Optimization of Subcritical Water Extraction of Polysaccharides from *Inonotus Obliquus* and their Antioxidant Activities

Xi Yuan[1], Ling Li[1], Hongyi Sun[1], Shuang Sun[1] & Zhenya Zhang[1]

[1] Graduate School of Life and Environmental Sciences, University of Tsukuba, Ibaraki, Japan

Correspondence: Zhenya Zhang, Graduate School of Life and Environmental Sciences, University of Tsukuba, Ibaraki 305-8577, Japan. Tel: 81-29-853-4712. E-mail: zhang.zhenya.fu@u.tsukuba.ac.jp

Abstract

Subcritical water extraction (SWE) of *Inonotus Obliquus* polysaccharides (IOP) was investigated using response surface methodology (RSM) with a design by Box–Behnken design (BBD). Results showed that the optimum SWE conditions for IOP production were as follows: extraction temperature 194°C, residence time 5.36 min and liquid-solid ratio 53 mL/g, yielding 168.80 ± 0.59 mg/g of IOP, which was in close agreement with the values predicted by the mathematical model. FT-IR spectra of the polysaccharides extracted by SWE and hot water extraction (HWE) were compared as well. Moreover, *in vitro* antioxidant assays revealed that SWE-IOP exhibited stronger scavenging activity that HWE-IOP. This investigation suggests that polysaccharides of *Inonotus Obliquus* extracted by SWE could be further developed as a potential antioxidant resource for dietary supplements of functional food.

Keywords: subcritical water extraction, optimization, *Inonotus Obliquus*, polysaccharides, antioxidant activity

1. Introduction

Nowadays, the interest in natural antioxidants considered as functional ingredients for pharmaceuticals, functional foods, dietary supplements, animal feed, cosmetics and other products, is getting momentum due to not only the generalizability of their action in various redox systems but also consequently broad spectra of possible applications (Augustyniak et al., 2010). As one of the vital natural antioxidants, polysaccharides are an important class of polymeric carbohydrate molecules composed of long chains of monosaccharide units joined together by glycosidic linkages. Studies showed that polysaccharides from *Inonotus obliquus* possess a wide range of pharmacologic and health-promoting properties including immune enhancement (Kim et al., 2005; Wasser, 2002), anti-oxidation (Cui, Kim, & Park, 2005), anti-diabetes (C. Wang, Chen, Pan, Gao, & Chen, 2017), and anti-tumor (Yiyong Chen et al., 2010).

In recent decades, subcritical water extraction (SWE, also called hot-compressed water or hydrothermal extraction) has been applied in the isolation of valuable components, especially polysaccharides from plants. By definition, SWE refers to water at a temperature ranging from 100°C (the boiling point of water) to 374°C (the critical point of water) under sufficient pressures (1 - 22.1 MPa) that high enough to maintain its liquid state (Ramos, Kristenson, & Brinkman, 2002). Specifically, the water polarity affected by dielectric constant decreases as the temperature increase, which makes it possible to selectively extract polar, bipolar, and nonpolar substances (Anekpankul, Goto, Sasaki, Pavasant, & Shotipruk, 2007). Besides, ionization constant of the subcritical water substantially increases with temperature, catalyzing chemical reactions such as hydrolysis and degradation without any additional catalyst (Öztürk, Irmak, Hesenov, & Erbatur, 2010). Additionally, water density, viscosity, diffusivity, electric conductance, and solvent ability are all changed (Chao, Ri-fu, & Tai-qiu, 2013). Therefore, SWE has been attracted considerable attention due to its temperature-dependent selectivity, environmentally friendly nature, high-efficiency, and low-cost, compared with the conventional methods (Chao et al., 2013).

Response surface methodology (RSM), as an effective statistical analysis technique, has been employed to optimize complex process parameters, including the extraction of polysaccharides, by evaluating interactions among various factors, as well as simultaneously estimating the effects of several process variables and their interactions on response variables (Maran, Manikandan, Thirugnanasambandham, Nivetha, & Dinesh, 2013). BBD-based statistical modeling, as one of the several statistical designs belonging to RSM represents an independent quadratic design that does not contain an embedded factorial or fractional factorial design (Maran et

al., 2013). It has been distinguished as a simplified design to cover three levels of experimental factors with less number of experiments (Zhao et al., 2011).

To the best of our knowledge, there is few literature investigated the *Inonotus obliquus* polysaccharides through subcritical water extraction. Hence, the objective of this chapter was to optimize the operational parameters (extraction temperature, residence time and liquid-solid ratio) of SWE to achieve the maximum yield of IOP by using RSM with a BBD design and their antioxidant activities were compared to with polysaccharides obtained by hot water extraction (HWE).

2 Materials and Methods

2.1 Chemicals and Reagents

Hydrogen peroxide, ascorbic acid, sodium hydroxide, sodium acetate trihydrate, chloride ferric, ferrous sulphate, dibasic sodium phosphate, sodium dihydrogen phosphate, sodium salicylate, ethanol, chloroform, n-butano, potassium persulphate, phenol, and D-glucose were purchased from Wako Pure Chemical (Osaka, Japan). 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO, USA). All the chemical reagents were of analytical grade.

2.2 Preparation of I. obliquus

In this study, I. obliquus was obtained from the same batch in the manufacturing process. Samples were dried at 60°C for 5 h to remove residual water and then ground in a high-speed disintegrator (IFM-800, IWATANI, JAPAN) to obtain a fine powder (250 μm). Ground I. obliquus was refluxed twice with ethanol (volume fraction 85%) at 70°C in a water bath for 4 h to defat, deactivate enzymes and remove some interference materials (colored materials, oligosaccharides, and some small molecule materials). The ethanol mixture extract was centrifuged (3000 rpm, 20 min). After removing supernatant, precipitation was vacuum dried at 50°C for 12 h and vacuum packed to reduce lipid oxidation.

