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Sheeza Rasheed
Jiangsu University
Xiaoai Wang
Jiangsu University
Ahmad Abubakar Rilwanu
Jiangsu University
Jingyu Wei
Jiangsu University
Chenchen Yao
Jiangsu University
Kwasi Yeboah Asuama
Jiangsu University
. Mufzala
Yangzhou University
Haihui Zhang
Jiangsu University
Kai Hu
Jiangsu University
Yuqing Duan
dyq101@ujs.edu.cn
Jiangsu University

Research Article

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Single-, dual-, and triple-frequency ultrasound, alone or combined with subcritical water, governs selective phenolic recovery and isoflavone profiles in purified defatted soybean meal extracts

Sheeza Rasheed [a], Xiaoai Wang [a], Ahmad Abubakar Rilwanu [a], Jingyu Wei [a], Chenchen Yao [a], Yeboah Asuama Kwasi [a], Mufzala [c], Haihui Zhang [a], Kai Hu [a], Yuqing Duan [a, b, *]

[a] *School of Food Science and Engineering, Jiangsu University, Zhenjiang 212013, China.*

[b] *Institute of Food Physical Processing, Jiangsu University, Zhenjiang 212013, China.*

[c] *College of Food Science and Engineering, Yangzhou University, Yangzhou 225127, PR China.*

Corresponding authors:

Yuqing Duan, Ph.D., Professor, School of Food Science and Biological Engineering, Jiangsu University, Zhenjiang 212013, China; Email: dyq101@ujs.edu.cn

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ABSTRACT

Defatted soybean meal is a rich but underutilized source of phenolic compounds, yet conventional extraction gives low recovery and poor selectivity. This study compared single-frequency (SF; 60 kHz), dual-frequency (DF; 28/60 kHz), and triple-frequency (TF; 20/28/60 kHz) ultrasound, applied alone or followed by subcritical water extraction (SWE; 140 °C, 25 min) as SUSWE, DUSWE, and TUSWE, with hot-water extraction (HWE) and SWE alone as references. Compared with HWE, TF doubled phenolic recovery (77.69 vs. 38.66 mg GAE/100 g meal), gave the highest total phenolic content (38.45 mg GAE/g), and increased DPPH radical-scavenging activity from 28.93% to 51.06%. SUSWE gave the highest purified extract yield (2.49%) and flavonoid recovery (92.76 mg QE/100 g meal), whereas DF gave the highest flavonoid concentration (41.79 mg QE/g). The benefit of SWE depended on the preceding ultrasound step: it improved recovery after SF, added little after DF, and, after TF, reduced phenolic recovery by 28.7% and DPPH activity by 73.0%. UPLC-ESI-QTRAP-MS/MS profiling showed that TF raised the daidzein/daidzin ion-signal ratio from 0.143 (HWE) to 0.590 and the glycitein/glycitin ratio from 0.365 to 1.173 at only 45 °C, whereas TUSWE lowered them to 0.195 and 0.297, respectively, indicating that the aglycone-rich fraction released by TF was thermally degraded or diluted by newly extracted glycosides during the 140 °C step. Ultrasound frequency configuration therefore determines both extract composition and the value of a subsequent hydrothermal step, and the sequential process is beneficial only when SWE complements, rather than overrides, the selectivity established by ultrasound. These findings provide a direct process reference and theoretical basis for the green, targeted preparation of phenolic and isoflavone ingredients from soybean meal and other oilseed by-products.

Keywords: Defatted soybean meal; multi-frequency ultrasound pretreatment; subcritical water extraction; Soybean isoflavones; phenolic recovery

1. INTRODUCTION

Defatted soybean meal is the main by-product of soybean-oil processing. China alone crushes nearly 100 million tons of soybeans per year (USDA FAS, 2024), and the resulting meal, which contains more than 40% protein, is used mainly as a low-value feed ingredient. Yet defatted soybean materials also retain phenolic acids, flavonoids, and especially isoflavones (Singh et al., 2017) that can be recovered for food, nutraceutical, and functional-ingredient applications (Rimbach et al., 2008). Using this fraction more effectively would add value to a material that is otherwise exploited largely for protein. Previous work has shown that soybean meal and related soybean-processing residues contain recoverable phenolics and isoflavones (Freitas et al., 2018; Nile et al., 2022; Tyug et al., 2010), and recent studies continue to explore greener routes for recovering daidzein and genistein from soybean meal (Santos et al., 2025). However, conventional hot-water or single-solvent extraction generally gives low total phenolic recovery and poor control over the conversion of isoflavone glycosides into aglycones, so the composition of the final product fluctuates and is difficult to standardize for functional-ingredient production.

