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Optimization of ultrasound-assisted enzymatic hydrolysis extraction of tea polyphenols from green tea and their antioxidant activities

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Summary

Production of natural extracts requires suitable processing conditions to facilitate the accumulation and preservation of bioactive ingredients. This study aimed to optimize the conditions for extracting tea polyphenols (TPs) from green tea using ultrasound-assisted compound enzymatic extraction (UACEE) technology with response surface methodology (RSM), based on a three-level, four-variable central composite rotatable design (CCRD). Extracted TPs yields were in the range of 16.48% to 28.77%; the experimental results were fitted to a second-order quadratic polynomial model and showed a good fit to the proposed model ($R^2 > 0.90$). Compared with other extraction methods, UACEE exhibited significant advantages in the TPs extraction rate and preservation of catechins composition. The antioxidant activities of these extracts were also analyzed using reducing power and DPPH radical scavenging activity; all extracts showed excellent antioxidant activity in a dose-dependent manner, and UACEE extracts showed the strongest antioxidant activity *in vitro*.

Keywords: tea polyphenols, catechin, antioxidant, ultrasound-assisted, compound enzymatic

Introduction

Tea (*Camellia sinensis*) is the second most widely consumed non-alcoholic beverages in the word, and can be divided into three major types according to the manufacturing process: non-fermented green tea, semi-fermented oolong tea, and fully fermented black tea (Wang et al., 2018). Tea polyphenols (TPs) are secondary metabolites with one aromatic ring and one or more hydroxyl groups; green tea contains approximately 18-36% TPs on a dry weight basis. Its chemical constituents include flavanols, flavanones, glycosids and their aglycons of plant pigments, and phenolic acids (Khan and Mukhtar, 2007). Among these, catechins are a major component of flavanols, which account for nearly 60% of the total TPs content. Catechins are a group of natural polyphenols found in tea and the health benefits of green tea are mainly attributed to their presence (Nkhili et al., 2009). The most abundant green tea polyphenols are the flavanols catechin (C), epicatechin (EC), gallocatechin (GC), catechingallate (CG), epigallocatechin (EGC), gallocatechin gallate (GCG), epicatechingallate (ECG) and epigallocatechin gallate (EGCG) (Fig. 1). They possess a wide variety of biological activities, such as anti-oxidant, anti-obesity and anti-inflammatory effects, as well as prevention of cardiovascular and cerebrovascular diseases, cancer and fatty liver (Higdon and Frei, 2003; Khan and Mukhtar, 2007; Xiang, 2018; Zhu et al., 2020). Compounds of the catechins family have been widely reported to exert the most beneficial effects on the human health. Currently, extraction of catechins has attracted great attention and many techniques have been developed and modified to extract these valuable compounds (Pasrija and Anandharamakrishnan, 2015). Green tea is manufactured from fresh tea leaves. Tea leaves are fermented through a process that for the most part prevents oxidation and polymerization of TPs (Yusuf et al., 2007). TPs are a kind of efficient, plant-derived, and safe antioxidants that possess significant anti-oxidative (Ong, 2017), anti-Alzheimer's (Afzal et al., 2015), anti-inflammatory (Sajilata et al., 2008), anti-carcinogenic (Yang et al., 2009), neuroprotective (Schimidt et al., 2017), and obesity-reducing properties (Pan et al., 2016). They have important research values and application prospects in tea comprehensive processing, commodities, food, and nutraceuticals. Currently, many techniques have been exploited to extract secondary metabolites including poly-

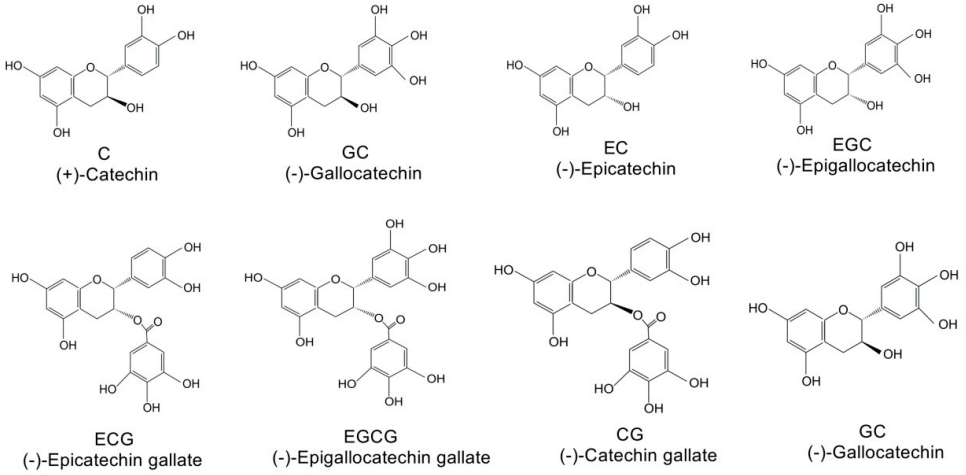

Fig. 1: Chemical structure of 8 major catechins.

