Carbohydrate Polymer Technologies and Applications 10 (2025) 100817

Contents lists available at ScienceDirect

Carbohydrate Polymer Technologies and Applications

journal homepage: www.sciencedirect.com/journal/carbohydrate-polymer-technologies-and-applications

Modulating ultrasound-assisted extraction of polysaccharide-protein complexes from β-glucan-rich medicinal mushrooms for enhanced antioxidant activity

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ARTICLE INFO

Keywords:
Ultrasound-assisted extraction
Polysaccharide-protein complexes
Medicinal mushroom
Antioxidant activity
Response surface methodology
β-glucan
Extraction optimization

ABSTRACT

The growing nutraceutical interest in polysaccharide-protein complexes (PSPs) from β-glucan-rich medicinal mushrooms necessitates efficient extraction methods. This study examined the impact of various ultrasound-assisted extraction (UAE) parameters, including ultrasound power, temperature, mushroom particle size, and solid-to-liquid ratio, on the composition and antioxidant activities of PSPs extracts from *Lentinula edodes* (*L. edodes*) and *Grifola frondosa* (*G. frondosa*). Using a Box–Behnken design and response surface methodology, second-order polynomial models were developed to correlate UAE parameters with PSPs antioxidant activity, demonstrating strong model fit with high *F*-values and low *p*-values (*F*-value = 8.64, *p*-value = 0.0143 for *L. edodes;* *F*-value = 42.41, *p*-value = 0.0003 for *G. frondosa*). The models showed a high degree of correlation between observed and predicted values, with variations depending on mushroom species. Optimal UAE conditions for achieving the highest antioxidant PSPs were identified for *L. edodes* (55 °C, 6.21 W/cm^2 ultrasound power, particle size <600 μm) and *G. frondosa* (70 °C, 6.21 W/cm^2 ultrasound power, particle size of 600–850 μm). A higher protein-to-polysaccharide ratio in PSPs was correlated with enhanced antioxidant activity. UAE presents a promising technique for enhancing the antioxidant properties of PSPs in pharmaceutical formulations, potentially broadening their use in extracting bioactive compounds from other medicinal mushrooms to promote health and wellness.

1. Introduction

Mushrooms, as macro-fungi with distinct fruiting bodies visible to the naked eye, are not only a popular and nutritious food but also renowned for their medicinal properties. Polysaccharides (PS) and polysaccharide-protein complexes (PSPs) are crucial components of mushrooms, exhibiting significant anti-tumor activities and immune system modulation (Zhao et al., 2012; Matloub et al., 2016; Yang et al., 2022; Zhao et al., 2023). Additionally, mushroom polysaccharides demonstrate antioxidant, antiviral, anti-inflammatory, and anti-hyperglycemic effects (He et al., 2020; Motta et al., 2021; Guo et al., 2024; Mizuno & Minato, 2024). In Asian countries, β-glucan-rich mushroom species such as *Lentinus edodes* (*L. edodes*) (Shiitake), *Grifola frondosa* (*G. frondosa*) (Maitake), and *Coriolus versicolor* (Yunzhi), are extensively studied for their health benefits. PS and PSPs derived from these mushrooms have shown promise in clinical cancer therapy (Halpern, 2007; Zhang et al., 2007; T.Y. Wang et al., 2022; Y. Wang et al., 2024). Notable examples include Lentinans from *L. edodes*, polysaccharide d-fraction and MD-fraction from *G. frondosa*, and PSPs from *C. versicolor* (Hobbs, 2000; Cui & Chisti, 2003).

Abbreviations: BBD, Box–behnken design; BSA, Bovine serum albumin; DMEM, Dulbecco's modified eagle medium; HWE, Hot-water extraction; FBS, Fetal bovine serum; PSPS, Polysaccharide-protein complexes (PSPs); RSM, Response surface methodology; UAE, Ultrasound-assisted extraction.

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https://doi.org/10.1016/j.carpta.2025.100817

Available online 28 April 2025

The significant medicinal values of PSPs in mushrooms have stimulated extensive research into optimizing the isolation processes to enhance their bioactivities. Different extraction methods and conditions significantly influence the yield, composition, and bioactivity of mushroom extracts. The most common method nowadays for obtaining bioactive water-soluble PSPs from medicinal mushrooms is hot-water extraction (HWE) (Zhang et al., 2007; Yang & Zhang, 2009). However, the protein constituents of PSPs are sensitive to high temperatures and can be denatured during HWE, which lowers their medicinal values. HWE also has drawbacks such as low process efficiency and slow extraction rates (Cheung et al., 2012).

To address these limitations, ultrasound-assisted extraction (UAE) has been investigated for the extraction of bioactive compounds in medicinal mushrooms. UAE offers advantages such as simple operation, convenient processing equipment, low-temperature conditions, and high process efficiency (Chemat et al., 2008; Glisic et al., 2011). Ultrasound irradiation provides mechanical energy to the solvent, disrupting mushroom particles and cells, and promoting mass transfer. Electron microscopy has shown that power ultrasound can disrupt mushroom cell walls during water extraction (Toma et al., 2001), enhancing solvent access to polysaccharide molecules and increasing extraction yield and rate. For example, UAE has demonstrated superior efficiency over HWE for PSPs extractions form *G. frondosa* and *L. edodes*, with PSPs yields increased by 10.3 % and 88.7 %, respectively (Cheung et al., 2012). Extraction rates also improved significantly, with increases of 102 % for *G. frondosa* and 86.7 % for *L. edodes* (Cheung et al., 2012). These findings highlight the potential of UAE as a more efficient extraction method, particularly for large-scale applications where higher yields and faster processing times are critical.

While other modern techniques, such as enzymatic extraction and microwave-assisted extraction, have been explored, UAE stands out due to its ability to preserve heat-sensitive bioactive compounds, reduce energy consumption, low cost and simplify processing. Previous studies have demonstrated that PSPs extracted using UAE differ in chemical composition, molecular weight distribution, and antioxidant activities compared to those extracted using HWE (Cheung et al., 2012, 2013; Cheung & Wu, 2013). Specifically, PSPs from UAE exhibit higher protein content and stronger antioxidant activity than those from HWE.

However, while UAE has shown promise, there is no established method for maximizing PSPs yields and bioactivity in medicinal mushrooms. This lack of systematic optimization limits the broader application of UAE for medicinal mushroom extraction. This study evaluated the effect of various UAE parameters, including ultrasound power intensity, temperature, mushroom particle size, and solid-to-liquid ratio, on the molecular composition and antioxidant activities of PSP extracts from two medicinal mushrooms, *L. edodes* and *G. frondosa*. By modulating UAE parameters, we aim to develop optimized UAE methods that maximize the antioxidant activity of PSPs in both mushrooms. This research is crucial for advancing the efficient extraction of bioactive compounds from medicinal mushrooms, potentially leading to enhanced therapeutic applications.