Greener techniques such as ultrasound-assisted extraction and subcritical water extraction (SWE) have therefore been introduced, but most studies still use total phenolic yield as the sole evaluation criterion, and the regulatory effect of different ultrasound frequency combinations on a subsequent SWE step has not been clarified. This matters because extract yield alone is a poor measure of product quality. Soybean isoflavones occur as aglycones, β-glucosides, acetyl-glucosides, and malonyl-glucosides, with daidzein/daidzin, glycitein/glycitin, and genistein/genistin as the major structural families, each with different polarity, extractability, thermal stability, and antioxidant behaviour. Because each form behaves differently during processing, the extraction conditions determine both how much is recovered and which form dominates the final product. Temperature-dependent processing, specifically SWE, changes conversion kinetics and solubility of isoflavones based on temperature (Aguiar et al., 2012; An et al., 2023; Mathias et al., 2006).

Two points of consensus have emerged for the technologies used to influence this balance. The first concerns ultrasound: acoustic cavitation disrupts plant cell walls and enhances solvent penetration, thereby accelerating mass transfer from plant matrices (Chemat et al., 2017; Rostagno et al., 2003; Vilkhu et al., 2008). Ultrasound frequency controls not only the magnitude and spatial distribution of these effects but also the accessibility of specific compounds to the solvent and, hence, the composition of the recovered fraction (Kentish & Ashokkumar, 2011; Mason & Peters, 2002). Mean cavitation-bubble size decreases as frequency increases (Brotchie et al., 2009); low frequencies such as

20 and 28 kHz therefore produce fewer but larger bubbles whose violent collapse generates strong shear and microjets, whereas higher frequencies such as 60 kHz produce more numerous, smaller bubbles that collapse more often but less violently.

Simultaneous multi-frequency irradiation can raise cavitation yield above that of single-frequency sonication by increasing the number of active cavitation events and producing a more uniform acoustic field (Feng et al., 2002; Tatake & Pandit, 2002), and single- and multi-frequency modes have been reported to give different recoveries and structures of polyphenols. However, it remains unresolved whether moving to a multi-frequency system merely intensifies extraction or also alters phenolic selectivity and, consequently, final product quality. The second point of consensus concerns subcritical water, i.e., liquid water held between 100 and 374 °C under sufficient pressure to keep it in the liquid state. As water is heated beyond its boiling point, its dielectric constant and viscosity decrease, while its diffusivity increases. This allows compounds with broader polarity to be solubilized (Cheng et al., 2021; García-Marino et al., 2006; Zhang et al., 2020). In soybean systems, hydrothermal environments improve mass transfer. In addition, it redistributes conjugated and aglycone isoflavones. Studies on okara and soybean isoflavones (An et al., 2023; Barea et al., 2025; Nkurunziza et al., 2019) have demonstrated efficient isoflavone recovery alongside hydrolytic conversion under subcritical water conditions. More recent kinetic work further shows that individual soybean isoflavone forms respond differently to temperature, so SWE can be used to steer the aglycone/glycoside balance under green conditions.

Despite these points of consensus, a clear gap remains. Studies combining ultrasound with SWE generally assume that the combination is synergistic, yet the value of the second step cannot be assumed in advance. Ultrasound may expose compounds that SWE can subsequently recover. SWE can act on both remaining solid and on compounds previously released by ultrasound. So, SWE can add or modify the molecular forms that were selectively released during ultrasound. The practical question is therefore not simply whether a combined treatment gives a higher yield, but whether the second step improves the composition and function of the purified fraction produced by the first. Ultrasound pretreatments with different frequency configurations may establish different initial phenolic compositions, so a subsequent SWE step could either reinforce aglycone formation or degrade target compounds and override the selectivity created by ultrasound. To our knowledge, no study has yet addressed the core scientific question of how single-, dual-, and triple-frequency ultrasound, by reshaping the initial release of phenolics from soybean meal,

93 governs the efficiency and selectivity of glycoside-to-aglycone conversion and phenolic recovery during a subsequent
94 SWE step. Without this knowledge, the development of coupled processes remains largely empirical.