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phenols from various tea sources (Dai and Row, 2019; Zhang et al., 2012).

Effective extraction of TPs from tea and using them to develop healthy food and medicines can enhance the physical health of humans as well as create good economic and social benefits. Conventionally, the solvents usually used in the extraction of crude TPs are water and ethanol, and several methods including microwave-assisted water extraction (Tsubaki et al., 2010), solvent-based extraction (Pasrija et al., 2015), high hydrostatic pressure extraction (Xi et al., 2009), and ultrasound-assisted extraction (Afroz et al., 2019), have been applied. In general, physical and enzymatic methods are regarded as alternative methods to solve problems related to the environment and safety.

Although water is a low-cost solvent, its extraction rate is low and the products contain high amounts of water-soluble impurities. Use of ethanol as a solvent requires too much ethanol, and is high-cost, time-consuming, with the possibility of losing effective constituents. Enzymatic extraction of TPs is based on the traditional solvent extraction method. Based on the plant cell wall structure, choosing the corresponding enzymes, hydrolyzing and degrading the cell wall components, fully exposing the effective constituents, and dissolving and suspending them in the extraction solvent can facilitate improved TPs extraction rates. Meanwhile, ultrasonic extraction is simple to operate, does not have toxic side effects, and can maximally retain biological activity in the extracted natural products. It has a higher extraction efficiency, lower energy consumption, shorter extraction time, and is free from high temperature. Simultaneously, it does not alter the structures of TPs and catechins. Response surface methodology (RSM) is a statistical method that uses reasonable experimental design, adopting multiple quadratic regression equations fitting the function relationship between factors and response values. Being different from the widely used orthogonal experimental method, it has a number of advantages including a shorter test period, higher regression equation accuracy, and can thus be applied to study the interactions among multiple factors etc. (Mangang et al., 2020).

Although TPs extraction has been reported (Pasrija et al., 2015; Spigno et al., 2009; Xi et al., 2009), no study has examined the use of ultrasound-assisted compound enzymatic extraction (UACEE) for the extraction of TPs from green tea. Thus, the present study aimed to optimize the processing parameters for TPs extraction from green tea in combination with the ultrasound-assisted extraction, under the condition of single factor experiment, using RSM. A central composite rotatable design (CCRD) (4 factors and 3 levels) was applied to study the effects of enzyme concentration, pH value, extraction temperature and ultrasonic power on the TPs extraction yield. We also compared UACEE with other extraction methods including heat reflux extraction, microwave-assisted water extraction, and ultrasound-assisted ethanol extraction. The antioxidant scavenging effects of TPs were then evaluated by an *in vitro* antioxidant assay including reducing power and DPPH radical scavenging activity.

Materials and methods

Materials

Green tea (moisture 5.5% in weight) was supplied by Duyunmaojian Co., Ltd. (Duyun, Guizhou Province, China). The green tea was ground using a micromill (FZ-102, Wuhan Gelaimo Testing Equipment Co., Ltd., Wuhan, Hubei, China), screened with a sieve, and particles with a 0.42×10^{-3} m diameter were selected.

(+)-catechin (C), (−)-gallocatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG), (−)-gallocatechin gallate (GCG); (−)-catechin gallate (CG) and (−)-gallocatechin (GC) standards were acquired from the National Research Center of Engineering Technology for Utilization of Botanical Functional Ingredients (Changsha, Hunan, China) and identified in their laboratory for analysis. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Chemical Co. (Sigma, USA). Ultrapure water was purified using a Milli-Q system (Millipore, Bedford, MA, USA) with a resistivity of ≥ 18.2 MΩm. Pectinase (1000 U/mg) was obtained from Shanghai Jinsui Biotechnology Co., Ltd. (Shanghai, China). Cellulase (1,500 U/mg) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). SBI-54DT ultrasonic equipment was purchased from Ningbo Xingzhi Biotechnology Co., Ltd. (Ningbo, Zhejiang, China). All the solvents and chemicals used were of chromatographic grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Determination of tea polyphenols, total catechins and catechin components content

The tea polyphenols content of the green tea extracts was determined using the Folin-Ciocalteu method (Spigno et al., 2009). Briefly, 0.5 mL of soluble extract was mixed with 2.5 mL of 10% Folin-Ciocalteu phenol reagent in the test tube and allowed to react for 5 min at 25 °C. Then, 2 mL of 7.5% sodium carbonate solution was added and allowed to stand for 60 min at 25 °C before the absorbance of the reaction mixture was read at 765 nm using a spectrophotometer. The measurements were carried out in triplicate and the calculations were done using the calibration curve plotted using gallic acid. TPs were expressed as mg of gallic acid equivalents per gram of green tea on a dry basis. The yield of TPs (%) was calculated as follows:

$$\text{Tea polyphenols extraction yield \% (w/w)} = \frac{\text{tea polyphenols content (g)}}{\text{dried green tea weight (g)}} \times 100 \quad (1)$$

