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Antioxidant activities of sunflower protein hydrolysates treated with dual-frequency ultrasonic: Optimization study

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Abstract

In this study, ultrasound pretreatments of sunflower-meal protein (SMP) to yield high antioxidant capacity from its hydrolysates by response surface methodology were optimized. Optimization of experimental conditions was achieved to examine the impact of temperature, solvent-solid ratio and sonication time on antioxidant capacities of SMP hydrolysates with Box–Behnken's design. Quadratic models of DPPH-scavenging activity (DPPHSA), hydroxyl-radical scavenging activity (HRSA), and Cu^{2+} and Fe^{2+} chelating activity (Cu^{2+}-CA and Fe^{2+}-CA) were developed, and their coefficients observed from multiple-regression analysis. ANOVA indicated that time was highly significant ($p < .01$) on all experimental responses. The best experimental point of DPPHSA, HRSA, Cu^{2+}-CA, and Fe^{2+}-CA was accessed at 42.50°C, 18.16 mL/g and 26.52 min and the predicted data for these responses were 52.09, 70.05, 50.85, and 43.35%, respectively. Outcome of verification experiment was reliable with predicted data for all responses. Additionally, DPPHSA, HRSA, Cu^{2+}-CA, and Fe^{2+}-CA of pretreated hydrolysate improved ($p < .05$) by 17.41, 20.00, 14.72, and 26.41%, respectively over nonsonicated hydrolysate. Amino acid content and hydrophobicity of SMP hydrolysate at the optimum sonication condition were analyzed. Analyses indicated that ultrasonication could facilitate the releasing/unfolding of hydrophobic amino acids from SMP over nonsonicated samples during enzymolysis with high antioxidative capacity.

Practical applications

Sonication pretreatment has been presented to have notable impacts on the antioxidant's capacities of protein hydrolysates. Sunflower-meal protein is an abundant and low-cost residue of oil industries, and is considered a potential bioactive peptides source, such as antioxidants. In this research, sonication pretreatment has been illustrated to be an effectual technique for the production of hydrolysates with antioxidant capacity from SMP. So, optimization of ultrasonication conditions is vital to examine the antioxidant capacities in SMP hydrolysates, which could be applied in large-scale food system and future pharmaceutical activity research.

1 | INTRODUCTION

Antioxidant is commonly utilized to avoid lipid oxidation in food products to prevent the creation of undesirable flavors and odors and toxic components (Lin & Liang, 2002). In addition, oxidative stress is implicated in presence of some diseases including diabetes, cancer, aging, hypertension, and Alzheimer's (Hajieva & Behl, 2006). Some artificial antioxidants including butylated hydroxytoluene and hydroxyanisole are widely utilized to delay deterioration and discoloration of foods. Nevertheless, due to health hazards, utilization of these artificial antioxidants is limited

(Park, Jung, Nam, Shahidi, & Kim, 2001). Therefore, there is growing attention in search for antioxidants from natural and safe resources that can improve the human body's antioxidant defenses to prevent oxidative damage (García-Moreno et al., 2014).

Using cheap agricultural residues to develop new foods has important economical and nutritional benefits. For example, sunflower meals, a coproduct of oil extraction, because of its protein content up to 40–50% (González-Pérez et al., 2002), has been recognized as a vital and low-cost raw material of protein for livestock feed and food ingredients. For human benefits, huge efforts have been done to evolve efficient procedures for preparation of acceptable products from sunflower meal (SM). With rich protein content, therefore, enzymolysis of SMP is an appropriate procedure to produce bioactive peptides, which can be applied in pharmaceutical and nutraceutical fields. In this respect, numerous sunflower protein hydrolysates have revealed several bioactivities including antihypertensive (Megías et al., 2004, 2009a), antihypocholesterolemic (Megías et al., 2009b), antimicrobial (Taha, Mohamed, Wagdy, & Mohamed, 2013), and antioxidant (Megías et al., 2007; Taha et al., 2013). However, the poor solubility of sunflower proteins cause barrier between enzymes and proteins, leading to reduction in the affinity between them, which impactfully decrease the bioavailability of SMP and restrict the release of functional peptides. Consequently, the development of a more effective method to overcome these drawbacks is a matter of great attention to food researchers.

Ultrasonication technology is usually considered to be simple, cheap, environmentally friendly, and safe method (Zou et al., 2016), that make its utilization have vital advantages over other technologies. Ultrasonic has been applied for different purposes in food system such as changing enzyme activity (Wei & Ye, 2011), facilitating enzymolysis (Dabbour, He, Mintah, Tang, & Ma, 2018), and improved biological properties of proteins (Yu et al., 2012). The cavitation impacts produced in solution by sonication waves could lead to degradation of proteins and changes in protein conformation, expose further hydrophobic sites and groups, additionally enhancing protein solubility and increasing the binding between matrix and enzyme. This enhances the hydrolysis efficacy. To the best our knowledge, no research on ultrasonic pretreatment of sunflower-meal protein to prepare antioxidant hydrolysates has been reported. Therefore, the antioxidant capacities of sunflower-meal protein hydrolysate were assessed comprehensively by DPPHSA, HRSA, Cu^{2+}-CA, and Fe^{2+}-CA.

In this context, the purpose of the current research was to examine and optimize ultrasonic pretreatments using response-surface methodology (RSM) to observe the highest antioxidant capacities for SMP hydrolysates. This work provided a theoretical basis to yield sunflower-meal protein hydrolysates with better antioxidant activities. The optimized hydrolysate could be applied as natural and safe antioxidants for pharmacological and nutraceutical fields.

2 | MATERIALS AND METHODS

2.1 | Materials and chemicals

SM, with 29.31% protein, which was utilized in our work was collected from Xinjiang Jinhai Oils Co., Xinjiang, (China) as a residue of extraction of sunflower oil. The meals were ground by portable mill and passed through a 60-mesh sieve, then maintained in zip lock bags at temperature of 4°C. All chemicals utilized in this work were of analytical-grade:1,1-diphenyl-2-picrylhydrazyl (DPPH), iron (II) sulfate ($FeSO_4$), hydrogen peroxide (H_2O_2), methanol, salicylic acid ($C_7H_6O_3$), iron (II) chloride ($FeCl_2$), ferrozine ($C_{20}H_{13}N_4Na_2O_6S_2$), pyridine ($C_5H_5N$), pyrocatechol violet ($C_{19}H_{14}O_7S$) were bought from Sinopharm Chemical Reagent Co., Ltd. (China). Enzyme alcalase 2.4 LFG (activity 150,000 U/mL) was acquired from Novozymes Biotech. Co., Ltd. (China).

2.2 | Sonication pretreatments and enzymolysis of SMP

Dual-frequency ultrasound (Meibo Biotech. Co., China) was applied as the source of sonication in this experiment (detailed in our earlier study [Dabbour, He, Ma, & Musa, 2018]). Sonication power (220 W), time and dual-frequency (20/40 kHz) in a combined operating mode (pulsed on and off-time of 5 and 2 s, respectively) were controlled from the equipment control panel and temperature was controlled using a digital-thermostatic bath. The ultrasonic probes were submerged two centimeters into solution. The suspensions were pretreated at various temperature (30–50°C), solvent-solid ratio (14–26 mL/g), and sonication time (10–30 min).

