Green production of diosgenin from *Discorea nipponica* Makino tubers based on pressurized biphase acid hydrolysis via response surface methodology optimization

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ABSTRACT
In this study, a novel strategy was established to produce diosgenin from *Discorea nipponica* Makino tubers, in which pressurized biphase acid hydrolysis and Soxhlet extraction of total saponins were incorporated for the first time. The hydrolysis conditions were optimized by response surface methodology after the preliminary investigation on affecting factors by single-factor experiments and L\(_{16}(4^5)\) orthogonal experiments. Under the optimal conditions, namely dioscin (eq. 8 g of the DNM tubers) was hydrolyzed in a biphase system composed of 50 mL of 6 \(\mu\)L/mL H\(_2\)SO\(_4\) solution and 50 mL of petroleum ether (90–120\(^\circ\)C) at 140\(^\circ\)C for 2 h, the highest yield of diosgenin has been achieved at 1.87\%, that was 85.1\% higher than the conventional method while the H\(_2\)SO\(_4\) consumption reduced by 92.5\%. The results demonstrated that the newly developed approach was more effective and cleaner, and promising value-added technology for the extraction of diosgenin from DNM tubers in industrial application.

1. Introduction
Diosgenin is the most preferred precursor for the commercial synthesis of cortisone, pregnenolone, progesterone and many other steroid drugs in the pharmaceutical industry (1). Diosgenin also shows a wide range of bioactivities including against diabetics (2,3), cancer (4,5), thrombosis (6), inflammation (7) and Alzheimer's disease (8), as well as the functions to regulate renal proximal tubular fibrosis (9), relieve goiters (10) and improve vascular function (11). As an important medicinal compound, more than 4000 tons of diosgenin are demanded annually in the world (12).

Diosgenin naturally exists in tubers of many *Dioscorea* or *Costus* genus plants and seeds of *Trigonella foenum-graecum* L., but it presents in the form of saponins where glucose (glc) or/and rhamnose (rha) is attached to the aglycone by C–O glycosidic bonds (cf. Figure 1)(13). Among them, *Discorea nipponica* Makino (DNM), a tuberous herbaceous perennial liana, is widely used as materials for diosgenin production in industries (14).

There were several conventional methods applied to release diosgenin from the DNM tubers, which caused severe pollution problems. For instance, direct acid
hydrolysis (15) and biphase acid hydrolysis (16,17) were developed. However, these methods were associated with intrinsic drawbacks such as high consumption of inorganic acid, low yield of diosgenin and serious organic pollution from concurrent hydrolysis of abundant polysaccharides. Therefore, many attempts have been made to develop cleaner processes of diosgenin production in recent decades. Some strategies including biotransformation (18), enzymic hydrolysis (19), physical separation (20), ultrasonic-assisted hydrolysis, low-pressure steam expansion pretreatment (21,22) and waste water treatment (23,24) were investigated to reduce acid consumption and pollution. Recently, the biotransformation and enzymatic hydrolysis are becoming increasingly attractive because enzymes replaced acid to release diosgenin, but low efficiency and tedious procedures limit practical application of the approaches in industries.

In our previous work, a novel method based on pressurized biphase acid hydrolysis (PBAH) (25) after the physical separation of cellulose and starch was established. By this strategy, H₂SO₄ consumption and organic pollution in waste water were largely reduced and the yield of diosgenin was greatly increased compared with conventional methods (cf. Figure 2). It suggested that the removal of polysaccharides from raw material before acid hydrolysis can contribute to moderation of pollution from the processes of diosgenin production. However, the established approach cannot be arbitrarily applied in the diosgenin production from the tubers of DNM.

In this study, a novel process was established to produce diosgenin cleanly by combining the pressurized biphase acid hydrolysis with Soxhlet extraction of total saponins dioscin from the DNM tubers (cf. Figure 3). To develop a green approach with higher diosgenin yield and lower pollution than conventional methods, the response surface methodology (RSM) was used to optimize hydrolysis conditions.
2. Material and methods

2.1. Material and reagents

The fresh tubers of *Discorea nipponica* Makino were harvested in June 2016, from the Changbai mountains in Huadian, Jilin Province, People Republic of China. A voucher specimen (DNM160901) has been deposited and authenticated by Prof. Jun Chen, Pharmacognosy Research Center, the School of Pharmacy, Jiangsu University, Zhenjiang, Jiangsu Province, People’s Republic of China. After cleaning with water, the tubers were cut to thin slices and then stored in a freezer at −70°C.