2. Materials and methods

2.1. Fungal materials

Two important medicinal mushrooms, *L. edodes* (shiitake) and *G. frondosa* (maitake), were selected in this study. The dried L. *edodes* were obtained from Zhejiang Fangge Pharmaceutical Co., Ltd. (Qingyuan County, Zhejiang, China), while the dried *G. frondosa* were purchased from HK JEBN Limited (Hong Kong, China). Both mushrooms were packaged in plastic bags and stored at 25 °C before use.

2.2. Ultrasonic processors and power measurement

A 20 kHz ultrasonic processor with a 13 mm tip diameter probe and a maximum output power of 750 W (Model VCX-750, Sonics & Materials Inc., Newton, USA) was used. The amplitude (%) displayed on the controller panel was adjusted to control the power level of the ultrasonic processor. The actual power P (in W) transferred to the treatment sample was determined by calorimetric method (Mason et al., 1992; Cheung & Wu, 2013). Briefly, 100 ml fixed amount of water was added in an insulated polycarbonate flask. The water was then sonicated with the probe horn for 15 min. In between the sonication process, the water temperature was recorded and the actual power was derived from the following equation,

$$P = C_p W \left(\frac{dT}{dt}\right)_{t=0} \quad (1)$$

where C_p is the heat capacity (4.18 kJ/kg °C) and W is the mass of water used for extraction (100 g), and $(dT/dt)_{t=0}$ is the initial slope of temperature (T) versus time (t) plot.

The ultrasound intensity I (in W/cm^2) is determined by the power per unit area of the probe tip,

$$I = \frac{P}{\pi r^2} \quad (2)$$

where r is the radius of the tip surface.

2.3. Ultrasound-assisted extraction of PSPs from mushrooms

Various UAE parameters, including ultrasound (US) power intensities (6.21 W/cm^2, 11.34 W/cm^2 and 21.62 W/cm^2), temperature (40 °C, 55 °C, 70 °C), mushroom particle sizes (<600 μm, 600–850 μm and 850–1180 μm), and solid-to-liquid ratios (1:30, 1:50 and 1:70), were used for the extraction of PSPs from L. *edodes* and *G. frondosa*. To assess the effect of US power intensity during UAE on PSP compositions, the US temperature (55 °C), mushroom particle size (<600 μm), and solid-to-liquid ratio (1:30) were kept constant. To examine the effect of temperature during UAE on PSP compositions, the US power intensity (11.34 W/cm^2), mushroom particle size (<600 μm), and solid-to-liquid ratio (1:30) were kept constant. To evaluate the effect of mushroom particle size during UAE on PSP compositions, the US power intensity (11.34 W/cm^2), temperature (55 °C), and solid-to-liquid ratio (1:30) were kept constant. To determine the effect of solid-to-liquid ratio during UAE on PSP compositions, the US power intensity (11.34 W/cm^2), temperature (55 °C), and mushroom particle size (<600 μm) were kept constant.

To prepare the extraction, the dried mushrooms were ground into powder using an electric mill. Different sizes of mushroom powder were collected by passing through a series of mesh sieves with pore dimensions of 1180 μm, 850 μm and 600 μm. This process facilitated the classification of the mushroom powder into three particle size ranges: <600 μm, 600–850 μm and 850–1180 μm. To extract the PSPs from mushrooms, a solid-to-liquid ratio of 1:30 was achieved by mixing 3 g of mushroom powder with 90 ml distilled water in a 250 ml plastic centrifuge bottle. For those conditions using 1:50 and 1:70 solid-to-liquid ratio extractions, the volume of water was increased while maintaining the solid mass constant at 3 g. The ultrasonic probe was inserted into the sample liquid about 2 cm deep. The UAE was performed at a selected ultrasound power for different periods of time (5 min, 10 min, 15 min and 30 min). During sonication, the extraction bottle was placed in a water bath to maintain a constant temperature during the UAE process. Following extraction, the liquid extract was isolated from the solid mushroom residues via centrifugation at 3000 rpm for 15 min. This liquid extract was then subjected to suction filtration. PSPs were isolated from the resultant filtrates by precipitation with five volumes of 95 % ethanol, yielding a final concentration of approximately 80 % v/v, and left to precipitate overnight (Leung et al., 2009). The precipitates were subsequently collected through centrifugation at 3000 rpm for 15 min and lyophilized, resulting in the crude PSP fraction. All extractions

were conducted using a one-factor-at-a-time test.

2.4. Polysaccharide and protein contents in PSPs

The total polysaccharide content of the crude PSPs was determined by the Anthrone test, using glucose as a reference, while the total protein content was ascertained by the Lowery method, using bovine serum albumin (BSA) as a reference (Leung et al., 2009). Initially, 10 mg of crude PSPs were dissolved in 10 ml water and centrifuged at 4000 rpm for 15 min to obtain the PSP solution. The undissolved pellet were oven-dried and its dry weight recorded. The PSP solution was then reacted with an Anthrone solution (comprising 98 % v/v sulfuric acid and 2 % w/v Anthrone) at 1:5 ratio at ambient temperature for at least 45 min. The solution was subsequently heated in a boiling water bath for 10 min. The absorbance of both the glucose standard solution and the reacted sample solution was measured at a wavelength of 620 nm under UV irradiation. For protein content analysis, the PSP solution was reacted with Lowry reagent at a 1:10 ratio for at least 1 h, followed by a reaction with Folin reagent for 30 min. The absorbance of the BSA standard solutions and the reacted PSP sample solution was measured at a wavelength of 750 nm. The polysaccharide and protein contents in the crude PSPs were represented as weight percentages (wt.%) and calculated using the following formulas:

$$\text{Polysaccharide content in PSPs } (wt.\%) = \text{mass of polysaccharide in PSPs solution} / (\text{original mass of crude PSPs} - \text{mass of undissolved PSPs}) \; x100\% \quad (3)$$

$$Protein\, wt.\% = mass\, of\, protein\, in\, PSPs\, solution / (original\, mass\, of\, crude\, PSPs - mass\, of\, undissolved\, PSPs) \times 100\% \quad (4)$$

2.5. Antioxidant activity assays

The antioxidant activities of PSP extracts were evaluated using a cytoprotective activity test against H_2O_2-induced cell injury in rat pheochromocytoma PC12 cell culture, as described in previous publication (Huang et al., 2013). Briefly, PC12 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM l-glutamine. The cells were maintained in an incubator set at 37 °C with 5 % CO_2. For the antioxidant activity experiment, PC12 cells were seeded at a concentration of 5×10^4 cells/well in supplemented DMEM in a 96 well-plate and incubated for 24 h before the experiment. H_2O_2 solution were freshly prepared and the crude PSPs were pre-dissolved in the supplemented DMEM medium. Both H_2O_2 solution and PSPs were added to each well to achieve final concentration of 300 μM and 1 mg/mL, respectively. For the control group, an equal volume of DMEM medium was added along with the H_2O_2 solution. All cells were incubated for an additional 24 h. Cell viability was determined using the MTT reagent, and absorbance was measured at wavelengths of 570 nm and 650 nm.