Following sonication treatment, pH of sunflower-meal protein solutions was adjusted to 9.0 using 1 mol/L sodium hydroxide. Subsequent to 15 min preheating in digital-thermostatic bath at temperature of 50°C, enzyme alcalase was added (0.343 g/L) to initiate the enzymatic reaction. The pH value of solutions was maintained at 9.0 by adding 1 mol/L sodium hydroxide through the enzymolysis (90 min). The reaction was inactivated after 90 min of hydrolysis by putting the hydrolysates in boiling water for 10 min. Afterward, the suspensions were subjected to centrifugation (4,500 g, 10 min) to obtain supernatants, and kept at −20°C for further investigations. The sonication parameters with various temperature, solvent-solid ratio, and time combinations are displayed in Table 1. Control (classical enzymolysis) was carried out using an impeller-agitator device under the same parameters but without sonication. Under optimum condition, supernatant was frozen, lyophilized by a freeze dryer and stored at −20°C to investigate the amino acid composition and surface hydrophobicity of SMP hydrolysate.

2.3 | Experimental design

To maximize the antioxidants activity in sunflower protein hydrolysates pretreated by ultrasonic, RSM technique was utilized. The best sonication treatment was accessed by Box–Behnken's design (BBD). Three-level-three-variable of temperature (°C, X_1), solvent-solid ratio (mL/g, X_2), and sonication time (min, X_3) were employed (Table 1). The experimental design comprised of 17 experiments; and DPPHSA (%, Y_1), HRSA (%, Y_2), Cu^{2+}-CA (%, Y_3), and Fe^{2+}-CA (%, Y_4) were selected as responses. Average values of these responses observed from three replicates were analyzed by Design-Expert 8.0.6.1 and fitted to a quadratic-polynomial equation as:

TABLE 1 BBD for experimental factors (temperature, solvent-solid ratio and time) and the responses data

Run	Temperature (°C)	Solvent-solid ratio (mL/g)	Time (min)	DPPHSA (%)	HRSA (%)	Cu^{2+}-CA (%)	Fe^{2+}-CA (%)
1	40	20	20	55.86	64.21	45.50	41.96
2	40	20	20	58.33	65.50	47.28	40.58
3	40	26	10	48.88	55.30	38.40	29.85
4	40	20	20	57.75	66.11	46.91	39.50
5	50	14	20	37.09	80.40	45.50	42.62
6	30	14	20	37.69	55.90	41.90	30.50
7	50	20	30	40.87	65.00	52.37	46.33
8	40	14	30	37.70	77.49	50.74	42.30
9	50	20	10	49.06	59.75	39.95	29.10
10	30	20	30	48.48	74.71	45.57	32.81
11	40	20	20	56.40	65.95	48.20	40.22
12	30	20	10	54.83	50.15	38.20	24.13
13	40	20	20	58.87	64.90	47.50	41.32
14	30	26	20	38.81	72.76	40.13	27.20
15	40	26	30	41.01	59.12	38.20	36.72
16	50	26	20	37.99	55.84	35.21	35.83
17	40	14	10	44.37	47.48	32.66	31.67

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where Y represents predicted response; β_i, β_{ii}, and β_{ij} signifies linear, squared (second-order) and combination coefficients for independent variables (X_1, X_2, and X_3), β_0 denotes intercept, and X_j and X_i representing experimental factors.

2.4 | Antioxidants activity

2.4.1 | DPPHSA assay

DPPH radical-scavenging activity was performed as outlined by Zhang, Li, Miao, and Jiang (2011) with slight alterations (García-Moreno et al., 2014). Suitable dilution of SMP hydrolysate (0.2 mL) was mixed with 3.4 mL of 0.1 mM DPPH radical dissolved in methanol (95%) and with 50 mM (pH 7.3) Tris buffer (0.40 mL), agitated and incubated (0.5 hr, 25°C) in dark. The absorbance of reactants was monitored using spectrophotometer at wavelength of 517 nm against distilled water instead of SMP hydrolysate (blank). Sample-control was done for every hydrolysate sample using methanol in place of DPPH. The DPPHSA (%) was estimated as:

$$\text{Scavenging}\,(\%) = \left(1 - \frac{\text{Absorbance of sample} - \text{Absorbance of sample\_control}}{\text{Absorbance of blank}}\right) \times 100 \quad (2)$$

2.4.2 | HRSA

The HRSA was investigated according to Wang, Wang, Dang, Zheng, and Zhang (2013) with some alterations. Aliquots (1 mL) of diluted samples were mixed with 6 mM $FeSO_4$ (1 mL) and 1 mL of 6 mM H_2O_2, agitated and incubated for 12 min. Then the resultant solution was mixed with 9 mM salicylic acid (1 mL), vortexed and allowed to react for 0.5 hr at temperature of 37°C. The absorbance of mixtures was read at wavelength of 510 nm and distilled water (1 mL) in place of hydrolysate sample was utilized as a blank. HRSA (%) was determined as:

$$\text{HRSA}\,(\%) = \left(1 - \frac{\text{Sample absorbance} - \text{Sample without salicylic acid absorbance}}{\text{Blank absorbance}}\right) \times 100 \quad (3)$$

2.4.3 | Fe^{2+}-CA

Fe^{2+}-CA of hydrolysates was assessed as described by Zhang et al. (2009) with slight modifications (García-Moreno et al., 2014). One milliliter of an appropriate dilution of hydrolysate suspension was added to deionized water (3.5 mL). Thereafter, 2 mM $FeCl_2$ (0.10 mL) was added and subsequent to 3 min the resultant mixtures were mixed with 5 mM ferrozine (0.20 mL) and then shaken vigorously. The absorbance was detected at wavelength of 562 nm after mixtures incubation (20 min, 25°C), and distilled water (1 mL) instead of hydrolysate were applied as blank. Sample-control was done for every hydrolysate but without ferrozine. The Fe^{2+}-CA (%) was measured as in Equation (2).

2.4.4 | Cu^{2+}-CA

The technique of Zhu, Jie, Tang, and Xiong (2008) with alterations was utilized to investigate Cu^{2+}-CA of SMP hydrolysate. Briefly,

2 mM $CuSO_4$ (1 mL) was added to 0.1% pyrocatechol violet (20 μL) and 10% pyridine (1 mL). After adding diluted sample (1 mL), the blue color disappearance was observed by analyzing the absorbance after 5 min of reaction at wavelength of 632 nm with spectrophotometer. Deionized water (equivalent volume) was applied for blank instead of hydrolysate. Sample-control was done for every hydrolysate but without pyrocatechol violet. Cu^{2+}-CA of SMP hydrolysate (%) was measured as in Equation (2).