2.3 Subcritical Water Extraction (SWE) and Hot Water Extraction (HWE)

The SWE was performed in a subcritical device (MMS-200, OMLABO, JAPAN) equipped with a temperature controller and a pressure gauge (Figure 1). Sample (1.0 g) was extracted with distilled water under the predesigned conditions. The extracts were centrifuged at 7500 rpm for 20 min and filtrated (0.45 μm, Millipore, USA) to collect the supernatant. The supernatant were concentrated by a rotary evaporator into a certain volume under reduced pressure at 60°C, and then precipitated by the addition of absolute ethanol to a final concentration of 80% (v/v) overnight at 4°C. The precipitates were collected as crude IOP after centrifugation at 7500 rpm for 15 min, washed triple times using dehydrated ethanol. After being re-dissolved in ultrapure water, the aqueous solution was subjected to remove proteins by using Sevag reagent (chloroform and n-butanol in a 4:1 ratio), dialyzed with deionized water for 72 h, concentrated under reduced pressure. Finally, the IOP product was collected after lyophilization. The obtained crude polysaccharides were re-dissolved in doubly distilled water for polysaccharides yield and antioxidant capacity determination. The polysaccharides content of crude IOP was determined based on the phenol-sulfuric acid method (D. Zhang, Li, Xiong, Jiang, & Lai, 2013). The results were expressed as the polysaccharides content in per gram of dry soybean residue using the following equation:

$$\text{OP yield (mg/g)} = C \times N \times V/W \qquad (1)$$

where C is the concentration of polysaccharides as calculated from the calibrated regression equation (mg/mL); N is the dilution factor; V is the total volume of extraction solution (mL); and W is the weight of the sample (g).

For comparison, in hot water extraction, a 5.0 g Ground *I. Obliquus* was extracted using 150 mL of distilled water at 100°C for 2 h.

2.4 Single-Factor Experiment Design

A series of single-factor experiments was conducted to identify the independent variables and investigate the preliminary BBD range of extraction factors including extraction temperature ranging from 160 to 200°C, residence time ranging from 0 to 20 min and liquid-solid ratio ranging from 30 to 80 mL/g. The extraction yield of IOP was the dependent variable. Each experimental factor was determined while others were retained constant. Each experiment was performed in triplicate.
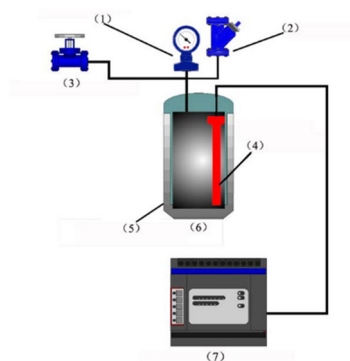
Figure 1. Experimental schematic diagram of subcritical water extraction. (1) Pressure gauge, (2) Safety head, (3) Pressure release value, (4) Temperature sensor, (5) Insulation lagging, (6) SWE reactor, (7) Temperature controller and thermocouple

2.5 Experimental Design of RSM

On the basis of results of the former single-factor experiments, BBD with RSM was applied to optimize the extraction conditions of IOP. Three independent variables (X_1, extraction temperature; X_2, residence time and X_3 liquid-solid ratio) and their levels followed in the second stage have been shown in Table 1. For the statistical calculation, process factors are coded according to Eq. (2):

$$x_i = (X_i - X_0)/\Delta X \quad i = 1, 2, 3 \tag{2}$$

where x_i is the coded value of the independent factor; X_i is the actual value of the independent variable, X_0 is the actual value of X_i at the central point, and ΔX is the step change value.

Table 1. Independent variables and their levels used in the response surface design (BBD)

Independent variables	Symbol	Levels		
		-1	0	1
Temperature (°C)	X_1	180	190	200
Residence time (min)	X_2	0	5	10
Liquid-solid ratio (mL/g)	X_3	40	50	60

All these runs were measured in a random order to maximize the effect of unexplained variability in the observed response due to extraneous factors and minimize the impact of non-controlled variables. Data from three repeated experiments were analyzed by multiple regressions to fit into the general form of a quadratic polynomial model as showed in the following Eq. (3),

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \tag{3}$$

where Y is the predicted response variable associated with each three levels combination (IOP yield), B_0 is the constant coefficient, B_i is the liner coefficient, B_{ii} is the quadratic coefficient, and B_{ij} is the two-factor interaction coefficient. X_i and X_j are the levels of the independently coded variables ($i \neq j$).

The experimental design, data analysis, and model building of the previous study were performed using Design-Expert software (8.0.5 Statease Inc., Minneapolis, USA). All trials were performed in triplicate.

2.6 Fourier Transform Infrared Spectroscopy of IOP

FT-IR was designed to identify the obtained IOP, which was carried out with the KBr-disk method (S. Li & Shah, 2014) using a Jasco FTIR 3000 spectrometer (Jasco, Wakayama, Japan). The dried OP was mixed thoroughly with potassium bromide (KBr) powder, ground and pressed into 1-mm pellet for spectrometric measurement at a frequency range of 400 - 4000 cm-1.