2.6. Evaluation and optimization of UAE process factors on PSPs antioxidant properties

The major UAE process factors influencing the antioxidant activities of PSP extracts for both *G. frondosa* and L. *edodes* were evaluated using Box–Behnken design (BBD) statistical experimental design and response surface methodology (RSM). BBD is a widely used experimental optimization technique that allows for efficient evaluation of multiple variables and their interactions while minimizing the number of experimental runs. Combined with RSM, it provides a robust statistical framework for modeling and optimizing the UAE parameters. Three process factors were selected as experimental variables: temperature (°C, X_1), US power intensity (W/cm^2, X_2), and mushroom particle size (μm, X_3). The levels used were as follows: temperature (40 °C, 55 °C, and 70 °C), US power intensity (6.21 W/cm^2, 11.34 W/cm^2, and 21.62 W/cm^2) and the particle sizes (<600 μm, 600–850 μm, and 850–1180 μm). The response measured was cell viability which representing the antioxidant activity of PSPs (%, Y). A three-level, three-factor BBD statistical method was employed, resulting a total of 15 experiment runs, including three replicates at the central point, for each mushroom (Tables 1 and 2). The following equation was used to predict the correlations of the response (Y) to the three UAE process factor variables:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{23}X_2X_3 + B_{11}{X_1}^2 + B_{22}{X_2}^2 + B_{33}{X_3}^2 \quad (5)$$

where Y is the response, B_0 is the constant term, and B_i and B_{ii} ($i = 1, 2, 3$) are the coefficients of the relative terms in the regressions.

The data and coefficients were analyzed using Design Expert® 13.0 software, which was also used for the analysis of variance (ANOVA), regression analysis, and plotting of response surface figures of the dependent variables.

2.7. Statistical analysis

All the experiments and assays were performed in triplicates, and the results were represented by their mean ± SD (standard deviation).

Table 1
Experiment design (coded and actual values) in the RSM and cell viability response (Y_1, %) in optimizing antioxidant activities in L. *edodes* extract using UAE.

Run	Independent variables						Response
	Coded			Actual			Cell viability (Y_1, %)
	X_1	X_2	X_3	Temperature (°C)	Power (W/cm^2)	Particle size (μm)	
1	−1	−1	0	40	6.21	600–850	80.04
2	1	−1	0	70	6.21	600–850	75.88
3	−1	1	0	40	21.62	600–850	71.90
4	1	1	0	70	21.62	600–850	70.65
5	−1	0	−1	40	11.34	<600	82.43
6	1	0	−1	70	11.34	<600	61.90
7	−1	0	1	40	11.34	850–1180	70.65
8	1	0	1	70	11.34	850–1180	76.80
9	0	−1	−1	55	6.21	<600	87.00
10	0	1	−1	55	21.62	<600	75.60
11	0	−1	1	55	6.21	850–1180	82.66
12	0	1	1	55	21.62	850–1180	75.50
13	0	0	0	55	11.34	600–850	77.10
14	0	0	0	55	11.34	600–850	73.05
15	0	0	0	55	11.34	600–850	76.75

Table 2
Experiment design (coded and actual values) in the RSM and cell viability response (Y_2, %) in optimizing antioxidant activities in *G. frondosa* extract using UAE.

Run	Independent variables						Response
	Coded			Actual			
	X_1	X_2	X_3	Temperature (°C)	Power (W/cm²)	Particle size (μm)	Cell viability (Y_2, %)*
1	−1	−1	0	40	6.21	600–850	73.21
2	1	−1	0	70	6.21	600–850	84.32
3	−1	1	0	40	21.62	600–850	75.16
4	1	1	0	70	21.62	600–850	63.34
5	−1	0	−1	40	11.34	<600	79.30
6	1	0	−1	70	11.34	<600	71.80
7	−1	0	1	40	11.34	850–1180	70.80
8	1	0	1	70	11.34	850–1180	79.61
9	0	−1	−1	55	6.21	<600	83.95
10	0	1	−1	55	21.62	<600	75.33
11	0	−1	1	55	6.21	850–1180	83.34
12	0	1	1	55	21.62	850–1180	73.40
13	0	0	0	55	11.34	600–850	74.30
14	0	0	0	55	11.34	600–850	72.35
15	0	0	0	55	11.34	600–850	71.15

* All results are the average of the determinations performed ($n = 3$).

3. Results and discussion

3.1. Effect of various UAE parameters on the polysaccharide and protein compositions in mushroom PSP extracts

3.1.1. Power intensity

The intensity of UAE power significantly influences the molecular composition of PSP extracts in both mushrooms (Fig. 1). For L. *edodes*, UAE power intensity of 6.21 W/cm² and 11.34 W/cm² resulted in PSPs with a higher protein content relative to polysaccharide content (Fig. 1a-b). At the lower UAE power intensity of 6.21 W/cm², a slight increase in polysaccharide content was observed over the extraction time, accompanied by a slight decrease in protein content. When the UAE power was elevated to an intermediate level of 11.34 W/cm², both polysaccharides and proteins were extracted more efficiently, and the protein-to-polysaccharide ratio was higher compared to the lower level of 6.21 W/cm². An optimal UAE extraction time of 15 min was identified, yielding PSPs composed of 35.6 % polysaccharides and 54.4 % proteins. A high protein-to-polysaccharide ratio in PSPs was found at 11.34 W/cm² UAE power intensity across all the time points, ranging from 1.53 to 1.75. However, when the UAE power intensity was further increased to a high level of 21.62 W/cm², the protein content in the PSPs was significantly affected, resulting in a similar protein and polysaccharide percentages (Fig. 1c). At the same UAE extraction time of 15 min, PSPs extracted at a US power intensity of 21.62 W/cm² contained 35.7 % polysaccharides and 32.4 % proteins. This suggests that high UAE power may degrade protein during the extraction process, an effect not observed in polysaccharides. Therefore, high UAE power extraction appears to favor the extraction of polysaccharides over proteins.

For *G. frondosa*, at the lower UAE power intensity of 6.21 W/cm², the PSPs contained significantly more protein than polysaccharides throughout the entire extraction period (Fig. 1d). The protein-to-polysaccharide ratio in PSPs ranged from 1.46 to 1.88 across different time points. The protein content in the PSPs increased from 39.9 % at 5 min to 47.7 % at 30 min, while the polysaccharide content decreased slightly from 27.4 % at 5 min to 25.3 % at 30 min. At the medium UAE power intensity of 11.34 W/cm², both polysaccharides and protein were extracted more efficiently (Fig. 1e). An optimal UAE extraction time of 15 min was identified, yielding PSPs composed of 46.2 % polysaccharides and 49.6 % protein. However, the protein-to- polysaccharide ratio in PSPs was reduced, ranging from 1.07 to 1.44, compared to the UAE power intensity of 6.21 W/cm². Furthermore, at a high UAE power intensity of 21.62 W/cm², the protein content in the PSPs decreased between 15 and 30 min, suggesting protein degradation at high UAE power, an effect not observed with polysaccharides (Fig. 1f).

