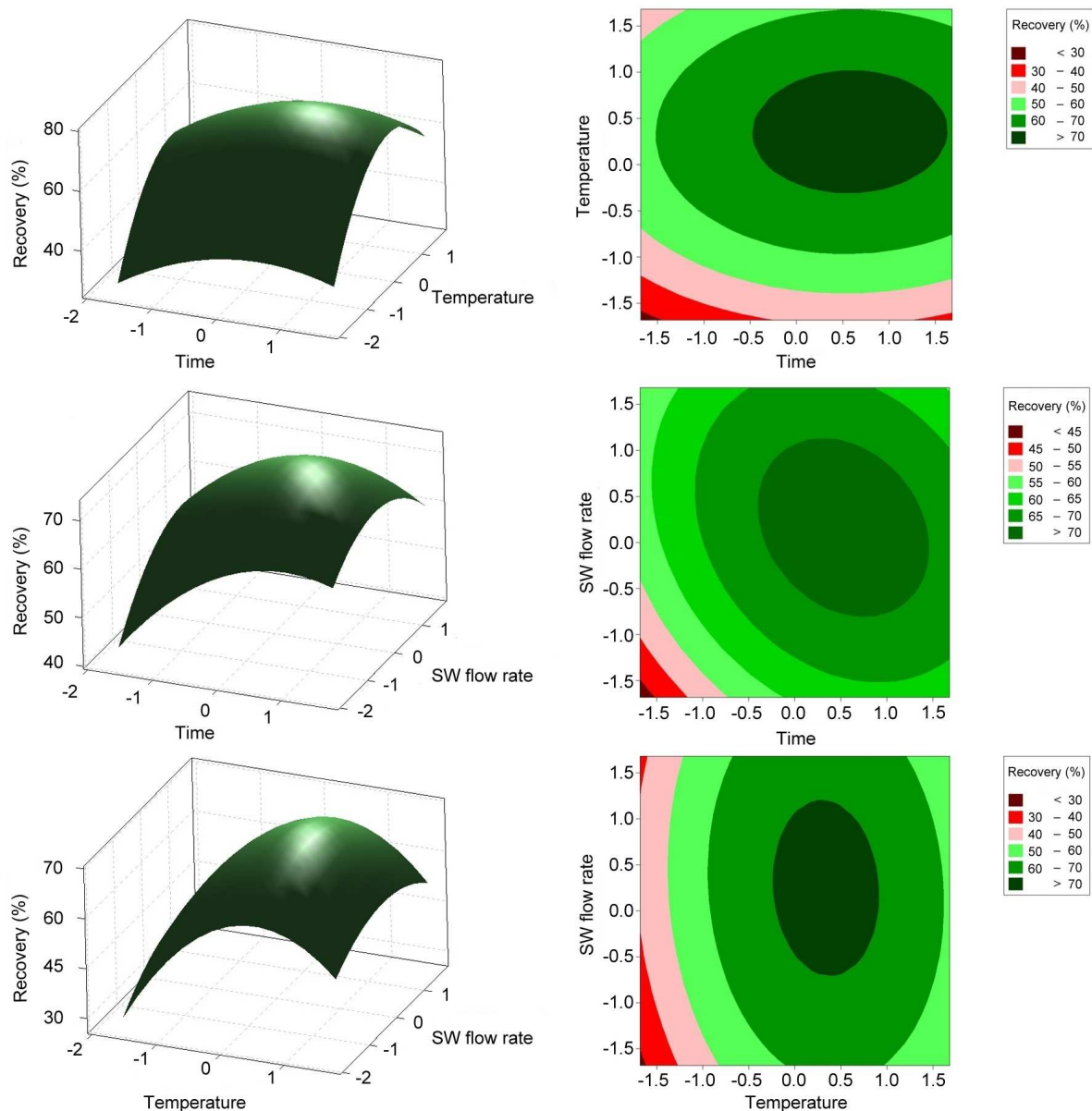


Figure 1. Response surface and contour plots of the target compound yield affected by extraction time, extraction temperature, and SW flow rate. (A) The interactive effect of extraction temperature and time on the AL&IAL yield; (B) the interactive effect of extraction SW flow rate and time on the AL&IAL yield; (C) the interactive effect of extraction SW flow rate and temperature on the AL&IAL yield.