95 Against this background, the present study used defatted soybean meal as a fixed matrix and systematically compared
96 single-frequency (SF; 60 kHz), simultaneous dual-frequency (DF; 28/60 kHz), and simultaneous triple-frequency (TF;
97 20/28/60 kHz) ultrasound, applied both alone and as pretreatments before SWE, i.e., single-, dual-, and triple-
98 frequency ultrasound–SWE (SUSWE, DUSWE, and TUSWE, respectively), with hot-water extraction (HWE) and
99 SWE alone as references. All extracts were prepared at the same solid-to-liquid ratio and purified identically with AB-
100 8 macroporous resin, which has been used successfully for soybean isoflavone and genistein purification (Li et al.,
101 2012; Tran et al., 2022). This allowed the treatments to be compared on the basis of the purified product rather than
102 crude extract yield.

103 Paired comparisons (SF vs. SUSWE, DF vs. DUSWE, and TF vs. TUSWE) were used to isolate the contribution of
104 the SWE step, and UPLC-ESI-QTRAP-MS/MS was used to follow the aglycone/glycoside balance of the major
105 isoflavone pairs, linking frequency configuration to phenolic recovery and antioxidant activity at the molecular level.
106 The central hypothesis was that ultrasound frequency configuration determines the chemical state of the phenolic
107 fraction entering the hydrothermal step and, therefore, whether SWE adds value or overrides the selectivity established
108 by ultrasound. The novelty of this work lies in (i) the first side-by-side evaluation, to our knowledge, of single-, dual-,
109 and triple-frequency ultrasound as pretreatments for SWE of soybean meal, and (ii) the molecular-level evidence that
110 frequency configuration, rather than bulk temperature, can redirect the aglycone/glycoside balance of soybean
111 isoflavones. The results provide a process reference for the targeted green preparation of soybean meal phenolics and
112 a transferable framework for two-step extraction of active components from other oilseed by-products.

2. MATERIALS AND METHODS

2.1. Materials and reagents

Defatted soybean meal was obtained from the Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences (Hubei, China). The material was ground, passed through a 60-mesh sieve, and stored at -20°C until extraction.

Gallic acid, quercetin, Folin-Ciocalteu reagent, DPPH, ABTS, tannic acid, phytic acid assay reagents, and other analytical-grade chemicals were purchased from Shanghai China National Medicines Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Characterization of the starting soybean meal

Moisture was determined by oven drying at 105 °C. Crude protein was measured by the Kjeldahl method using a nitrogen conversion factor of 6.25. Initial total phenolic content, tannin, and phytic acid were determined using methods described below.

The starting soybean meal contained 12.8% moisture and 45.61% crude protein. Its initial TPC, tannin, and phytic acid contents were 9.11 ± 0.43 mg GAE/g, 4.96 ± 0.05 mg/g, and 28.91 ± 0.42 mg/g, respectively.

2.3. Preliminary selection of SWE conditions

SWE conditions were selected through single-factor screening. Extraction temperature (100, 120, 140, 160, and 180 °C), extraction time (10, 15, 20, 25, and 30 min), and soybean-meal-to-water ratio (1:5, 1:10, 1:15, 1:20, and 1:25, w/v) were evaluated separately.

Based on these screening experiments, 140 °C, 25 min, and a 1:20 solid-to-liquid ratio were used in the comparative extraction study.

2.4. Preliminary selection of ultrasound conditions

Ultrasound temperature, treatment time, power, and frequency configuration were screened independently using TPC as the response. The common ultrasound conditions selected for the main experiment were 45 °C, 15 min, and 240 W. A frequency of 60 kHz was selected for SF, 28/60 kHz for DF, and 20/28/60 kHz for TF.

The complete single-factor screening data are provided in Supplementary Table S1 and Figure S1.

2.5. Experimental extraction design

All extraction treatments were conducted at a soybean-meal-to-water ratio of 1:20 (w/v).