Table 2. Box-Behnken design matrix of the three variables (coded and uncoded units) and results for the extraction rate of IOP

Run	Temperature (°C) -X_1	Residence time (min) -X_2	Liquid-solid ratio (mL/mg) -X_3	Polysaccharide content (mg/g)	
				Experimental	Predicted
1	-1 (180)	-1 (0)	0 (50)	83.21	83.32
2	1 (200)	-1 (0)	0 (50)	151.98	151.89
3	-1 (180)	1 (10)	0 (50)	144.41	144.52
4	1 (200)	1 (10)	0 (50)	132.72	132.62
5	-1 (180)	0 (5)	-1 (40)	119.66	119.63
6	1 (200)	0 (5)	-1 (40)	144.14	144.32
7	-1 (180)	0 (5)	1 (60)	127.08	126.91
8	1 (200)	0 (5)	1 (60)	158.86	158.89
9	0 (190)	-1 (0)	-1 (40)	119.57	119.49
10	0 (190)	1 (10)	-1 (40)	138.09	138.02
11	0 (190)	-1 (0)	1 (60)	127.91	127.98
12	0 (190)	1 (10)	1 (60)	151.29	151.38
13	0 (190)	0 (5)	0 (50)	163.67	164.64
14	0 (190)	0 (5)	0 (50)	164.36	164.64
15	0 (190)	0 (5)	0 (50)	165.73	164.64
16	0 (190)	0 (5)	0 (50)	165.05	164.64
17	0 (190)	0 (5)	0 (50)	164.36	164.64

2.7 Evaluation of Antioxidant Capacity

2.7.1 DPPH Radical Scavenging Activity Assay

DPPH radical scavenging activity of extractions was evaluated according to a literature procedure with slight modifications (Sun et al., 2013). Aliquots (0.5 mL) of various concentrations (0.3125-10.00 mg/mL) of solutions were mixed with 3 mL (25 µg/mL) of a MeOH solution of DPPH and then were shaken vigorously and allowed to stand in the dark for 30 min. The absorbance was measured with a spectrophotometer at 517 nm against a blank. A decrease of the DPPH solution absorbance indicated an increase in the DPPH radical scavenging activity. Ascorbic acid was invoked as positive controls. The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = (1 - A_1/A_0) \times 100\% \tag{4}$$

where A_0 is the absorbance without samples and A_1 the absorbance containing the samples.

2.7.2 SOD-like Activity Assay

The SOD-like activity of the extracts was measured by the SOD Assay Kit-WST according to the technical manual provided by Dojindo Molecular Technologies, Inc. Firstly, 20 µl of the sample solution was added to blank 2 well and each sample, meanwhile, 20 µl of double distilled water was added to each blank 1 and blank 3 well in a 96-well plate. After that, 200 µl of WST working solution was added to each well, mixed waiting for further processing. Then, 20 µl of dilution buffer was added to each blank 2 and blank 3 well, and 20 µl of enzyme working solution was added to each sample and blank 1 well. The plate was incubated at 37°C for 20 min and the optical density (OD) was measured at 450 nm using a microplate reader (Bio-Rad Model 550, USA). SOD-like activity was calculated by the following equation:

$$\text{SOD activity (inhibition rate \%)} = \left\{ \frac{(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})}{(A_{blank1} - A_{blank3})} \right\} \times 100\% \tag{5}$$

A_{blank1}, A_{blank2}, A_{blank3} and A_{sample} were the absorbances of blank 1, blank 2, blank 3 and sample wells. 1 Unit of SOD activity was defined as the amount of enzyme having a 50% inhibitory effect on WST-1.

IC_{50} value (mg/mL) was the effective concentration at which SOD activity was scavenged by 50% and was obtained by interpolation from a linear regression analysis.

2.7.3 Hydroxyl Radical Scavenging Activity Assay

Hydroxyl free radicals generated from $FeSO_4$ and H_2O_2 were detected by their ability to hydroxylate salicylate. The reaction mixture (2.5 mL) contained 0.5 mL of $FeSO_4$ (1.5 mM), 0.35 mL of H_2O_2 (6 mM), 0.15 mL of sodium salicylate (20 mM), and 1.0 mL of different concentrations of the IOP. Ascorbic acid was used as the positive control. The absorbance of the hydroxylated salicylate complex was measured at 562 nm after incubation at 37°C for 1 h. The scavenging effect on hydroxyl free radicals was calculated as Eq. (6).

$$\text{Scavenging activity (\%)} = [1 - (A_1 - A_2)/A_0] \times 100\% \tag{6}$$

where A_1 is the absorbance of the sample or ascorbic acid, and A_0 is the absorbance of the solvent control, whereas A_2 is the absorbance of the reagent blank without sodium salicylate.

2.8 Statistical Analysis

Analysis of the experimental design and data was performed using Design-Expert software of version 8.0.5 (Stat-Ease Inc., Minneapolis, USA). All experimental results were expressed as means ± SD of triplicate experiments. All analyses were performed using the Statistical Package for the Social Sciences (SPSS, 19.0). Statistically significant was assumed at $P < 0.05$.

3. Results and Discussion

3.1 Single Factor Experimental Analysis

3.1.1 Effect of Extraction Temperature on the Extraction Yield

The extraction temperature is a crucial parameter affecting the extraction efficiency and selectivity (Ye & Jiang, 2011). With an appropriate temperature, targets compounds tied on the sample matrices by means of physical adsorption and chemical interactions were released into extraction solvents (Qi et al., 2015) and even modify the characteristics of solvent like decreasing the viscosity and increasing the diffusion, which all make great contributions to the mass transportation of targeted ingredients. The effect of temperature (160, 170, 180,190 and 200°C) on the IOP yield is illustrated in Figure 2a when the other factors, residence time and liquid-solid ratio, were fixed at 20 min and 80 mL/g, respectively. As outlined, as the temperature changed from 160 to 190ºC, the extraction efficiency increased rapidly and the polysaccharides yield reached a maximum of 132.62 ± 2.63 mg/g at 190°C. Upon further temperature increased, the polysaccharide yield decreased sharply, indicating that excessive temperature may lead to degradation and decomposition of polysaccharides induced by browning reaction, whose chemical products are not detected in a phenol–sulphuric assay and, then, decrease the concentration of IOP yield (Xu et al., 2016). In terms of extraction efficiency and energy consumption, an extraction temperature of 190ºC was employed in subsequent extraction experiments.

