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Contributed Paper

Multi-response Optimization of Ultrasonic Assisted Enzymatic Extraction of Antioxidant Polysaccharides from Waste *Ginkgo biloba* Sarcotesta

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ABSTRACT

The combination of Plackett–Burman design (PBD) and rotatable central composite design (RCCD) was used to optimize ultrasonic assisted enzymatic extraction (UAEE) for achieving maximal recovery of antioxidant polysaccharides from waste *Ginkgo biloba* sarcotesta (GBSP). After the combination of PBD and RCCD, the resulting optimal UAEE conditions were as follows: UAE power of 239 W, time of 31 min, cellulase concentration of 3.2%, UAE temperature of 60°C, pH of 4.5, solvent-to-solid of 30 mL/g and sample particle size of 60 mesh. Under the optimum conditions, the experimental yield of GBSP was 7.71 ± 0.53 % (n = 3) and ferric-reducing antioxidant capacity values of GBSP was 22.43 ± 1.93 mmol Fe^{2+}/g (n = 3), which were close to the above predicted values. Moreover, the antioxidant activities of $GBSP_{UAEE}$ obtained by UAEE compared with $GBSP_{HWE}$ obtained by hot water extraction (HWE), the antioxidant activities obtained using $GBSP_{UAEE}$ were significantly higher than those $GBSP_{HWE}$. Additionally, the carbohydrate content, monosaccharide composition and IR spectrum of the $GBSP_{UAEE}$ were also investigated. Therefore, the present study provided an advisable method of UAEE for extraction of antioxidant GBSP from waste *Ginkgo biloba* resource.

Keywords: *Ginkgo biloba* sarcotesta, polysaccharides, ultrasonic assisted enzymatic extraction, response surface methodology, antioxidant activity

1. INTRODUCTION

Ginkgo biloba L. (*G. biloba*) was widely distributed in extratropical, warm and subtropical zones [1]. *G. biloba* has existed on the earth for two hundred million years, but its true value has induced a range of attentions all around the world till the recent five decades [2]. Male and female flowers grew on different plants, and female plants bore a yellowish-green plum-like "fruit" (aril), which was indeed the seed of *G. biloba* in gymnosperms [3]. The seeds of *G. biloba* have been used in traditional Chinese medicine and as a foodstuff for centuries throughout Asia.

However, the sarcotesta of *G. biloba* (GBS), the outer malodorous layer of mature seeds, was peeled from the seeds of *G. biloba*. GBS was treated as waste and discarded abundantly in soil and water, thus it polluted soil and poisoned fish in rivers and lakes [4]. GBS, the primary by-products of ginkgo seeds processing, has become an urgent problem to the local enterprises and governments of the planting area for its poison to the environment. Recently, there has been an increasing concern for the preservation of the environment and sustainability of resources. Hence, the utilization of natural resources was receiving renewed interest as an alternative to non-renewable resources in material technology [5]. Modern studies indicated that GBS rich in polysaccharide which possessed a range of biologic activities [4, 6, 7] such as enhancing immunity, protecting neuro function, anti-tumor and antiaging. As a matter of fact, most of these effects were mainly due to the antioxidant activity of GBS polysaccharides (GBSP) [7]. The development of an efficient utilization of the antioxidant capacity of GBSP might be of great importance for minimizing potential environmental impact of GBS. Thus, maximum extraction of antioxidant activity of GBSP might be an efficient strategy to solve the problem of utilization of waste GBS.

Many extraction techniques including hot water extraction (HWE) [8], ultrasonic assisted extraction (UAE) [9, 10], microwave assisted extraction (MAE) [10] and ultrasonic assisted enzymatic extraction (UAEE) were employed to extract polysaccharides. Comparatively speaking, MAE and UAE were better than other traditional extraction methods, which could reduce extraction time and the usage of solvents, optimize extraction parameters as well as increase yield of target compounds [11, 12]. Particularly, UAE was a simple, rapid, inexpensive and efficient method for extracting polysaccharides [13-15]. From the previous reports, UAE exhibited the best mass transfer, cell disruption, solvent penetration and capillary effect, and it was the economical technique to scale up for industrial production [16]. Additionally, the release of intracellular contents was also promoted by breaking the cell wall when enzymes such as cellulases and proteases were added in the solution of UAE [13-15]. So, the combinational usage of UAE and enzyme would be more effective probably during the extraction of polysaccharides. In the process of UAEE, many factors, including ultrasound power, extraction time, cellulase concentration etc., could influence the extraction process, individually and collectively. Therefore, the optimization of the extraction parameters was required to obtain the maximum yield of antioxidant activity of GBSP. Plackett-Burman designs (PBDs) combined with response surface method (RSM) was a useful method for evaluating multiple parameters and their interactions based on quantitative data, and might effectively overcome the drawback of classic "one-factor-at-a-time" or "full-factors" approach [17]. Additionally, PBDs-RSM was frequently used to optimize the extraction parameters of target compounds from plant materials [18].

The objectives of this study were to establish an optimized simple, safe and low-cost process of UAEE for the industry to obtain maximum yield of antioxidant activity of GBSP from waste *Ginkgo biloba* sarcotesta.

2. Materials and Methods

2.1 Materials and Samples

The *Ginkgo biloba* sarcotesta were collected from Taizhou, Jiangsu, China. Samples were ground and passed through 60 mesh. D-glucose (Glc), glucuronic acid (GlcA) galactose (Gal), xylose (Xyl), rhamnose (Rha), mannose (Man), arabinose (Ara), inositol, cellulase from *Trichoderma viride* (11000 U/mg), iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), phenol, 1,1-diphenyl-2-picrylhydrazxyl (DPPH), 1,10-phenanthroline and 2,2-azinobis (3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Deionized water was prepared by a Milli–Q water purification system

(Millipore, Bedford, MA, USA). All other reagents and chemicals were of analytical grade.

