Quality Assurance and Safety of Crops & Foods, 2019; 11 (5): 479-490

Wageningen Academic Publishers

# Optimisation of green tea polysaccharides by ultrasound-assisted extraction and their *in vitro* antidiabetic activities

**A. Karadag[1*], E. Pelvan[2], K. Dogan[1], N. Celik[3], D. Ozturk[3], K. Akalın[3] and C. Alasalvar[2]**

*[1]Department of Food Engineering, Yıldız Technical University, 34210, Esenler-Istanbul, Turkey; [2]TÜBİTAK Marmara Research Center, Food Institute, P.O. Box 21, 41470 Gebze-Kocaeli, Turkey; [3]TÜBİTAK Marmara Research Center, Genetic Engineering and Biotechnology Institute, P.O. Box 21, 41470 Gebze-Kocaeli, Turkey; karadaga@yildiz.edu.tr*

Received: 27 February 2019 / Accepted: 30 June 2019


**RESEARCH ARTICLE**

## Abstract

Tea polysaccharides have attracted scientific interest due to their antidiabetic effects, and lower quality tea leaves have more polysaccharide in their content compared to higher grade tea leaves. The aim of this study was to optimise the ultrasound-assisted extraction (UE) conditions of polysaccharides from low-grade green tea (GTPS) by Box-Behnken response surface design on the desired response (yield). The optimal extraction parameters were determined as follows: extraction temperature (80 °C), extraction time (60 min), ultrasound power (400 W), and liquid to solid ratio (22 ml:g). The experimental yield of GTPS (4.65±0.29%) obtained under these conditions were well agreed with the value predicted by the model. Without applying ultrasound, while the other extraction conditions were the same (CE), the extraction yield was lower (1.83±0.04%). Fourier transform-infrared spectroscopy (FT-IR) was used for the identification of functional groups present in GTPS and gel permeation chromatography was used to determine the molecular weight distribution of samples. The molecular weight of GTPS obtained by UE was lower, probably some polysaccharide degradations occurred due to ultrasound application. The IR spectrum of GTPS obtained by UE and CE had very similar absorption bands typical for the polysaccharides. Although ultrasound application significantly increased the yield compared to classical hot water extraction, it reduced antioxidant and α-glucosidase inhibitory activity of GTPS.

**Keywords:** green tea polysaccharide, ultrasound-assisted extraction, response surface methodology, α-glycosidase inhibitory activity

## 1. Introduction

Diabetes mellitus is one of the most important public health challenges for the last century around the world, whereas type II diabetes, accounts for 90-95% of all cases, is the most common form. Therefore, the research in safe and effective antidiabetic bioactive compounds has become an important topic due to the undesirable adverse effects affiliated with the synthetic hypoglycaemic drugs (Shori, 2015; Wang and Zhu, 2016)

It is well known that coarse tea leaves have traditionally been used for the treatment of diabetes, particularly in China, Korea, and Japan. However, the majority of researches about tea have been concentrating on comprehension of physicochemical characterisation and bioactivities of tea polyphenols (Cao, 2013; Nie and Xie, 2011). Low-quality tea shoots are poor in polyphenols, caffeine, and catechins, whereas they are rich in polysaccharides Over the last decade, tea polysaccharides have also attracted scientific interest due to their antidiabetic effects (Chen *et al.,* 2016; Xiao and Jiang, 2015).

Classical hot-water extraction (CE), is a popular method for polysaccharides extraction which is usually associated with higher temperature, longer extraction time but lower extraction yield. The use of ultrasound in the extraction of polysaccharides have been studied and offered a feasible alternative to the classical extraction process. In comparison to CE, the most important advantage of ultrasound-assisted

extraction (UE) is to improve the yield. Tea polysaccharides exist as structural constituents of the cell wall, therefore disruption of cell wall structure by the ultrasonic cavitation will facilitate mass transfer and their dissolution (Chen *et al.,* 2016; Ebringerová and Hromádková, 2010).

As a class of response surface methodology (RSM), Box-Behnken (BBD) is a rotatable second-order design and requires three levels of each factor, which the treatments are at the midpoints of edges of the design space and at the centre. RSM has been satisfyingly practiced in the process optimisation in food and pharmaceutical field (Hanrahan and Lu, 2006; Yolmeh and Jafari, 2017).

There are some studies for RSM optimisation of UE conditions in plant polysaccharides (Feng *et al.,* 2014; Jiang *et al.,* 2014; Zhang *et al.,* 2016; Zhu and Liu, 2013). However, not only UE parameters but also the structural properties of the polysaccharides (polymerisation degree, molecular weight, the types of sugar chains and glycosidic linkage) could substantially and/or differently affect the extraction ability and bioactivity of polysaccharides (Yip *et al.*, 2016). There is only one study related to the application of ultrasound extraction from tea (*Camellia sinensis*) flower polysaccharides (Wei *et al.,* 2010). To the best of our knowledge, there has not been any study about tea leaves polysaccharides by taking into consideration of extraction methods on structural properties and desired antidiabetic activity. Therefore, alternative use of low-quality tea as raw material for dietary supplement and to demonstrate the extraction methods with higher yield are of great interest for both producers and consumers.

The aims of this study were to optimise the UE conditions of GTPS by using three levels and four factors (ultrasonic power, extraction temperature, extraction time, and liquid to solid ratio) BBD on the extraction yield and to compare the structures, *in vitro* antidiabetic activities, compositional characteristics, and antioxidant activities of GTPS obtained by both UE and CE.

## 2. Materials and methods

### Materials

Green tea (Antik Yeşil I) was procured from Çaykur (state-owned tea enterprise, Rize, Turkey). *m*-hydroxyl biphenyl was purchased from Fluka-Honeywell (Seelze, Germany) and α-glucosidase and p-nitrophenyl glucopyranoside (pNPG), Coomassie brilliant blue G-250, bovine serum albumin, iron(II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) were purchased from Sigma Aldrich (St. Louis, MO, USA). All other reagents and solvents were of analytical reagent grade and used without further purification unless otherwise noted. All aqueous solutions were prepared using deionised water.