GSH-0.5 high-pressure reaction kettle coated with polytetrafluoroethylene (PTFE) film on the surface of inner vessel was supplied by Weihai Kun Chang Chemical Machinery Co., Ltd., China. The Alpha 1-2LD lyophilizer was provided by Christ, Germany. Acetonitrile (ACN) of HPLC grade was purchased from Honeywell, Korea. Diosgenin standard (purity > 98.0% by HPLC-UV, B/N: 20120828) was provided by Gold Wheat Biotechnology Co., Ltd., China. All other chemicals used in the study were of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd., China.

2.2. Soxhlet extraction of dioscin

Before acid hydrolysis, total saponins were extracted from dry powder of the DNM tubers. In details, tuber slices were subjected to lyophilization and then were ground. The fine powder (<40 mesh) was collected and stored in a dry cabinet at room temperature. In total 26.7 g of powder was accurately weighed, followed with Soxhlet extraction with 400 mL of 70% ethanol in boiling water bath for 24 hrs. The ethanol in the obtained solution was recycled at 50°C under reduced pressure condition, and the concentrated extract was transferred into a 100-mL volumetric flask and diluted to scale with distilled water.

2.3. HPLC conditions and calculations

Quantitative analysis of diosgenin was performed on a Shimadzu Prominence HPLC instrument, Japan. Data acquisition and processing were conducted by N2000 SP1 software (Zhejiang University, China). Twenty-micro liters of sample solution or standard solution was loaded on a Waters SunFire RP18 column (150 mm × 4.6 mm, 5 μm, Ireland), eluted by mobile phases consisting of ACN and water (85:15, v/v). A flow rate of 1.0 mL/min and the column temperature at 35.0°C was kept constant throughout the HPLC analysis. The UV detection wavelength was set at 203 nm.

In total 15.50 mg diosgenin was dissolved in 10 mL of EtOH, and a series of dilution was performed. Then the calibration curve was constructed within the concentration ranging from 1.211 × 10⁻² to 1.550 mg/mL. Before manual injection, all sample solutions were filtered through a 0.45 μm PTFE membrane. Peak area (Y) versus concentration of diosgenin (X) was plotted to obtain correlation equation (26).

The diosgenin concentration (C) of the sample solution was calculated according to the calibration curve based on peak area, and the diosgenin yield (Z%) was calculated by the following equations:

\[ Z_1\% = \frac{(C \times 100 \text{ mL})}{1000 \times \text{Sample quantity}} \times 100\% \]

\[ Z_2\% = \frac{(C \times 250 \text{ mL})}{1000 \times \text{Sample quantity}} \times 100\% \]

\[ I\% = \frac{(Z_1 - Z_2)}{Z_2} \times 100\% \]

Z₁, Z of proposed strategy and Z₂: Z of the conventional method.

2.4. Conventional method

To evaluate the newly established strategy in this study, a conventional method was carried out for comparison. Specifically, DNM tubers powder (10 g) was hydrolyzed in 60 mL of water containing 5 mL of H₂SO₄ for 3.0 h,
and the sample after hydrolysis was filtered and the residue was washed using 100 mL of hot water. Then, the residue was dried at 80°C and then extracted in a Soxhlet extractor using 80 mL of petroleum ether (90–120°C) for 4 h. The extraction solution was transferred into a 250-mL volumetric flask, and the petroleum ether was added to the scale line.

2.5. PBAH for diosgenin

Thirty milliliters of dioscin solution (8 g of dry DNM tubers) and 20 mL of H2O were added into a high-pressure reaction kettle, followed by adding 200 µL of H2SO4 and 50 mL of petroleum ether (90–120°C). Hydrolysis was reacted at 150°C for 2.0 h with constant stirring at 100 rpm, and the inner pressure was ca. 0.5 MPa during the hydrolysis.

After the acid hydrolysis, the reaction system was cooled down to room temperature by tap water, and two phases were transferred into a separation funnel. Then, the water phase was discarded and the petroleum ether phase was neutralized by washing with water, and the petroleum ether phase was transferred into a 100 mL volumetric flask and added more petroleum ether to scale line.

2.6. Preparation of sample solutions

Ten milliliter of the above solution was pipetted into a round bottom flask and the petroleum ether was removed at reduced pressure below 50°C. The residue was dissolved in ethanol with 10 mL, which was filtered through a 0.45 μm membrane before quantification using HPLC.