The total catechins content of the green tea extracts was determined using the hydrochloric acid-vanillin colorimetry method. Briefly, 20 μL of soluble extract was mixed with 1 mL of 95% ethanol reagent in the test tube. Then, 5 mL of 1% hydrochloric acid-vanillin solution was added and allowed to stand for 40 min at 25 °C before the absorbance of the reaction mixture was read at 500 nm using a spectrophotometer. The measurements were carried out in triplicate and the calculations were done using the formula 2. The yield of total catechins (%) was calculated as follows:

$$\text{Total catechins extraction yield \% (w/w)} = \frac{\text{A} \times 72.84}{10} \times \frac{L_1}{L_2 \times \text{dried green tea weight(g)}} \quad (2)$$

where A represent the absorbance value of test sample at 500 nm, L_1 represent the total volume of tea polyphenol extract solution (mL), L_2 represent the volume of solution used in the measurement (mL). When the absorbance value is 1.0, it is equivalent to 72.84 μg of total catechins in the tested extract liquid.

High-performance liquid chromatography (HPLC, Agilent 1200, Agilent Technologies, Santa Clara, CA, USA) was employed to analyze catechin components content, and with a C_{18} reverse-phase column (Hy Persil ODS2, 4.6 mm × 250 mm, 5 μm). Solvent A was consisted of 0.1% (v/v) trifluoroacetic acid in ultra-pure water and solvent B was methanol; the eluant was monitored at 278 nm with a 25 °C column temperature; and sample injection volume was 10 μL. The gradient elution profile was as follows (solvent B): 0-12 min, 8% constant; 12-25 min, from 8% to 20%; 25-35 min, from 20% to 30%; 35-40 min, from 30% to 35%; 40-60 min, from 35% to 8%; followed by 8% for 5 min, and the solvent flow rate was 0.8 mL/min. Eluted compounds were identified by comparing their retention times and absorption spectra with those of authentic standards. The compounds were quantified using curves constructed from the catechin standards.

Single-factor design for tea polyphenols (TPs) extraction

The single-factor design was used to determine the preliminary range of extraction factors. For single factor experiment, one factor was

changed in a certain range while all other factors were kept constant. The extraction parameters were ratio of compound enzymes (pectinase:cellulase = 0.5:1, 1:1, 1.5:1, 2:1, 2.5:1, 3:1 and 3.5:1), enzyme concentration (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 %), pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0), extraction temperature (50, 55, 60, 65, 70, 75 and 80 °C), extraction time (30, 40, 50, 60, 70, 80 and 90 min), ultrasonic power (300, 400, 500, 600, 700, 800 and 900 W), ratio of water to raw material (10:1, 15:1, 20:1, 25:1, 30:1, 35:1 and 40:1 mL/g), of which the single factor experiment was investigated in this study, respectively.

Experimental design

RSM was applied to examine the effect of independent variables including enzyme concentration, X_1 (1.0-2.0%); pH, X_2 (4.5-5.5); extraction temperature, X_3 (65-75 °C) and ultrasonic power, X_4 (600-800 W), on the extraction yield of TPs (%). CCRD at three levels was performed with four independent variables (Tab. 1). The RSM was applied to statistically analyze the experimental data using a commercial statistical package, Design-Expert 8.0 (Stat-Ease Inc., USA). The complete design consisted of 29 experimental points, including five replications of the center points, and triplicates were performed for all the design points in a randomized order (SAMAVATI et al., 2013). The TPs extraction yield was expressed as a second-order polynomial as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X^2{}_i + \sum\sum X_i X_j \quad (3)$$

where Y is the predicted value of the TPs yield, β_0 is a constant, β_i is a linear coefficient, β_{ii} is a quadratic coefficient, β_{ij} is an interactive term coefficient, and X_i and X_j represent five independent variables.

Tab. 1: Independent variable values of the process and their corresponding levels.

Independent variable	Levels		
	-1	0	1
Enzyme concentration (X_1) / %	1.0	1.5	2.0
pH (X_2)	4.5	5.0	5.5
Extraction temperature (X_3) / °C	65	70	75
Ultrasonic power (X_4) / W	600	700	800

Comparison of different extraction methods

In addition to UACEE extraction, other extraction methods including microwave-assisted water extraction (NKHILI et al., 2009), ultrasound-assisted ethanol extraction (SONG et al., 2011), and heat reflux extraction (JIANG et al., 2010) were taken as references and compared. We determined the TPs yields obtained with different extraction methods, and analyzed the catechin components of TPs using HPLC method.

Microwave-assisted water extraction (MAWE)

Green tea powder samples (2.0 g) were infused in 40 mL of water at 100 °C in the microwave oven (Galanz G70D20CN1P-D2, Guangdong, China) for 60 min, the irradiation power was set at 600 W. The extracted solution was centrifuged at 8,000 rpm for 15 min, and the supernatant was collected and analyzed under the conditions described above.