2.2 Extraction Procedures

The extraction process was performed with an ultrasonic device (SY-5200T, Shanghai Shenyuan Ultrasonic Instrument Co. Shanghai, China). Using the method of Soxhlet, 10 g dry GBS powder was extracted by petroleum ether at 80°C for 3 h to remove lipids and some colored materials. After being vacuum dried, the defatted powder (1 g) was mixed with cellulase solution. This mixture was ultrasonicated at various factors such as concentrations of cellulase, solvent-to-solid ratios, times, powers and temperatures under several sets of designed UAEE conditions. After the ultrasonic extraction, the UAEE solution was concentrated, and then precipitated by adding ethanol to a final concentration of 80% (v/v) for 12 h at 4°C. The precipitate was collected and dried to obtain crude polysaccharides which were re-dissolved in distilled water for polysaccharides yield and antioxidant capacity determination. For comparison, 3.0 g GBS powder was extracted using 60 mL of deionized water at 90°C for 4 h using the method of hot water.

The polysaccharides content was measured by phenol-sulfuric acid method using D-glucose as a standard. The yield (%) of GBSP was calculated as formula:

$$y(\%) = c/w \times 100\% \quad (1)$$

where c was the content of polysaccharides, and w represented dried sample weight.

The ferric-reducing antioxidant capacity (FRAC) of GBSP was detected by the 1,10-phenanthroline with slight modification. Briefly, 0.3 mL of each GBSP solution mixed with 1.0 mL of 0.2% $FeCl_3$ and 0.5 mL of 0.5% 1,10-phenanthroline, followed by adjusting the final volume up to 10 mL with 50% methanol, mixing each adequately, keeping in the dark for 0.5 h and measuring the absorbance at 510 nm. $FeSO_4 \cdot 7H_2O$ was selected as standard, and the antioxidant capacity of GBSP was expressed as Fe^{2+} equivalent per gram of weight material (mmol Fe^{2+}/g).

2.3 Experimental Design of UAEE Conditions

2.3.1 Plackett–Burman design (PBD)

The PBD was used to screen out the multifactor and derive valid and robust statistical significant factors that influence the applied procedure [19]. In this work, PBD was performed to evaluate the significance of seven variables affecting the UAEE procedures, including UAE power (A), time (B), temperature (C), cellulase concentration (D), pH (E), solvent-to-solid (F) and sample particle size (G). Table 1 shows the seven factors and their levels. The design matrix is presented in Table 2. The selected 7 parameters were realized with the yield and FRAC values of GBSP taken as the responses. PBD was based on the first order polynomial model shown as standard equatin Eq. (2):

$$Y_i = C_0 + \sum_i C_i X_i \quad (2)$$

where Y_i is the xperimental response, X_i is the indeenden variables, and C_0 and C_i are the regression coefficients for intercept and linear terms, respectively.

2.3.2 Rotatable central composite design (RCCD)

To optimize the significant independent variables from PBD, a RCCD was applied to obtain maximum the yield and FRAC values of GBSP. The significant independent variables were individually modified while the others without significance were maintained at the 0 conditions. The data from the RCCD were analyzed by multiple regressions to fit the following quadratic equation:

$$Y = \varphi_0 + \sum_{i=1}^{n} \varphi_i x_i + \sum_{i=1}^{n} \varphi_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \varphi_{ij} x_i x_j . \quad (3)$$

where Y, φ_0, φ_i, φ_{ii}, φ_{ij} , and indicate the predicted

Table 1. Real values of the variables in the Plackett-Burman design and experimental data of the yield of GBSP and FRAC values of GBSP (n = 3).

Run	Factors							Responses	
	A [a] (W)	B (min)	C (°C)	D (%)	E	F (mL/g)	G (mesh)	Y_1 [b] (%)	Y_2 (mmol Fe^{2+}/g)
1	100.00	10.00	30.00	4.00	4.00	40.00	60.00	4.65	16.5
2	300.00	40.00	30.00	4.00	6.00	40.00	20.00	6.79	19.2
3	100.00	40.00	80.00	0.50	6.00	40.00	60.00	3.68	15.1
4	300.00	10.00	30.00	0.50	6.00	10.00	60.00	5.21	17.8
5	300.00	40.00	30.00	0.50	4.00	40.00	20.00	5.87	18.3
6	100.00	10.00	80.00	0.50	6.00	40.00	20.00	3.16	14.2
7	100.00	10.00	30.00	0.50	4.00	10.00	20.00	3.05	14.7
8	300.00	10.00	80.00	4.00	6.00	10.00	20.00	6.21	18.7
9	100.00	40.00	30.00	4.00	6.00	10.00	60.00	5.18	16.4
10	100.00	40.00	80.00	4.00	4.00	10.00	20.00	5.26	17.3
11	300.00	40.00	80.00	0.50	4.00	10.00	60.00	6.03	18.5
12	300.00	10.00	80.00	4.00	4.00	40.00	60.00	6.31	18.7

[a] **A**: UAE power; **B**: UAE time; **C**: UAE temperature; **D**: cellulase concentration; **E**: pH; **F**: solvent-to-solid; **G**: sample particle size.
[b] $\mathbf{Y_1}$: The yield of GBSP; $\mathbf{Y_2}$: FRAC values of GBSP.

Table 2. Analysis of variance and regression analysis of Plackett-Burman design data for the prediction of significant extraction variables.

Source	Regression data							
	Y_1 (The yield of GBSP)				Y_2 (FRAC values of GBSP)			
	Effect	F-value	P-value	Inference	Effect	F-value	P-value	Inference
Model		67.12	0.0006	Significant		33.17	0.0022	Significant
A	1.90	299.82	<0.0001	Significant	2.84	175.15	0.0002	Significant
B	0.70	40.80	0.0031	Significant	0.70	10.69	0.0308	Significant
C	0.02	0.02	0.8870		0.06	0.097	0.7711	
D	1.24	125.45	0.0004	Significant	1.36	40.75	0.0031	Significant
E	0.16	2.02	0.2279		0.44	4.10	0.1130	
F	0.08	0.53	0.5078		0.24	1.19	0.3370	
G	0.12	1.19	0.3371		0.10	0.22	0.6647	

response, the intercept term, the linear coefficient, the squared coefficient and the interaction coefficient, respectively. Model adequacy was evaluated using F ratio and coefficient of determination (R^2) represented at 5% level of significance, accordingly.

2.4 Chemical Composition and Structural Characteristics of GBSP

2.4.1 Chemical composition analysis of GBSP

The total carbohydrate and protein contents were determined by phenol–sulfuric acid colorimetric method [20] and Lowry method [21], respectively. Uronic acid content was measured by photometry with m-hydroxydiphenyl at 525 nm using GlcA as the standard [22].