2.7. Optimization of PBAH conditions

2.7.1. Single factor experiment

Four varieties including temperature (100–150°C, 4 g, 4 µL/mL, 2 h), tubers amount (140°C, 1–8 g, 4 µL/mL, 2 h), acid concentration (140°C, 6 g, 2–8 µL/mL, 2 hrs) and hydrolysis duration (140°C, 6 g, 4 µL/mL, 0.5–2.0 h) were investigated accordingly by single factor experiment, and each experiment was performed in triplicates. The results from the single factor experiment were used for the orthogonal design.

2.7.2. Orthogonal experiment design

Based on the results of the single factor experiment, an orthogonal experiment design L16(4)5, showed in Table 1, was used to further optimize and to investigate significance of the four factors on the diosgenin yield.

2.7.3. Design of RSM

RSM was applied to optimize the extraction condition of diosgenin. The experiment was designed using the Box–Behnken Design (BBD) based on three-level three factor by the Design-expert 8.0.6 software. The yield of diosgenin was used as index for the optimization. Based on the results from single factor and orthogonal experiments, total saponins (8 g of the DNM tubers) in 50 mL of H2SO4 solution and 50 mL of petroleum ether was fixed. And as shown in Table 2, three other varieties including temperature (X1, °C), extraction duration (X2, h), and acid concentration (X3, µL/mL) were selected for further investigation. Lower, middle and higher value of three levels were presented using variable code (−1, 0, 1). The regression coefficients were obtained by fitting to the second-order polynomial model based on the experimental data. The generalized second-order polynomial model used in the response surface analysis as follows:

\[ Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j}^{k} \beta_{ij} X_i X_j \]

where Y is the diosgenin yield, and \( \beta_0, \beta_i, \beta_{ii} \) and \( \beta_{ij} \) are there regression coefficients for intercept, linearity, square and interaction. \( X_i \) and \( X_j \) are the independent coded variables \( (i \neq j, i \ and \ j \ range \ from \ 1 \ to \ k) \), and \( k \) is the number of tested variables \( (k = 3) \).

3. Results and discussion

3.1. Single factor experiments

3.1.1. Temperature

As shown in Figure 4(A), the yield of diosgenin reached the highest point at 140°C. When the temperature was
increased to 150°C, the yield decreased, which suggested that the kinetic energy of molecule and the diffusion rate were increased with the temperature escalated so that more reactant molecules would collide each other per unit volume and the dioscin hydrolysis reaction was promoted (27). However, the rate of side reactions would increase and the chemical structure of diosgenin might be destroyed when reacting at a higher temperature.

3.1.2. Tubers amount
As shown in Figure 4(B), the yield of diosgenin kept stable at a high level when the tubers amount was between 3–7 g and it reached the highest while the saponins from 6 g of the DNM tubers were hydrolyzed in 4 μL/mL H₂SO₄ solution for 2 h. Low tubers amount (< 3 g) did not result in a higher yield as the saponins of small amount could have been exposed to too many H⁺ ions that can damage the product. On the other hand, high tubers amount (>7 g) is also not preferred as it could have caused an incomplete hydrolysis.

3.1.3. Acid concentration
The results were illustrated in Figure 4(C). When the acid concentration was between 4 and 8 μL/mL, the yield of diosgenin remained at a stable and high level. And, the highest yield has been achieved while the hydrolysis was carried out in 4 μL/mL H₂SO₄ solution. And the yield of diosgenin was much lower when the acid concentration was less than 4 μL/mL, implying an incomplete hydrolysis of dioscin.

3.1.4. Hydrolysis duration
It can be seen from Figure 4(D), the yield of diosgenin has been rapidly increased from 1.5% to 1.7% with the hydrolysis lasted from 0.5 to 1 hr. Then, it stayed at this high level and the highest yield was achieved while the saponins were hydrolyzed for 2.0 h. This indicates that dioscin has been completely converted to the desired diosgenin within 2 h, with the dioscin was continuously consumed, the rate of hydrolysis and the yield of diosgenin were both increased slowly. Simultaneously, due to the accumulation of diosgenin, the rate of side reactions to deoxygenating diosgenin was also increased (27). An extended hydrolysis for half an hour could not achieve a higher yield but even might have led to the destruction of diosgenin.