### Preliminary characterisation of green tea polysaccharides

#### *Analytical methods of components*

Total polysaccharides were determined by the phenol-sulphuric acid method with D-glucose as standard (DuBois *et al.,* 1956; Xi *et al.,* 2010). Briefly, green tea polysaccharides (GTPS) was dissolved in water and, mixed with sulphuric acid (98%) and then phenol (5%) was added. The mixture was kept at 90 °C for 5 min, after cooling to room temperature, the absorbance of the solution was measured at 490 nm. For standard glucose, the calibration curve was prepared between 5-150 nmol. The soluble protein was determined by the method of Bradford, 1976 by using bovine serum albumin as a standard. The calibration curve for the standard was prepared between 0.1-0.01 mg/ml. 100 mg protein reagent (Coomassie Brilliant Blue G-250) was dissolved in 50 ml ethanol (95%) and 100 ml phosphoric acid (85%) and diluted with distilled water to a final volume of 1 litre. Then, it was filtered and stored at dark 4 °C. GTPS dissolved in water and mixed with protein reagent solution. The absorbance of the mixture was measured at 595 nm. The uronic acid content was determined by the *m*-hydroxyl biphenyl method (Blumenkrantz and Asboe-Hansen, 1973) with galacturonic acid as standard. The calibration curve for the standard was prepared between 0.01-0.3 mg/ml. A portion of GTPS solution (0.2 ml) was mixed with 0.0125 M tetraborate in sulphuric acid solution (1.2 ml) and kept in a crushed ice bath. After heating tubes at 100 °C for 5 min, the reagent (0.15% *m*-hydroxyl biphenyl in 0.5% NaOH) was added. Tubes were well shaken and the absorbance was measured at 520 nm. Blank samples were prepared with the addition of 0.5% NaOH instead of the *m*-hydroxyl biphenyl reagent. The content of total phenolic compounds was determined according to the procedure described by Horszwald and Andlauer (2011) using the Folin-Ciocalteu phenol reagent and gallic acid as standard. Diluted samples were mixed with the reagent and incubated for 15 min, and then sodium carbonate (20%, w/v) was added to the mixture, and the absorbance was measured at 755 nm. The content of total phenolic compounds was expressed as grams of gallic acid equivalents (GAE) per 100 g of sample.

#### *Determination of antioxidant activities*

Antioxidant activity was assessed by the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity and ferric reducing antioxidant potential (FRAP) methods (Cheng *et al.,* 2013). ABTS radical cation was prepared by mixing the ABTS stock solution (7 mM) with 2.45 mM potassium persulfate solution. GTPS was dissolved in water (0.1 ml), and mixed with ABTS solution (2 ml), and the absorbance was measured at 734 nm. The FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine solution and 20 mM $FeCl_3 \cdot 6H_2O$ in a 10:1:1 ratio just before use.

100 µl sample was mixed with 900 µl $H_2O$ and 2 ml FRAP reagent. The samples incubated at room temperature for 30 min, and the absorbance was measured at 593 nm. The results were expressed as milligrams of Trolox equivalents (TE) per 100 milligrams of the sample for ABTS assay and mg of $FeSO_4 \cdot 7H_2O$ equivalents per 100 mg sample for FRAP assay.

*Determination of the molecular weight of tea polysaccharides*

A high performance liquid chromatography (HPLC) system (LC-20AD pump, RID-10A RID detector, SIL-20A HT autosampler, CTO-2OAC column oven, DGU-20A5 degasser, and CMB-20A communications bus module; Shimadzu Corp., Kyoto, Japan) was used with a column (Ultrahydrogel Linear 10 µm, 7.8×300 mm, Waters, Tokyo, Japan) and a guard column (Ultrahydrogel, 125Å, 6 µm, 6×40 mm, Waters). The samples were dissolved (10 mg/ml) in the mobile phase solution and passed through a 0.45 µm filter (Cai *et al.,* 2013). The detailed conditions were as follows: mobile phase, 0.1 M $NaNO_3$, flow rate 1 ml/min, column temperature 45 °C, and injection volume 10 µl. The calibration curve was established by using dextran of different molecular weights from Sigma (molecular weight: 1000, 5,000, 12,000, 25,000, 50,000, 80,000, 150,000, 270,000, 410,000 and 670,000 Da). Depending on the retention time of dextran standards, the molecular weight of tea polysaccharide was determined.

*Determination of monosaccharide compositions*

The derivatisation of monosaccharides was performed with PMP (1-phenyl-3-methyl-5-pyrazolone) as given by Dai *et al.,* 2010, after hydrolysis of polysaccharides with 4 M trifluoroacetic acid for two hours. After incubation at 70 °C for 100 min, the mixture was neutralised with 0.3 M hydrochloric acid. The resultant solution was evaporated to dryness and extracted with water and chloroform, in which the extraction repeated for three times and the chloroform layers were discarded. Samples, dissolved in water, were injected to Shimadzu HPLC system (LC-20AD pump, SPDM20A DAD detector, SIL-20A HT autosampler, CTO-2OAC column oven, DGU-20A5 degasser, and CMB-20A communications bus module; Shimadzu Corp., Kyoto, Japan) with a Shiseido column (Capcell Pak C18, 250 mm × 4.6 mm, 5 µm particles, Macclesfield, UK). The mobile phase was a mixture of 0.1 M phosphate buffer (pH 6.7) and acetonitrile in a ratio of 83:17 (v/v, %) and the flow rate was 1 ml/min. The quantitation of monosaccharides was based on the calibration curves built for each of the standards at 245 nm. Mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, xylose, arabinose, and fucose were used for building calibration curves between 0.1-3 mg/ml concentration range.

*Fourier transform infrared spectrophotometric analysis*

The infrared (IR) spectra of the polysaccharides were recorded with a Fourier transform infrared (FT-IR) spectrophotometer (Perkin Elmer Spectrum 400, Waltham, MA, USA). The finely powdered sample was pressed into the sample holder for FT-IR measurement in the frequency range of 4,000-650 $cm^{-1}$.