The findings from both L. *edodes* and *G. frondosa* indicate that the power intensity of UAE significantly affects the composition of PSPs and highlights species-specific differences. L. *edodes* exhibited a higher protein-to-polysaccharide ratio at intermediate UAE power intensity compared to low power intensity, whereas *G. frondosa* showed a higher protein-to-polysaccharide ratio at lower UAE power intensity compared to intermediate intensity. These observed differences warrant further investigation and may be attributed to biological or structural variations between the two mushroom species. Overall, lower UAE power intensity results in higher protein percentages within the PSPs, likely due to the increased shear forces, mechanical energy, cavitation, and enhanced protein solubility. Conversely, higher power intensity leads to reduced protein percentages, probably due to more pronounced sonochemical

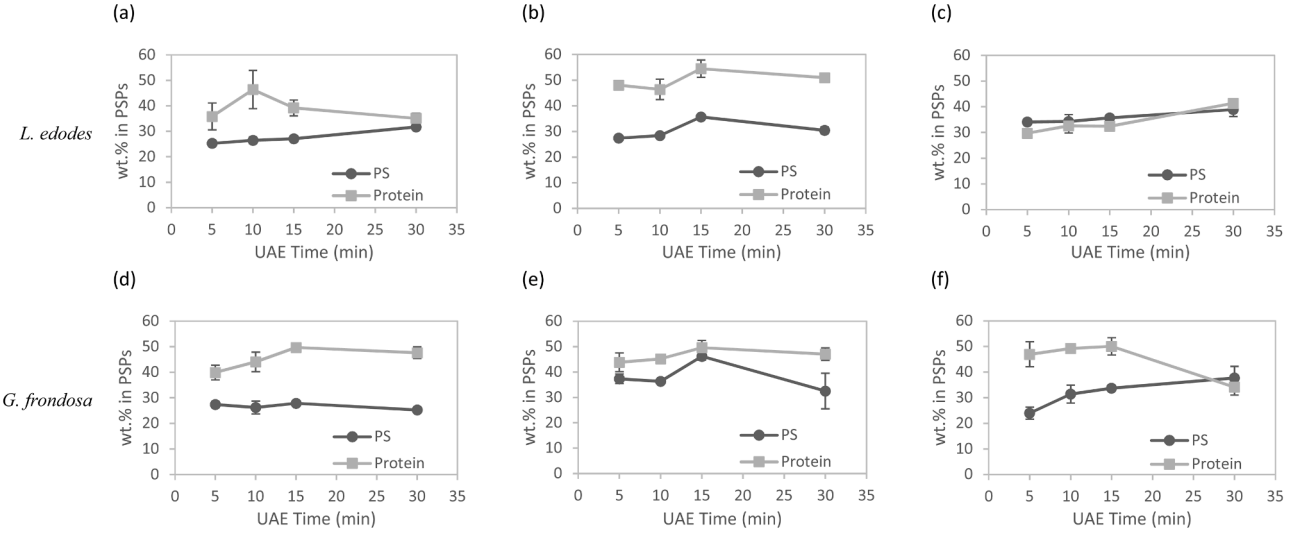

Fig. 1. Effect of ultrasound power intensity on the polysaccharide and protein compositions in mushroom PSP extracts: (a) and (d) at 6.21 W/cm²; (b) and (e) at 11.34 W/cm²; (c) and (f) at 21.62 W/cm². The ultrasound temperature (55 °C), mushroom particle size (<600 μm), and solid-to-liquid ratio (1:30), were kept constant throughout the experiments. PS: polysaccharide, PSPs: polysaccharide-protein complexes, UAE: ultrasound assisted extraction, wt.%: weight percentage.

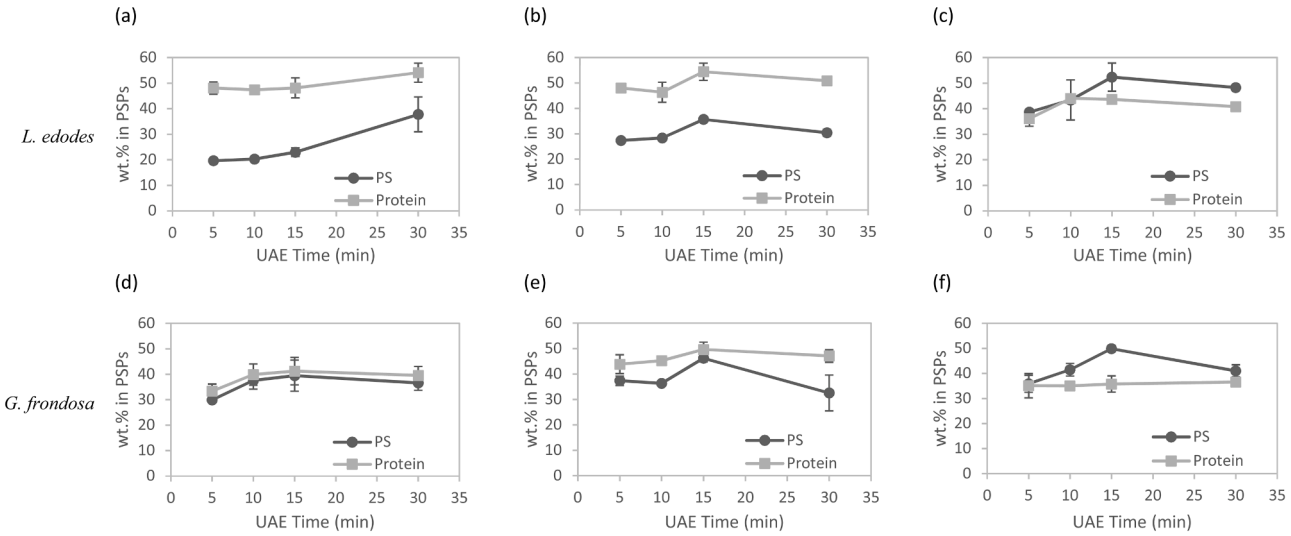

Fig. 2. Effect of ultrasound temperature on the polysaccharide and protein compositions in mushroom PSP extracts: (a) and (d) at 40 °C; (b) and (e) at 55 °C; (c) and (f) at 70 °C. The ultrasound power intensity (11.34 W/cm^2), mushroom particle size (<600 μm), and solid-to-liquid ratio (1:30) were kept constant throughout the experiments. PS: polysaccharide, PSPs: polysaccharide-protein complexes, UAE: ultrasound assisted extraction, wt.%: weight percentage.

effects, such as the breakage of peptide chains, protein denaturation, hydrolysis or aggregation due to the formation of disulfide bridges through oxidation of sulphur-containing amino acids in the protein induced by hydroxyl-free radicals generated at high US power (Su & Cavaco-Paulo, 2021; Wang et al., 2021). The degradation of protein content in PSPs from other mushrooms under high-intensity UAE has been previous reported (Cheung, Liu, Wang, & Wu, 2015).

