Subcritical water extraction of Diosgenin from *Dioscorea nipponica* Makino and its antioxidant activity

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Abstract. Subcritical water extraction (SWE) was used as an alternative and environmentally friendly method for extraction of diosgenin from *Dioscorea nipponica* Makino (DNM). The effects of extraction temperature (75–175 °C) and time (15–45 min) on the diosgenin yield and antioxidant activity of extracts from DNM were analyzed. The profiles of extraction compounds at different temperatures were determined by HPLC. Results showed that diosgenin extraction was significantly (*P* < 0.05) affected by the extraction time and temperature and their interaction. The antioxidant activity was also affected by treatment time and temperature. The high antioxidant capacity was correlated with the high diosgenin content. Subcritical water may be a potential medium for diosgenin production.

1. Introduction

Diosgenin is a kind of important steroidal saponins in *Dioscorea nipponica* Makino (DNM). Diosgenin exhibits therapeutic effects on a variety of pathologies, including metabolic diseases (diabetes, obesity, and dyslipidemia, such as hypercholesterolemia) [1, 2], inflammatory diseases [3], and cancer [4, 5]. Furthermore, diosgenin can be used to synthesize many useful steroid hormones and contraceptive drugs [6]. An efficient method for the preparation of diosgenin from medicinal plants should be established considering that scientific research and clinical application of diosgenin require substantial amounts of diosgenin. Diosgenin exists in plant cells as ligand of steroidal saponins. Several technologies are available for the hydrolysis of steroidal saponins into diosgenin. The most common production method is acid hydrolysis of steroidal saponins from plants directly. Acid hydrolysis can produce high yields of hydrolysate, but, the heavy consumption of inorganic acids and organic solvents results in severe environmental pollution [7]. To overcome the drawbacks of this method and obtain a remarkable environmentally friendly process, enzymatic hydrolysis is extensively studied [8]. High hydrolysate yields can be obtained by using enzymatic hydrolysis. However, to date, the economic feasibility of this process is uncertain. Other potential techniques include microbial transformation [9], microwave-assisted extraction [10], and a saccharification–membrane retrieval–hydrolysis process [11]. All these methods exhibit advantages and disadvantages, but they are all still under initial stage of development.

In recent years, many studies reported that subcritical fluid, a method widely used to extract compounds from biological substrates [12]. In this process, the high temperature and high pressure
change the dielectric constant (polarity) to a value similar to that of the organic solvent, so that a green solvent can be effectively used to extract various compounds [13].

In particular, subcritical water, an environmentally friendly solvent, used as a substitute for organic solvents. The results indicate that subcritical water had significant effects on the chemical and physical properties, functional properties and biological properties of the extract [14]. In addition, it can degrade (hydrolyze) biomass (with or without acid catalyst) to value-added products [15, 16]. Therefore, it is effective to increase the hydrolysis process when using pressurized solvents under high temperature. Many changes in water properties occur at elevated temperature, like a decline in polarity, viscosity, and surface tension [17]. The self-ionization property of water also changes drastically at increasing temperature. The ionic produce of water in subcritical region is considerably high. Under high temperature and pressure, subcritical water is corrosive as an acid/base catalyst that can degrade or hydrolyze many organic compounds [18]. Such properties indicated that subcritical water technology can be an alternative route for bioactive compound extraction and biomass hydrolysis. Nevertheless, there is a decreasing yield because the thermally unstable bioactive compounds decompose during subcritical water extraction (SWE) or hydrolysis under high temperatures. Hence, the most efficient conditions into aglycone form should be determined and optimized to minimize degradation of for the hydrolysis of steroidal saponins.

The present work used subcritical water in hydrolyzing dioscin without the use of an acid catalyst. The effect of temperature and extraction time on the yield of diosgenin was investigated in this study. The stability and antioxidant activity of the diosgenin extracted were further determined by the SWE from DNM.