2.5 | Amino acid analysis

Composition of amino acid of sonicated and nonsonicated hydrolysates at the optimum condition was investigated as outlined by Marino et al. (2010) with slight alterations. Hydrolysate of SMP for control and ultrasonication pretreatment was hydrolyzed in 6 mol/L HCl for 24 hr at temperature of 110 ± 2°C in Pyrex microcapillary tubes. Then, volume of resultant mixture was set to 50 mL with deionized water. One milliliter of diluted hydrolysates was filtered (syringe filter 0.22 μm), lyophilized at temperature of 60°C using vacuum concentrator. Quantification was done with an amino acid analyzer (S-433D, Sykam GmbH Co., Germany).

2.6 | Surface hydrophobicity (H_0)

The protocol of Kato and Nakai (1980) was utilized to estimate the hydrophobicity of hydrolysate with ANS as a probe of fluorescence. SMP hydrolysate was dissolved in phosphate buffer (pH 8.0, 10 mmol/L) to attain different concentrations (0.04–0.1 mg/mL). Aliquot (4 mL) of hydrolysate suspension was added to ANS (20 μL) (8.0 mmol/L in the same buffer) and the resultant suspension was kept (3 min) in dark. Fluorescence capacity (FC) of hydrolysate suspension was estimated at wavelength of 270 nm (excitation, slit 5.0 nm) and the scanning speed of 2 nm/s with a Cary Eclipse spectrophotometer (Varian, Inc., Palo Alto). The emission spectrum was quantified at 485 nm wavelength. The initial slope (H_0) was determined using linear regression analysis.

3 | RESULTS AND DISCUSSION

3.1 | Model fitting

In this investigation, the empirical models were established to examine the impact of the interaction between various parameters (temperature, solvent-solid ratio, and sonication time) on the antioxidant's activities (DPPHSA, HRSA, Cu^{2+}-CA, and Fe^{2+}-CA) of SMP hydrolysate. To assess the adequacy of DPPHSA, HRSA, Cu^{2+}-CA, and Fe^{2+}-CA function in the experimental setup, the ultrasonication parameters were optimized. Additionally, the linear, second-order and interaction impacts of experimental factors were analyzed. Nevertheless, the suitability of these quadratic models is confirmed with the determination-coefficient (R^2), adjusted *R*-squared, model significance and significance of lack-of-fit. The closer the adjusted *R*-squared and *R*-squared values to unity, the more appropriate the empirical models fit the experimental values (Chen et al., 2018). The R^2 and adjusted R^2 values (Table 2) were 0.975 and 0.942, 0.991 and 0.978, 0.960 and 0.908, and 0.979 and 0.952 for DPPHSA, HRSA, Cu^{2+}-CA, and Fe^{2+}-CA of SMP hydrolysates, respectively, which demonstrated a strong correlation degree among the predicted and actual values. In conclusion, ANOVA showed that the quadratic models are dependable for the prediction of antioxidant capacities.

TABLE 2 ANOVA (*p*-value, R^2 and Adj.R^2) of the fitted quadratic models for the examined parameters versus each response

	p-value			
Source	DPPHSA	HRSA	Cu^{2+}-CA	Fe^{2+}-CA
Model	<.0001	<.0001	.0004	<.0001
X_1	.0360	.0928	.1750	<.0001
X_2	.1289	.0021	.0056	.0033
X_3	.0014	<.0001	.0001	<.0001
X_1X_2	.9581	<.0001	.0401	.2577
X_1X_3	.6628	.0002	.1797	.0194
X_2X_3	.7752	<.0001	.0010	.2260
X_1^2	.0002	.0351	.1942	.0004
X_2^2	<.0001	.2486	.0004	.0121
X_3^2	.0796	.0002	.0579	.0021
Lack-of-fit	.0895	.0638	.0691	.1155
R-squared	.975	.991	.960	.979
Adj *R*-squared	.942	.978	.908	.952

Note. *p*-value <.05 and <.01 are statistically significant and highly significant, respectively.

3.2 | Influence of experimental factors on the antioxidant's activities of SMP hydrolysates

3.2.1 | DPPHSA of SMP hydrolysate

It is reported that antioxidants function through various mechanisms. So, it is suggested to use various procedures to assess antioxidant capacity (Frankel & Meyer, 2000). In this work, DPPHSA, HRSA, Cu^{2+}-CA, and Fe^{2+}-CA of SMP hydrolysate treated by sonication were evaluated and optimized (Table 1). To evaluate free-radical scavengers of antioxidants, a broadly recognized procedure is the DPPHSA (Li, Jiang, Zhang, Mu, & Liu, 2008). DPPH is a stable free-radical, which decolorizes when decreased by proton donor substances, resulting in a decrease in absorptivity at wavelength of 517 nm. The reduction in color is an index of analyte scavenging activity. The actual data of DPPHSA via different experimental factors were displayed in Table 1. The DPPHSA ranged from 37.09 to 58.87% and the maximum value of DPPHSA (58.87%) was found at sonication temperature of 40°C, solvent-solid ratio of 20 mL/g and time of 20 min (run No. 13). The observed data were analyzed (regression analysis) to examine the impact of experimental factors and their interactions on the DPPHSA (%). The observed coefficients were utilized to create the following regression equation.