Ultrasound-assisted ethanol extraction (UAEE)

Green tea powder samples (2.0 g) were infused in 40 mL of 70% ethanol at 70 °C in the ultrasonic for 50 min, the ultrasonic power was set at 700 W. Then, the extracted solution was centrifuged at 8,000 rpm for 15 min, and the supernatant was collected and analyzed under the conditions described above.

Heat reflux extraction (HRE)

Green tea powder samples (2.0 g) were infused in 40 mL of 50% ethanol in water, the mixture was poured into a round bottom flask, connected to a condenser tube device and stirred at about 85 °C for 45 min in a constant state. The extracted solution was centrifuged at 8,000 rpm for 15 min, and the supernatant was collected and analyzed under the conditions described above.

Antioxidant activity

Reducing power

The reducing power was assessed as studied by DENG et al. (2017) with moderately modified. Various concentrations (10-120 μg/mL) of green tea extracts (1.0 mL) were mixed and moderately modified with 0.1 mL of pH 6.5, 0.2 mol/L phosphate buffer, and 0.1 mL of 1% $K_3Fe(CN)_6$. The reaction solution was incubated at 50 °C for 20 min. Subsequently, the mixture was added to 0.1 mL of trichloroacetic acid and then centrifuged for 10 min at 3,000 rpm. The supernatant (0.1 mL) was diluted in 0.1 mL distilled water and then added with 25 μL of 0.1 % $FeCl_3$. Absorbance at 700 nm was recorded using ascorbic acid (Vc) as the positive control. An increase in the absorbance of the mixture indicated an increase in reducing power.

DPPH radical scavenging activity

This assay was performed using a previously described method with moderately modified (MOLAN et al., 2008). Briefly, 0.1 mL of crude tea polyphenols extracts at variable concentrations (10-120 μg/mL) was added to 2.9 mL of DPPH solution (0.1 mol/L in ethanol) as the free radical source. The reaction mixture was incubated at 25 °C in a dark room for 30 min, the absorbance was read at 517 nm against the blank. The DPPH radical scavenging activity was calculated using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{A_{sample}}{A^{control}}\right) \times 100 \quad (4)$$

where $A_{control}$ is the absorbance of the DPPH radical solution with ethanol and A_{sample} is the absorbance of the DPPH radical solution with the tested samples.

Statistical analyses

All experiments were performed in triplicate and centered. Analysis of variance (ANOVA) was performed. $P < 0.05$ and $P < 0.01$ were regarded as significant and extremely significant, respectively. Design-Expert 8.0 was used for the experimental design and the regression analysis of experimental data.

Results and discussion

Optimization of UACEE by RSM

Based on the results of a single-factor study (Fig. 2), four key parameters that remarkably affected the extraction yield of TPs were selected to be optimized by RSM, and were adopted for RSM experiments as follows: enzyme concentration, 1.0-2.0%; pH values, 4.5-5.5; extraction temperatures, 65-75 °C; and ultrasonic powers, 600-800 W. The other experimental conditions were held constant for all reactions in this set of experiments as follows: ratio of compound enzymes (pectinase : cellulase, 2:1; enzyme treatment time, 70 min; ultrasonic time, 60 min; and ratio of water to raw material, 30:1 (mL/g).