2. Materials and methods

2.1. Materials
DNM samples were purchased from a local drugstore in Qingdao. The samples were cleaned, ground to a particle size of 1-5 mm that used a high-speed mixer (Q6J3-W1000A, Taisite Instrument Co., Ltd., Tianjin, China), and stored in air-tight containers in refrigerator until further use. Diosgenin (≥98%) was purchased from Weikeqi Biological Technology Co., Ltd. (Sichuan, China). DPPH free radical was obtained from Sigma-Aldrich (St. Louis, MO, USA), and HPLC-grade methanol was purchased from Merck (Germany).

All other reagents were of analytical grade and used without further purification. Deionized water was used throughout the experiments.

2.2. Subcritical water hydrolysis
The SWE of diosgenin was operated in batch-type high-pressure extractor, which was assembled in our laboratory. In this system, the extraction was conducted in a 1.5 L double-jacketed stainless-steel vessel (108 mm in internal diameter and 150 mm in useful height), equipped with a thermal probe and a pressure control valve. The medium temperature was maintained at a constant level by circulation of heat transfer oil in the double jacket of the extractor and controlled by a PID regulator (Hongrun Precision Instruments Co., Ltd., Fujian, China). The pressure was introduced to the extraction vessel by an air compressor.

In all experiment runs, 20.0 g of samples and 200 mL of deionized water were introduced into the extractor. The extraction was conducted at different temperatures and times, but the pressure inside the extractor was held constant at 1.0 MPa. After treatment under control statuses, the extractor was speedily cooled to room temperature, and air was discharged from the extractor. A sample aliquot was collected and diluted with methanol to a concentration that allowed its analysis. The prepared solution was filtered by a 0.45μm Millipore filter and injected into HPLC immediately to analyze the target compound content and extract composition. The prepared solution was filtered by a 0.45 μm Millipore filter and injected into HPLC immediately to analyze the target compound content and extract composition.
2.3. **HPLC analysis**

Analysis of diosgenin and other hydrolyzed products was carried out on the suspension that was obtained after subcritical water treatment. The DIONEX U300HPLC system consisted of a binary pump, an autosampler, a column heater, and a variable wavelength detector. The analysis was executed on a Hypersil ODS C18 analytic column (200 mm×4.6 mm, 5 μm). The column temperature was 35 °C. Elution was carried out with 70% methanol and 30% water. Mobile phase's flow rate was 1.0 mL/min. The detection wavelength was 210 nm. Diosgenin was quantified based on the calibration curves obtained with standards. Diosgenin yield (DY) was expressed as g of diosgenin per 100 g dry weight.

2.4. **DPPH radical scavenging activity (DPPH-RSA)**

The assay for free radical scavenging capacity was conducted according to the method previously reported by Blois [19] with some modifications. In brief, 1.5 mL of each sample extract was mixed with 2.5 mL of 0.018 mg/mL methanolic solution of DPPH. The mixture is placed in the dark at room temperature for 30 minutes, then under 517 nm by measuring the absorbance blank as a control. All measurements were performed in triplicate. The percentage of DPPH-RSA was calculated using the following equation:

\[
\text{DPPH scavenging activity (\%) = (Ac – As) / Ac \times 100,}
\]

where As is the absorbance of the test sample, and Ac is the absorbance of the control (contained extraction solvent instead of the sample).

2.5. **Ferric-reducing power (FRP)**

The FRP of all samples was determined through utilizing the potassium ferricyanide–ferric chloride method [20]. Approximately 1.0 mL of sample (2.5 mg/mL) was mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide. The mixtures were incubated at 50 °C for 25 min, and 2.5 mL of 10% (w/v) trichloroacetic acid was added. Afterward, 2.5 mL of the mixture was obtained and thoroughly mixed with 2.5 mL of distiller water and 1.0 mL of 0.1% FeCl₃ (w/v). The intensity of blue green color was gauged at 700 nm under blank control. The blank samples were prepared for the same statuses without any extract's addition. Increased absorbance of the reaction mixture that was indicated increased reducing power. The values are presented as the means of triplicate analyses.