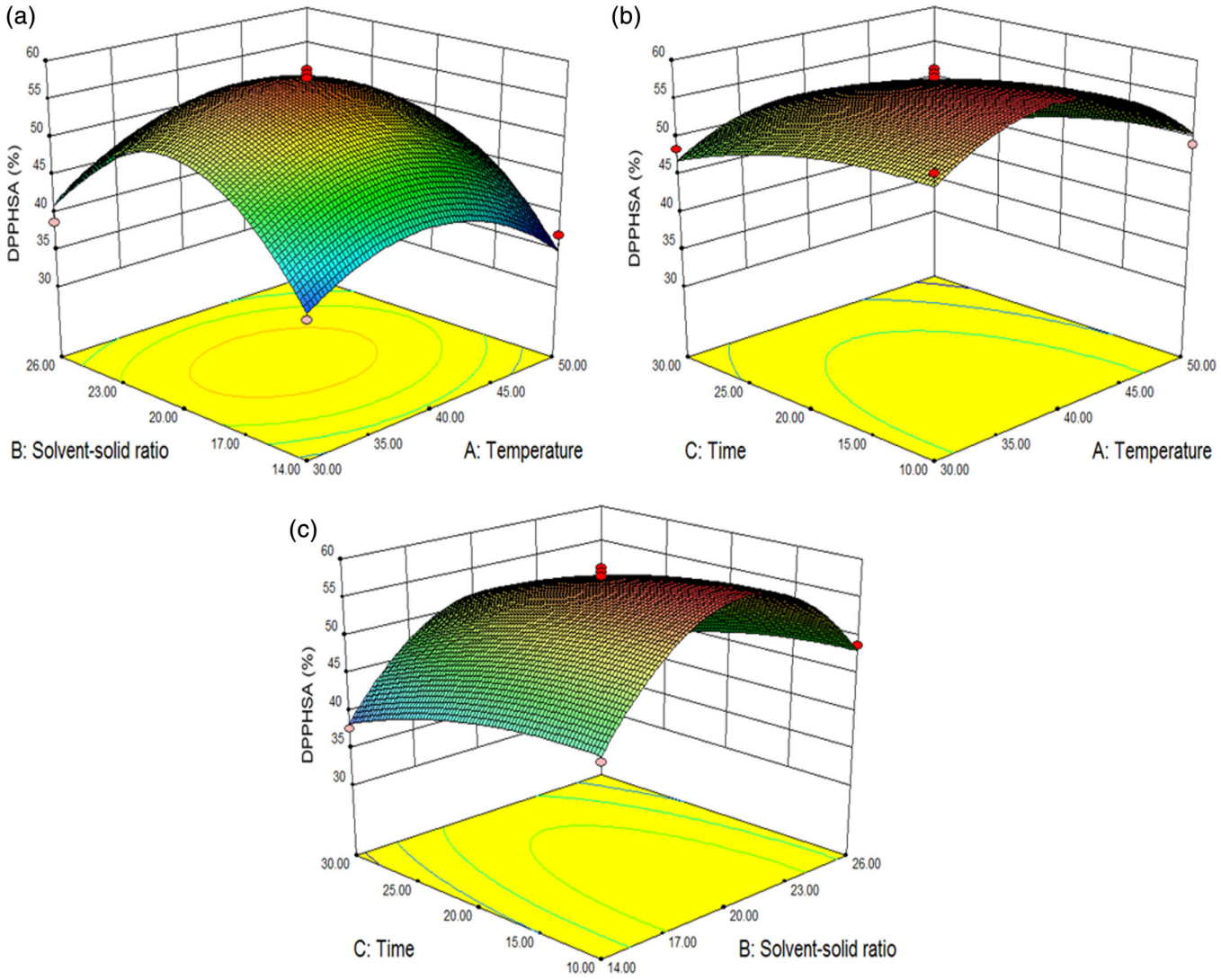

FIGURE 1 3D-response curves representing the combination impact of experimental parameters on DPPHSA (%) of SMP hydrolysates

$$Y_1(\text{DPPHSA}) = -198.44 + 5.62X_1 + 14.16X_2 + 0.73X_3 - 0.0009X_1X_2 - 0.005X_1X_3 - 0.005X_2X_3 - 0.071X_1^2 - 0.345X_2^2 - 0.02X_3^2 \quad (4)$$

Statistical analysis (analyzed by BBD) in Table 2 showed that the DPPHSA model was remarkably significant ($p < .01$), while the lack-of-fit was nonsignificant ($p = .0895$), suggesting that this mathematical model was fit with strong prediction (R-squared = 0.975; Adj. R-squared = 0.942). The linear impact of temperature and ultrasonication time (X_1 and X_3) were significant ($p < .05$)—positive effect on DPPHSA (%). Additionally, the terms of X_1^2 and X_2^2 had highly significant ($p < .01$)—negative correlation on DPPHSA of SMP hydrolysate (Table 2 and Equation (4)), whereas the other-terms were ($p > .05$) nonsignificant.

Based on Equation (4), 3D-curves were plotted (Figure 1a–c) to characterize the influences of the experimental variables and their interaction on DPPHSA and to realize the best experimental point of the response. Figure 1a illustrates the influence of the interaction of temperature and solvent-solid ratio and clarified that maximal DPPHSA was observed at 40°C, 20 mL/g and 20 min. DPPHSA (%) improved gradually with increasing temperature. Yet, when the temperature was elevated to around 40°C, the DPPHSA reduced, as exhibited in Figure 1b. Figure 1c exhibits combination effect of X_2X_3 and explained that DPPHSA was raised as solvent-solid ratio rises, whereas, when solvent-solid ratio was raised to around 20 mL/g, the DPPHSA rapidly reduced. Comparable result on the impact of temperature on DPPHSA has been indicated in literatures but for bioactive compound (Sood & Gupta, 2015; Tabaraki, Heidarizadi, & Benvidi, 2012).

3.2.2 | HRSA of SMP hydrolysate

Hydroxyl radical is highly reactive oxygen-radical that can react with various biomolecules including DNA, carbohydrates, lipids, amino acids, protein, and nucleotide (You, Zhao, Regenstein, & Ren, 2010) and can lead to intense harm to cell tissues. Consequently, elimination of hydroxyl radical may be efficient defense versus different diseases in vivo. Evaluation of HRSA gives valuable information on antioxidants capacities. The observed