2.4.2 Determination of monosaccharide composition of GBSP

The monosaccharide compositions of GBSP were determined by GC-MS as previous method [23] with some modifications. Briefly, GBSP (5 mg) was hydrolyzed separately with CF_3COOH (2M, 4 mL) at 110°C for 6 h and concentrated to dry residue which was washed again by methanol for removing CF_3COOH. The acetylation was carried out with 6 mg inositol, 10 mg hydroxylamine hydrochloride and 1 mL pyridine by heating in a water bath for 30 min at 90 °C. After incubation, the tubes were removed from the heat block, allowed to cool to room temperature, and then 1 mL of acetic anhydride was added and mixed. The tubes were sealed and incubated in a water bath shaker set at 90 °C for 30 min again to produce alditol acetate derivatives, which were analyzed by GC-MS (model 7890/5975C-GC/MSD, Agilent Technologies; Santa Clara, CA, USA) equipped with a DB-5MS capillary column (30 m × 0.25 mm × 0.25 mm, Agilent). Aldononitrile acetate derivatives of monosaccharide standards with inositol as internal standard were prepared and subjected to GC-MS analysis the same way. The monosaccharide compositions of the GBSP were determined by comparing the retention times with those of standards.

2.4.3 Infrared (IR) spectroscopy analysis of GBSP

Dried GBSP (2 mg) was mixed with KBr powder and pressed into a pellet. The spectrum of the samples was recorded from KBr pellet on a Fourier transform IR spectrophotometer (FT-IR) (NEXUS-870, Nicolet Instrument Co., USA) in the range of 400–4000 cm^{-1}.

2.5 Antioxidant Activity of GBSP

The antioxidant activities of $GBSP_{UAEE}$ and $GBSP_{HWE}$ obtained from UAEE and HWE were respectively evaluated using many methods including DPPH, ABTS, and reducing power.

2.5.1 DPPH assay

The DPPH radical scavenging activity of the GBSP was performed as described previously with slight modification [7]. Briefly, 1.0 mL GBSP water solution (5–50 mg mL^{-1}) was added in 2.0 mL freshly prepared DPPH methanol solution (0.1 mM). After 30 min at room temperature in the dark, the absorbance was detected at 517 nm and expressed as radical scavenging capacity (RSC (%)). RSC was calculated by following equation:

$$RSC = 100 - (A_{sample} \times 100) / A_{blank} \quad (4)$$

where A_{sample} and A_{blank} were the absorbance of sample and control solution, respectively. This activity was also expressed as the inhibitory concentration at RSC value 50% (IC_{50}, the concentration of test solution required to obtain 50% of radical scavenging capacity).

2.5.2. ABTS assay

The ABTS radical scavenging activity of GBSP was performed as described previously with slight modification [24]. Briefly, the stock solution was diluted to make the absorbance of $ABTS^+$ working solution (0.710 ± 0.05 at 734 nm). For the assay, 2.9 mL $ABTS^+$ working solution and 100 mL GBSP water solution (5–50 mg mL^{-1}) was mixed and the absorbance was immediately

detected at 734 nm after 6 min of incubation under room temperature. The ABTS radical scavenging activity was calculated according to Eq. (4) with ABTS solution instead of DPPH.

2.5.3 Reducing power assay

The reducing power of the GBSP was determined through the transformation of Fe^{3+} to Fe^{2+} by previous reports with slight modification [7]. Briefly, 1.0 mL GBSP water solution (50–500 mg mL^{-1}) was added in 2.5 mL 1% potassium ferricyanide and 2.5 mL phosphate buffer (pH 6.6, 0.2 M). After incubating at 50°C for 20 min, 2.5 mL 10% trichloroacetic acid was added to the mixtures and centrifuged at 5000 rpm for 20 min. Finally, 2.5 mL the supernatant solution was mixed with the same volume of distilled water and 0.5 mL 1% ferric chloride water solution. After 10 min, the absorbance was detected at 700 nm.

2.6 Statistical Treatment of Data

Design Expert software (version 8.0, Minneapolis, USA) was used for designing experiments as well as for regression analysis of the experimental data obtained.

3. Results and Discussion

3.1 Screening of Significant Factors using PBD

Two stages might be considered in method optimization: (a) a screening step, where many factors were studied to identify those with the significant effects on critical variables, and (b) the optimization, where the factors were further examined to determine the best useful conditions [25]. In this study, a simple and useful PBD was selected as a method of screening design by a relatively few experiments.

Based on the preliminary tests and previous reports on the UAEE, seven variables including UAE power (A), time (B), temperature (C), cellulase concentration (D), pH (E), solvent-to-solid (F) and sample particle size (G) were determined and listed in Table 1. The experiment design matrix with Y_1 (the yield of GBSP) and Y_2 (FRAC values of GBSP) as responses were also listed in Table 1, and the result and analysis of variance (ANOVA) were presented in Table 2. In general, variables of a large coefficient with a small *p*-value (<0.05) were considered as significant influence. The results indicated that UAE power (A), time (B) and cellulase concentration (D) were considered as significant for responses (Y_1 and Y_2). From the previous reports, pareto chart could present the effect of factors on responses and check the statistical significance. In the pareto chart, two limit lines including Bonferroni limit line (5.746) and *t*-value limit line (2.776) were used to determine the extremely significant, significant and insignificant coefficients of different factors when *t*-value of effect above the Bonferroni limit line, between Bonferroni limit line and *t*-value limit line, and below *t*-value limit line, respectively [26]. Thus, the pareto chart could intuitively provide significant factors and it was employed to identify the significant factors in this study. The result of pareto chart plotted by the *t*-value of effect versus each parameter is shown in Figure S1. From Figure S1A, the *t*-value of UAE power (A), time (B) and cellulase concentration (D) were above Bonferroni limit line, it indicated that UAE power (A), time (B) and cellulase concentration (D) were the extremely significant factors to the yield of GBSP (Y_1). As shown in Figure S1B, the *t*-value of UAE power (A) and time (B) were above Bonferroni limit line, and the *t*-value of cellulase concentration (D) was between Bonferroni limit line and *t*-value limit line, both of which indicated that the three variables were considered as significant factors for FRAC values of GBSP (Y_2). The R^2 values of Y_1 and Y_2 were 99.16% and 98.31%, respectively. The result indicated that the description of pareto charts were dependable. The initial first order model equations developed by PBD for Y_1 and Y_2 was generated by the Design-Expert 8.5 software according to the following equation:

$$\Upsilon_1 = 2.188 + 0.01A + 0.023B - 0.0003C + 0.352D - 0.078E - 0.003F + 0.003G \quad (5)$$

$$\Upsilon_2 = 14.072 + 0.014A + 0.023B - 0.001C + 0.39D - 0.217E - 0.008F + 0.002G \quad (6)$$