3.2. Orthogonal analysis
The result of the L₁₆(4)⁵ orthogonal experiments was summarized in Table 3, and range analysis was carried out using diosgenin yield as evaluation index. It can be
Table 3. Result and analysis of the orthogonal experiment.

| No. | A (°C) | B (g) | C (µL/mL) | D (h) | E (blank) | Yield of diosgenin (%) |
|-----|--------|-------|-----------|-------|-----------|------------------------|
| 1   | 120    | 5     | 2         | 0.5   | 1         | 1.11                   |
| 2   | 120    | 6     | 3         | 1.0   | 2         | 1.40                   |
| 3   | 120    | 7     | 4         | 1.5   | 3         | 1.77                   |
| 4   | 120    | 8     | 5         | 2.0   | 4         | 1.70                   |
| 5   | 130    | 5     | 3         | 1.5   | 4         | 1.64                   |
| 6   | 130    | 6     | 2         | 2.0   | 3         | 1.55                   |
| 7   | 130    | 7     | 5         | 0.5   | 2         | 1.59                   |
| 8   | 130    | 8     | 4         | 1.0   | 1         | 1.68                   |
| 9   | 140    | 5     | 4         | 2.0   | 2         | 1.67                   |
| 10  | 140    | 6     | 5         | 1.5   | 1         | 1.69                   |
| 11  | 140    | 7     | 2         | 1.0   | 4         | 1.27                   |
| 12  | 140    | 8     | 3         | 0.5   | 3         | 1.57                   |
| 13  | 150    | 5     | 5         | 1.0   | 3         | 1.39                   |
| 14  | 150    | 6     | 4         | 0.5   | 4         | 1.70                   |
| 15  | 150    | 7     | 3         | 2.0   | 1         | 1.79                   |
| 16  | 150    | 8     | 2         | 1.5   | 2         | 1.58                   |

\[ k_1 = 1.492 \times 1.452 \times 1.377 \times 1.493 \times 1.568 \]
\[ k_2 = 1.615 \times 1.585 \times 1.600 \times 1.435 \times 1.560 \]
\[ k_3 = 1.550 \times 1.605 \times 1.705 \times 1.670 \times 1.570 \]
\[ k_4 = 1.615 \times 1.630 \times 1.590 \times 1.675 \times 1.575 \]
\[ R = 0.123 \times 0.186 \times 0.328 \times 0.240 \times 0.015 \]

R refers to the result of extreme difference.

seen that the influence on the yield of diosgenin decreased in the order: C > D > B > A according to the R values of each factor. Variance analysis was also performed, and as shown in Table 4, four factors could significantly affect the diosgenin yield. Under the optimal conditions, namely, \( A_0 B_0 C_0 D_0 \), the highest yield was increased to 1.80% to simplify the subsequent RSM experiments and handle the most saponins in each experiment, tubers amount was arbitrarily fixed at 8 g, and the other three factors including temperature, acid concentration and hydrolysis duration were selected as the independent variables of BBD.

3.3. Model fitting

The designed experiment was carried out and the diosgenin yield was collected as evaluation index for fitting a model, subsequently. The model was then applied to optimize the extraction conditions based on the second-order polynomial equation. The results showed that the developed model was highly significant and well fitted, with low lack of fit (0.4567) and high value of \( R^2 \) (0.9989). As shown in Table 5, the temperature (\( X_1 \)), hydrolysis duration (\( X_2 \)) and acid concentration (\( X_3 \)) were found to have a significant effect on the yield of diosgenin. Meanwhile, A, B, C, A^2, B^2, C^2 and AB^2 were extremely significantly different (\( P < 0.0001 \)).

The ANOVA for the lack of fit test indicated that the model could adequately fit the experimental data (\( P > 0.05 \)) for all response variables. As shown in Figure 5, response surface 3D graphs were generated for each response, which were kept at a higher end to show the interaction between independent variables better.

The regression coefficients for dependent variables were also obtained by multiple linear regressions as shown in Table 6. These results demonstrated that the model prediction for the diosgenin yield was suitable as indicated by ANOVA lack-of-fit analysis (\( P > 0.05 \); non-significant). Furthermore, the regression model for the diosgenin yield was highly significant (\( P < 0.001 \)). The non-significant variables were removed and the fitted second-order polynomial equation was showed as follows:

\[ Y = 1.66 + 0.28X_1 + 0.12X_2 + 0.32X_3 + 0.069X_1X_2 - 0.30X_1X_3 - 0.21X_2X_3 - 0.18X_1^2 - 0.14X_2^2 - 0.17X_3^2 - 0.38X_1X_2^2 \]

Table 4. Variance analysis of the orthogonal experiment.