3.1.2 Effect of Residence Time on the Extraction Yield

Extraction time is another important parameter influencing the extraction rate and quality of yielded compounds. It usually takes polysaccharides some time to contact the release medium, where solvent permeated into the dried materials so that they could be dissolved and diffused out subsequently from the materials (C.-p. Zhu, Zhai, Li, Wu, & Li, 2015). Apart from that, extraction time is crucial in minimizing energy and production cost, which is especially noticeable in the extraction process. Thus, considering economic efficiency and energy consumption, the effect of the extraction time on the yield of IOP was investigated and the extraction process was performed for different extraction time from 0 to 20 min when the other factors were as follows: liquid to solid ratio 80:1 mL/g and temperature 190ºC, respectively. As illustrated in Figure 2b, the IOP yield was positively correlated with an increased duration, and it increased up to a maximum amount of 151.09 ± 2.77 mg/g in 5 min, which meant a great number of IOP was released to exterior solvent in this early extraction stage. Thereafter, IOP began to continually decrease, which can be attributed to the degradation and hydrolysis of polysaccharides exposed to high temperature for a long extraction time, especially under subcritical water conditions. For this reason, the extraction time was controlled in the range of 5 min.

3.1.3 Effect of Liquid-Solid Ratio on the Extraction Yield

In classical extraction methods, the solid-liquid ratio is recognized as one of the major parameters for affecting the effectiveness of the procedure and the quality of the extracts. Various liquid-solid ratio can directly impact the concentrations between the interior plant cells and the exterior solvent, significantly affecting the yield of the extracted polysaccharides (Yuzhen Chen, Zhao, Liu, & Zuo, 2012). An inappropriate liquid-solid ratio may result in the incompleteness of polysaccharides or solvent waste. Therefore, different liquid-solid ratio including 30, 40, 50, 60, 70 and 80 mL/g was tested while the other two extraction variables (extraction temperature and residence time) were set in the central points as follows: 190ºC and 5 min, respectively. As presented in Figure 2c, the

extraction yield of IOP initially increased slightly with increasing the liquid to solid ratio and reached a maximum yield (157.17 ± 0.65 mg/g) when the liquid-solid ratio was 50 mL/g. And then, as the liquid-solid ratio continued to increase, the yield tended to decrease. The reason of this is some certain liquid-solid ratio can promote diffusivity of the solvent into the cells and enhance the leaching-out rates of polysaccharides therefrom. However, an excess of solvent could consume cavitation energy from the extraction system and complicate extraction procedure, resulting in a lower IOP yield. Hence, to minimize electricity and time costs, a liquid-solid ratio of 50 mL/g was selected to ensure the extraction yield.
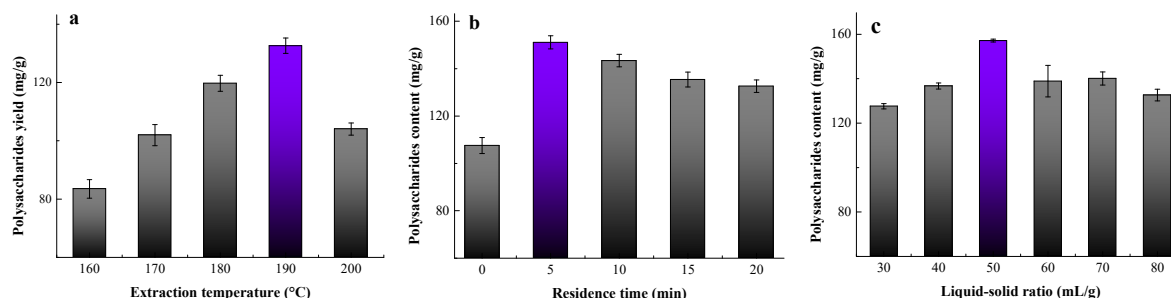

Figure 2. Effect of extraction temperature (a), residence time (b), and liquid-solid ratio (c) on the extraction yield of IOP. (a) 20 min of residence time and 80 mL/g of liquid-solid ratio; (b) 190°C of temperature and 80 mL/g of liquid-solid ratio; (c) 5 min of residence time and 190°C of temperature. Values are means ± S.D. (n = 3)

3.2 Box–Behnken Design (BBD) for Optimization of Extraction Parameters

3.2.1 Statistical Analysis and the Model Fitting

Based on single factor experiments mentioned above and according to BBD design matrix, a number of 17 experimental runs (12 runs for various conditions and 5 center point runs for the measurement of the process stability and inherent variability) with three factors and three levels were performed to study the reciprocal influence of three independent variables (extraction temperature, residence time and liquid-solid ratio) on IOP extraction yield and establish the optimal SWE conditions. The variables of the actual and coded levels and the experimental results performed three times were represented in Table 1 and Table 2, respectively. As could be seen, the IOP yield ranged from 83.21 to 165.73 mg/g. Using multiple regression analysis of the experimental data using Design Expert software version 8.0.5, the data in Table 2 were carried out. The fitted model for the IOP yield (mg/g) was correlated according to the following second-order polynomial equation:

$$Y = 164.64 + 14.17X_1 + 10.48X_2 + 5.46X_3 - 20.12X_1X_2 + 1.82X_1X_3 + 1.21X_2X_3 - 16.67X_1^2 - 19.89X_2^2 - 10.53X_3^2 \quad (7)$$

Where Y is the yield of polysaccharides, X_1, X_2, and X_3 are the coded variables for extraction temperature, residence time and liquid-solid ratio, respectively.