*Inhibition assay α-glucosidase activity*

The effects of extracts on α-glucosidase activity were determined by modifying the method described by Wei *et al.,* 2010, using α-glucosidase from *Saccharomyces cerevisiae* (Sigma G5003, Dorset, UK). The substrate solution p-nitrophenyl glucopyranoside (pNPG) was prepared in 20 mM phosphate buffer with 6.7 mM sodium chloride at pH 6.9. A 75 µl of α-glucosidase (1.0 U/ml) was pre-incubated with 50 µl of the different concentrations of the extracts for 5 min. Then, a 75 µl of 3.0 mM pNPG as a substrate was added to start the reaction. The reaction mixture was incubated at 37 °C for 20 min and stopped by adding 2 ml of 0.1 M $Na_2CO_3$. The α-glucosidase activity was determined by measuring the yellow-colored para-nitrophenol released from pNPG at 405 nm. One unit of α-glucosidase activity is defined as the amount of enzyme required to produce one µmole of p-nitrophenol from pNPG (3 mM) in sodium phosphate buffer (20 mM) with 6.7 mM NaCl, pH 6.9 at 37 °C. Percentage inhibition was calculated as follows:

$$\%\text{Inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{extract}}}{\text{Abs}_{\text{control}}} \times 100 \quad (1)$$

where $\text{Abs}_{\text{control}}$ represents the absorbance of the control and $\text{Abs}_{\text{extract}}$ denotes the absorbance of the experimental sample.

*Extraction of GTPS and determination of the yield*

Green tea was mixed with 80% ethanol (1:10; w:v) for overnight to remove most of the polyphenols, monosaccharides, and small molecule impurities. After filtration, the tea leaves were dried in air and submitted to UE with a designated extraction time, ratio of water to the material, extraction temperature, and ultrasound power. Experiments were performed using a 20 kHz ultrasonic device (Model CV33, Cole-Palmer, Vernon Hills, IL, USA) with a 2.5 cm flat tip probe and, the probe was directly submerged into the suspension in the double-walled cylindrical chamber which water was circulated through the jacket of the chamber to help maintain the temperature. After extraction, solutions were centrifuged at 5,870×*g* for 10 min and the supernatant was filtered through a filter paper (pore size 20-25 µm). The filtrate was collected and concentrated to 1/4 of the original volume with a rotary evaporator at 50 °C under vacuum. Then it was mixed

with four volumes of dehydrated ethanol (1:4; v:v) and kept at 4 °C at overnight. The mixture was centrifuged for 20 min at 15,000×*g* at 4 °C. The precipitate was washed with acetone and ethanol. After that, it was solubilised in water, applied to Sevag reagent (butanol: chloroform, 1:4; v:v) to remove free proteins. The aqueous phase was dialyzed against distilled water for a day, and lyophilised to obtain crude polysaccharide-rich extracts of green tea (GTPS). The percentage yield (Y%) was calculated by the following equation:

$$Y\ (\%) = 100 \times (W_p / W_i) \quad (2)$$

$W_p$ is the weight of the crude polysaccharide powder and $W_i$ is the weight of green tea.

## Experimental design

### *Single factor experiments*

The single factor experiments were performed with various ratio of water to raw material (liquid:solid (LS) ratio, from 10 to 30 ml:g), extraction temperature (from 50 to 90 °C), extraction time (from 20 to 60 min), and ultrasound power (from 100 to 500 W). One factor was changed, while the others kept constant in each experiment, and each single factor experiment was repeated thrice. The effect of each factor was evaluated by determining the yield of crude polysaccharide (Y%).

### *Optimisation of extraction conditions by BBD*

Based on the single factor experiment results, independent process variables and their ranges were determined and the independent variables (four factors) were coded at three levels (–1, 0, and +1). Then the BBD (Minitab®17, 2016, Minitab Inc., State College PA, USA) was applied to study and optimise the influence of process variables on the maximum extraction yield of GTPS. The range of process variables and their levels were presented (Table 1). In order to predict the optimised conditions, a second-order polynomial model was fitted to correlate the relationship between the process variables and the response.

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i<j=2}^{4} \beta_{ij} X_i X_j \quad (3)$$

Y is the response, $X_i$ and $X_j$ are independent process variables, $\beta_0$, $\beta_i$, $\beta_{ii}$ and $\beta_{ij}$ are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively. A total number of 30 experiments with six replicates (used to estimate experimental error) at the centre point were conducted (Table 1).

**Table 1. Box-Behnken experimental design and the results for extraction yield.**

| Run | Independent variables[1] | | | | |
|---|---|---|---|---|---|
| | $X_1$ (extraction temperature, °C) | $X_2$ (extraction time, min) | $X_3$ (liquid to solid ratio, ml:g) | $X_4$ (ultrasonic power, W) | Experimental yield (%) |
| 1 | –1 | 0 | 0 | +1 | 4.338 |
| 2 | +1 | +1 | 0 | 0 | 4.069 |
| 3 | 0 | +1 | +1 | 0 | 3.834 |
| 4 | 0 | 0 | +1 | +1 | 4.470 |
| 5 | 0 | 0 | 0 | 0 | 3.873 |
| 6 | +1 | 0 | +1 | 0 | 3.638 |
| 7 | 0 | 0 | 0 | 0 | 3.730 |
| 8 | 0 | 0 | –1 | –1 | 2.918 |
| 9 | –1 | –1 | 0 | 0 | 3.334 |
| 10 | –1 | 0 | +1 | 0 | 3.798 |
| 11 | +1 | 0 | –1 | 0 | 3.425 |
| 12 | 0 | –1 | +1 | 0 | 3.469 |
| 13 | 0 | –1 | –1 | 0 | 3.194 |
| 14 | +1 | 0 | 0 | +1 | 4.496 |
| 15 | 0 | 0 | 0 | 0 | 3.845 |
| 16 | +1 | 0 | 0 | –1 | 3.541 |
| 17 | 0 | 0 | –1 | +1 | 4.077 |
| 18 | 0 | 0 | 0 | 0 | 3.680 |
| 19 | 0 | +1 | –1 | 0 | 3.552 |
| 20 | –1 | 0 | –1 | 0 | 2.918 |
| 21 | –1 | 0 | 0 | –1 | 3.244 |
| 22 | 0 | +1 | 0 | +1 | 4.430 |
| 23 | 0 | 0 | 0 | 0 | 3.663 |
| 24 | 0 | –1 | 0 | –1 | 3.211 |
| 25 | 0 | 0 | 0 | 0 | 3.713 |
| 26 | 0 | +1 | 0 | –1 | 3.529 |
| 27 | 0 | –1 | 0 | +1 | 4.093 |
| 28 | 0 | 0 | +1 | –1 | 3.621 |
| 29 | –1 | +1 | 0 | 0 | 3.713 |
| 30 | +1 | –1 | 0 | 0 | 3.269 |