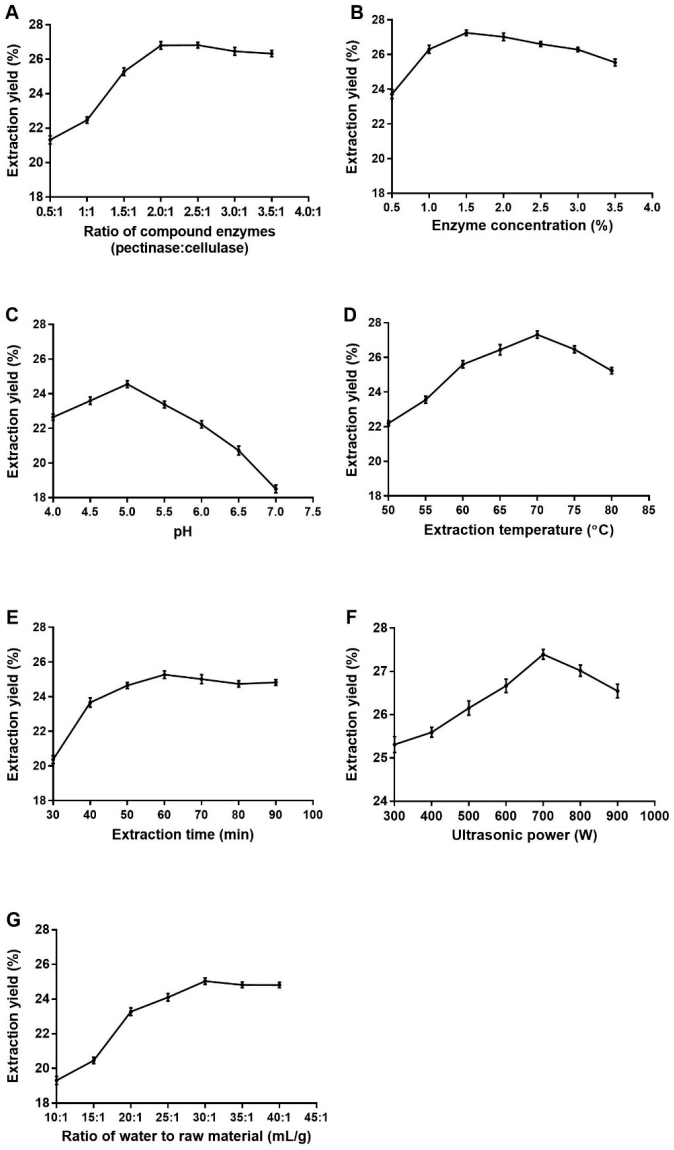

Fig. 2: Effect of different extraction factors (A) ratio of compound enzymes, (B) enzyme concentration, (C) pH value, (D) extraction temperature, (E) extraction time, (F) ultrasonic power, (G) ratio of water to raw material on extraction yield of TPs.

Tab. 2: Central composite design with the observed responses and predicted values for yield of tea polyphenol.

Run	Enzyme concentration X_1 (%)	pH X_2	Extraction temperature X_3 (°C)	Ultrasonic Power X_4 (W)	TPs extraction yield (%)	
					Experimental	Predicted
1	1.0	5.0	65	700	16.48	16.64
2	1.5	5.0	70	700	28.55	28.47
3	2.0	5.0	70	600	19.72	20.00
4	1.5	5.5	75	700	25.53	25.01
5	1.0	5.5	70	700	19.51	19.46
6	1.5	5.0	70	700	28.65	28.47
7	1.5	5.0	75	800	26.05	25.85
8	2.0	5.0	70	800	21.84	21.72
9	2.0	5.0	75	700	21.58	22.02
10	1.5	4.5	65	700	18.55	19.37
11	1.5	5.5	70	600	21.74	22.46
12	1.5	5.0	75	600	23.06	21.99
13	1.0	5.0	75	700	21.09	21.56
14	1.5	4.5	70	800	21.42	21.30
15	1.5	5.5	65	700	23.09	22.53
16	1.5	5.0	70	700	28.53	28.47
17	1.5	4.5	70	600	19.45	19.80
18	1.0	5.0	70	800	19.07	19.10
19	1.5	5.0	65	800	20.27	20.43
20	1.0	5.0	70	600	17.45	17.90
21	1.5	5.0	70	700	27.85	28.47
22	1.5	5.5	70	800	23.62	23.88
23	2.0	4.5	70	700	20.05	19.20
24	2.0	4.5	65	700	20.77	20.90
25	1.5	4.5	75	700	22.07	22.93
26	1.5	5.0	70	700	28.77	28.47
27	2.0	5.5	70	700	22.46	22.62
28	1.5	5.0	65	600	22.09	21.37
29	1.0	4.5	70	700	18.71	17.64

Fitting the model

The effects of four processing variables (i.e. enzyme concentration (X_1), pH value (X_2), extraction temperature (X_3), and ultrasonic power (X_4)) were studied during experimentation. The response of interest was the extraction yield of TPs. The results of 29 runs using CCRD design are presented in Tab. 2, which include the design, observed responses and predicted values. All the results showed a close agreement between experimental and predicted values. In addition, the extraction yields ranged from 16.48% to 28.77%. The corresponding variables would be more significant when the absolute F-value become greater and the p-value became smaller (WANG et al., 2007). The regression coefficients of the linear, quadratic, and interaction terms of the model were calculated using the least square technique (BOUATAY et al., 2019) and are given in Tab. 3. The variables with the largest effect were the linear terms of enzyme concentration (X_1), pH (X_2), extraction temperature (X_3), ultrasonic power (X_4) and the quadratic term of enzyme concentration (X_1^2), pH (X_2^2), extraction temperature (X_3^2), ultrasonic power (X_4^2) followed by the interaction effects of enzyme concentration and extraction temperature (X_1X_3), and extraction temperature and ultrasonic power (X_3X_4). Regression analysis of test results was performed with fitting for the extraction yield of TPs in the quadratic multinomial regression model as follows:

$$Y = 28.47 + 1.18X_1 + 1.31X_2 + 1.51X_3 + 0.73X_4 + 0.40X_1X_2 - 0.95X_1X_3 + 0.13X_1X_4 - 0.27X_2X_3 - 0.022X_2X_4 + 1.2X_3X_4 - 5.46X_1^2 - 3.28X_2^2 - 2.73X_3^2 - X_4^2$$

Where Y is the extraction yield of TPs, and X_1, X_2, X_3 and X_4 are the coded values for enzyme concentration, pH value, extraction temperature, and ultrasonic power, respectively.