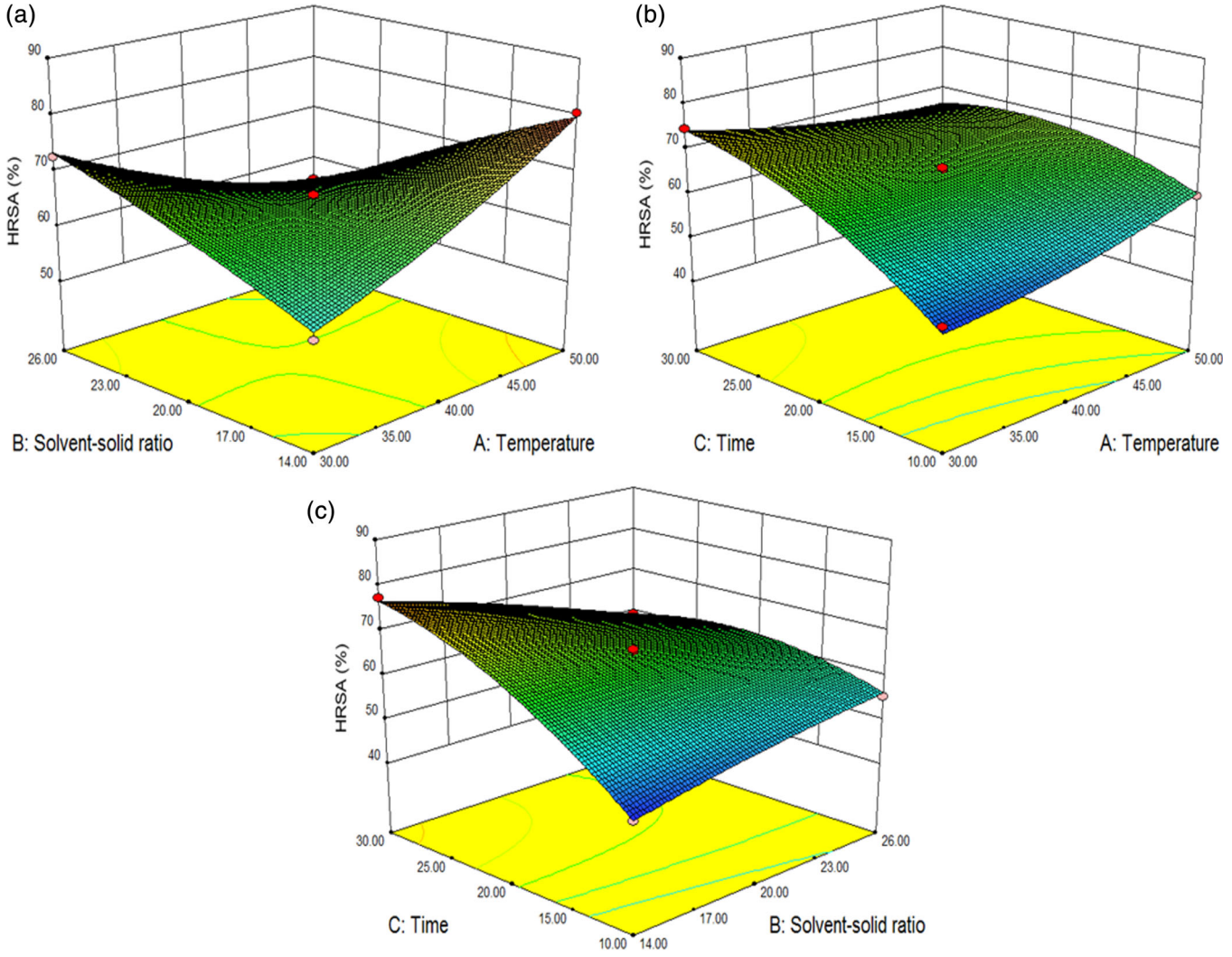

FIGURE 2 3D-response curves representing the combination impact of experimental parameters on HRSA (%) of SMP hydrolysates

data of HRSA (%) at various experimental variables were exhibited in Table 1. The maximum HRSA value was 80.40% and found via experimental parameters of 50°C, 14 mL/g and 20 min (run No. 5), while the minimum HRSA (47.48%) was found in combination of sonication temperature of 30°C, solvent-solid ratio of 20 mL/g and time of 10 min (run No. 17). The fitted regression model of HRSA (%) is as follows:

$$Y_2(\text{HRSA}) = -167.34 + 3.13X_1 + 9.63X_2 + 6.77X_3 - 0.173X_1X_2 - 0.048X_1X_3 - 0.109X_2X_3 + 0.017X_1^2 - 0.023X_2^2 - 0.047X_3^2 \quad (5)$$

The HRSA model showed a remarkably significant ($p < .01$), while the lack-of-fit illustrated a nonsignificant ($p = .0638$), confirming that the current model was suitable to describe the actual HRSA data ($R^2 = 0.991$ and Adj.$R^2 = 0.978$) (Table 2). As displayed in Table 2, solvent-solid ratio and sonication time exhibited highly significant ($p < .01$)—positive correlation on HRSA. The impacts of X_1^2 and X_3^2 were significant ($p < .05$). Furthermore, all combined factors (X_1X_2, X_1X_3, and X_2X_3) presented a noticeably significant ($p < .01$)—negative impact on HRSA of sunflower-meal protein hydrolysates.

Figure 2a–c shows the interaction impacts of temperature, solvent-solid ratio and sonication time on HRSA (%) of SMP hydrolysates. Figure 2a displays that HRSA was obtained high at high levels of solvent-solid ratio (X_2) and temperature (X_1) and the maximal value was obtained at low level of solvent-solid ratio (X_2) and high level of temperature (X_1). Combined influence of temperature and sonication time was exhibited in Figure 2b and clarified HRSA enhanced steadily by increasing sonication time. Moreover, Figure 2c illustrates mutual effect of X_2X_3 and explained that a higher sonication time (30 min) and a lower solvent-solid ratio (14 mL/g) would provide a higher HRSA of SMP hydrolysates. Similar finding on the influence of sonication time on HRSA has been reported in literature but for different sample (Zou & Hou, 2017).

3.2.3 | Cu^{2+}-CA of SMP hydrolysate

Transition metal-ions, including Cu^{2+} and Fe^{2+}, can accelerate the reactive oxygen species formation, leading to DNA harm and lipid oxidation (Stohs & Bagachi, 1995). Particularly, Fe^{2+} produces hydroxyl-radical

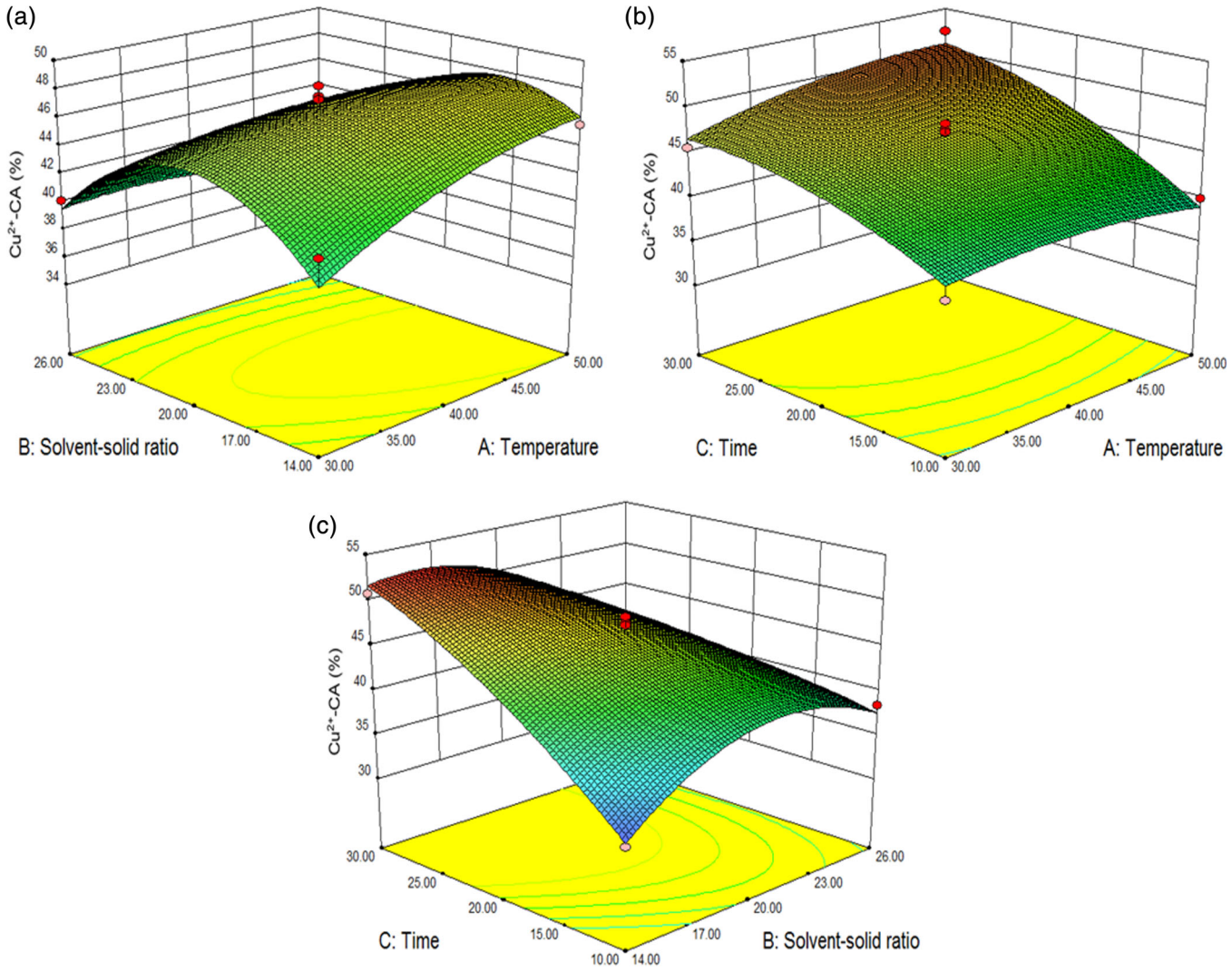

FIGURE 3 3D-response curves representing the combination impact of experimental parameters on Cu^{2+}-CA (%) of SMP hydrolysates