Where Υ_1, Υ_2, A, B, C, D, E, F and G represent the predicted yield of GBSP, FRAC values of GBSP, the values of UAE power, time, temperature, cellulase concentration, pH, solvent-to-solid and sample particle size, respectively.

From the final results of PBD, UAE power (A), time (B) and cellulase concentration (D) were comprehensive considered as three important parameters for further UAEE experiments of maximum the yield of GBSP (Y_1) and FRAC values of GBSP (Y_2), whereas the rest 4 factors (UAE temperature, pH, solvent-to-solid and sample particle size) contributed non-significantly. In this study, the effects of non-significant factors including UAE temperature (C), pH (E), solvent-to-solid (F) and sample particle size (G) were also investigated in the preliminary experiments. The results indicated that a relatively large solvent-to-solid might help increase the contact chance between GBS and extraction solvent, and raw materials with small particle size contributed to enlarge contact area between powders and extraction solvent. Therefore, larger solvent-to-solid and smaller particle size might contribute to enhance the yield of GBSP. Additionally, the appropriate rise of extraction temperature was advantageous to the molecular motion, the penetration of solvent and dissolution of GBSP. Considering the yield of GBSP and FRAC values of GBSP, the conditions were selected as following: UAE temperature of 60°C, pH of 4.5, solvent-to-solid of 30 mL/g and sample particle size of 60 mesh.

3.2 Optimization of Significant Factors using RCCD

The significant factors chosen from PBD *viz.* UAE power (A), time (B) and cellulase concentration (D) were considered for further optimization using RCCD. The levels chosen for the factors were set by the previous PBD analysis. The RCCD with a = 1.68 has been carried out on 20 experimental runs ($2^3 + (2 \times 3) + 6$), including 8 (2^3) vertex points, 6 (2×3) axis points and 6 center points. Multiple regression analysis was performed on the experimental data (Table 3) to evaluate for significance.

3.2.1 Optimization of UAEE conditions for maximum yield of GBSP

RCCD was applied to research the yield of GBSP using significant variables of A, B and D (Table 3). An initial response surface model of GBSP yields from UAEE was generated by the Design–Expert 8.5 software. ANOVA was then performed to retain the significant terms ($p < 0.05$) and exclude the non-significant terms ($p > 0.05$). The result of ANOVA indicated that A, B, D, A^2, B^2 and D^2 were significant. Consequently, a simplified model can thus be expressed as:

$$\Upsilon_1 = -9.314 + 0.038A + 0.446B + 3.479D - 0.00007A^2 - 0.006B^2 - 0.535D^2 \quad (7)$$

Where Υ_1, A, B and D represent the predicted yield of GBSP, the values of UAE power, time and cellulase concentration, respectively. The model F-value of 76.59 implied the model to be significant ($p < 0.05$). The lack of fit ($p = 0.1857$) suggested that it was an adequate model to accurately predict the response variable. The regression coefficient $R^2 = 0.9857$ also indicated that the resulting model was a good fit for GBSP extraction.

Drawing RSM was regarded as the best pathway to visualize the influence of the independent variables. In this study, 3D response surfaces were developed by the fitted Eq. (7) and plotted by the response (Z-axis) according to two factors (X and Y coordinates), holding the other one factor at zero (0-level). From Figure 1A, when the UAE power (A) was fixed, the yield of GBSP rapidly increased with the rapidly increase of

Table 3. RCCD with experimental values for the yield of GBSP and FRAC values of GBSP, ANOVA for response surface quadratic model, and fit statistics for the response values (n = 3).

CCD experiments						Analysis of variance (ANOVA)				
Run	A	B	D	Y_1	Y_2	Source	Y_1		Y_2	
	(W)	(min)	(%)	(%)	(mmol Fe^{2+}/g)		*F*-value	*P*-value	*F*-value	*P*-value
1	300.00	40.00	4.00	6.82	19.4	Model	76.59	< 0.0001	23.19	< 0.0001
2	368.18	30.00	3.00	6.91	18.9	A	238.56	< 0.0001	31.59	0.0002
3	200.00	13.18	3.00	5.57	15.8	B	18.08	0.0017	6.59	0.0280
4	300.00	40.00	2.00	6.71	17.5	D	5.42	0.0422	8.08	0.0175
5	200.00	30.00	3.00	7.73	22.9	AB	2.84	0.1230	7.15	0.0233
6	200.00	30.00	3.00	7.85	23.5	AD	0.018	0.8949	0.028	0.8706
7	100.00	40.00	2.00	5.51	16.5	BD	0.76	0.4040	1.37	0.2692
8	200.00	30.00	3.00	7.67	22.5	A^2	203.26	< 0.0001	73.71	< 0.0001
9	200.00	30.00	3.00	7.59	21.8	B^2	178.88	< 0.0001	64.48	< 0.0001
10	200.00	30.00	3.00	7.43	21.3	D^2	123.94	< 0.0001	45.71	< 0.0001
11	200.00	46.82	3.00	5.95	17.6	Lack of fit	2.34	0.1857	1.30	0.3906
12	200.00	30.00	4.68	6.41	18.3					
13	200.00	30.00	1.32	5.72	16.7					
14	100.00	20.00	2.00	4.52	14.6					
15	100.00	40.00	4.00	5.38	19.1	**Credibility analysis of the regression equations**				
16	300.00	20.00	4.00	6.49	19.3					
17	200.00	30.00	3.00	7.62	22.1	**Index mark**	**CV%**	**R^2**	**Adj.R^2**	**Pre. R^2**
18	31.82	30.00	3.00	4.37	13.8					
19	100.00	20.00	4.00	4.82	15.3	**Y_1**	2.87	0.9857	0.9728	0.9164
20	300.00	20.00	2.00	6.36	18.3	**Y_2**	4.51	0.9543	0.9131	0.7749