Analysis of variance (ANOVA) and F-test was employed to evaluate the significance and to analyze the adequacy and the goodness-of-fit of the response surface quadratic model (Table 3). The high quadratic regression model significance was identified by its high F-value (F = 2408.99) and low P-value (P < 0.0001). The goodness-of-fit can be demonstrated by the determination coefficient (R^2), adjusted determination coefficient (R_{adj}^2) and coefficient of variance (C.V.) (L. Zhang & Wang, 2017). R^2 offers the proportion of the total variation in the response predicted by the model and indicates the ratio of sum of squares because of regression to the total sum of squares (Y. Zhang et al., 2016). R^2 coefficient high closed to unity indicates a satisfactory adjustment of the quadratic model to the experiment statistics (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008). In this study, the determination coefficient (R^2) was 0.9997, implying that 99.97% of the variations could be interpreted by the fitted model. Besides, a high degree of correlation between the observed and predicted values was confirmed since the adjusted determination coefficient ($R_{adj}^2 = 0.9993$) for the model had no obvious difference with R^2. Moreover, a low coefficient of variation (C.V. = 1.07) indicated that the degree of precision and reliability of experimental values of the regression model was highly significant. The P-value was used as a tool to not only reveal the significance of each coefficient but also explain the interaction pattern of independent variables (Xu et al., 2016). The smaller the value of P with respect to 0.05, the more significant the corresponding coefficient.

As all the regression coefficients listed in Table 3, it could be concluded that all the liner coefficient, the quadratic term coefficients, and the cross product coefficients with small P-value (P < 0.05) were significant for IOP

extraction. This finding manifested that all the independent parameters analyzed in this study were important factors affecting the yield of extracted IOP. In addition, the significance of the model was also judged by a lack-of-fit test, meaning the failure of the model to represent the data in the experimental domain at points that are not included in the regression (Ghafari, Aziz, Isa, & Zinatizadeh, 2009). The ANOVA showed that lack of fit (F-value = 0.070 and P-value = 0.9728) was not significantly relative to the pure error, implying that it was not significant and just a 2.72% chance could occur due to noise. Consequently, it was determined that proposed mathematical model and extraction parameters could be used to predict the yield of IOP under any combination of the values of different variables under SWE conditions.

Table 3. Analysis of variance (ANOVA) of the response surface quadratic model for the effects of extraction temperature (X_1), residence time (X_2) and liquid-solid ratio (X_3) on the extraction.

Source	Sum of Squares	Df	Mean Square	F-value	P-value	Significant
Model	8019.19	9	891.02	2408.99	< 0.0001	***
X_1	1605.59	1	1605.59	4340.93	< 0.0001	***
X_2	878.95	1	878.95	2376.36	< 0.0001	***
X_3	238.63	1	238.63	645.17	< 0.0001	***
X_1X_2	1618.54	1	1618.54	4375.92	< 0.0001	***
X_1X_3	13.29	1	13.29	35.92	0.0005	***
X_2X_3	5.90	1	5.90	15.96	0.0052	**
X_1^2	1169.43	1	1169.43	3161.70	< 0.0001	***
X_2^2	1665.12	1	1665.12	4501.85	< 0.0001	***
X_3^2	467.17	1	467.17	1263.06	< 0.0001	***
Residual	0.22	7	0.03			
Lack of Fit	0.13	3	0.043	0.070	0.9728	*
Pure Error	0.06	4	0.01			
Cor Total	69.23	16				
SD	1.78					
Mean	166.76					
C.V. (%)	1.07					
Press	267.87					
R^2	0.9997					
Adj. R^2	0.9993					
Pred. R^2	0.9993					
Adequate precision	43.0765					

**Significant, $p < 0.05$

***Highly significant, $p < 0.001$

*Non-significant, $p > 0.05$

3.2.2 Response Surface Plot

To represent a visual interpretation of the interactions between the independent and dependent variables, three-dimensional response surface and two-dimensional (2D) contour plots (Figure 3) were achieved using Design-Expert software. Generally, 3D response surface plot can display the mutual influences of independent variables on the response variable and further assistance in finding the maximum, minimum and saddle points of the responses (Matos et al., 2015). For 2D response contour plots, not only do 2D response contour plots interpret the reciprocal interactions between independent variables, also reflect the significance of reciprocal interactions between the variables (Guo, Zou, & Sun, 2010).

The three-dimensional response surface (Figure 3a) and contour plots (Figure 3b) illustrated the effects of extraction temperature as well as residence time on the yield of IOP at a fixed liquid-solid ratio of 50 mL/g. The IOP yield increased significantly as extraction temperature together with residence time increased in the range of 180.00ºC - 193.98ºC and 0 - 5.36 min, respectively. However, when these two parameters beyond the peak points, the yield decreased gradually, which probably due to excessive extraction time and temperature could lead to the hydrolyzation or degradation of polysaccharides. According to the elliptical shape of the contour plot, the