2.5.1. Hot-water extraction

HWE was carried out at 100 °C for 20 min. Soybean meal was mixed with deionized water at a 1:20 (w/v) ratio and boiled at 100 °C for 20 min. It was then vacuum-filtered, and the supernatant was collected for purification and freeze-drying.

2.5.2. Subcritical-water extraction

SWE was performed using a subcritical-water reactor. Soybean meal was mixed with deionized water in a 1:20 ratio at 140 °C for 25 min. This was then vacuum filtered, purified, and freeze-dried for final characterization.

2.5.3. Single-, dual-, and triple-frequency ultrasound

SF was applied at 60 kHz, DF at simultaneous 28/60 kHz, and TF at simultaneous 20/28/60 kHz. All ultrasound treatments were conducted at 45 °C and 240 W for 15 min in pulsed mode, using a 10 s ultrasound period followed by a 5 s rest period.

2.5.4. Sequential ultrasound–SWE treatments

For SUSWE, DUSWE, and TUSWE, SF, DF, and TF treatments were applied first, and then the whole suspension was transferred for SWE. The SWE stage was identical for all sequential treatments: 140 °C for 25 min at a 1:20 solid-to-liquid ratio. After extraction, the suspension was vacuum-filtered, and the supernatant was collected for purification.

2.6. Purification with AB-8 macroporous resin

All extraction supernatants were purified using the same AB-8 macroporous resin procedure. Crude extracts were purified using AB-8 macroporous adsorption resin. Approximately 50–60 g of resin was packed into a glass column with distilled water, and the column volume (CV) was determined from the packed resin bed volume. Extract corresponding to 2 CV was loaded onto the column at a flow rate of 2–4 mL/min, after which the column was allowed to stand for 30 min to facilitate adsorption. The resin was subsequently washed with 4 CV of distilled water at the same flow rate to remove unadsorbed components. Adsorbed compounds were then eluted with 5 CV of 70% (v/v) ethanol, and the eluate was collected as the purified fraction. The collected eluate was concentrated under reduced pressure using a rotary evaporator and subsequently freeze-dried for further analyses. After use, the AB-8 resin was regenerated using 95% ethanol and 1 M HCl.

2.7. Purified extract yield

Purified extract yield was calculated from the mass of the final AB-8-purified, freeze-dried powder:

$$\text{Purified extract yield (\%)} = \left[\frac{\text{mass of purified freeze-dried extract}}{\text{mass of soybean meal used}}\right] \times 100$$

2.8. Total phenolic content

The phenolic content of purified freeze-dried extract was determined using the procedure of (Mehmood et al., 2019; Singleton et al., 1999) with some modifications. Briefly, 200μL sample extract was mixed with 2 mL of Folin–Ciocalteu reagent and incubated in the dark for 1 min. Then 2mL Na_2CO_3 (20 % w/v) was added to it and incubated in the dark again for 30 min. Finally, a UV-1601 UV-Vis spectrophotometer (Beijing Rayleigh Analytical Instrument, China) was used to check the absorbance at 765 nm. Results were expressed as mg gallic acid equivalents (GAE) per gram of extract (mg GAE/g extract).

2.9. Total flavonoid content

TFC was determined using the aluminum chloride colorimetric method (Chang et al., 2002). Extract (1 mL) was mixed with 4 mL of distilled water and 0.3 mL of 5% $NaNO_2$. After 5 min, 0.3 mL of 10% $AlCl_3$ was added, followed after

6 min by 2 mL of 1 M NaOH. Absorbance was measured at 510 nm. Results were expressed as mg quercetin equivalents per g of purified extract (mg QE/g).

2.10. Phenolic and flavonoid recovery from soybean meal

Phenolic and flavonoid recovery were calculated for each independent extraction replicate before statistical analysis.

TPC recovery = TPC (mg GAE/g purified extract) × purified extract yield (%)

TPC recovery was expressed as mg GAE/100 g soybean meal.

Similarly,

TFC recovery = TFC (mg QE/g purified extract) × purified extract yield (%)

TFC recovery was expressed as mg QE/100 g soybean meal.