Table 3. Analysis of variance (ANOVA) for the fitted quadratic polynomial model

Source	Degree of freedom	Sum of squares	Mean square	F value	p-Value
Model	9	1572.22	174.691	25.1	0.000
Linear	3	583.79	194.597	27.96	0.000
Square	3	968.53	322.843	46.39	0.000
2-Way Interactions	3	19.9	6.634	0.95	0.452
Error	10	4.43	6.959	–	–
Lack of fit	5	69.59	8.814	1.73	0.282
Pure error	5	44.07	5.104	–	–
Total	19	25.52	–	–	–

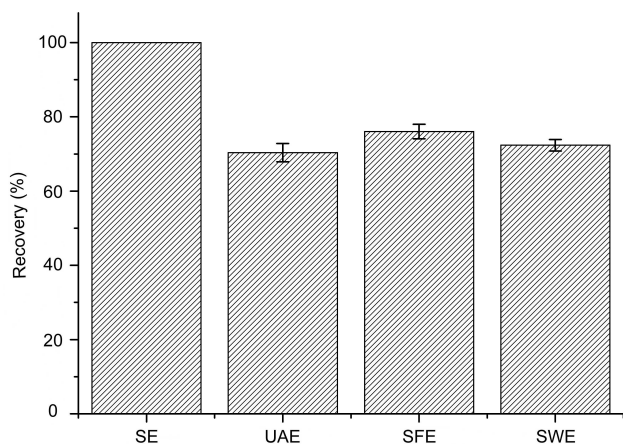


Figure 2. Comparison of the recovery of AL&IAL extracted by different methods. (The recovery of AL&IAL by SE was considered as 100%)

ficient ($Adj.R^2$) were 95.76% and 91.95%, respectively, suggesting the model reflects the actual experimental data appropriately.

The predicted total recovery of AL & IAL was 73.42%, with the examined variables as extraction time, 45.61 min, extraction temperature, 190.70 °C, and SW flow rate, 3.12 mL/min. For the convenience of operation, the actual parameters were adjusted as extraction time, 45 min, extraction temperature, 190 °C, and SW flow rate, 3.0 mL/min. To further verify the accuracy of the model, experiments were carried out based on the adjusted conditions. The average total recovery of AL & IAL was calculated as $71.97 \pm 1.54\%$ ($n=3$), which demonstrated that the model accurately and adequately reflected the expected optimization of the recovery process.

2.2 Effect of operating parameters on the recovery of SWE process

SWE selectivity and extraction efficiency are affected by various parameters, such as pressure, temperature, extraction time and flow rate.^[22–24] Adjustment of pressure can alter the aqueous phase. At the extraction temperature, the pressure is usually maintained between 1 and 8 Mpa for keeping the water in the liquid phase.^[22] However, previous studies revealed that the recovery and extraction efficiency of SWE could not be improved by adjusting the pressure.^[21,24, 28–29] In this study, we maintained the pressure at 5 Mpa through the extraction process for keeping the water in the liquid phase.

Temperature influenced the extraction selectivity and efficiency significantly.^[30–35] High temperatures can change the dielectric constant of water, and enhance the solubility of less polar compounds in water, thus improving the extraction efficiency.^[22,24] However, at the higher temperature, the compounds may be degraded via hydrolysis or oxidation.^[35–37] In this study, the extraction temperature greatly affected the extraction recovery of AL&IAL. The linear and quadratic terms of temperature significantly influenced the response investigated ($p < 0.01$), and a positive linear (5.32) and a negative

quadratic (-7.65) effect on the AL&IAL recovery was found (Table 2). Analysis of the response surface and contour plots (Figure 1), suggested that a higher recovery of AL&IAL can be achieved by raising the temperature, especially at the temperature range of 180–200 °C. According to the previous studies, the extraction of the targeted compounds can be improved at a higher temperature as at elevated temperature the diffusion coefficient of the solvent is improved, facilitating the solvent penetration into the matrix, thereby improving the solubility and diffusion rate of the analytes, and reducing the viscosity and surface tension of the solvent.^[30–35,38] However, in this study, the recoveries of the targeted compounds were observed to decrease at a temperature higher than 200 °C, which may due to the temperature-dependent degradation process.

In the dynamic extraction mode of SWE, the extraction time depended largely on extraction temperature, matrix properties, and target compounds.^[24] As shown in Table 2, the extraction time showed a positive linear effect (3.43) and a negative quadratic effect (-3.03) on AL&IAL recovery. Recovery was significantly improved while prolonging the extraction time (Figure 1). However, the trend was not lasting while further increasing the extraction time. Moreover, prolonging the extraction time would increase the volume of the extraction solvent. A dynamic extraction time of 45 min was sufficient to get the maximum yield.