2.6. **Experimental design and statistical analysis**

Response surface methodology (RSM) was used to evaluate the impact of treatment time and temperature on the DY of extracts from DNM. The obtained data were dealt with the conditions that were described in Section 2.2 and modeled with the following equation:

\[
R_i = \beta_0 + \beta_1 T + \beta_2 t + \beta_3 Tt + \beta_4 t^2 + \beta_5 T^2
\]

where Ri is the response representing DY; T and t are independent factors representing temperature and time; \(\beta_0\) is the constant; and \(\beta_1, \beta_2, \beta_3, \beta_4, \beta_5\) are regression terms.

The model and 3D surface response plots are expressed as fitted polynomial regression equations with the software package Design-Expert 6.0.6 (Stat-Ease Inc., Minneapolis, MN, USA). Experiments were performed in triplicate, and results are presented as means ± standard deviation.

3. **Results and discussion**

3.1. **HPLC profiles of extracts obtained from DNM**

The HPLC profiles of extracts obtained at five different temperatures (75 °C, 100 °C, 125 °C, 150 °C, and 175 °C) for 45 min are presented in Fig 1. The retention time of diosgenin was approximately 12.66 min (Fig 1a). The chromatograms of the extraction samples obtained at different temperatures are shown in Fig. 1b–1f. Although the number and height of peaks were different based on their treatment temperatures, all the samples obtained at all operational temperatures were composed of
diosgenin. Notably, the extracts obtained at 100 °C and 125 °C contained large proportions of diosgenin. With increased temperature, the diosgenin content deceased, and the contents of other compounds began to increase. The results indicate that, in addition to diosgenin, other hydrolysates were produced in subcritical water as temperature changes.

Glycosides are unstable at elevated temperatures and long extraction times in the SWE. For instance, hydro thermolysis of steroid saponins occurs to heating in water at temperature higher than 150 °C [21] or at lower temperature (100 °C-140 °C), but for with the extension of time (17-90 h) [22]. According to current research, dioscin hydrolysis occurred at relatively low temperature and short time of 100 °C and 45 min, respectively. This result is probably due to that the rhamnose of dioscin possesses a deoxy sugar molecular structure, which is unstable and easily degraded in the SWE [23].

3.2. Effect of treatment temperature and time
The effects of temperature and time were investigated at five different temperatures (75 °C, 100 °C, 125 °C, 150 °C, and 175 °C). The time was held constant at 15, 30, and 45 min for each experiment. Experimental results of the total extraction yield (TEY), DY, DPPH-RSA assay, and FRP obtained under different SWE conditions are presented in Table 1.
Table 1. Effects of subcritical water extraction on total extraction yield (TEY), diosgenin yield (DY), DPPH radical scavenging activity (DPPH-RSA), and ferric-reducing power (FRP).

| Temperature (°C) | Time (min) | TEY (%) | DY (%)  | DPPH-RSA (%) | FRP (OD)  |
|------------------|------------|---------|---------|--------------|-----------|
| 75               | 15         | 9.7±0.5 | 1.19±0.38 | 79.63±0.79  | 0.494±0.033 |
| 25               | 11.2±1.2   | 1.81±0.63 | 87.97±1.57  | 0.427±0.036 |
| 45               | 14.3±1.0   | 2.81±0.42 | 50.83±1.54  | 0.349±0.019 |
| 100              | 15         | 10.2±0.7 | 1.56±1.23 | 82.27±2.35  | 0.413±0.002 |
| 25               | 13.1±1.7   | 2.30±0.42 | 91.92±0.79  | 0.396±0.057 |
| 45               | 15.6±0.6   | 3.11±0.26 | 89.15±1.58  | 0.352±0.032 |
| 125              | 15         | 10.7±0.8 | 3.85±0.51 | 95.07±2.90  | 0.513±0.089 |
| 25               | 13.6±1.3   | 3.45±0.20 | 93.26±1.02  | 0.444±0.009 |
| 45               | 16.3±0.9   | 3.31±0.35 | 75.24±1.34  | 0.402±0.005 |
| 150              | 15         | 11.3±2.3 | 2.82±0.09 | 87.32±0.49  | 0.442±0.071 |
| 25               | 14.2±1.7   | 2.80±0.34 | 84.31±0.73  | 0.412±0.071 |
| 45               | 16.8±1.6   | 2.07±0.45 | 66.02±1.34  | 0.397±0.038 |
| 175              | 15         | 13.5±2.1 | 2.79±0.58 | 71.35±1.05  | 0.430±0.093 |
| 25               | 16.4±0.7   | 1.69±1.26 | 56.09±1.49  | 0.345±0.001 |
| 45               | 18.9±0.4   | 1.40±0.72 | 37.97±1.90  | 0.316±0.023 |