Upon analysis of variance (ANOVA) for the model, the coeffcient of determination (R^2) of the predicted model was 0.9783, suggesting a good fit. The predicted model appeared to reasonably represent the observed values. Thus, the response was suffciently explained by the model. To determine the optimally combined parameters at different factors and levels of response values, the first derivative was used to solve the regression equation and assigned to 0. After having arranged these, we can obtain the equations whose solutions are the corresponding code values of single factors. According to the transformation of code values, the theoretical optimal process conditions can be obtained as follows: enzyme content, 1.84%; pH, 5.12; extraction temperature, 70.09 °C; and ultrasonic power, 715 W. Under theoretical optimum parameters, the theoretical predictive value of the TPs yield was 27.16%. However, considering the actual experiments, the theoretical parameter was adjusted as follows: enzyme content,

Tab. 3: Results of ANOVA of regression model for the extraction yield of tea polyphenols.

Source	Sum of squares	DF	Mean square	F-Value	P-value
Model	342.90	14	24.4928	45.0435	< 0.0001**
Linear					
X_1	16.59	1	16.5910	30.5118	< 0.0001**
X_2	20.54	1	20.5408	37.7757	< 0.0001**
X_3	27.39	1	27.3914	50.3743	< 0.0001**
X_4	6.39	1	6.3948	11.7604	0.0041**
Interaction					
X_1X_2	0.65	1	0.6480	1.1918	0.2934
X_1X_3	3.61	1	3.6100	6.6390	0.0220*
X_1X_4	0.06	1	0.0625	0.1149	0.7396
X_2X_3	0.29	1	0.2916	0.5363	0.4761
X_2X_4	0.00	1	0.0020	0.0037	0.9522
X_3X_4	5.78	1	5.7840	10.6371	0.0057**
Quadratic					
X_1^2	193.61	1	193.6087	356.0569	< 0.0001**
X_2^2	69.77	1	69.7665	128.3044	< 0.0001**
X_3^2	48.20	1	48.1957	88.6345	< 0.0001**
X_4^2	72.02	1	72.0180	132.4451	< 0.0001**
Residual	7.61	14	0.5438	-	-
Lack of Fit	7.10	10	0.7096	5.4921	0.0575
Pure Error	0.52	4	0.1292	-	-
Cor Total	253.41	28	-	-	-
C.V.%			3.3		
R^2			0.9783		
Adj-R^2			0.9566		

*$P < 0.05$ significant.
**$P < 0.01$extremely significant.

1.85%; pH, 5.1, extraction temperature, 70 °C; and ultrasonic power, 700 W. Under these optimized conditions, the experimental yield of TPs was 27.12%.

Analysis of response surface

Response surface graphics is the surface figure of three-dimensional space that is created by response value Y against various experimental factors. The parameters of the optimal process can be determined by observing the 3D response surface figure that reflects the degrees for various factors with influencing effects (Simić et al., 2016). According to the fitting function, the response surface of the yield and contour plot can be drawn on every two factors, considering how the various factors qualitatively affect the yield, dealing with the zero level when the other two factors are fixed (Wu et al., 2015). Fig. 3A-F intuitively reflects the influences of various factors on the response values. In case of the extraction yield of TPs, the enzyme concentration (X_1), pH value (X_2), extraction temperature (X_3) and ultrasonic power (X_4) used had quadratic effects on tea polyphenol extraction. When the extraction parameter was kept at one level, the extraction yield of TPs increased with the increasing extraction parameter within certain a range and then decreased upon extending it. Hence, the interaction effect of any two independent factors was remarkable.

Verification of predictive model

The suitability of the model equation for predicting the optimum response values was tested under the selected optimal conditions. The experimental yield of TPs was 27.12% whereas the predicted values were in close agreement with the experimental values and were found