UAE time (B) until reaching a maximum and then slowly decreased. Similarly, UAE power (A) caused an initial rapidly increase and then slightly decrease in the yield of GBSP. In the UAEE process, more bubbles were formed and collapsed with larger amplitude ultrasound waves traveling through extraction solvent under high power. As a result, violent shock wave and high-shear gradients might be created to cause the destruction of the cell walls. This facilitated the release of target compounds significantly and enhanced the mass transfer rate simultaneously, thus leading to high yield of GBSP. However, the degradation of GBSP might occur under too high UAE power, which could explain the reason why the yield of GBSP decreased. Additionally, the whole extraction process needed enough time for penetration of extraction solvent into GBS, dissolution and diffusion of GBSP from plant matrix to outside solvent. The increasingly small improvement rate or even non-improvement of Y_1 as the further increasing UAE time was because that the time of extraction was enough for almost complete extraction of GBSP from GBS. From Figure 1B, the surface was relatively flat. As the UAE power (A) increased, the yield of GBSP increased significantly and then decreased slightly. When the UAE power (A) was at a certain value, the similar result was also achieved in cellulase concentration (D). The incomplete extraction may be occurred in a low cellulase concentration, and higher cellulase concentration might promote the

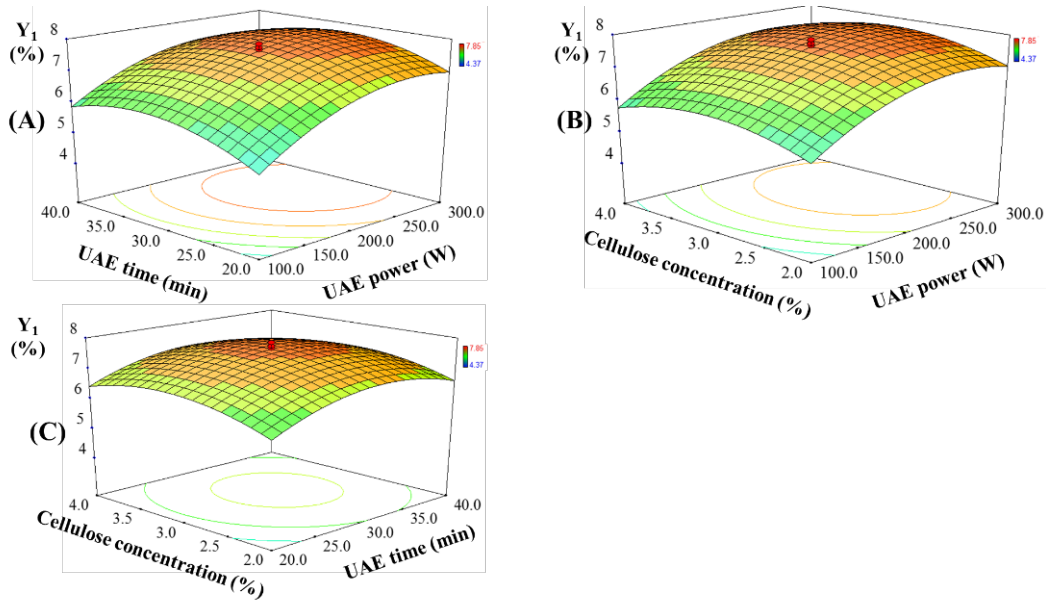

Figure 1. Three-dimensional contour plots showing the experimental factors and their mutual interactions: (A) effect of UAE power and time on the yield of GBSP, (B) effect of UAE power and cellulase concentration on the yield of GBSP, and (C) effect of UAE time and cellulase concentration on the yield of GBSP.

release of intracellular GBSP by breaking the cell wall and lipid bodies, resulting in the continual enhancement on the yield of GBSP in the first period. Therefore, a relatively higher cellulase concentration contributed to positive influence on the yield of GBSP. However, the relative constant yield of GBSP was due to the cellulase concentration used was enough for extraction of GBSP. Figure 1C shows the effects of UAE time (B) and cellulase concentration (D) on the yield of GBSP. When the UAE time (B) was fixed, the value of Y_1 increased with the increase of cellulase concentration (D) until reaching a maximum and then decreased. Similarly, cellulase concentration (D) caused an initial increase and then decrease in Y_1. This result indicated that UAE power (A), time (B) and cellulase concentration (D) were important variables for extracting GBSP from GBS. The maximum yield of GBSP from GBS was calculated as 7.86% in the following optimum UAEE conditions: UAE power of 248 W, UAE time of 31.6 min, cellulase concentration of 3.17%, UAE temperature of 60°C, pH of 4.5, solvent-to-solid of 30 mL/g and sample particle size of 60 mesh.

3.2.2 Optimization of UAEE Conditions for Maximum FRAC Values of GBSP

To simultaneously optimize two responses of the yield of GBSP and FRAC values of GBSP, a RCCD (20 runs) was also used for the optimization of effective parameters on UAEE for the maximum FRAC values of GBSP. The experimental data show in Table 3. ANOVA was then performed to remove the insignificant terms (p >0.05), resulting in the following model:

$$Y_2 = -23.252 + 0.114A + 1.188B + 8.744D - 0.0008AB - 0.0002A^2 - 0.018B^2 - 1.507D^2 \quad (8)$$

Where Y_2, A, B and D represent the predicted

FRAC values of GBSP, the values of UAE power, time and cellulase concentration, respectively. The combination of independent variables, result and analysis of variance (ANOVA) were also listed in Table 3. According to the ANOVA results, there was a good agreement between the predicted values and observed data points ($R^2 = 0.9543$ for FRAC values of GBSP). Herein, R^2 value of 0.9543 implied that 95.43% of the total variations for the FRAC values of GBSP were attributed to the above three independent variables (UAE power, time and cellulase concentration). The *p*-value of lack of fit for FRAC values of GBSP was found to be 0.3906, indicating the good fitness of the model (Table 3).