relationships between extraction temperature and residence time were significant. Figure 3c and Figure 3d showed the effects of extraction temperature and liquid-solid ratio on IOP yield when residence time was fixed at a zero level. A maximum IOP was obtained at 193.98℃ with a liquid-solid ratio of 52.98 mg/L. As shown in Figure 3e and Figure 3f, the IOP yield with varying residence time and liquid-solid ratio at a fixed ratio of extraction temperature (0 level) had been evaluated. The elliptical shape of the 2-D contour plot indicated that the mutual interaction between residence time and liquid-solid ratio was significant.
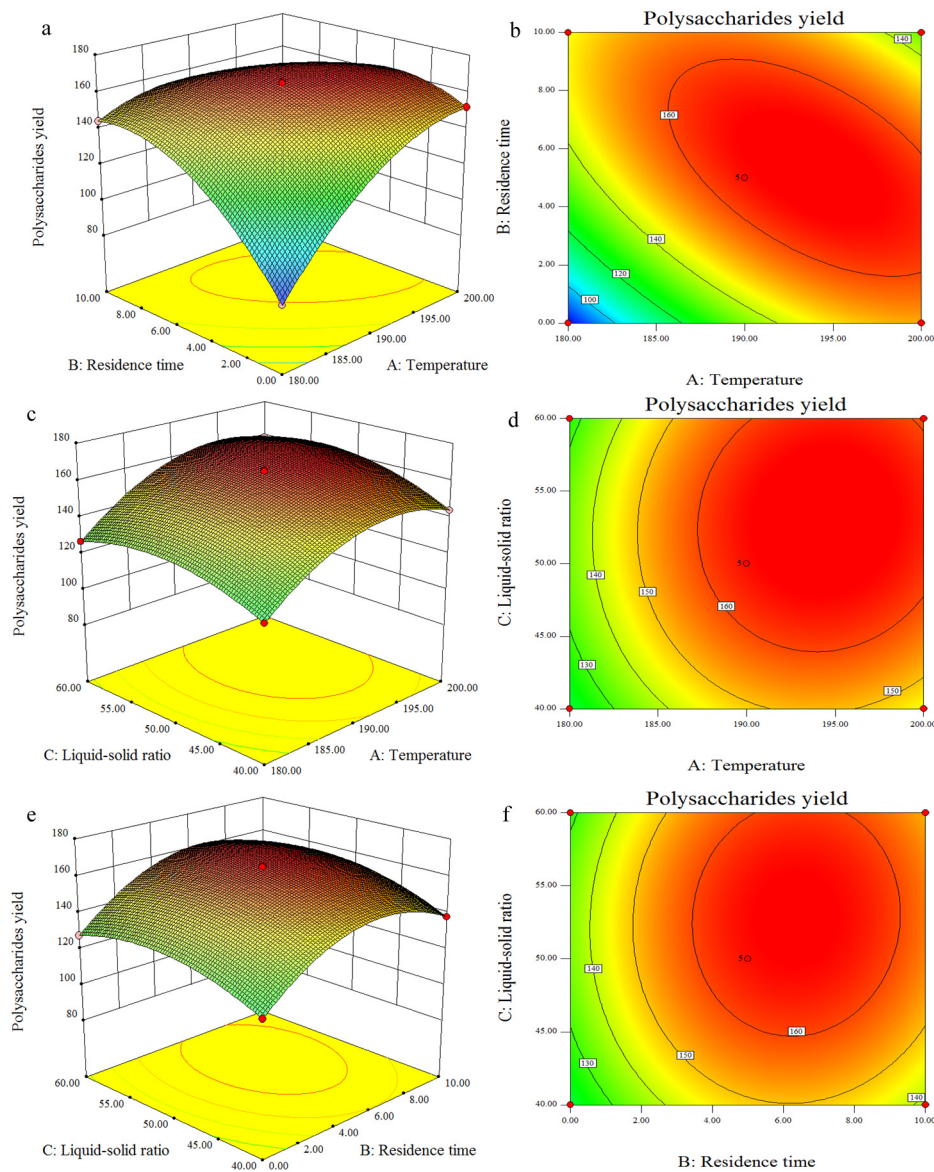

Figure 3. Response surface plots (a, c, and e) and contours plots (b, d, and f) for the effects of extraction temperature (X_1), residence time (X_2) and liquid-solid ratio (X_3) on the yield of IOP

3.2.3 Optimization of Extraction Parameters and Validation of the Model

The optimal values of independent variables and response variable for the proposed extraction were analyzed by Design-Expert software and recommended as follows: extraction temperature of 193.98℃, residence time of 5.36 min, and liquid-solid ratio of 52.98 mL/g and the predicted value for IOP yield was 168.65 mg/g. To validate the adequacy of the model equations, a verification experiment should be tested using the estimated optimal conditions. However, considering feasible function of the apparatus, the parameters were modified slightly in the verification experiment as follows: extraction temperature of 194℃, residence time of 5.36 min, and liquid-solid ratio of 53 mL/g. Under these extraction conditions, triplicate confirmatory experiments were carried out and the average

extraction yield of IOP was 168.80 ± 0.59 mg/g, which agreed closely with the predicted value. A good correlation between predicted and experimental values revealed that the regression model was accurate and adequate for the extraction of IOP by SWE method. Therefore, the model was suitable for the optimization of SWE extraction procedure for IOP.
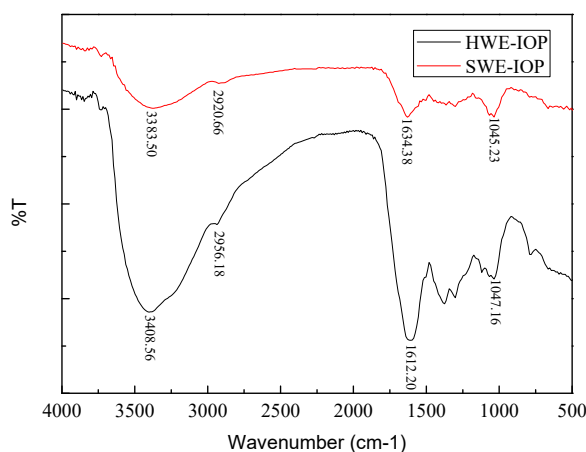
3.3 FT-IR Spectroscopy of IOP