[1] The actual levels for Box-Behnken design that corresponds to coded values –1, 0 and +1 are 60, 70 and 80 °C for extraction time; 30, 45, and 60 min. for extraction time; 15:1, 22.5:1 and 30:1 ml:g for liquid to solid ratio and 200, 300 and 400 W for ultrasonic power, respectively.

## Statistical analysis

Each sample was replicated in three times. The value was expressed as the mean ± standard deviation. Differences were considered to be significant at $P<0.05$. Differences for analyses were estimated by Tukey multiple comparison tests. Experimental design and all statistical analyses were performed using the Minitab®17 (2016, Minitab Inc.)

## 3. Results and discussion

### Effect of ultrasound power on GTPS yield

As shown in the Figure 1A, when ultrasound temperature varied from 100 to 500 W, the extraction yield increased rapidly, while the other extraction parameters were fixed as follows: liquid:solid ratio (20:1), temperature (70 °C), and extraction time (30 min), and after 400 W further increase in power did not increase in the yield. At the same extraction conditions without applying ultrasound, the extraction yield was found to be 1.83±0.04%, whereas applying only 100 W ultrasound power increased the yield around 30%. It was doubled when the power was 400 W, and it started to decrease beyond that level.

The higher yield depending on the higher input power was in accordance with other reports for the extraction of polysaccharides (Jiang *et al.,* 2014; Maran and Priya, 2014; Zhang *et al.,* 2016). Due to the production of more cavitation bubbles that collapse near a solid surface, the structure of the plant material was disrupted, the pores of plant matrix were enlarged. Therefore, the solvent could penetrate through the cell deeper and mass transfer during extraction was accelerated.

### Effect of extraction temperature on GTPS yield

As shown in Figure 1B, the yield was increased with elevated extraction temperature. The polysaccharide production reached a maximum at 80 °C, and then no longer changed. The yield increased rapidly from ~3 to 4% when the extraction temperature ranged from 50 to 80 °C. Previous researches have also shown that increasing the temperature may proceed to swell and breaking up the structure of plant tissue, therefore the wetting of sample improved penetration of solvents. The increase in mass transfer between the plant matrix and surrounding medium would enhance the polysaccharides diffusion and the extraction yield (Ying *et al.,* 2011; Zhao *et al.,* 2015).

### Effect of extraction time on GTPS yield

The effect of extraction time (20 to 60 min) on the yield of polysaccharides was shown in Figure 1C. Polysaccharide yield upon the increase in extraction time has been observed in other studies (Jiang *et al.,* 2014; Zhu and Liu, 2013). The higher extraction yield due to longer extraction time was due to probable enlargement of the plant pores by time, exposure of greater amount of disrupted cells to extraction medium, and improved solvent penetration into the plant matrix. Therefore, polysaccharides inside the cells were more easily released into the exterior medium. After 50