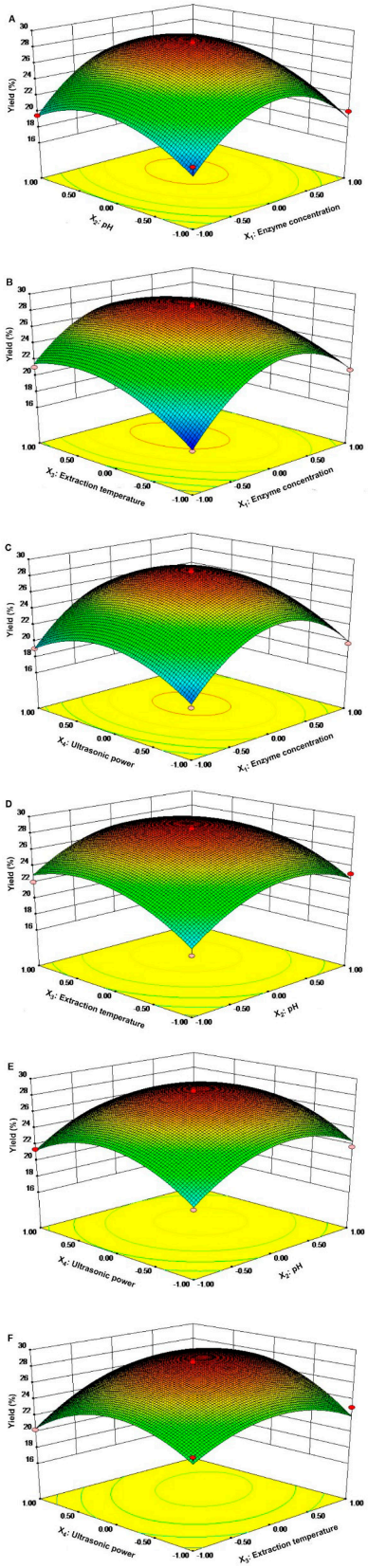

Fig. 3: Response surface analysis for TPs yield from green tea with ultrasonic-assisted compound enzymatic extraction with respect to enzyme concentration and pH (A); enzyme concentration and extraction temperature (B); enzyme concentration and ultrasonic power (C); pH and extraction temperature (D); pH and ultrasonic power (E); extraction temperature and ultrasonic power (F).

to be not significantly different ($P > 0.05$) (Tab. 4). The predicted response values were slightly deviated from the experimental data. From the preliminary data, the normal probability at the residuals indicated no abnormality in the methodology adopted. A strong correlation between the actual and predicted results confirmed that the response of the regression model was adequate and accurate to reflect the expected optimization (YANG et al., 2010).

Tab. 4: Predicted and experimental yield of tea polyphenols at optimum conditions.

Independent variable	Theoretical parameter	Adjusting parameter	Predicted yield (%)	Experimental yield (%)
Enzyme concentration (%)	1.84	1.85	27.164	27.12 ± 0.17a
pH	5.12	5.1		
Extraction temperature (°C)	70.09	70		
Ultrasonic Power (W)	715	700		

[a] Values are means ± standard deviations of triplicate measurement.

Comparison of UACEE and other extraction methods

Green tea leaf compositions vary with territory, climate, season, tea variety, and age of the leaf (TANIZAWA et al., 1984). To compare the results of UACEE with those obtained using other extraction methods, we performed all the experiments using the same green tea, and the extraction methods (microwave-assisted water extraction, MAWE; ultrasound-assisted ethanol extraction, UAEE; heat reflux extraction, HRE) used were the same as those described in literature. The results are shown in Tab. 5, among the extraction methods selected in this study, UACEE extracted TPs with the highest content, reaching 27.12% ($P < 0.05$). There was no significant difference between the MAWE and UAEE methods, while TPs extracted by HRE was the lowest, only 22.56%. For total catechins content (Tab. 4), we found that the maximum yield was 14.82% with UACEE, whereas the minimum yield was 11.37% with HRE. Similarly, for the proportion of total catechins accounted for by the proportion of tea polyphenols (total catechins : TPs), UACEE and HRE showed the maximum and minimum value of 54.89% and 50.40%, respectively. The TPs obtained by different extraction methods were compared for the content of catechin components (Fig. 4). We found that UACEE obtained the highest content of catechin components, especially, EGCG, GCG and CG were significantly different compared with other extraction methods ($P < 0.05$). These results indicated the effective components, catechins, extracted by UACEE from TPs were the most reserved in the process of extraction and this method provided a higher extraction rate for the effective components. We also found that under the extraction condition at high temperature for a long time, TPs in tea, especially catechins, may be oxidized easily, and could generate tea cheese with caffeine, protein and metal ions, which are present in the extracting solution, thus affecting the biological activity of TPs (IKEDA et al., 2017; KANAKIS et al., 2011).

Tab. 5: Comparison of the results of UACEE and other extraction methods.

Extraction method	TPs (%)	Total catechins (%)	Total catechins : TPs (%)
MAWE	25.89±0.37 [b]	12.97±0.49 [b]	50.08±1.17 [b]
UAEE	25.42±0.19 [b]	13.19±0.39 [b]	51.87±1.17 [b]
HRE	22.56±0.18 [a]	11.37±0.34 [a]	50.40±0.47 [b]
UACEE	27.12 ±0.17 [c]	14.82±0.28 [c]	54.89±0.49 [a]

MAWE, microwave-assisted water extraction; UAEE, ultrasonic-assisted ethanol extraction; HRE, heat reflux extraction; UACEE, ultrasonic-assisted compound enzymatic extraction.
Values are means ± standard deviations of triplicate measurement. For different extraction methods, means in every column with different letters.