Figure 4. FT-IR spectrum of the polysaccharides extracted from *Inonotus obliquus* by using SWE (SWE-IOP) and HWE (HWE-IOP)

FT-IR spectroscopy is a practical technique for identification of characteristic organic groups and exhibition of structural features in polysaccharides (W. Chen et al., 2014). As shown in Figure 4, the FT-IR spectra of two polysaccharides (the black line for hot water extraction; the red line for subcritical water extraction) were almost same. Given the fact that the signals within the range of 3600 - 3300 cm^{-1}, 3000 - 2800 cm^{-1}, and 1100 - 1000 cm^{-1} are the characteristic absorption peaks of polysaccharides (C. Li, Fu, Luo, & Huang, 2013), both HWE-IOP and SWE-IOP spectra could fit the typical pattern of polysaccharides well, indicating that the main structure of polysaccharides was not destroyed under subcritical water conditions. A strong and broad peak at approximately 3400 cm^{-1} (HWE-IOP: 3408.56 cm^{-1}; SWE-IOP: 3383.50 cm^{-1}) corresponded to a characteristic hydrogen bond O-H stretching vibration (Cheng et al., 2013). The absorption peak at around 2940 cm^{-1} (HWE-IOP: 2956.18 cm^{-1}; SWE-IOP: 2920.66 cm^{-1}) assigned to the C-H asymmetric stretching vibration including CH-, CH_2- and CH_3- (R. Wang, Chen, Jia, Tang, & Ma, 2012). The bands at approximately 1630 cm^{-1} (HWE-IOP: 1612.20 cm^{-1}; SWE-IOP: 1634.38 cm^{-1}) suggested the presence of the stretching vibration of C=O or C=C group in structure (Shi, Zhang, & Yang, 2013). A range from 1000 cm^{-1} to 1200 cm^{-1} of extensive absorption bands indicated the stretching vibrations of C-O-H side groups, and C-O-C glycosidic band vibrations were observed in the spectra (Kacurakova, Capek, Sasinkova, Wellner, & Ebringerova, 2000). The main absorptions of C-O stretching (HWE-IOP: 1047.16 cm^{-1}; SWE-IOP: 1045.23 cm^{-1}) showed that the characteristics of sugar structures were pyranose configuration (Y. Zhu et al., 2014). Overall, these results showed that polysaccharides extracted by HWE and SWE exhibited all of the typical absorption peaks of a polysaccharide.

3.4 Antioxidant Activity

3.4.1 DPPH Radical Scavenging Activity

DPPH assay has been widely used as a substrate to evaluate the antioxidant activity of samples, including natural polysaccharides (Yuan, Zhang, Fan, & Yang, 2008). The scavenging activities of polysaccharides extracted by SWE and HWE against the DPPH radicals were shown in Figure 5A. Ascorbic acid was as the positive control to verify the validity and authenticity of the assay. In this study, ascorbic acid showed DPPH radical-scavenging activity of $97.80 \pm 0.23\%$ at the initial concentration of 0.3125 mg/mL, exhibiting excellent scavenging ability on DPPH radicals. And altered concentrations of all the SWE-extracts (0.3125, 0.625, 1.25, 2.5, and 5 mg/mL) showed DPPH scavenging activities in a dose-dependent manner. As shown, both IOP samples exhibited DPPH radical scavenging activities at a concentration range of 0.3125 - 5 mg/mL. Specifically, when the sample concentration ranging from 0.3125 - 5 mg/mL, the scavenging activity of SWE-IOP increased from 20.21% to 90.66% whereas the scavenging activity of HWE-IOP grew 13.57% to

75.78%. The half inhibition concentration (IC_{50}) increased in the order of SWE-IOP (0.86 mg/mL) and HWE-IOP (1.78 mg/mL), which indicated that the thermal processing condition for samples processed under subcritical water conditions significantly improved the scavenging ability to DPPH radicals. Moreover, these findings implied that IOP can donate an electron or hydrogen atom to scavenge DPPH radical.
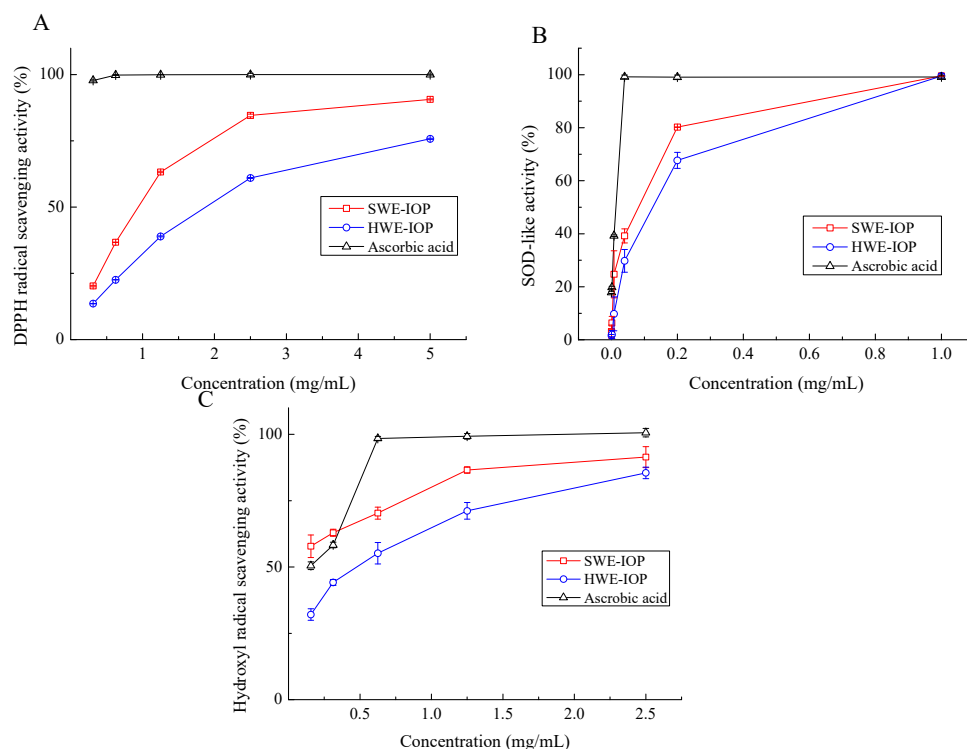