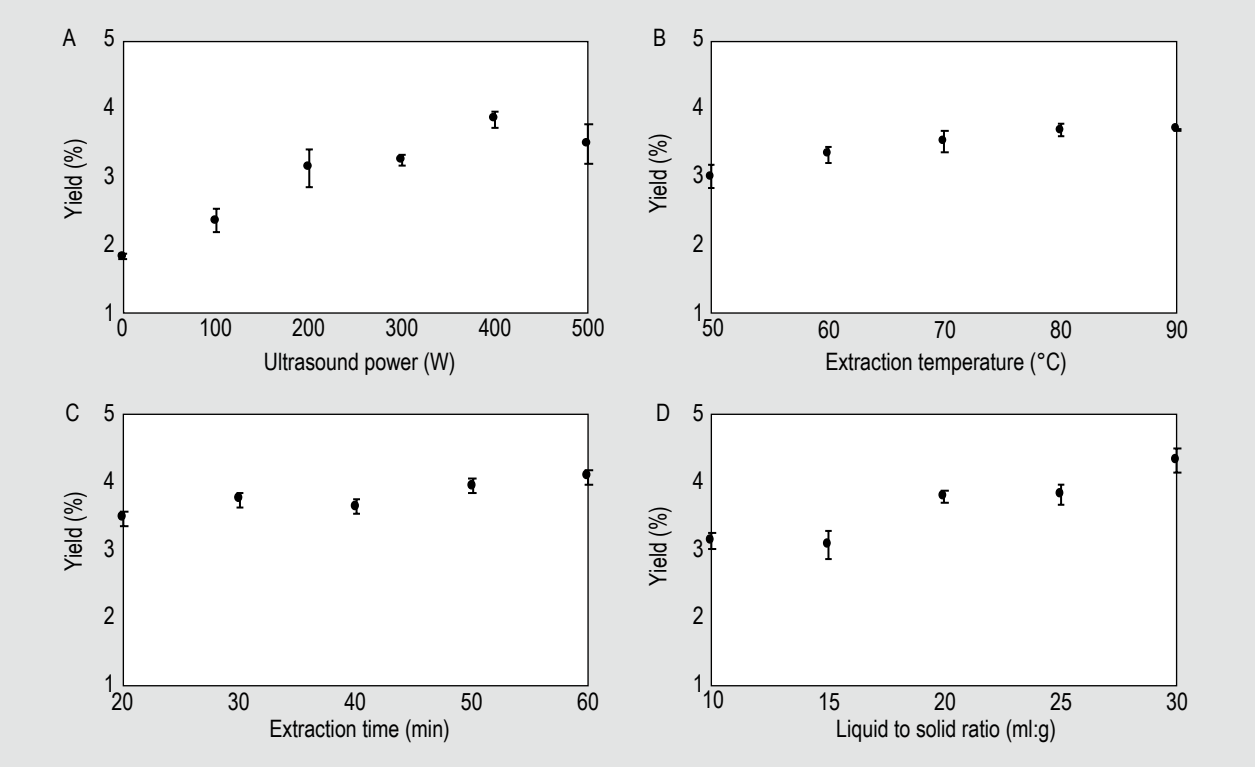


**Figure 1. Effects of (A) ultrasound power, (B) extraction temperature, (C) extraction time and (D) liquid:solid (LS) ratio, on the yield (%) of green tea polysaccharides (GTPS). Ultrasound power (300 W), extraction temperature (70 °C), time (30 min) and LS ratio was 20.**

min, increasing the extraction time slightly improved the yield, which indicated that a dynamic equilibrium was maintained.

### Effect of liquid to solid ratio on GTPS yield

The yield was determined at different LS ratios (10:1 to 30:1 ml:g), while other parameters kept constant and the results were shown in Figure 1D. The extraction yield was found to improve with higher LS ratio. The increasing solvent ratio by 3 folds improved extraction yield by 40%. This increase in the yield could be expected when mass transfer principles were considered since the more volume of solvent creates a higher concentration gradient between the interior plant cell and the surrounding environment. On the other hand, from an economic point of view, less solvent consumption for the extraction was entirely reasonable and practical. Although the solvent (water) used for polysaccharide extraction was cheaper, extracts should have a specific concentration before precipitating polysaccharides with pure ethanol (Wang *et al.,* 2012). Therefore, a considerable amount of water needs to be removed from the extract solution that would be both energy and time-consuming. Thus, the amount of solvent used in polysaccharide extraction needs to be optimised.

### Optimisation of the extraction parameters by BBD

The calculated coefficient values and their significances were presented in Table 2. A high value of determination coefficient ($R^2$=0.963) in present model ensured a satisfactory adjustment of the quadratic model to the experimental data, indicating that only 3.70% of the total variations was not explained by our model. The value of the adjusted determination coefficient (adjusted $R^2$=0.928) also suggested that a total variation of 92.8% for the yield was accounted for independent process variables. It was also demonstrated that the model chosen to represent the relationship between the responses and independent variables were well-correlated (Table 2).

F-value for the lack of fit was found not significant ($P$>0.05), confirming that the model was adequate for predicting the polysaccharide yield under any combination of the independent variable values. The $P$-value was used to check the significance of each coefficient. The smaller was the value of $P$, the more significant was the corresponding coefficient. In the present study, linear coefficients of $X_1$, $X_2$, $X_3$, and $X_4$, quadratic coefficients of $X_3^2$ and $X_4^2$, and interaction coefficients of $X_1.X_3$ values were significant ($P$<0.05) model terms. The final equation obtained in terms of coded factors was given below only with significant variables:

$$Y(\%) = 4.998 + 0.139\,X_1 + 0.285X_2 + 0.322X_3 + 0.649X_4 - 0.265X_3^2 + 0.272X_4^2 - 0.272X_1 \times X_3$$

**Table 2. Analysis of variance for the fitted quadratic polynomial model of extraction of green tea polysaccharides.[1]**

| | Coefficient | *P*-value | Model fit | |
|---|---|---|---|---|
| | | | $R^2$ | $R^2_{adj}$ |
| Yield (%) | | | 0.963 | 0.928 |
| constant | 3.750 | 0.000 | | |
| temperature | 0.104 | 0.007 | | |
| temperature$^2$ | -0.089 | 0.063 | | |
| time | 0.213 | 0.000 | | |
| time$^2$ | -0.081 | 0.088 | | |
| LS ratio | 0.242 | 0.000 | | |
| LS ratio$^2$ | -0.198 | 0.000 | | |
| power | 0.487 | 0.000 | | |
| power$^2$ | 0.203 | 0.000 | | |
| temperature × time | 0.105 | 0.090 | | |
| temperature × LS ratio | -0.205 | 0.003 | | |
| temperature × power | -0.035 | 0.556 | | |
| time × LS ratio | 0.002 | 0.975 | | |
| time × power | 0.005 | 0.935 | | |
| power × LS ratio | -0.078 | 0.201 | | |
| Lack-of-fit | 0.209 | | | |
| Model | 0.000 | | | |

[1] LS = liquid:solid.

Figure 2 shows the 3D response surface and 2D contour plots of the graphical representations of regression equations. They provide a method to visualise the relationship between responses and experimental levels of each variable and the type of interactions between two test variables. Each figure exhibited the effect of two factors on the tea polysaccharide yield, while the other two factors were kept at zero level (centre value of the testing range). The plane projection of the response surface reflected the intensity of the interaction between two factors. In our model, only the interaction between LS ratio and the temperature was significant ($P$=0.003). In Figure 2C, the change of the yield would require a small change along with the dependent variables compared to Figure 2A and 2D. The steep response surface obtained resembled as a peak (Figure 2c), where yield was maximum when the LS ratio was more than 20 and the temperature was higher than 70 °C. In Figure 2A, GTPS yield increased with both temperature and time ($P$=0.09), and the maximum yield was obtained higher than 70 °C and 55 min, and in Figure 2D, the yield was highest at LS ratio higher than 22.5 and longer than 50 min. When contour plots were circular as in Figure 2B, 2E, and 2F the mutual interactions between the corresponding variables were negligible. These figures showed that GTPS yield increased when ultrasound power