3.1.2. Temperature

The temperature conditions during the UAE significantly impacts the molecular composition of PSP extracts in both mushrooms (Fig. 2). For L. *edodes*, UAE at 40 °C and 55 °C resulted in PSPs with a higher protein content relative to polysaccharide content (Fig. 2a and b). Specifically, the protein-to-polysaccharide ratio in PSPs extracted at 40 °C ranged from 1.43 to 2.44, while at 55 °C, this ratio ranged from 1.53 to 1.75 across different time points. At 40 °C, both polysaccharide and protein contents exhibited a gradual increase over time. When the UAE temperature was raised to 55 °C, an optimal UAE extraction time of 15 min was identified, yielding PSPs composed of 35.6 % polysaccharides and 54.4 % protein. However, at 70 °C, the protein content in the PSPs was significantly reduced, resulting in PSPs with a higher polysaccharide content than protein (Fig. 2c). This suggests that elevated temperatures may degrade protein during the extraction process, favoring polysaccharides over proteins.

For *G. frondosa*, at both 40 °C and 55 °C, polysaccharide and protein contents in PSPs exhibited a gradual increase over time, reaching a plateau at 15 min (Fig. 2d-e). Both protein and polysaccharide contents were extracted more efficiently at 55 °C. An optimal UAE extraction time of 15 min at 55 °C was identified, yielding PSPs composed of 46.2 % polysaccharides and 49.6 % protein. However, a high protein-to-polysaccharide ratio was not observed under either 40 °C or 55 °C UAE conditions, with all extracts exhibiting a ratio of <1.5. At 70 °C, the protein content in the PSPs was significantly reduced, resulting in PSPs with a higher polysaccharides than protein (Fig. 2f).

The results from both L. *edodes* and *G. frondosa* indicate that the temperature condition during UAE significantly affects PSP

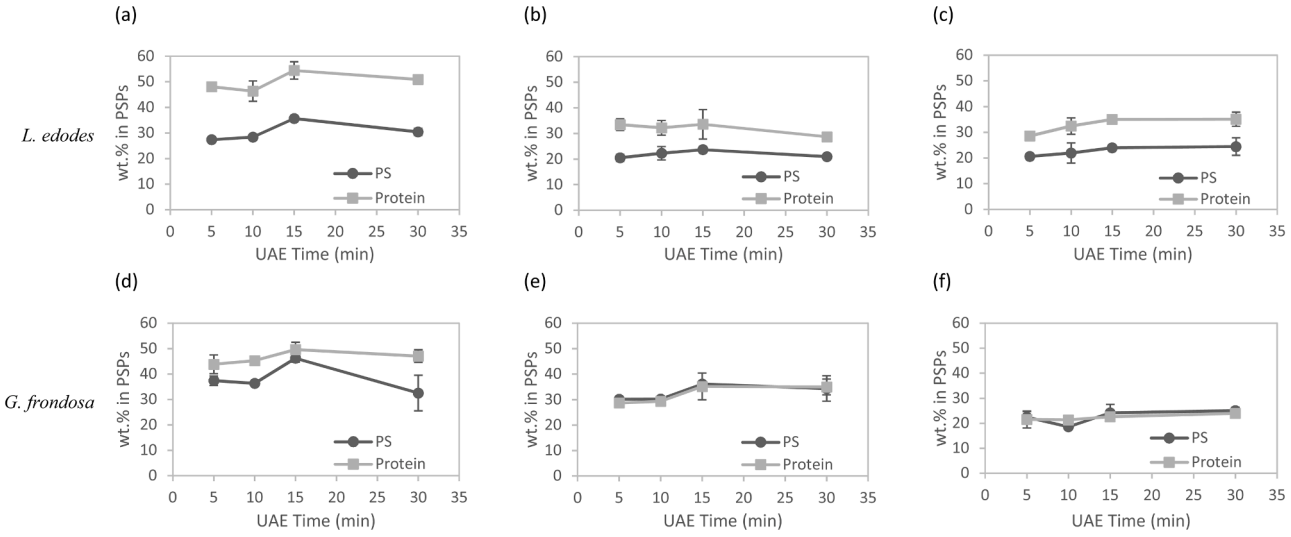

Fig. 3. Effect of mushroom particle size on the polysaccharide and protein compositions in mushroom PSP extracts: (a) and (d) at <600 μm; (b) and (e) at 600–850 μm; (c) and (f) at 850–1180 μm. The US power intensity (11.34 W/cm^2), temperature (55 °C), and solid-to-liquid ratio (1:30) were kept constant throughout the experiments. PS: polysaccharide, PSPs: polysaccharide-protein complexes, UAE: ultrasound assisted extraction, wt.%: weight percentage.

composition. UAE temperatures of 40 °C and 55 °C yield higher protein percentages in PSPs due to increased diffusion rates, reduce the surface tension of the solutions, and the release of matrix-bound compounds. Conversely, a high temperature of 70 °C results in higher polysaccharide percentages in PSPs but reduce protein contents due to protein denaturation. Other studies have also shown that if the extraction temperature rises above 60 °C, chemical bonds begin to break, leading to molecular structure collapse and precipitation, thereby decreasing the protein extraction yield (Dong et al., 2019).

3.1.3. Mushroom particle size

Both L. *edodes* and *G. frondosa* demonstrated that when the mushroom particle size was <600 μm in UAE, polysaccharides and proteins were extracted most efficiently, compared to UAE using particle sizes of 600–850 μm and 850–1180 μm (Fig. 3). For L. *edodes*, all three different particle sizes used in UAE yielded PSP extracts containing a higher protein percentage than the polysaccharide content percentage. Utilizing smaller mushroom particle sizes, specifically <600 μm, resulted in increased contents of both polysaccharide and protein in PSPs. A comparatively high protein-to-polysaccharide ratio in PSPs was found when the particle size was <600 μm, ranging from 1.53 to 1.75. An optimal UAE extraction time of 15 min using a mushroom size of <600 μm was identified to yield the highest composition of polysaccharides and proteins, 35.6 % and 54.4 % respectively. A similar observation was made for *G. frondosa*, where a mushroom size of <600 μm for UAE for 15 min resulted in the highest percentage contents of both polysaccharide and protein (46.2 % and 49.6 %) in PSPs. However, a high protein-to-polysaccharide ratio in *G. frondosa* PSPs was not found across all the time points of different mushroom particle sizes. The protein-to-polysaccharide ratio in all the extracts was <1.5, indicating that mushroom particle size does not significantly contribute to the extraction selection of the molecular composition of the PSPs in *G. frondosa*.