The response surfaces were plotted (Figure S2) to investigate the interaction among the variables and to obtain the best UAEE conditions of each variable to obtain maximum FRAC values of GBSP. The effect of UAE power (A) and time (B) was presented in Figure S2A. The FRAC values of GBSP increased rapidly when UAE power (A) and time (B) were increased in the range of 31.8–230.9 W and 13.2–31.2 min, respectively. But beyond 230.9 W and 31.2 min, the FRAC values of GBSP decreased with increasing UAE power (A) and time (B), respectively. As shown in Figure S2B, the FRAC values of GBSP increased rapidly when UAE power (A) and cellulase concentration (D) were increased in the range of 31.8–230.9 W and 1.3–3.2%, respectively. On the contrary, FRAC values of GBSP decreased slowly with increasing of UAE power (A) from 230.9 W to 368.2 W, and Y_2 was also decreased slowly with increasing of cellulase concentration (D) from 3.2% to 4.7%. The interaction relationship UAE time (B) and cellulase concentration (D) on the FRAC values of GBSP is shown in Figure S2C. The response Y_2 increased with increasing UAE time (B) from 13.2 min to 31.2 min. However, upon exceeding 31.2 min of UAE time (B), FRAC values of GBSP was decreased. Similarly, cellulase concentration (D) caused an initial increase and then decrease in the FRAC values of GBSP. Therefore, it was concluded that maximum FRAC values of GBSP (the predicted value of 22.62 mmol Fe^{2+}/g) could be achieved when UAE power (A) was 230.9 W, time was 31.2 min, cellulase concentration was 3.2%, UAE temperature was 60°C, pH was 4.5, solvent-to-solid was 30 mL/g and sample particle size was 60 mesh.

3.2.3 Optimization and model validation

In this study, considering the fact of maximum the yields and antioxidant capacity of GBSP, the Design–Expert 8.5 software was used for simultaneous optimization of the multiple responses by the desirability function. The desirability approach was a popular method that assigned a given score to a set of responses and then a factor setting that maximizes the overall score would be chosen [27]. In this study, the value of desirability was 0.97 using Design–Expert 8.5, and the simultaneous optimal values of significant factors for the yields of GBSP and FRAC values of GBSP were provided as follows: UAE power of 238.6 W, time of 31.1 min, cellulase concentration of 3.2%. Furthermore, the other non-significant factors including UAE temperature, pH, solvent-to-solid and sample particle size were also investigated in the preliminary experiments and described in section "3.1". Finally, for operational convenience, the following optimum conditions were selected: UAE power of 239 W, time of 31 min, cellulase concentration of 3.2%, UAE temperature of 60°C, pH of 4.5, solvent-to-solid of 30 mL/g and sample particle size of 60 mesh which predicted the yields of GBSP and FRAC values of GBSP as 7.85% and 22.61 mmol Fe^{2+}/g, respectively. Under the optimum conditions, the experimental yield of GBSP was 7.71 ± 0.53 % ($n = 3$) and FRAC values of GBSP was 22.43 ± 1.93 mmol Fe^{2+}/g ($n = 3$), which were close to the above predicted values. These results suggested that the models developed through desirability function proved adequate for predicting yields and antioxidant capacity of GBSP in this study. Additionally, comparing the hot water extraction (HWE) with

ultrasonic-assisted extraction, UAEE exhibited more extraction yield and better antioxidant capacity with increase of 12.8% and 25.3%, respectively. These results indicated that ultrasonic-assisted method was suitable for extraction antioxidant polysaccharides from GBS.

Normally, conventional extraction techniques such as hot water or boiling extraction and novel extraction methods including UAE, MAE and pressurized water extraction (PWE). Compared with the conventional extraction method, UAE was an effective extraction technique to improve the extraction efficiency by increasing the yield and shortening time. Moreover, enzyme-assisted extraction, a mild, efficient and environmental friendly extraction method, was successfully used in the extraction of polysaccharides from plants. The previous studies showed that the introduction of ultrasonic energy during enzymatic treatment resulted in significant improvement [5, 9, 16]. Therefore, UAEE was employed to extract antioxidant GBSP in this study.

3.3 Antioxidant Activity of GBSP

Antioxidant activity was the common effect in many traditional Chinese medicines including *G. biloba*, *Gardenia jasminoides* Ellis, *Rehmannia glutinosa* Libosch., *Eucommia ulmoides* Oliv. and *Achyranthes bidentata* Blume [28]. The antioxidant activities of the two GBSP ($GBSP_{UAEE}$ and $GBSP_{HWE}$ were obtained by UAEE and HWE, respectively) were evaluated by DPPH radical scavenging, ABTS radical scavenging and reducing power assay methods. The results showed that the antioxidant activities obtained using UAEE were significantly higher than those using hot water extraction method (Figure 2).