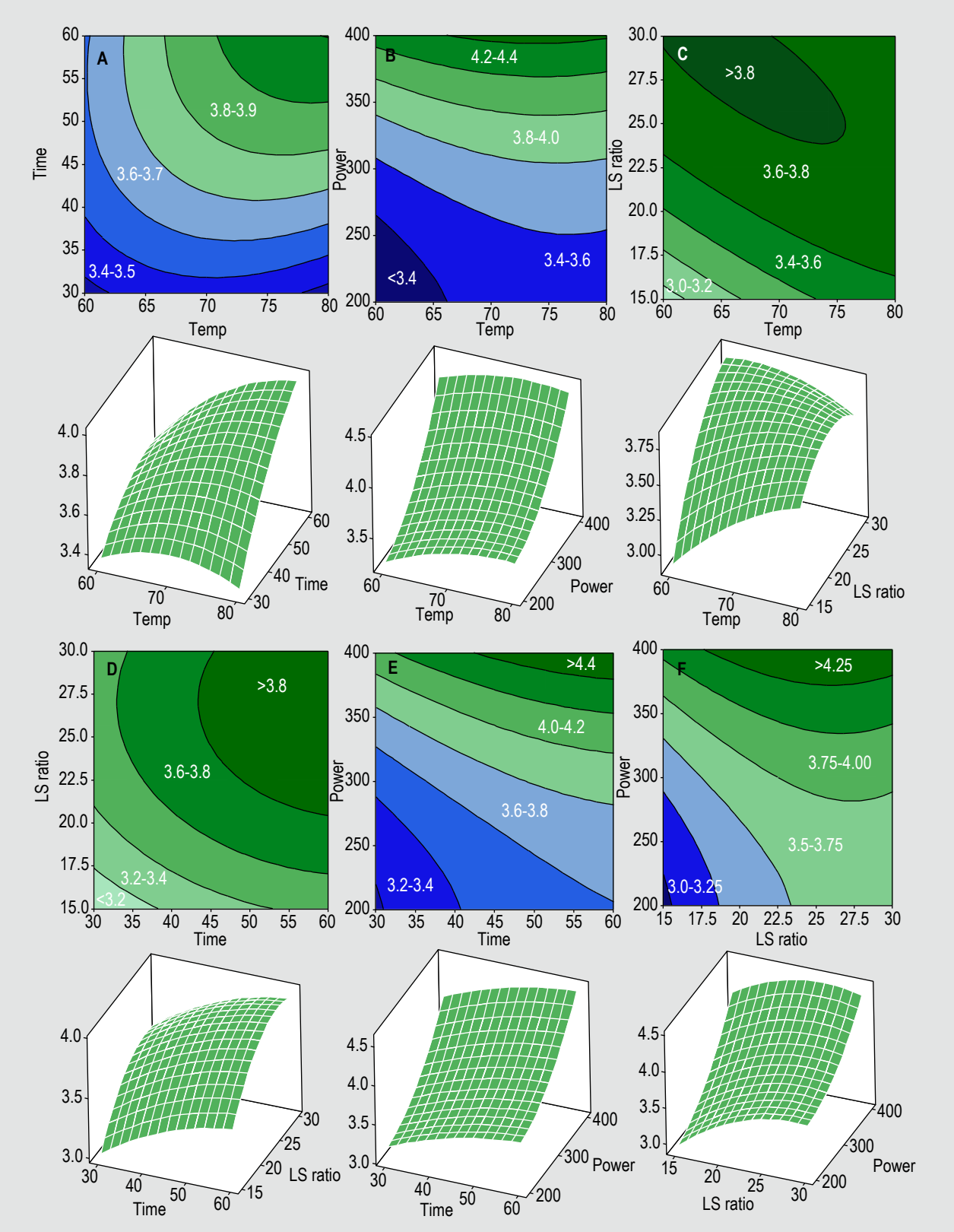


**Figure 2. Response surface and contour plots showing the effects extraction temperature (°C), time (min), liquid:solid (LS) ratio (ml:g), ultrasound power (W), and their interactive effects on the extraction yield of tea polysaccharides (%).**

was at 400 W, the temperature was higher than 70 °C, LS ratio was higher than 20, and the extraction time was longer than 50 min.

### Verification of the model

The suitability of the model equation for predicting the optimum response values was tested using the selected optimal conditions. By using the response optimiser (Minitab®17, 2016, Minitab Inc.) for the model developed by RSM, optimum extraction conditions were determined as extraction time (60 min), extraction temperature (80 °C), ultrasound power (400 W), ratio of liquid to solid material (22 ml:g), and the model predicted a maximum response (4.66%). Additional experiments with the predicted optimum extraction conditions were carried out, and a mean value of 4.65±0.29% (n=5) obtained from real experiments which demonstrated the validation of the RSM model. The results confirmed that the response model was satisfactory and accurate for reflecting the expected optimisation.

The yield, carbohydrate compositions, and antioxidant activities of GTPS obtained by CE and UE were given in Table 3. Ultrasound application enhanced the extraction yield around 50% and the major components of GTPS differed only in terms of total carbohydrate content, where UE provided a higher content of total carbohydrate. In the study of He *et al.* (2018), total carbohydrate content of *Dendrobium officinale* polysaccharides was higher when UE was applied. In terms of monosaccharide compositions, GTPS extracted with UE showed higher glucose level than CE. Polyphenol and protein contents did not show any significant differences between the two extraction methods ($P$>0.05), whereas antioxidant activities of extraction methods differed and GTPS obtained by CE showed higher antioxidant activities than UE ($P$<0.05) (Table 3). The effects of the extraction methods, on the composition and antioxidant activity of crude polysaccharides, were also studied by Kang *et al.,* 2019, and it was found that the antioxidant activity of *Ganoderma lucidum* polysaccharides obtained by conventional hot water extraction was higher than those obtained by ultrasound-assisted extraction. They also stated that the monosaccharide composition of polysaccharides was also different for two extracts. The composition of GTPS showed that it had a protein moiety and acidic heteropolysaccharide mainly composed of galactose, arabinose, and glucose with the presence of glucuronic and galacturonic acid. Our results were similar to the studies in the literature showing that crude tea polysaccharides had arabinogalactan structures (Chen *et al.,* 2018; Scoparo *et al.,* 2016).