2.11. DPPH radical-scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of the purified freeze-dried extract was calculated using the method described by (Blois, 1958; Yaqoob et al., 2024) with some modifications. First, DPPH (0.1 mM) was prepared in MeOH. 120 µL of the sample was mixed with 4.2 mL DPPH solution. Then the solution was rested for 30 min at RT, and the absorbance was measured at 517 nm. The sample extract without DPPH solution was used as a control, and the scavenging activity was measured by following the formula:

DPPH scavenging activity (%) = $\frac{Control-sample}{Control}$ x 100

2.12. ABTS radical-scavenging activity

The ABTS activity was determined by the method reported by (Mehmood et al., 2019) with slight modification. Briefly, ABTS radical stock solution was prepared by adding ABTS (7 mM) to $K_2S_2O_8$ and stored in the dark for 16 h. The ABTS radical solution was diluted with PBS (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. 120 µL of sample extract was mixed with 4.2 mL of ABTS radical working solution in the dark, and absorbance was measured at 734 nm after 10 min. Sample extract was used as a control without ABTS solution, and the scavenging activity was measured by following the formula:

ABTS scavenging activity (%) = $\frac{Control-sample}{Control}$ x 100

2.13. Tannin and Phytic Acid Contents

Tannins were determined according to the Chinese National Standard Method GB/T 27985-2011. For tannin extraction, 0.2 g of sample powder was mixed in 6mL water and was water-bathed at 100 ℃ for 30min. Then it was centrifuged (6000rpm/5min). Then 1ml of the supernatant was diluted twice. For determination, 200 μL of sample extract was mixed with 1mL water, 200 μL Folin-Denis reagent, and 600 μL of Na_2CO_3 (10.6%) and was allowed to react for 40min. Then the absorbance was measured at 765nm. The tannic acid curve was used to calculate results that were expressed as mg/g purified extract.

Total phytic acid content was determined using the modified method (Gao et al., 2007). For extraction, 0.35 g of the sample powder was mixed with 7 mL of HCl (2.4%) and shaken at 210 rpm for 2 h. Then it was centrifuged (1000 × g, 20 min, 10 ℃), and the supernatant was collected. Then, 0.7 g of NaCl was added to the supernatant, and it was shaken again at 350rpm for 20min. Then, the mixture was allowed to stand at -20 ℃ for 20min before being centrifuged again to collect the supernatant. For determination, the sample was diluted 25 times. Then, 5 mL of solution was mixed with 4 mL of Wade reagent (0.3% $FeCl_3 \cdot 6H_2O$, 3%sulfosalicylic acid) and allowed to react for 20 min. Finally, the absorbance was measured at 500 nm. A standard curve of sodium phytate was prepared, and the results were expressed as mg/g purified extract.

2.14. Scanning electron microscopy

The morphology of the purified freeze-dried extracts was examined using a scanning electron microscope (S-3400N, Hitachi, Japan). Samples were mounted on carbon tape and observed at approximately ×3000 magnification with a 10 μm scale bar.

2.15. Fourier-transform infrared spectroscopy

FTIR spectra were recorded using a Fourier Transform Infrared Spectrometer (Thermo Scientific, Germany) over 4000-400 cm^{-1} using KBr pellets (1:100 sample: KBr ratio).

2.16. UPLC-ESI-QTRAP-MS/MS profiling

A 50 mg portion of each freeze-dried extract was mixed with 1.0 mL methanol/acetonitrile/water (1:2:1, v/v/v), vortexed for 30 s, and homogenized with a bead in a mixer mill for 10 min at 45 Hz. Samples were then sonicated in an ice-water bath for 10 min and kept at -20 °C for 1 h. After centrifugation at 12,000 rpm and 4 °C for 15 min, 300 µL of supernatant was passed through a 0.22 µm membrane filter.

A quality-control sample was prepared by combining 10 µL from each analytical sample.

Chromatographic separation was carried out using a Waters Acquity I-Class PLUS UPLC system fitted with a Waters HSS-T3 column (1.8 µm, 2.1 × 100 mm). Mobile phase A was water containing 0.1% formic acid and 5 mM ammonium acetate, and mobile phase B was acetonitrile containing 0.1% formic acid. The gradient started at 98% A/2% B and was held for 1.5 min, changed to 50% A/50% B by 5 min and to 2% A/98% B by 9 min, held for 1 min, and then returned to the initial composition. The flow rate was 0.35 mL/min, the column temperature was 50 °C, and the injection volume was 4 µL.