through Fenton reaction, thus accelerating the chain reaction of lipid oxidation. Additionally, Fe^{2+} catalyzes the lipid peroxides decomposition, resulting in off-flavor creation. So, chelating of Cu^{2+} and Fe^{2+} with antioxidant could delay the oxidation reaction. The Cu^{2+}-CA data (%) of SMP hydrolysate of all 17 experimental runs were in the range of 32.66–52.37% (Table 1). Run No. 17 (40°C, 14 mL/g and 10 min), resulted in the lowest Cu^{2+}-CA (%) value, while run No. 7 (50°C, 20 mL/g and 30 min) provided the maximal value. Cu^{2+}-CA results were analyzed (multiple regressions) for fitting the regression model as follows:

$$Y_3\left(Cu^{2+}-CA\right) = -91.17 + 1.50X_1 + 8.34X_2 + 2.24X_3 - 0.036X_1X_2 + 0.013X_1X_3 - 0.076X_2X_3 - 0.012X_1^2 - 0.145X_2^2 - 0.019X_3^2 \quad (6)$$

Statistical significance of the experimental factors and their combination were displayed in Table 2. The Cu^{2+}-CA model was highly significant ($p < .01$) and correlated with the values of R^2 (0.960) and Adj. R^2 (0.908), while lack-of-fit was nonsignificant ($p = .0691$). These observations proved the mathematical model is satisfactory for prediction of Cu^{2+}-CA values. Solvent-solid ratio (X_2) and sonication time (X_3) were highly significant ($p < .01$) and had positive impact on Cu^{2+}-CA of SMP hydrolysate. The effect of X_2^2 was highly significant ($p = .0004$) and had negative influence on Cu^{2+}-CA. Additionally, combination of temperature and solvent-solid ratio (X_1X_2) and solvent-solid ratio and sonication time (X_2X_3) had a significant effect ($p < .05$)—negative correlation on Cu^{2+}-CA.

3D-curves plots were utilized to investigate the influence of combination of temperature (X_1), solvent-solid ratio (X_2), and ultrasonication time (X_3) on Cu^{2+}-CA (%) of SMP hydrolysates (Figure 3a–c). The interaction impacts of X_1X_2, X_1X_3, and X_2X_3 were examined. Figure 3a presents the combination impact of X_1X_2 and elucidated that Cu^{2+}-CA was enhanced as temperature and solvent-solid ratio rises, while, when solvent-solid ratio was raised to around 20 mL/g, the Cu^{2+}-CA reduced. Moreover, temperature had a little influence on Cu^{2+}-CA compared with ultrasonication time as exhibited in Figure 3b and the highest value of Cu^{2+}-CA was observed at high levels of temperature (50°C) and sonication time (30 min). Combined influences of solvent-solid ratio and time is presented in Figure 3c

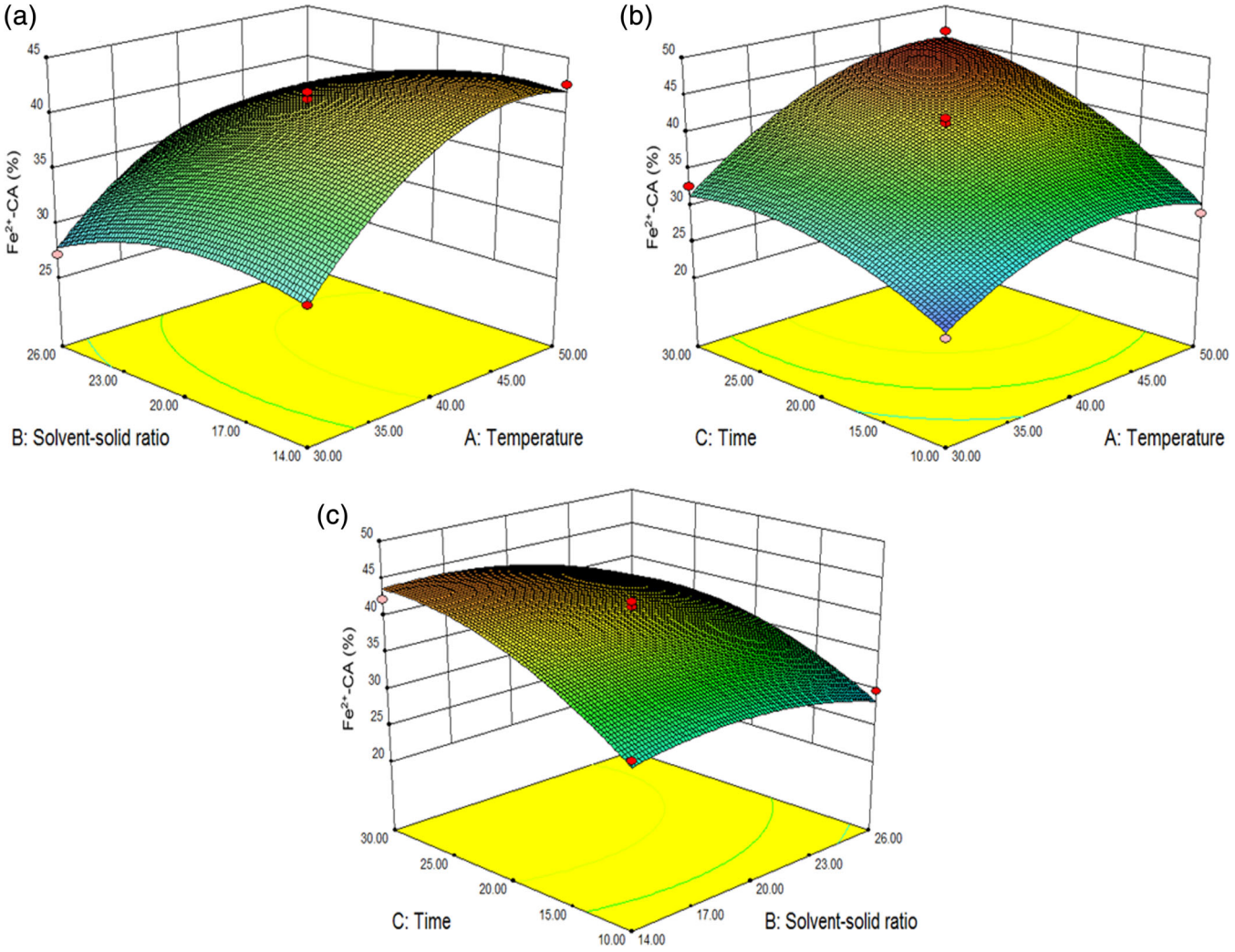

FIGURE 4 3D-response curves representing the combination impact of experimental parameters on Fe^{2+}-CA (%) of SMP hydrolysates

which indicates a longer sonication time (30 min) and a lower solvent-solid ratio (14 mL/g) would yield a greater Cu^{2+}-CA.