**Table 3. Yields, compositional characteristics, and antioxidant activities of green tea polysaccharides (GTPS) obtained by classical hot water extraction (CE) and ultrasound-assisted extraction (UE).[1]**

| Parameter[2] | CE | UE |
|---|---|---|
| Yield (%) | 3.05±0.15[a] | 4.65±0.29[b] |
| Total carbohydrate (%) | 34.33±2.00[a] | 42.80±2.55[b] |
| Protein (%) | 2.21±0.05[a] | 2.28±0.27[a] |
| Polyphenol (g GAE/100 g GTPS) | 5.80±0.52[a] | 5.86±0.55[a] |
| Uronic acid (%) | 15.11±1.55[a] | 14.39±2.46[a] |
| ABTS (mg TE/100 mg GTPS) | 42.51±1.03[a] | 39.79±1.16[b] |
| FRAP (mg $FeSO_4 \cdot 7H_2O$/100 mg of GTPS) | 19.86±1.52[a] | 15.94±1.51[b] |
| Monosaccharide composition (mol ratio %) | | |
| Mannose | 3.97±0.00 | 2.09±0.01 |
| Ribose | 5.95±0.00 | 3.30±0.02 |
| Rhamnose | 3.06±0.00 | 2.24±0.03 |
| Glucuronic | 2.02±0.26 | 2.03±0.36 |
| Galacturonic | 0.95±0.23 | 2.17±0.01 |
| Glucose | 16.61±0.00 | 23.75±0.08 |
| Galactose | 40.22±0.02 | 38.30±0.13 |
| Xylose | 1.37±0.02 | 1.74±0.01 |
| Arabinose | 24.13±0.01 | 22.98±0.08 |
| Fucose | 1.72±0.03 | 1.40±0.10 |

[1] Data are expressed as the mean ± SD (n=3). Means ± SD followed by the same letter are not significantly different ($P$>0.05). UE and CE have the same conditions in terms of extraction time (60 min), temperature (80 °C), LS ratio (22 ml:g), and only differed in terms of ultrasound application.
[2] ABTS = 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); FRAP = ferric reducing antioxidant potential; GAE = gallic acid equivalents; TE = trolox equivalents.

### Molecular weight distribution

The molecular weight distributions and peak pattern of crude GTPS extracts are illustrated in Figure 3, which differed between CE and UE methods. Ultrasound application narrowed molecular weight distribution, partially disappearing of shoulders and the lower intensities of the peaks were observed. Moreover, weight-average molecular weight (Mw) value, calculated by the regression equation [log (Mw) = $-0.081x^3 + 2.215x^2 - 20.965x + 72.956$; $R^2$=0.998 obtained by dextran standards and retention time], reduced by ultrasound application. This suggested that UE likely caused in the degradation of certain polysaccharides with higher molecular weights. Previous studies also revealed that natural polysaccharides with higher molecular weights were more susceptible to depolymerisation (Yip *et al.,* 2016; Zhang *et al.,* 2013). Therefore, UE might degrade these polysaccharides and, thereby, change their bioactivities.

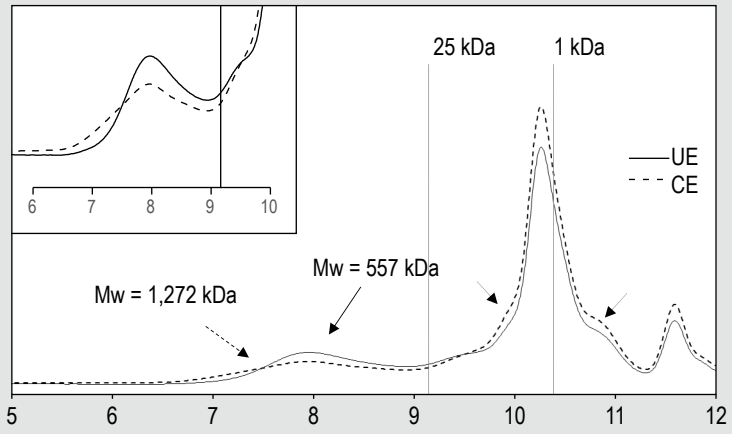


**Figure 3. GPC chromatograms of green tea polysaccharides obtained by classical hot water extraction (CE) and ultrasound-assisted extraction (UE) methods.**

### FT-IR analysis of GTPS

The FT-IR spectra of green tea polysaccharide extracts obtained by CE and UE were shown in Figure 4. The IR spectrum of GTPS obtained by UE and CE had very similar absorption bands. Peaks at 1000-1,200 $cm^{-1}$, 2,800-3,200 $cm^{-1}$, and 3,200-3,600 $cm^{-1}$ were characteristics for polysaccharides, the broadly intense peak at 3,310 $cm^{-1}$ represented the stretching vibrations of O-H, while the weak band at 2,920 $cm^{-1}$ was attributed to the C-H stretching and bending vibrations. In the study of Cai *et al.* (2015), tea polysaccharides were characterised by using FT-IR spectra. The samples showed the characteristic strong broad absorption in the range of 1,659-1,600 $cm^{-1}$ corresponding to stretching vibration of sugar rings, whereas in our sample a relatively strong absorption peak at 1,627 $cm^{-1}$ was observed. The peaks at ground 1,400-1,200 $cm^{-1}$ was also the characteristic absorptions of C-H bands. The broadband at 1,107 and 1,020 $cm^{-1}$ suggested the presence of C-O-H side groups and C-O-C glycosidic band vibration and should be the characteristic absorption bands of pyran glycosides. The peaks around 910 and 800 $cm^{-1}$ corresponded to skeletal modes of pyranose rings, which was characteristic of α-type glycosidic linkages between the sugar units (Cai *et al.,* 2013; Chen *et al.,* 2009). The samples prepared by CE and UE did not show any differences in terms of infrared spectra and showed characteristic peaks for polysaccharides.