Mass spectrometric detection was performed using an Applied Biosystems QTRAP 6500+ system. The source temperature was 550 °C. Ion-spray voltage was +5500 V in positive mode and -4500 V in negative mode. GSI, GSII, and curtain gas were set to 50, 55, and 35 psi, respectively. MRM acquisition was performed using compound-specific de-clustering potentials and collision energies. Metabolites were identified using a self-built database (364 compounds) based on Q1, Q3, and retention time.

2.17. Statistical analysis

Purified extract yield, TPC, TFC, TPC/TFC recovery, DPPH, ABTS, tannin, and phytic acid are reported as mean ± SD from three independent extraction replicates and were evaluated by one-way ANOVA followed by Tukey's multiple-comparison test at $p < 0.05$. Pearson and Spearman correlation coefficients between the phenolic indices (purified extract yield, TPC, TFC, and TPC/TFC recovery) and antioxidant activities (DPPH and ABTS) were calculated from OriginPro 2024 with a two-tailed test.

UPLC-MS/MS data were used descriptively to compare relative ion-signal patterns among pooled treatment extracts. Hierarchical clustering was based on row-wise, standardized values.

3. RESULTS AND DISCUSSION

3.1. Purified extract yield did not predict phenolic enrichment or recovery

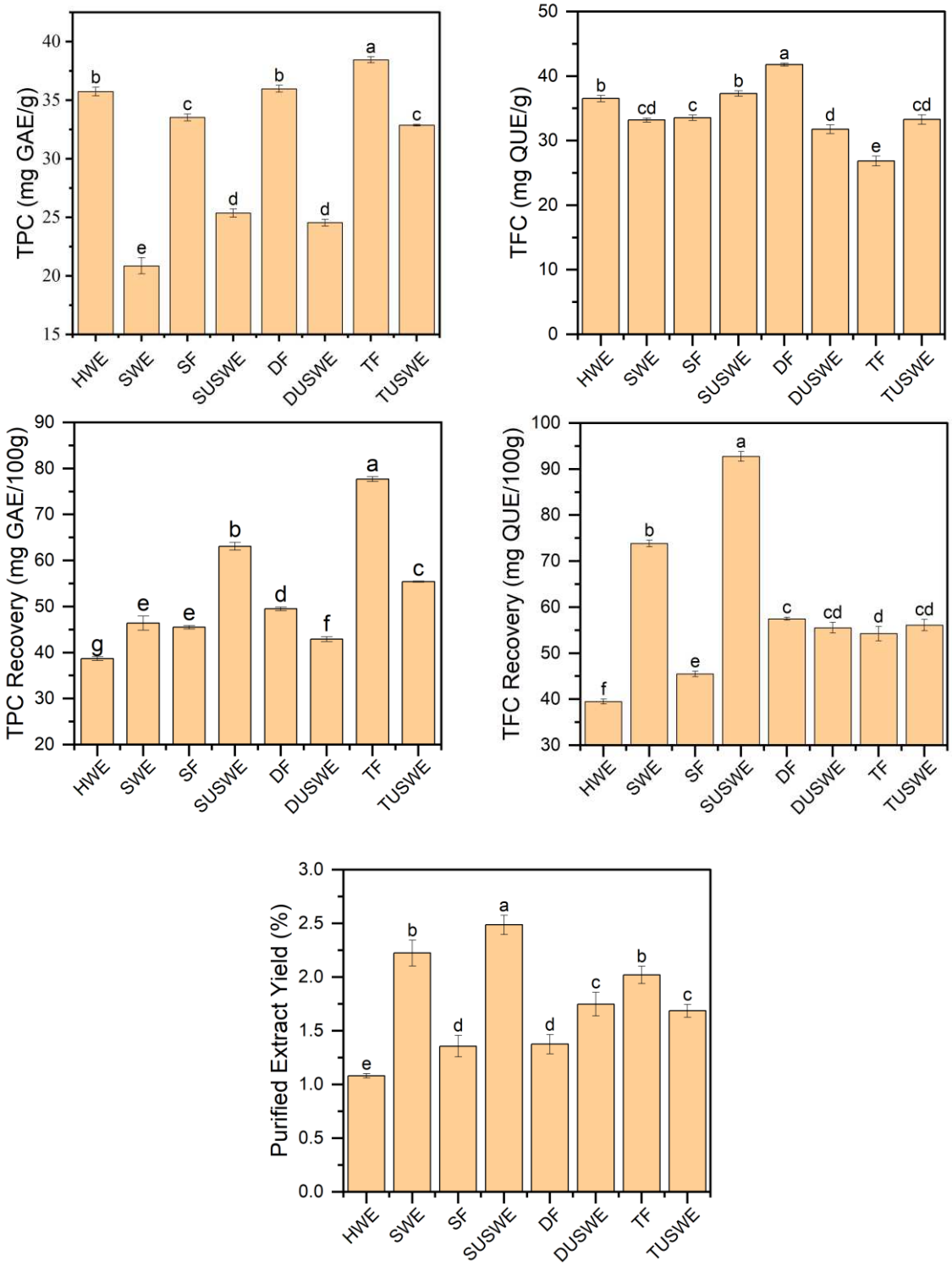


Fig. 1 *Shows (a) TPC mg GAE/g), (b) TFC (mg QUE/g), (c) TPC Recovery (mg GAE/100g), (d) TFC Recovery (mg QE/100g), (e) Purified Extract Yield (%) of all treatments*