TEY: weight of extracts (g)/dry weight of samples (g)×100%.

3.2.1. Effects of temperature and time on TEY. The results showed, an increase in TEY, as the extraction temperature and time increased. The highest yields reached 18.9% at 175 °C and 45 min. Thermal energy heightened the extraction efficiency by increasing the dissolution capacity of the solvent system and disrupting the plant matrix interactions as a result of the van der Waals forces [24, 25]. Additionally, other plant ingredients, such as starch and proteins, might be thermally degraded in the state of increased temperature. The reason for high TEY obtained at high temperatures may be partly due to the high solubility of the decomposition compounds in SWE [26].

3.2.2. Effects of temperature and time on DY. As shown in Table 1, the DY increased with increasing temperature and reached the maximum of 3.85% at 125 °C and 30 min. With further increased temperature, the yield began to decrease significantly at three different treatment times. This decrease was most probably in part due to the thermal degradation of dioscin to other compounds when the extraction temperature exceeded 125 °C. This result is shown in the HPLC profiles in Fig 1. The extraction rate was 64% lessen for anthocyanins extracted from red grape skins, when the temperature increases from 110 °C to 160 °C for simple flavonoids degraded in water [27]. Therefore, the mass transfer and solubility enhancement impacts linked to SWE extraction will rise with temperature and bring about high extraction productions. Moreover, the extraction efficiency might be adversely impacted if thermal degradation exists on elevated temperatures. The impact on extraction time was observed at three dissimilar time spans.

As shown in Table 2, the highest DY was obtained at 125 °C, but no significant changes were observed throughout the entire treatment time range of 15–45 min. At 75 and 100 °C, the increase of time from 15 min to 45 min resulted in an increase in the DY. However, when the extraction was performed at high temperatures (150 and 175 °C), the yields were decreased as a role of time. Consequences indicated that time exerted an important influence on diosgenin's establishment. The importance of organizing the experimental design in time ranges and a carefully selected and temperature were also indicated by this result.
Table 2. ANOVA analysis for the model developed to describe the influence of temperature (T) and time (t) on the diosgenin extraction yield from *Dioscorea nipponica* Makino.

| Source        | df | Sum of squares | Mean square | F value | P value | Significance |
|---------------|----|----------------|-------------|---------|---------|--------------|
| Model         | 5  | 1.31           | 0.26        | 141.88  | <0.0001 | ***          |
| t             | 1  | 0.011          | 0.011       | 5.75    | 0.0477  | *            |
| T             | 1  | 0.018          | 0.018       | 9.83    | 0.0165  | *            |
| t × T         | 1  | 0.14           | 0.14        | 77.96   | <0.0001 | ***          |
| t²            | 1  | 0.39           | 0.39        | 213.38  | <0.0001 | ***          |
| T²            | 1  | 0.88           | 0.88        | 475.09  | <0.0001 | ***          |
| Residual error| 7  | 0.013          | 1.842E-003  |         |         |              |
| Lack-of-fit   | 3  | 7.164E-003     | 2.388E-003  | 1.67    | 0.3099  | no           |
| Pure error    | 4  | 5.733E-003     | 1.433E-003  |         |         |              |
| Total         | 12 | 1.32           |             |         |         |              |

***very significant difference (*P* < 0.001); **highly significant difference (*P* < 0.01); *significant difference (*P* < 0.05); $R^2 = 0.9902$, $R_{adj}^2 = 0.9833$.