When the extraction time is kept constant, the amount of extracted solute is proportional to the volume of water passing through the matrix, and hence the SW flow rate.^[24] According to Table 2, the SW flow rate showed a positive linear effect (1.64) and a negative quadratic effect (-2.80) on the recovery. Figure 1 suggests that the recovery of AL&IAL can be improved by increasing the SW flow rate. However, the recovery declined at the SW flow rate faster than 3 mL/min due to the reduced contact time between the solvent and the matrix.^[22,24]

2.3. Comparison of the traditional extraction methods

In this study, four methods (SE, UAE, SFE, and SWE) for AL & IAL extraction from *I. racemose* were compared (Figure 2). SE was the most basic method for extraction natural products from herbal medicine with a high target compound recovery.^[39] Similar to the previous literatures,^[40–42] SE was also found to yield the highest recovery (considered as 100% for the comparison). However, accompanied by this method was the longest extraction time (540 min) and the highest solvent consumption.

Compared to SE, UAE, SFE and SWE were conducted in a shorter time, only 50, 40 and 45 min, respectively. Meanwhile, solvent consumption of the three methods was less than SE. Especially the SFE and SWE consumed no organic solvent, which avoid the solvent residues. UAE yielded the lowest recovery of AL&IAL (70.36%), while SFE and SWE achieved a higher recovery (76.06% and 71.97%, respectively). SFE and SWE both offered advantages in the sesquiterpene lactones extraction with a short extraction time, good recovery and no organic solvent consumption. However, the cost of SWE was