Figure 5. Antioxidant activities of SWE-IOP, HWE-IOP, and ascorbic acid: (A) DPPH radicals scavenging activity, (B) SOD-like activity and (C) hydroxyl radicals scavenging activity. Values are means ± S.D. (n = 3)

3.4.2 SOD-like Activity

Superoxide dismutases (SODs), as metalloenzymes catalyzing the dismutation of superoxide anion to oxygen and hydrogen peroxide, ubiquitously exist in eukaryotes and prokaryotes (Liou & Storz, 2010). SOD-like is recognized as a non-enzyme-small molecule super antioxidant that has a similar antioxidant function with SOD (Yang, Wu, Liu, Chai, & Tseng, 2016). Hence, SOD-like activity assay is widely used to determine radical scavenging activity due to its ability to inhibit the auto-oxidation of pyrogallol. As showed in Figure 5B, at the initiate concentration of 0.3125 mg/mL, the activity of SWE-IOP (3.10 ± 2.49%) was almost as twice as that of HWE-IOP (1.58 ± 0.44%). With the increase of concentrations, SOD-like activity of both fractions increased dramatically, suggesting that IOP extracted by these two methods were sensitive to the expression of SOD-like. There was no difference between two kinds of IOP (SWE-IOP, 99.54± 0.16%; HWE-IOP, 99.53 ± 0.82%) and ascorbic acid (99.14± 0.42%), indicating that IOP may have potential effects on human cells. Moreover, SOD-like activities were in a dose-dependent manner. As shown in Table 4, the IC_{50} values of SWE-IOP and HWE-IOP were 0.039 mg/mL and 0.077 mg/mL, respectively.

3.4.3 Hydroxyl Radical Scavenging Activity

Hydroxyl radical, a detrimental oxidant, can react with bio-macromolecules functioning in living cells and induce damage to virtually all types of biomolecules, such as lipids, proteins, carbohydrates, and DNA. The hydroxyl radicals scavenging abilities of SWE-IOP and HWE-IOP are illustrated in Figure 5C. The results showed that IOP possessed a dose-dependent activity within the concentration of 0.3125 to 5 mg/mL. It can be easily observed that both kinds of IOP had lower scavenging abilities than ascorbic acid at all tested concentrations. However, when the concentration was 0.3125 mg/mL, SWE-IOP exhibited stronger scavenging activity even than that of ascorbic acid. As for SWE-IOP, the scavenging activity increased significantly from 57.82% to 91.41% at a concentration ranging from 0.3125 to 5 mg/mL. Compared with the results of SWE-IOP, HWE-IOP has a lower inhibition ability, which increased from 32.08% to 85.48% with the increase of sample concentration ranging from 0.3125 to 5

mg/mL. In addition, the IC$_{50}$ values of SWE-IOP and HWE-IOP were 0.13 mg/mL and 0.41 mg/mL, respectively (Table 4). The difference of scavenging activities between SWE-IOP and HWE-IOP may be attributed to the former research that a relatively elevated temperature exposure may exert an influence on bioactivity via modifying the molecular weight of polysaccharides (M. Zhang, Zhang, Cheung, & Ooi, 2004). There are two possible ways for polysaccharides eliminating radicals: polysaccharides supply the hydrogen to combine with radicals so that the radical chain reaction can be terminated; polysaccharides themselves integrate with radical ions directly. But up till now, the exact mechanism underlying the free-radical scavenging activity exerted by polysaccharides is still not fully understood. Consequently, *Inonotus Obliquus* polysaccharides obtained by hot water extraction showed lower DPPH radical scavenging activities than that achieved by subcritical water extraction, suggesting that elevated temperature might appropriately degrade the high-molecular-weight polysaccharides and enhance their antioxidant activities.

Table 4. Comparison of IOP obtained by HWE and SWE.

Method	Extraction conditions			Extraction yield (mg/g)	Antioxidant assay (IC$_{50}$, mg/mL)		
	Temperature (°C)	Residence time (min)	Liquid-solid ratio (mL/g)		DPPH	SOD-like	Hydroxyl
HWE	100	120	30	30.71 ± 0.43	1.780	0.077	0.413
SWE	194	5.36	53	168.80 ± 0.59	0.859	0.039	0.129

4. Conclusion

In this study, the conditions under subcritical water extraction for obtaining polysaccharides from *I. Obliquus* were optimized by response surface methodology with a Box-Behnken design. Based on the prediction test and adjustment of the actual procedure, the optimal conditions were as follows: extraction temperature of 194°C, residence time of 5.36 min, and liquid-solid ratio of 53 mL/g. Under these conditions, the experimental yield (168.80 ± 0.59 mg/g) agreed closely with the predicted yield. Moreover, the extraction yields as well as antioxidant activity were investigated. SWE-IOP had stronger antioxidant activities with a higher extraction yield, compared to those polysaccharides extracted by hot water extraction. This study shows the possibility of extracting polysaccharides from *I. Obliquus* by using subcritical water extraction. Certainly, further studies on the precise chemical structures and biological functions of IOP are currently in progress.

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