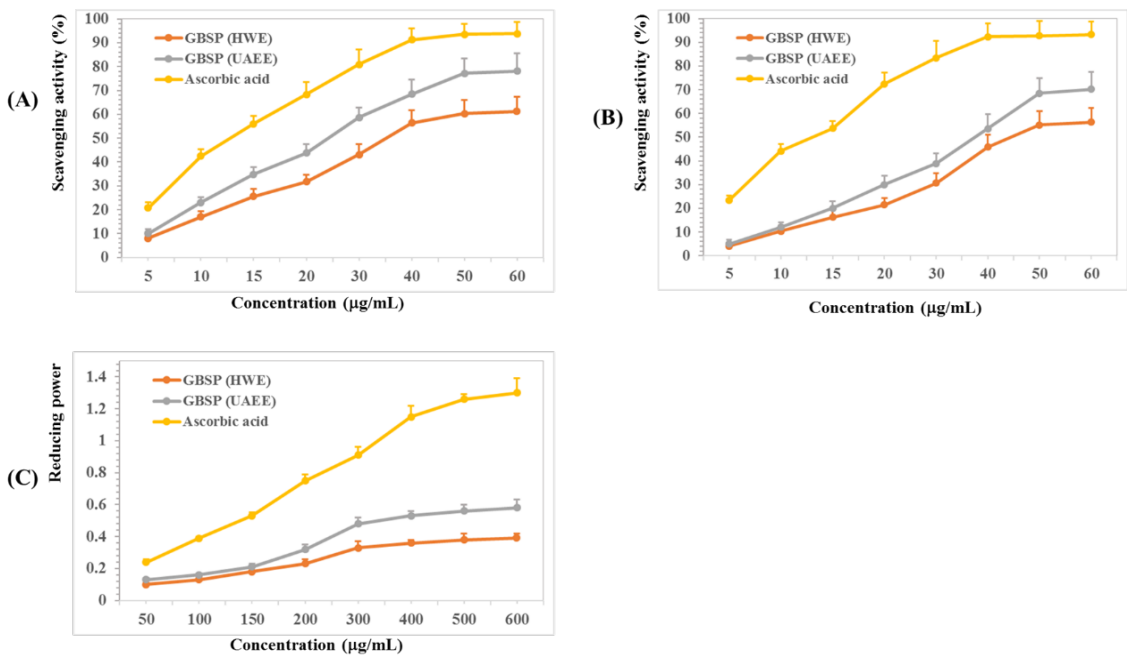

Figure 2. Antioxidant activities of $GBSP_{UAEE}$ obtained by UAEE and $GBSP_{HWE}$ obtained by HWE, (A) scavenging of DPPH radical; (B) scavenging of ABTS radical and (C) reducing power.

3.3.1 DPPH radical scavenging activity

DPPH were widely employed to investigate radical scavenging activity of polysaccharides from previous reports [27]. DPPH radical scavenging activities of $GBSP_{UAEE}$ and $GBSP_{HWE}$ were also tested in this study. And the results indicated that $GBSP_{UAEE}$ and $GBSP_{HWE}$ showed obvious scavenging activity on DPPH radical with a concentration-dependent manner at 5–50 mg mL^{-1} and 5–40 mg mL^{-1}, respectively. Additionally, from Figure 2A, $GBSP_{UAEE}$ exhibited a better scavenging activity compared with $GBSP_{HWE}$ although weaker than positive control of ascorbic acid at all tested concentration.

3.3.2 ABTS radical scavenging activity

According to the describes of previous publications, the ABTS method was a decolorization assay applicable to both lipophilic and hydrophilic antioxidants at various pH levels [20]. As shown in Figure 2B, the ABTS radical scavenging activities of $GBSP_{UAEE}$ and $GBSP_{HWE}$ increased with the increase of $GBSP_{UAEE}$ and $GBSP_{HWE}$ concentration until reaching a maximum. And all the test samples showed remarkable effect on ABTS radical scavenging activities. Furthermore, the ABTS radical scavenging activities of $GBSP_{UAEE}$ were higher than $GBSP_{HWE}$ at the same concentration. The result indicated that $GBSP_{UAEE}$ had a stronger ABTS radical scavenging activity than $GBSP_{HWE}$.

3.3.3 Reducing power assay

From Figure 2C, the reducing power of $GBSP_{UAEE}$ was higher than that of $GBSP_{HWE}$ in the concentration range of 50–600 mg mL^{-1}. Although the reducing power of $GBSP_{UAEE}$ was lower than that of positive control of ascorbic acid, it still reached 0.58 at the concentration of 600 mg mL^{-1}. The reducing property was generally associated with the capacity of reacting with certain precursors of peroxide and preventing peroxide formation. In the reducing power assay, the antioxidants would result in the reduction of the Fe^{3+}/ferricyanide complex to its ferrous form (Fe^{2+}) by donating an electron. Based on that theory, $GBSP_{UAEE}$ could possess a stronger ability to donate electrons and reduce peroxide than $GBSP_{HWE}$.

3.4 Chemical Composition and Structural Characteristics of $GBSP_{UAEE}$

The chemical composition of $GBSP_{UAEE}$ were determined. The results showed that $GBSP_{UAEE}$ contained 90.23 ± 2.98% of total carbohydrate, 1.31 ± 0.12% % of protein, and 20.35 ± 1.61 % of uronic acid, respectively.

The monosaccharide compositions of $GBSP_{UAEE}$ were analyzed by the GC-MS. By comparing the retention time with those of monosaccharide standards, the peaks in $GBSP_{UAEE}$ were identified. Results showed that $GBSP_{UAEE}$ was composed of Glc, Gal, Xyl, Rha, Man and Ara with molar ratio 7.86, 20.31, 1.52, 9.43, 2.86 and 5.31, respectively. Gal, Rha and Glc were the major monosaccharides of $GBSP_{UAEE}$. The monosaccharide compositions of $GBSP_{UAEE}$ were similar to the polysaccharides isolated from GBS in previous report [7].

The IR spectra of $GBSP_{UAEE}$ were showed in Figure S3. The strong absorption peak at around 3318.45 cm^{-1} was attributed to the stretching vibration of O-H, which was the main functional group of the polysaccharides. The small band at 2945.16 cm^{-1} represented the stretching vibration of C-H including CH, CH_2 and CH_3 [30]. The peak at 1629.28 cm^{-1} was corresponded to the bending vibration of O-H. The strong absorption peak at around 1033.67 cm^{-1} was attributed to the stretching of C-O-C or C-O-H that could also explain the existence of pyranoid ring conformation in the polysaccharides.

4. Conclusions

In the present study, the UAEE conditions were optimized for achieving maximal recovery of antioxidant polysaccharides from waste *Ginkgo biloba* sarcotesta using a PBD coupled with RSM, and the optimum extraction conditions were UAE power of 239 W, time of 31 min, cellulase

concentration of 3.2%, temperature of 60°C, pH of 4.5, solvent-to-solid of 30 mL/g and sample particle size of 60 mesh. Additionally, GBSP obtained by optimum UAEE condition showed higher DPPH, ABTS radical scavenging activity and reducing power compared with $GBSP_{HWE}$. This study indicated that the maximum extraction of GBSP with higher antioxidant activity which might be operational to produce GBSP nutraceuticals and pharmaceuticals in food and pharmaceutical industries under these optimal UAEE conditions.

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References

[1] Major R.T., *Science*, 1967; **157**: 1270-1273. DOI 10.1126/science.157.3794.1270.

[2] Zhou Z. and Zheng S., *Nature*, 2003; **423**: 821-822. DOI 10.1038/423821a.