3.2.4 | Fe^{2+}-CA of SMP hydrolysates

The impacts of temperature (X_1), solvent-solid ratio (X_2), and sonication time (X_3) on Fe^{2+}-CA (%) of SMP hydrolysate were examined by a BBD. Results of Fe^{2+}-CA (%) of all experimental runs increased from 24.13 to 46.33% (Table 1). Maximum value was found at the interaction of 50°C, 20 mL/g and 30 min (run No. 7), but the lowest value was observed at 30°C, 20 mL/g and 10 min (run No. 12). Relationship among Fe^{2+}-CA (%) and experimental variables (actual parameters) was determined as follows:

$$Y_4\left(Fe^{2+}-CA\right) = -91.85 + 3.84X_1 + 3.12X_2 + 1.31X_3 - 0.015X_1X_2 + 0.021X_1X_3 - 0.016X_2X_3 - 0.044X_1^2 - 0.064X_2^2 - 0.033X_3^2 \quad (7)$$

The statistical significance of the Fe^{2+}-CA model and the experimental factors were measured with p-value (Table 2). The model was highly significant ($p < .01$) for fitting the Fe^{2+}-CA (%) with high R^2 (0.979) and Adj.R^2 (0.952) values, but the lack-of-fit was insignificant ($p = .1155$), demonstrating that this mathematical model could properly clarify the relation among the Fe^{2+}-CA and the experimental variables. Data analysis of Fe^{2+}-CA (Table 2) showed differences in the temperature (X_1), solvent-solid ratio (X_2), and sonication time (X_3) were highly significant ($p < .01$), showed positive influence on Fe^{2+}-CA. Furthermore, three-quadratic terms (X_1^2, X_2^2, and X_3^2) were significant ($p < .05$)—negative impact on Fe^{2+}-CA. Combination impacts of X_1X_3 was significant ($p < .05$), while another combination (X_1X_2 and X_2X_3) illustrated nonsignificant ($p > .05$). For each term in the previous mathematical models, a low p-value demonstrate a more significant influence on the experimental parameter (Quanhong & Caili, 2005).

Figure 4a-c shows the interaction influences of X_1X_2, X_1X_3, and X_2X_3 on Fe^{2+}-CA (%) of SMP hydrolysates. Figure 4a exhibits the combination impact among X_1 and X_2 and elucidated that Fe^{2+}-CA was raised steadily as temperature (X_1) rises, and the lesser temperature (30°C) and a higher solvent-solid ratio (26 mL/g) provide a greater Fe^{2+}-CA. Moreover, maximal Fe^{2+}-CA value was observed at high levels of temperature and sonication time, while the least value was found at low levels of the same experimental variables (X_1 and X_3) as

TABLE 3 DPPHSA, HRSA, Cu^{2+}-CA, and Fe^{2+}-CA of SMP hydrolysate observed by optimal experimental variables of sonication and control treatment

	Responses (%)			
Treatment	DPPHSA	HRSA	Cu^{2+}-CA	Fe^{2+}-CA
Ultrasonic pretreatment	49.92 ± 2.56 a	71.83 ± 3.12 a	51.02 ± 1.92 a	44.87 ± 3.02 a
Control	41.23 ± 1.85 b	57.46 ± 1.27 b	43.51 ± 2.47 b	33.02 ± 0.98 b
Increased rate (%)	17.41	20.00	14.72	26.41

Note. Mean data with various letters in column are significantly ($p < .05$).

displayed in Figure 4b. Mutual influences of solvent-solid ratio and ultrasonication time is displayed in Figure 4c which demonstrates that sonication time had significant impacts more than solvent-solid ratio on Fe^{2+}-CA.

3.3 | Validation of predictive optimum experimental conditions

The antioxidants activities of SMP hydrolysates were investigated at the predicted optimum experimental variables to validate the developed models observed from RSM. This technique was applied in our study to access the best experimental conditions which could achieve the maximal antioxidants activities. The predicted levels of temperature 38.68°C, solvent-solid ratio 20.38 mL/g and time 11.49 min gave the highest DPPHSA (59.65%), and the maximal HRSA (82.26%) was realized under the predicted experimental parameters of 48.64°C, 14.12 mL/g and 29.73 min. Greatest value of Cu^{2+}-CA (53.75%) was achieved at 47.81°C, 14.21 mL/g, and 29.25 min, while the experimental factors of 47.77°C, 14.51 mL/g and 29.47 min provided maximum Fe^{2+}-CA value (45.25%). The optimal experimental point for all experimental responses was at 42.50°C, 18.16 mL/g and 26.52 min. Under this condition, the predicted DPPHSA, HRSA, Cu^{2+}-CA and Fe^{2+}-CA values were 52.09, 70.05, 50.85, and 43.35% respectively. These values are verified with the obtained results. The observed DPPHSA of 49.92%, HRSA of 71.83%, Cu^{2+}-CA of 51.02%, and Fe^{2+}-CA of 44.87% were insignificant ($p > .05$) with the predicted results. This powerfully demonstrates that the regression model is appropriate to predict DPPHSA, HRSA, Cu^{2+}-CA, and Fe^{2+}-CA at selected experimental variables.

3.4 | Comparison of sonication and control pretreatments

The results of antioxidant activity of SMP hydrolysates pretreated with sonication and control were compared (Table 3). Sonication at optimal treatment point remarkably raised ($p < .05$) antioxidant capacities over control. The values of DPPHSA, HRSA, Cu^{2+}-CA, and Fe^{2+}-CA of sonicated SMP hydrolysates enhanced ($p < .05$) by 17.41, 20.00, 14.72, and 26.41%, respectively compared with control. These phenomena may be attributed to sonication pretreatment motivated alterations to protein structure, resulting in a loosened molecular structure and rise in proteins surface-hydrophobicity (Zhang et al., 2016) and hydrophobic amino acids content. This was confirmed by the composition of amino acid and observation of hydrophobicity of SMP hydrolysate. Thus, an improvement in the alcalase activity to facilitate the enzymolysis and then release more active-peptides with high antioxidant capacities in suspension. Comparable observation has been indicated in previous works (Abdualrahman et al., 2016; Liang et al., 2017). Finally, sonication treatments over control were more helpful for enzymolysis of sunflower-meal protein to prepare hydrolysates with high antioxidants activities.