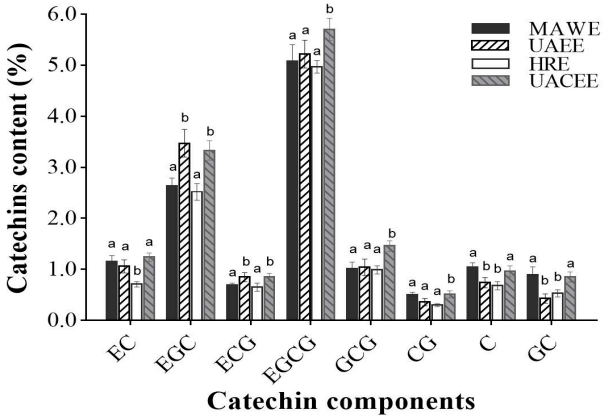

Fig. 4: Different extraction methods for catechin components content. MAWE, microwave-assisted water extraction; UAEE, ultrasonic-assisted ethanol extraction; HRE, heat reflux extraction; UACEE, ultrasound-assisted compound enzymatic extraction. different letters indicate statistically significant differences ($P < 0.05$) between extraction methods and the same catechins.
EC, (–)-epicatechin; EGC, (–)-epigallocatechin; ECG, (–)-epicatechingallate; EGCG, (–)-epigallocatechin gallate; GCG, (–)-gallocatechin gallate; CG, (–)-catechingallate; C, (+)-catechin; GC, (–)-gallocatechin.

Antioxidant activities

Reducing power

The correlation coefficient between absorbance and the tested concentration of UACEE, MAWE, UAEE, HRE extracts and ascorbic acid (Vc) were 0.993, 0.987, 0.995, 0.990, and 0.993, respectively, which indicated that the reducing power of all extracts and Vc followed a dose-dependent manner. Other studies also found that the positive correlation between the phenolic content and their antioxidant power (DENG et al., 2017; KUMAR et al., 2011). As shown in Fig. 5A, with the increase in concentration, the reducing power of the four extraction methods was close to that of Vc.

DPPH radical scavenging activity

As shown in Fig. 5B, all tea polyphenol samples showed an obvious scavenging effect on DPPH radical in a concentration-dependent manner. At 120 μg/mL, the scavenging activity of the UACEE, MAWE, UAEE, and HRE extracts were 85.67%, 78.30%, 74.35% and 70.09%, respectively. The IC_{50} values were 35.15, 36.17, 37.24, 40.28, and 32.13 μg/mL for UACEE, MAWE, UAEE, HRE extracts, and Vc, respectively. Their IC_{50} values were not statistically different ($P > 0.05$). The UACEE, WAWE, UAEE and HRE extracts thus had a similar activity to Vc.

Conclusions

In this study, RSM was used to optimize the extraction process of tea polyphenols from green tea. The optimum extraction conditions of UACEE were as follows: enzyme concentration 1.85%, pH 5.1, extraction temperature 70 °C, and ultrasonic power 700 W, resulting in a 27.12% yield for the total tea polyphenols. Furthermore, from HPLC analysis, 8 catechins compounds were found in both extracts

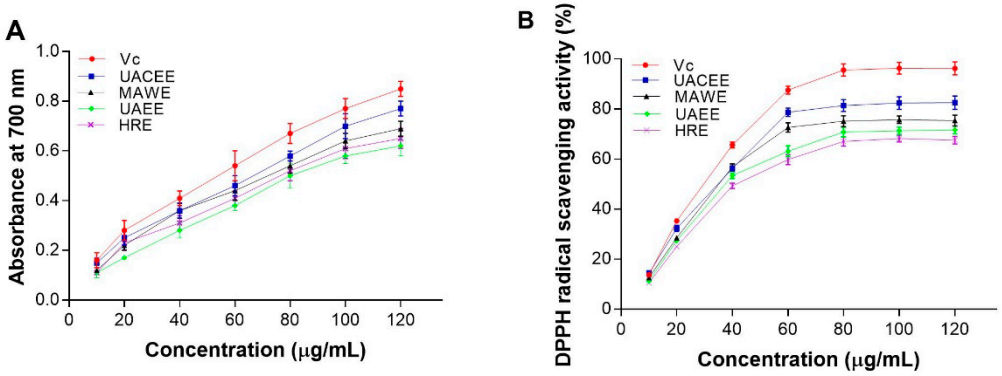

Fig. 5: Antioxidant activities of TPs from green tea obtained by UACEE, MAWE, UAEE and HRE extraction methods, respectively. reducing power (A); DPPH radical scavenging activity (B).
Each value is the mean ± standard deviations of triplicate measurements.
UACEE, ultrasound-assisted compound enzymatic extraction; MAWE, microwave-assisted water extraction; UAEE, ultrasonic-assisted ethanol extraction; HRE, heat reflux extraction.

and UACEE offered a higher yield (14.82%) than that of traditional extraction methods. All the extracts exhibited similar specific antioxidant activities including reducing power and DPPH radical scavenging activity, but the UACEE showed strong antioxidant activity *in vitro*. Therefore, the proposed UACEE method proved to be an innovative, effective, and environment-friendly method that may benefit food and medicinal industries.

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Conflict of interest
No potential conflict of interest was reported by the authors.

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