[3] Zhou G., Yao X., Tang Y., Yang N., Pang H., Mo X., Zhu S., Su S., Qian D., Jin C., Qin Y. and Duan J. *Chem. Phys. Lipids*, 2012; **165**: 731-736. DOI 10.1016/j.chemphyslip.2012.08.003.

[4] Wu X., Mao G., Zhao T., Zhao J., Li F., Liang L. and Yang L. *Carbohyd. Polym.*, 2011; **86**: 1073-1076. DOI 10.1016/j.carbpol.2011.04.069.

[5] Minjares-Fuentes R., Femenia A., Garau M.C., Meza-Velazquez L.A., Simal S. and Rossello C., *Carbohyd. Polym.*, 2014; **106**: 179-189. DOI 10.1016/j.carbpol.2014.02.013.

[6] Yang L., Xu Z., Wu X., Liu Q., Chen J. and Liu J., *Food Sci.*, 2004; **25**: 372-375.

[7] Chen J., Zhang T., Jiang B., Mu W. and Miao M., *Carbohyd. Polym.*, 2012; **87**: 40-45. DOI 10.1016/j.carbpol.2011.06.083.

[8] Liu H., Wang F. and Liu Y., *Food Chem.*, 2016; **202**: 104-109. DOI: 10.1016/j.foodchem.2016.01.129.

[9] Ponmurugan K., Al-Dhabi N.A., Maran J.P., Karthikeyan K., Moothy I.G., Sivarajasekar N. and Manoj J.J.B., *Carbohyd. Polym.*, 2017; **173**: 707-713. DOI: 10.1016/j.carbpol.2017.06.018.

[10] Chemat F., Rombaut N., Sicaire A.G., Meullemiestre A., Fabiano-Tixier A.S. and Abert-Vian M., *Ultrason. Sonochem.*, 2017; **34**: 540-560. DOI: 10.1016/j.ultsonch.2016.06.035.

[11] Zeković Z., Cvetanović A., Švarc-Gajić J., Gorjanović S., Sužnjević D., Mašković P., Savić S., Radojković M. and Đurović S., *Ind. Crop. Prod.*, 2017; **108**: 423-430. DOI: 10.1016/j.indcrop.2017.06.055.

[12] Mašković P., Veličković V., Đurović S., Zeković Z., Radojković M., Cvetanović A., Švarc-Gajić J., Mitić M. and Vujić J., *Phytomedicine*, 2018; **38**: 118-124. DOI: 10.1016/j.phymed.2017.11.010.

[13] Bai Y., Liu L., Zhang R., Huang F., Deng Y. and Zhang M., *Int. J. Biol. Macromol.*, 2017; **96**: 214-222. DOI: 10.1016/j.ijbiomac.2016.11.105.

[14] Chen F., Li D., Shen H., Wang C., Li E., Xing H., Guo L., Zhao Q., Shi J., Nguyen H. and Liu J., *Biomed Res. Int.*, 2017; **2017**: 6160785. DOI: 10.1155/2017/6160785.

[15] Liu Y., Gong G., Zhang J., Jia S., Li F., Wang Y. and Wu S., *Carbohyd. Polym.*, 2014; **110**: 278-284. DOI: 10.1016/j.carbpol.2014.03.040.

[16] Pezeshkpour V., Khosravani S.A., Ghaedi M., Dashtianba K., Zare F., Sharifi A., Jannesar R. and Zoladl M., *Ultrason. Sonochem.*, 2018; **40**: 1031-1038. DOI: 10.1016/j.ultsonch.2017.09.001.

[17] Zhou J., Xu J., Cong J., Cai Z., Zhang J., Wang J. and Ren Y., *J. Chromatogr. A*, 2018; **1532**: 20-29. DOI: 10.1016/j.chroma.2017.11.050.

[18] Ma L., Wang L., Tang J. and Yang Z., *Food Chem.*, 2016; **204**: 283-288. DOI: 10.1016/j.foodchem.2016.02.126.

[19] Panhwar A., Tuzen M. and Kazi T., *Talanta*, 2018; **178**: 588-593. DOI: 10.1016/j.talanta.2017.09.079.

[20] Chow P.S. and Landhäusser S.M., *Tree Physiol.*, 2004; **24**: 1129-1136. DOI: 10.1093/treephys/24.10.1129.

[21] Hajare A.A., More H.N. and Pisal S.S., *Curr. Drug Deliv.*, 2011; **8**: 678-690. DOI: 10.2174/156720111797635450.

[22] Zhang Z., Lv G., Song T., Jin Q., Huang J., Fan L. and Cai W., *Carbohyd. Polym.*, 2015; **132**: 397-399. DOI: 10.1016/j.carbpol.2015.06.006.

[23] Lv X., Wang C., Cheng Y., Huang L. and Wang Z., *Carbohyd. Res.*, 2013; **365**: 20–25. DOI: 10.1016/j.carres.2012.10.013.

[24] Raza A., Li F., Xu X. and Tang J., *Int. J. Biol. Macromol.*, 2017; **94**: 335-344. DOI: 10.1016/j.ijbiomac.2016.10.033.

[25] Zhou G., Fu L. and Li X., *Food Chem.*, 2015; **170**: 186-192. DOI: 10.1016/j.foodchem.2014.08.080.

[26] Chen F., Zhang Q., Fei S., Gu H. and Yang L., *Ultrason. Sonochem.*, 2017; **35**: 161-175. DOI: 10.1016/j.ultsonch.2016.09.015.

[27] Zhou G., Ma J., Tang Y., Wang X., Zhang J. and Duan J., *Molecules*, 2018; **23**: 1029. DOI: 10.3390/molecules23051029.

[28] Shan M., Yu S., Yan H., Guo S., Xiao W., Wang Z., Zhang L., Ding A., Wu Q. and Li S., *Molecules*, 2017; **22**: 1689. DOI: 10.3390/molecules22101689.

[29] Jahanbin K., *Int. J. Biol. Macromol.*, 2018; **107**: 1227-1234. DOI: 10.1016/j.ijbiomac.2017.09.100.

[30] Li X. and Wang L., *Int. J. Biol. Macromol.*, 2016; **107**: 270-276. DOI: 10.1016/j.ijbiomac.2015.11.060.