These results indicate that using smaller mushroom particle sizes in UAE extracts both polysaccharides and proteins most efficiently. This efficiency may be attributed to enhanced mass transfer, as smaller particle sizes lead to a greater surface area of the solid matrix, improving the interaction between the solvent and the solid material, shortening the average diffusion path within the solid matrix of the raw material, and thereby enhancing mass transfer during the extraction process (Elhag et al., 2019). While smaller particle sizes (<600 μm) enhance the extraction efficiency of both polysaccharides and proteins in PSPs for both L. *edodes* and *G. frondosa*, the protein-to-polysaccharide ratio is more significantly influenced in L. *edodes* than in *G. frondosa*.

3.1.4. Solid-to-liquid ratio

Both L. *edodes* and *G. frondosa* demonstrated that when the mushroom particles to water ratio (solid-to-liquid ratio) was 1:30 in UAE, both polysaccharides and proteins were extracted most efficiently, compared to the solid-to-liquid ratios of 1:50 and 1:70 (Fig. 4). For L. *edodes*, utilizing a more concentrated ratio, specifically at 1:30, resulted in increased contents of both polysaccharide and protein in PSPs. An optimal UAE extraction time of 15 min using a solid-to-liquid ratio of 1:30 was identified to yield the highest composition of polysaccharides and proteins, 35.6 % and 54.4 %, respectively. Similarly, for *G. frondosa*, an optimal UAE extraction time of 15 min using a solid-to-liquid ratio of 1:30 resulted in the highest percentage contents of both polysaccharide and protein, 46.2 % and 49.6 %, respectively.

These results indicate that using a more concentrated solid-to-liquid ratio in UAE extracts both polysaccharides and proteins most efficiently. This efficiency may be attributed to the enhanced concentration gradient between the solvent and the extraction matrix, which increases the mass transfer to the solid mushroom as the mass of the liquid is reduced. Notably, the solid-to-liquid ratio does not significantly contribute to the extraction selection of the molecular compositions of the PSPs in both L. *edodes* and *G. frondosa*, as the protein-to-polysaccharide ratio in PSPs remained relatively steady (Fig. 4). This suggests that while the solid-to-liquid ratio influences the overall efficiency of extraction, it does not preferentially affect the extraction of either polysaccharides or proteins, maintaining a consistent protein-to-polysaccharide ratio in the PSPs.

3.2. Evaluation and optimization of antioxidant activities in mushroom PSP extracts by modulating different UAE conditions using RSM

To optimize the antioxidant activities in PSP extracts of *L. edodes* and *G. frondosa*, different UAE process variables, including temperature (°C, X_1), US power intensity (W/cm^2, X_2), and mushroom particle size (μm, X_3), were studied using the RSM-BBD. The solid-to-liquid ratio was not included in this study because previous results demonstrated that a ratio of 1:30 yielded the highest polysaccharide and protein content in the PSPs, and different ratios did not significantly affect the composition ratio of polysaccharides and proteins (Fig. 4). The values of the studied independent variables and the corresponding response, cell viability (%, Y), representing the antioxidant activity of PSPs, are shown in Tables 1

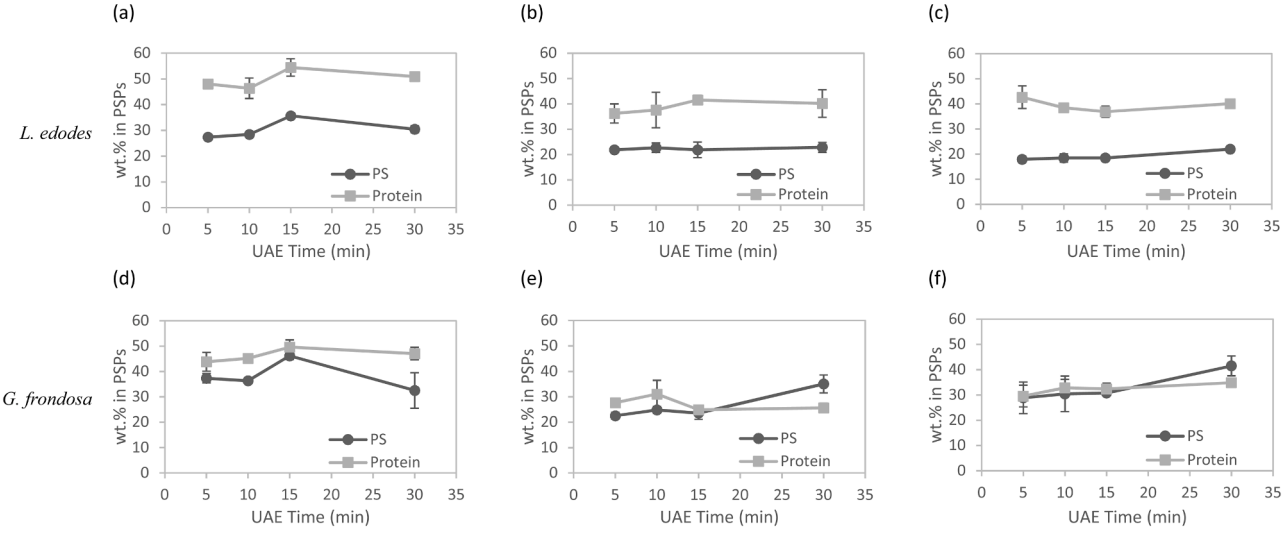

Fig. 4. Effect of solid-to-liquid ratio on the polysaccharide and protein compositions in mushroom PSP extracts: (a) and (d) at 1:30; (b) and (e) at 1:50; (c) and (f) at 1:70. The US power intensity (11.34 W/cm^2), temperature (55 °C), and mushroom particle size (<600 μm) were kept constant throughout the experiments. PS: polysaccharide, PSPs: polysaccharide-protein complexes, UAE: ultrasound assisted extraction, wt.%: weight percentage.

and 2 for L. *edodes* and *G. frondosa*, respectively.

The maximum antioxidant activity in L. *edodes* was observed in experiment 9, which utilized a temperature of 55 °C, a US power intensity of 6.21 W/cm^2, and a mushroom particle size of <600 μm (Table 1). High antioxidant activity was also noted in experiments 1 and 11, both of which employed a US power intensity of 6.21 W/cm^2. A comparison between experiments 5 and 6 revealed that increasing the temperature from 55 °C to 70 °C resulted in a decrease in antioxidant activity from 82.43 % to 61.90 %. Additionally, experiments involving high US power extraction (experiments 3, 4, 10, and 12) exhibited relatively low cell viability. Figs. 1 and 2 illustrate *that* L. *edodes* PSPs extracted at a US power intensity of 6.21–11.34 W/cm^2 and a temperature range of 40–55 °C yielded a higher protein-to-polysaccharide ratio. Conversely, a high US power intensity of 21.62 W/cm^2 and a temperature of 70 °C reduced the protein-to- polysaccharide ratio, suggesting that a higher protein-to-polysaccharide ratio in PSPs contributes to increased antioxidant activity.