3.5 | Amino acid analysis

Composition of amino acid was quantified for sonicated and non-sonicated hydrolysates (Table 4) to examine the influences of ultrasonication on the antioxidant potential of SMP hydrolysates. From Table 4, the untreated and pretreated samples were rich in Glu and Asp. Contents of hydrophobic amino acid (HAA) raised by 8.23%,

TABLE 4 Composition of amino acid (%) of nonsonicated and sonicated SMP hydrolysate

Amino acids	Control	Ultrasonic pretreatment
Asp	12.6 ± 0.19	12.0 ± 0.29
Thr	2.9 ± 0.06	3.5 ± 0.15
Ser	3.7 ± 0.04	3.4 ± 0.09
Glu	20.1 ± 0.62	22.2 ± 0.49
Pro	3.7 ± 0.09	3.6 ± 0.13
Gly	6.0 ± 0.21	5.4 ± 0.11
Ala	4.3 ± 0.17	4.9 ± 0.08
Cys	1.6 ± 0.02	1.6 ± 0.04
Val	3.6 ± 0.18	3.4 ± 0.15
Met	2.4 ± 0.03	2.7 ± 0.05
Ile	2.2 ± 0.07	3.0 ± 0.10
Leu	6.5 ± 0.25	6.7 ± 0.31
Tyr	2.5 ± 0.10	2.2 ± 0.04
Phe	4.4 ± 0.18	4.9 ± 0.11
His	2.7 ± 0.09	2.9 ± 0.08
Lys	1.4 ± 0.02	1.6 ± 0.05
Arg	3.5 ± 0.13	3.5 ± 0.07
HAA (%)	30.0	32.7

Note. HAA, hydrophobic amino acids (Val, Thr, Met, Ala, Ile, Phe, Pro, and Leu).

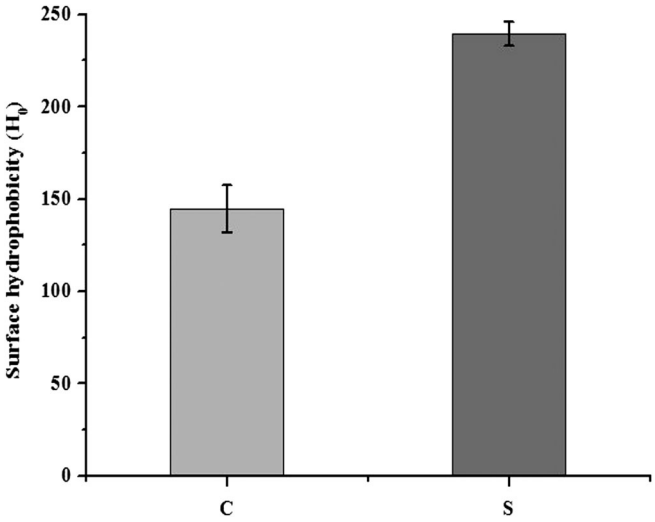

FIGURE 5 Surface hydrophobicity of nonsonicated (C) and sonicated (S) SMP hydrolysate

especially remarkable increase in Ile (26.67%) with sonication over nonsonicated hydrolysate. The finding is in accordance with the observation of Huang, Dai, Li, and Ma (2015). This phenomenon of the sonicated samples inclines to form peptides with C-terminal HAA (Ala, Val, Leu, Ile, Phe, Pro, Thr, and Met) more than untreated one during hydrolysis, since sonication results in more hydrophobic regions/sites and groups inside the molecules to be exposed to surrounding surface. Hydrolysates with high antioxidant activity are associated with HAA. Thus, more hydrophobicity may cause more antioxidant capacities of sonicated hydrolysates. Our findings suggested ultrasonication could be easy to produce hydrolysate with strong antioxidant potential.

3.6 | Surface hydrophobicity

To evaluate the conformational alterations in protein, hydrophobicity is important. It shows the groups on periphery of protein molecules (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011), that influences on protein functionality. Impact of ultrasonication pretreatment at the optimum points on H_0 of SMP hydrolysates was presented in Figure 5. Compared with control, H_0 of the sonicated SMP hydrolysate was improved (144.68 ± 12.83–239.55 ± 6.55, $p < .05$). Our observation agreed with Xiong et al. (2018). The improvement in H_0 of hydrolysate indicates that SMP hydrolysate molecules were stretched subsequent to ultrasonication. This may be attributed to the microstreaming and cavitation actions produced by sonication, leading to a rise in number of hydrophobic groups and sites/regions buried at the interior of hydrolysate; thus exposing the polar sites (Jakopović, Barukčić, & Božanić, 2016), and then enhancing the hydrophobicity of SMP hydrolysate. Combined with the findings of amino acids composition, these observations may offer a clarification for the obtained enhancement in antioxidant capacities of sonicated SMP hydrolysates.

4 | CONCLUSIONS

In this observation, RSM was efficaciously utilized for accessing the experimental variables for optimal antioxidants activities from SMP hydrolysates. Temperature, solvent-solid ratio and sonication time conditions significantly and positively affected DPPHSA, HRSA, Cu^{2+}-CA, and Fe^{2+}-CA. ANOVA presented a high R^2 and Adj.R^2 0.975 and 0.942 for DPPHSA, 0.991 and 0. 978 for HRSA, 0.960 and 0.908 for Cu^{2+}-CA and 0.979 and 0.952 for Fe^{2+}-CA, confirming a reasonable fit of the regression models with the measured data. Optimal experimental conditions for antioxidants activities were at 42.50°C, 18.16 mL/g and 26.52 min. This point provided DPPHSA of 52.09%, HRSA of 70.05%, Cu^{2+}-CA of 50.85% and Fe^{2+}-CA of 43.35%. For these responses, experimental data were noticed to be close to the predicted data realized by the quadratic models. At optimum sonication condition, antioxidant capacities of sonicated hydrolysate were improved ($p < .05$) over nonsonicated one. This enhancement concurred with the increase in the hydrophobic amino acids content and hydrophobicity of sonicated hydrolysate. According to the findings observed in this work, these results could be scaled to industries to produce antioxidants from SMP hydrolysates in large quantities.

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NOTATION

BBD	Box–Behnken's design
Cu^{2+}-CA	Cu^{2+} chelating activity (%)
DPPHSA	DPPH-scavenging activity (%)
Fe^{2+}-CA	Fe^{2+} chelating activity (%)
HAA	hydrophobic amino acids
H_0	surface hydrophobicity
HRSA	hydroxyl-radical scavenging activity (%)
RSM	response-surface methodology
SM	sunflower meal
SMP	sunflower-meal protein
X_1	temperature (°C)
X_2	solvent-solid ratio (mL/g)
X_3	sonication time (min)
Y_1	DPPHSA (%)
Y_2	HRSA (%)
Y_3	Cu^{2+}-CA (%)
Y_4	Fe^{2+}-CA (%)

GREEK SYMBOLS

β_i	linear coefficients for independent variables
β_{ii}	second-order coefficients for independent variables

β_{ij} combination coefficients for independent variables

β_0 Intercept

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