3.2.3. Effects of temperature and time on antioxidant activity. Considering the complex reactive activity, plant extracts' antioxidant activities cannot be evaluated through exclusively a single method. Hence, DNM extracts' antioxidant activity was determined by two different ways. The DPPH-RSA and RP values of the SWE extracts of DNM are presented in Table 1. The extracts' DPPH-RSA values also rose when the time and temperature increased; this trend was almost the same as that of diosgenin content. Although determining a simple trend is difficult, the SWE extracts at 125 °C for 15 min showed the highest FRP value. These consequences suggested that time and temperature influenced the antioxidant activities of the SWE extracts of DNM significantly. Moreover, the diosgenin content influenced the antioxidant activities of DNM extracts. Although little information is available about the direct antioxidant activity of diosgenin, Ghosh *et al.* [28] reported that the extracts of *Dioscorea bulbifera*, with diosgenin as major compound, display brilliant antioxidant and rid radical scavenging activities; this species can also be exploited as an expected source of herbal therapy for oxidative stress-induced diseases. Son *et al.* [29] found that diosgenin feeding to enhance the resistance to lymphocyte DNA damaged caused by an oxidant that is challenged with $H_2O_2$; the antioxidative enzyme activities are also influenced with diosgenin. Their results suggested that diosgenin might be a substantially helpful chemical compound to contain hypercholesterolemia through both modulating oxidative stress and improving the lipid profile. Our finding agrees with these reports.

3.3. Effect of the interaction between temperature and time on diosgenin extraction

SWE was performed at temperatures between 75 °C and 175 °C and processing times of 15−45 min using other preliminary experiments to access the impacts of both the independent variable and the interaction between them on the extraction procedure. Multiple regression analysis on according to the experimental data, the model for the predicted response could be expressed by the following quadratic polynomial equations (in the form of coded values):

\[
\text{Diosgenin yield (\%)} = 3.36 + 0.036 \, t + 0.048 \, T + 0.19 \, tT - 0.24 \, t^2 - 0.35 \, T^2.
\]
Figure 2. Response surface plot (a) and contour plot (b) of the diosgenin yield affected with temperature and time.

The regression equations for the DY ($P < 0.001$) was extremely significant, which demonstrated that the degree of fit was good on the border of the independent variables. Additionally, the quality of the regression equation was confirmed by the coefficient $R^2$ (0.9902). The ANOVA of the experimental results is presented in Table 2. The effects of time and temperature ($P < 0.05$) on the DY were significant. However, their interaction and the square terms of the temperature and time were highly significant ($P < 0.0001$). In addition, the linear term exhibited positive influence, whereas the square term showed a negative one, as indicated by the optimum value for temperature and time; higher than this value, the time raise or temperature exerted no substantial raise on the DY (Fig 2). In Fig 2, the response surface is represented with a 3D plot for the yield versus the extraction temperature and time.

4. Conclusion
As a green extraction method, subcritical water extraction requires less time and reduces the use of organic solvents and energy. Subcritical water was used to extract diosgenin from DNM. Results showed that the effects of temperature, time, and their interaction on the DY were significant. The optimal parameters for extracting diosgenin were 127 °C for 31 min. Temperature and time also affected the antioxidant activity of the SWE extracts of DNM. This research offered a reference for selecting the optimum condition for diosgenin extraction using subcritical water as solvent. This study also provided a valuable information regarding the hydrothermal degradation of dioscin from DNM.

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