In *G. frondosa*, the maximum antioxidant activity was achieved in experiments 2, 9, and 10, all of which employed a US power intensity of 6.21 W/cm^2 (Table 2). Comparing experiments 2 and 4, both of which used a US power intensity of 6.21 W/cm^2 and a particle size of 600–850 μm, an increase in US power intensity from 6.21 W/cm^2 to 21.62 W/cm^2 resulted in a decrease in cell viability from 84.32 % to 63.34 %. This indicates that US power intensity is a major factor influencing antioxidant activity in the PSPs of *G. frondosa*. Fig. 1 demonstrates that a US power intensity of 6.21 W/cm^2 yields the highest protein-to-PS ratio, contributing to high antioxidant activity. These results are consistent with those observed in L. *edodes* and previous studies (Leung et al., 2009; Cheung et al., 2012). Notably, unlike L. *edodes*, the use of high temperature during US extraction did not significantly affect antioxidant activity in *G. frondosa*, as demonstrated in experiments 2 and 8. This may be due to the inability to generate a high protein-to- polysaccharide ratio in PSPs by varying the UAE temperature (Fig. 2).

Results from both antioxidant activities experiments in PSP extracts of L. *edodes* and *G. frondosa* suggested that high antioxidant activities are correlated with a high protein-to-polysaccharide ratio in PSPs. These findings are consistent with other studies, which reported that PSPs with higher protein content in *C. versicolor, Cordyceps sinensis, G. frondosa*, and L. *edodes* exhibited greater antioxidant activities (Leung et al., 2009; Cheung et al., 2012). A reasonable explanation is that the protein molecules contain bioactive peptides or amino acid sequences that contributed to antioxidant activities (Ahmed et al., 2024).

The models associated with the UAE process and the antioxidant activities for L. *edodes* and *G. frondosa* were built from the design presented in Tables 1 and 2. The analysis of variance (ANOVA) is presented in Tables 3 and 4. Both models showed that the experimental data could be fitted with second-order equations, with $p = 0.0143$ for L. *edodes* and $p = 0.0003$ for *G. frondosa*. The high F-value and the low *p*-value indicate the significance of both models. Additionally, the lack of fit is not significant in either models, suggesting the models are appropriate for the data. The R^2 values for the L. *edodes* and *G. frondosa* models were 0.9396 and 0.9871, respectively, indicating a high degree of correlation

Table 3
Analysis of variance on the fitted quadratic response surface model employed to optimizing antioxidant activities in L. *edodes* extract using UAE. X_1 temperature, X_2 US power intensity, X_3 mushroom particle size, Sig significant, Non-sig non-significant.

Source	*F*-value	*p*-value	Decision*
Model	8.64	0.0143	Sig.
X_1	7.99	0.0368	Sig.
X_2	20.8	0.0061	Sig.
$X_1\,X_3$	29.04	0.0030	Sig.
X_1^2	10.28	0.0238	Sig.
Lack of fit	1.36	0.4500	Non-sig.

* Statistical significance was set at a *p*-value of 0.05.

Table 4
Analysis of variance on the fitted quadratic response surface model employed to optimizing antioxidant activities in *G. frondosa* extract using UAE. X_1 temperature, X_2 US power intensity, X_3 mushroom particle size, Sig. significant, Non-sig. non-significant.

Source	*F*-value	*p*-value	Decision*
Model	42.41	0.0003	Sig.
X_2	146.69	<0.0001	Sig.
$X_1\,X_2$	109.17	0.0001	Sig.
$X_1\,X_3$	55.23	0.0007	Sig.
X_2^2	19.43	0.0007	Sig.
X_3^2	46.34	0.0010	Sig.
Lack of fit	0.1273	0.9358	Non-sig.

* Statistical significance was set at a *p*-value of 0.05.

between the observed and predicted values. For the L. *edodes* model, the significant variables ($p < 0.05$) were X_1, X_2, ($X_1\,X_3$), and (X_1^2), while the insignificant variables ($p > 0.05$) were X_3, ($X_1\,X_2$), ($X_2\,X_3$), (X_2^2), and (X_3^2). For the *G. frondosa* model, the significant variables ($p < 0.05$) were X_2, ($X_1\,X_2$), ($X_1\,X_3$), (X_2^2), and (X_3^2), while the insignificant variables ($p > 0.05$) were X_1, X_3, ($X_2\,X_3$) and (X_1^2). The final equations in terms of the significant factors obtained for the antioxidant activity (Y) are as follows to predict the antioxidant activity of PSP extracts under different UAE conditions:

$$Y_1 = 75.6333 - 2.47375X_1 - 3.99125X_2 + 6.67X_1X_3 - 4.13042{X_1}^2 \quad (6)$$

$$Y_2 = 72.6 - 4.69875X_2 - 5.7325X_1X_2 + 4.0775X_1X_3 + 2.5175{X_2}^2 + 3.8875{X_3}^2 \quad (7)$$

The Design-Expert software was employed to determine the optimal UAE conditions for maximizing antioxidant activities in the mushroom extracts. For L. *edodes*, the optimal conditions were identified as a temperature of 55 °C, a US power intensity of 6.21 W/cm^2, and a mushroom particle size of <600 μm. For *G. frondosa*, the optimal conditions were a temperature of 70 °C, a US power intensity of 6.21 W/cm^2, and a mushroom particle size of 600–850 μm. Under these conditions, the software predicted maximum cell viability of 85.41 % for L. *edodes* and 84.51 % for *G. frondosa*. The experimental results yielded cell viabilities of 87.00 % and 84.32 % for L. *edodes* and *G. frondosa*, respectively. The experimental results closely aligned with the software's predictions, demonstrating the accuracy and reliability of the optimization process. Figs. 5 and 6 illustrate the response surface graphs, showing the relationship between antioxidant activity (%) and the independent variables (temperature, US power intensity and mushroom particle size) for *L. edodes* and *G. frondosa*, respectively. The response surface graphs for L. *edodes* indicates that increasing the temperature and US power intensity generally leads to a decrease in antioxidant activity (Fig. 5). The response surface graphs for *G. frondosa* shows that the US power intensity is the most significant factor affecting antioxidant activity (Fig. 6). Specifically, high US power intensity during UAE can drastically reduce antioxidant activity.