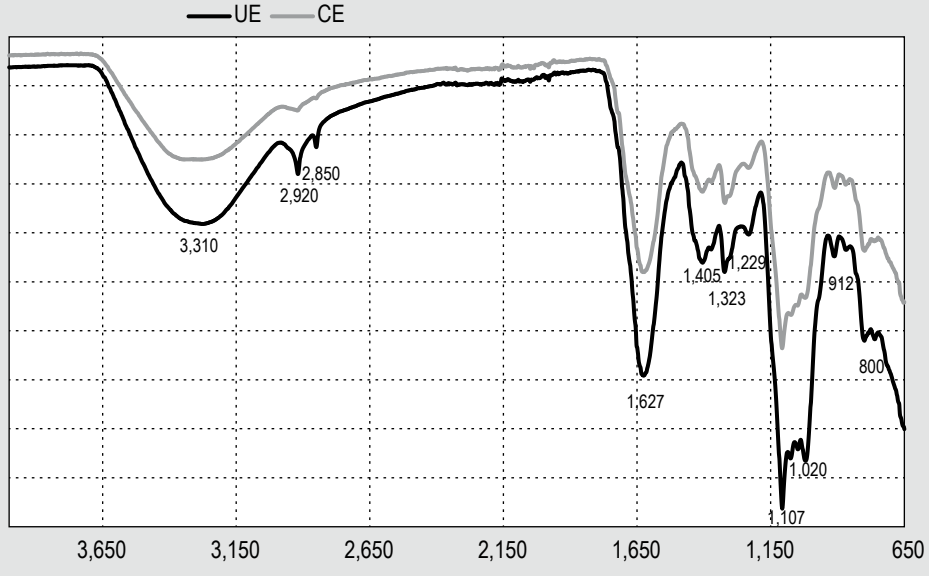


**Figure 4. FT-IR spectra of green tea polysaccharides obtained by classical hot water extraction (CE) and ultrasound-assisted extraction (UE) methods.**

### Inhibitory effects on α-glucosidase activity

The α-glucosidase inhibitors are current interest owing to their promising therapeutic potential for diabetic treatment as oral hypoglycaemic agents. The α-glucosidase inhibitors act on the brush border of intestinal mucosa to inhibit the post-meal blood glucose level from rising and decrease fasting blood glucose, to some extent, by delaying the carbohydrates digestion and absorbance at intestine (Wei *et al.,* 2010). As shown in Table 4, GTPS had a dose-dependent effect on α-glucosidase inhibitory activity and inhibition rose from 7.15 to 95.24% with the concentration increased from 6.25 to 625 µg/ml. The extraction method had a significant effect on α-glucosidase inhibitory activity and crude polysaccharides extracted by UE had lower inhibitory activity compared to samples obtained by CE. GTPS obtained by CE did not also show any significant differences ($P>0.05$) when its concentration in the reaction medium lowered from 625 to 125 µg/ml. Whereas, the sample prepared with UE showed a significant reduction ($P<0.05$) in terms of inhibitory activity when its concentration was reduced by half (Table 4). As a positive control, at 625 µg/ml of acarbose concentration, it showed only 50.98% ± 0.30 inhibition on α-glucosidase, much lower than the inhibitory activities of GTPS obtained by both extraction methods. Similar to our results, Wei *et al.* (2010) found that tea flower polysaccharides obtained by ultrasound-assisted extraction had very low inhibitory effects on α-glucosidase compared to the polysaccharides obtained by classical hot-water extraction (90 °C).

**Table 4. Inhibitory effects (%) of green tea polysaccharides on α-glycosidase activity.[1]**

| Concentration (µg/ml) | Classical hot water extraction | Ultrasound-assisted extraction |
|---|---|---|
| 625 | $95.25\pm3.13^{ax}$ | $93.64\pm2.36^{ax}$ |
| 312.5 | $93.41\pm3.30^{bx}$ | $87.22\pm4.37^{ay}$ |
| 125 | $91.86\pm0.04^{bx}$ | $87.57\pm1.58^{ay}$ |
| 31.25 | $43.77\pm1.73^{by}$ | $38.60\pm2.62^{az}$ |
| 6.25 | $7.15\pm5.25^{az}$ | $9.32\pm1.55^{ak}$ |

[1] Data are expressed as the mean ± SD (n=3). Means ± SD followed by the same letter are not significantly different ($P>0.05$). [a and b] refer to the comparison between columns (extraction method). [x, y, z and k] refer to a comparison between rows (concentration).

## 4. Conclusions

The process conditions for the UE method for GTPS extraction was optimised by using RSM design based on the single factor experiments. Low-quality green tea leaves were used for polysaccharide extraction and the compositional, antioxidant, structural, and antidiabetic properties of GTPS extracts prepared by optimum conditions of UE method were compared with the samples prepared with CE method. The results showed that the RSM model was satisfactory and accurate for reflecting the expected optimisation. Linear coefficients of all independent variables, quadratic coefficients of LS ratio, ultrasound power and interaction coefficients of extraction temperature and LS ratio, were significant model terms. Although UE application significantly increased the polysaccharide yield, the antidiabetic and antioxidant properties of GTPS was reduced, probably ultrasound application caused some polysaccharide depolymerisation and degradation. Therefore, we highly recommend that the studies aiming to improve the yield of bioactive polysaccharides by using UE should take its effects on the desired bioactivities into consideration.

## Conflict of interest

The authors declare that they have no conflicts of interest.

## Acknowledgments

This study is a part of a project funded by TÜBİTAK (under 1003 program-project no 105O037). We are grateful to ÇAYKUR Tea Enterprise for providing tea samples.

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