| Factors                  | SS      | df | F      | Significance |
|-------------------------|---------|----|--------|--------------|
| Temperature (A)         | 0.044793187 | 3  | 157.896233 | **           |
| Tubers amount (B)       | 0.079588187 | 3  | 280.548799 | **           |
| Acid concentration (C)  | 0.218447687 | 3  | 770.029302 | **           |
| Hydrolysis duration (D) | 0.186472687 | 3  | 657.317251 | **           |
| Error                   | 0.0002833687 | 3  | –        | –            |
| F_{0.05}(3,3) = 9.276628153 | F_{0.01}(3,3) = 29.4566951 |      |    |              |

Table 5. BBD matrix and response values for the extraction yield of diosgenin.

| No. | Temperature (°C) | Hydrolysis duration (h) | Acid concentration (µL/mL) | Yield of diosgenin (%) |
|-----|------------------|-------------------------|---------------------------|------------------------|
| 1   | 1                | –1                      | –1                        | 0                      | 1.32                   |
| 2   | 1                | 1                       | 0                         | 0                      | 0.98                   |
| 3   | 1                | –1                      | 1                         | 0                      | 1.55                   |
| 4   | 1                | 1                       | 1                         | 0                      | 1.49                   |
| 5   | 1                | –1                      | 0                         | –1                     | 0.41                   |
| 6   | 1                | 0                       | –1                        | 1                      | 1.57                   |
| 7   | –1              | 0                       | 1                         | –1                     | 1.64                   |
| 8   | 1                | 0                       | 1                         | –1                     | 1.61                   |
| 9   | 0                | –1                      | –1                        | 1                      | 1.62                   |
| 10  | 0                | 1                       | –1                        | 1                      | 1.40                   |
| 11  | 0                | –1                      | 1                         | 1                      | 1.72                   |
| 12  | 0                | 1                       | 1                         | –1                     | 1.64                   |
| 13  | 0                | 0                       | 0                         | 0                      | 1.66                   |
| 14  | 0                | 0                       | 0                         | 0                      | 1.64                   |
| 15  | 0                | 0                       | 0                         | 0                      | 1.70                   |
| 16  | 0                | 0                       | 0                         | 0                      | 1.67                   |
| 17  | 0                | 0                       | 0                         | 0                      | 1.64                   |

Table 6. Results of variance analysis for the RSM model.

| Factors                  | SS      | df | F      | Significance |
|-------------------------|---------|----|--------|--------------|
| Temperature (A)         | 0.044793187 | 3  | 157.896233 | **           |
| Tubers amount (B)       | 0.079588187 | 3  | 280.548799 | **           |
| Acid concentration (C)  | 0.218447687 | 3  | 770.029302 | **           |
| Hydrolysis duration (D) | 0.186472687 | 3  | 657.317251 | **           |
| Error                   | 0.0002833687 | 3  | –        | –            |
| F_{0.05}(3,3) = 9.276628153 | F_{0.01}(3,3) = 29.4566951 |      |    |              |


3.4. Validation of the developed method

The optimal values of the tested variables were predicted as follows: temperature (148.53°C), acid concentration (6 μL/mL) and hydrolysis duration (1.79 h). These predicted conditions were then applied to hydrolyze the total saponins to diosgenin, and the experiment was repeated in triplicate. However, the actual yield of diosgenin (1.67%, RSD = 1.06%, n = 3) was much lower than...
the predicted value (1.81%). Based on the results of the single factor experiment, temperature was then decreased to 140°C and the highest yield of diosgenin was achieved at 1.87%.

### 3.5. Comparison between the new strategy and the conventional method

To verify this new strategy, the conventional method was conducted, and the comparison of results was shown in Table 7. It can be seen that the yield of diosgenin for the new strategy was 85.1% higher than the conventional method while the H$_2$SO$_4$ consumption was reduced by 92.5%. In addition, the running cost has been estimated while the new approach was compared to the conventional method (cf. Table 8), and the cost could be reduced by 54.7% as predicted. Consequently, the result suggested the newly proposed strategy was more ecological and efficient than the conventional method.

### 4. Conclusions

In the present study, a novel method was established to produce diosgenin by pressurized biphase acid hydrolysis after Soxhlet extraction of total saponins dioscin from the DNM tubers. The hydrolysis conditions were optimized by RSM-BBD, and the validated yield of diosgenin was higher than the predicted after cautious adjustment. The newly developed strategy was demonstrated to be more efficient and cleaner than conventional method. In addition, the separated cellulose and starch can be easily collected for further EtOH production. Consequently, this approach is promising as a green and effective method for diosgenin production from Discerea nipponica Makino tubers in industrial application.

### Disclosure statement

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