These findings underscore the critical role of UAE parameters in determining the biological properties of PSPs. Recent advancements in the optimization of PSPs extractions from β-glucan-rich medicinal mushrooms have increasingly focused on enhancing bioactivity during the extraction process (Gong et al., 2020; Guo et al., 2025). Key strategies include preserving the structural integrity of polysaccharides and investigating the synergistic interactions between polysaccharides and proteins, which are known to enhance bioactivity. These synergistic interactions have been shown to improve the biocompatibility, stability, and immunomodulatory as well as antitumor properties of polysaccharides (Matloub et al., 2016; Yang et al., 2022; Zhao et al., 2023)

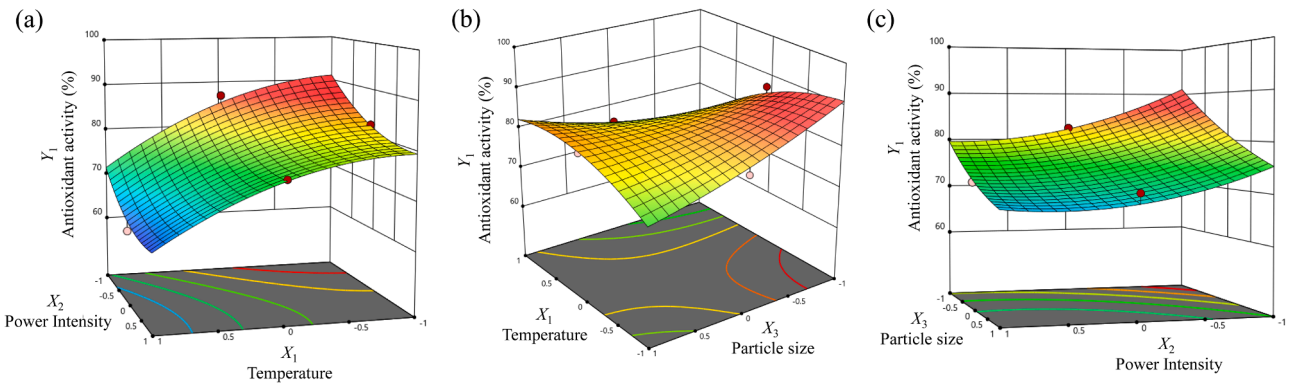

Fig. 5. Response surface plots of independent variables for the antioxidant activity of L. *edodes* under UAE, showing the maximum for each combination of variables. (a) Temperature and US power intensity using a constant mushroom particle size of <600 μm; (b) Temperature and mushroom particle size using a constant US power intensity of 6.21 W/cm^2; (c) US power intensity and mushroom particle size using a constant temperature of 40 °C.

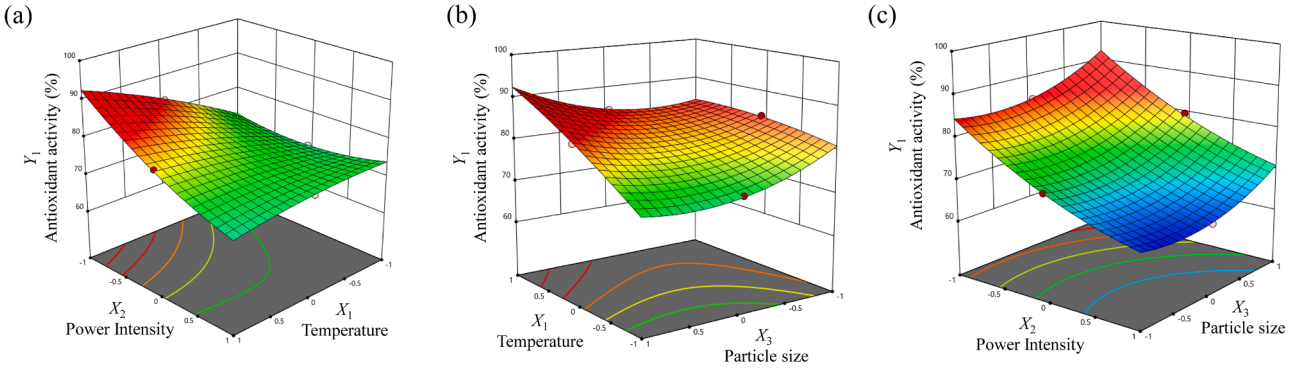

Fig. 6. Response surface plots of independent variables for the antioxidant activity of *G. frondosa* under UAE, showing the maximum for each combination of variables. (a) Temperature and US power intensity using a constant mushroom particle size of 850–1180 μm; (b) Temperature and mushroom particle size using a constant US power intensity of 6.21 W/cm^2; (c) US power intensity and mushroom particle size using a constant temperature of 70 °C.

4. Conclusion

This study demonstrates that UAE conditions significantly affect the composition of PSPs in mushroom extracts. The composition of PSPs is correlated with antioxidant activities, with a high protein-to-polysaccharide ratio in PSPs enhancing the antioxidant activity of the extracts. Additionally, second-order polynomial models were successfully developed to correlate UAE process factors with antioxidant activities, enabling the prediction of optimal UAE conditions for maximizing antioxidant activities in PSPs. Given the growing nutraceutical interest in the therapeutic effects of L. *edodes* and *G. frondosa*, including their anticancer, prebiotic, antioxidant, anti-inflammatory, antiviral, and neuroprotective properties, developing effective extraction methods is crucial for maximizing the bioactivity of these extracts. However, the interactions and ratios between mushroom polysaccharides and proteins are not yet fully understood. Further research is required to elucidate these interactions and their underlying antioxidation mechanisms. This study provides valuable insights into optimizing UAE conditions for extracting bioactive compounds from medicinal mushrooms. These findings offer a strong foundation for future research and practical applications, particularly in the development of pharmaceutical formulations aimed at enhancing the health benefits of mushroom extracts.

Funding declaration

The work described in this paper was fully supported by grants awarded to EWTT from the Research Grants Council of the Hong Kong Special Administrative Region, China (UGC/FDS25(16)/P03/19) and Hong Kong Metropolitan University Research Grants (RD/2022/1.7) and (PFDS/2024/19). FWNC acknowledges partial support from the Seed Fund provided by the Research Centre for Chinese Medicine Innovation (Project No. P0039173) at the Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong SAR, China.

CRediT authorship contribution statement

Emily Wan Ting Tam: Writing – original draft, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Yi Ching Cheung:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Wesley Chin Ho Lung:** Writing – original draft, Formal analysis, Data curation. **Ching Ching Hui:** Writing – review & editing, Formal analysis, Data curation. **William W.Y. Lam:** Writing – review & editing, Validation, Project administration. **Stella Siu Mui Ng:** Writing – review & editing, Supervision, Software. **Franklin Wang Ngai Chow:** Writing – review & editing, Validation, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Emily Wan Ting Tam reports financial support was provided by the Research Grants Council of the Hong Kong Special Administrative Region, China. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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