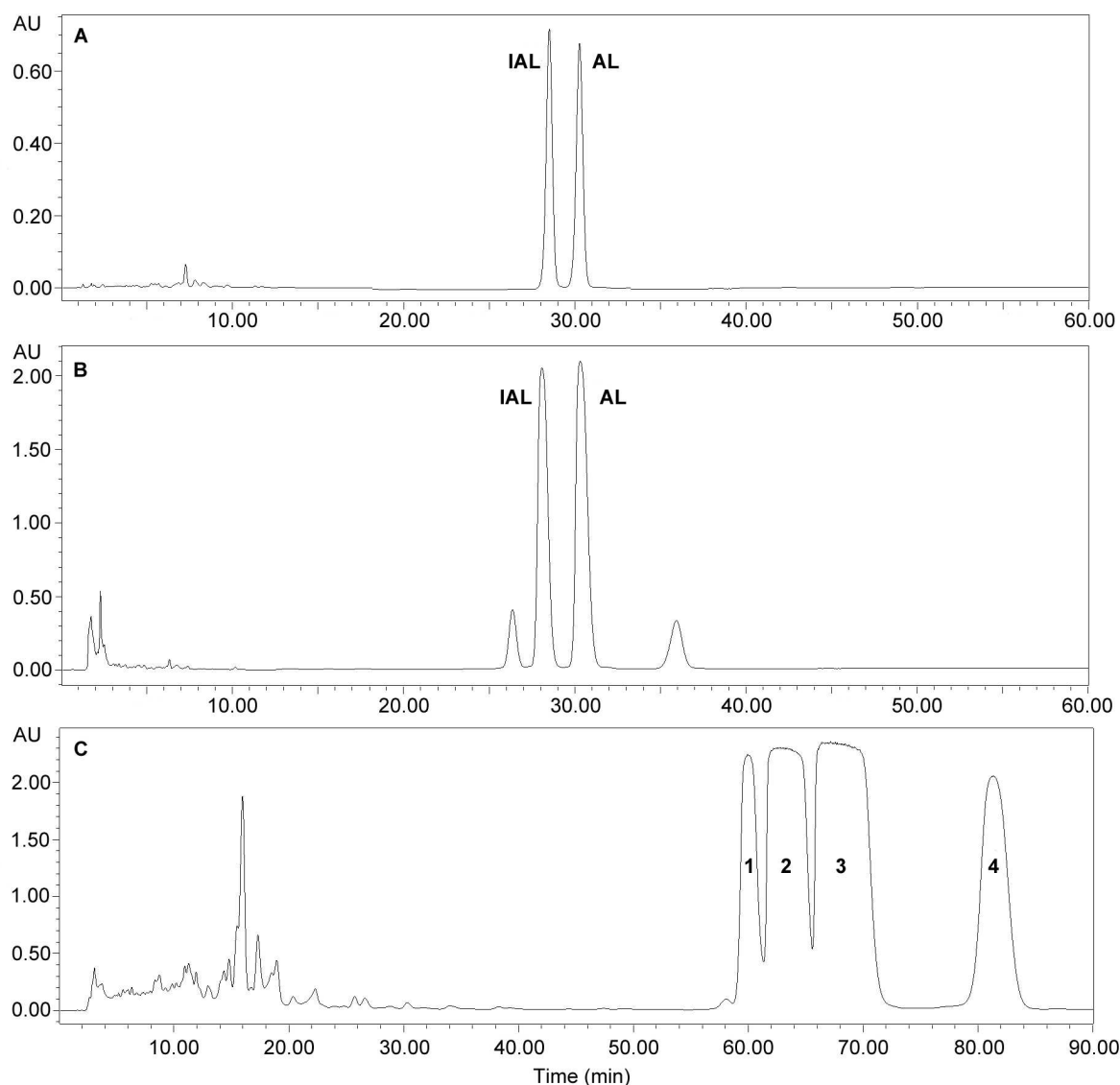


Figure 3. Chromatograms. (A) AL&IAL reference standard;(B) crude extracts of *Inula racemose* by SWE; (C) *prep*-HPLC separation of four sesquiterpene lactone isomers.

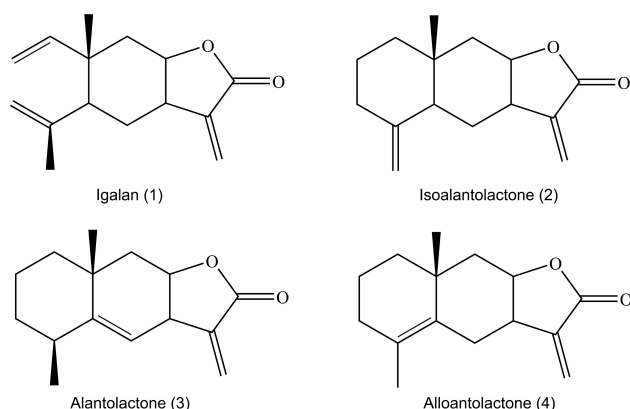


Figure 4. Molecular structures of four sesquiterpene lactone isomers

much lower than SFE. Therefore, SWE seems to have the advantage of a higher extraction efficiency than SE, a higher target compound yield than UAE and more economical than SFE.

2.4 Identification of sesquiterpene lactones

As shown in Figure 3, four main compounds were found in the SWE crude extract of which only AL & IAL could be identified. So, we performed *prep*-HPLC to purify these four compounds. In the *prep*-HPLC separation, the same mobile phase of HPLC analysis was used for a better separation spectrum, and the flow rate of *prep*-HPLC was calculated by linear magnifying methodology using Equation (3)

$$\frac{F_A}{F_P} = \frac{R_A^2}{R_P^2} \quad (3)$$

Where, F_A and F_P stand for the flow rates of the analytical HPLC and the *prep*-HPLC, respectively; R_A and R_P refer to the analytical HPLC and the *prep*-HPLC column diameters, respectively. Hence, 4.7 mL/min was used as the *prep*-HPLC flow rate after calculation.

Targeted compounds were collected using the *prep*-HPLC chromatography (Figure 3), and the structure of each peak fraction was identified by the MS, ^1H -NMR and ^{13}C -NMR data (see Supporting Information). Compared with reference data, peak 1, peak 2, peak 3, and peak 4 were accurately identified as igalan,^[43–44] isoalantolactone,^[45–47] alantolactone,^[45–47] and alloantolactone,^[46,48–49] respectively (Figure 4).

3. Conclusions

As an emerging technology, SWE is increasingly used for the extraction of natural products. SWE utilizes water as the solvent, which is cheap, green and recyclable. In the current research, SWE was employed for isolating sesquiterpene lactones from *I. racemosa*. RSM was introduced to optimize the parameters of SWE (time, temperature, SW flow rate) for higher recovery. The study showed that extraction temperature, extraction time and SW flow rate significantly affected the recovery of the targeted compounds. The maximum recovery was achieved at the following conditions: extraction time of 45 min, extraction temperature of 190 °C, and SW flow rate of 3.0 mL/min. Four sesquiterpene lactone isomers (igalan, isoalantolactone, alantolactone, alloantolactone) were obtained and identified from the SWE extracts. Compared to the traditional methods, SWE can obtain the targeted compounds in a shorter time and at a lower cost, without using toxic solvents. Meanwhile, present work exhibits that SWE combined with the relevant separation technologies can efficiently isolate natural products from the plant sources, which provided a new approach to obtain natural products from herbal plants efficiently.

Supporting Information Summary

The specific experimental procedures are attached in supporting information.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: *Inula racemosa* · Sesquiterpene lactones · Subcritical water extraction

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