Because the purified powder, rather than the crude extract, is the product of practical interest, purified extract yield was evaluated first, followed by TPC and TFC and their recoveries from the starting meal, which together show whether a high yield reflects genuine phenolic enrichment. Overall, the treatments that gave the most purified mass were not those that gave the most phenolics: SUSWE (2.49 ± 0.09%) and SWE (2.22 ± 0.12%) maximized purified extract yield, TF maximized phenolic concentration (38.45 ± 0.26 mg GAE/g) and recovery (77.69 ± 0.53 mg GAE/100g), and DF maximized flavonoid concentration (41.79 ± 0.23 mg QE/g) (Table 1; Fig. 1).

Table 1. Yield, phenolic enrichment and recovery of purified soybean-meal extracts

Treatment	Purified extract yield (%)	TPC (mg GAE/g)	TPC recovery (mg GAE/100 g meal)	TFC (mg QE/g)	TFC recovery (mg QE/100 g meal)
HWE	1.08 ± 0.02^{e}	35.74 ± 0.37^{b}	38.66 ± 0.40^{g}	36.51 ± 0.50^{b}	39.49 ± 0.54^{f}
SWE	2.22 ± 0.12^{b}	20.86 ± 0.69^{e}	46.39 ± 1.53^{e}	33.21 ± 0.32^{cd}	73.85 ± 0.70^{b}
SF	1.36 ± 0.10^{d}	33.53 ± 0.29^{c}	45.50 ± 0.39^{e}	33.54 ± 0.42^{c}	45.51 ± 0.57^{e}
DF	1.38 ± 0.09^{d}	35.98 ± 0.29^{b}	49.50 ± 0.40^{d}	41.79 ± 0.23^{a}	57.48 ± 0.32^{c}
TF	2.02 ± 0.08^{b}	38.45 ± 0.26^{a}	77.69 ± 0.53^{a}	26.85 ± 0.76^{e}	54.25 ± 1.54^{d}
SUSWE	2.49 ± 0.09^{a}	25.37 ± 0.34^{d}	63.09 ± 0.86^{b}	37.30 ± 0.43^{b}	92.76 ± 1.07^{a}
DUSWE	1.75 ± 0.11^{c}	24.55 ± 0.29^{d}	42.93 ± 0.51^{f}	31.77 ± 0.66^{d}	55.54 ± 1.16^{cd}
TUSWE	1.69 ± 0.06^{c}	32.86 ± 0.07^{c}	55.40 ± 0.12^{c}	33.27 ± 0.74^{cd}	56.09 ± 1.25^{cd}

Values are mean ± SD (n = 3). Different letters within a column indicate significant differences according to Tukey's test ($p < 0.05$).

As shown in Table 1 and Fig. 1, the two reference treatments without ultrasound established the baseline pattern. HWE (conventional extraction in boiling water at atmospheric pressure) gave the lowest purified extract yield (1.08 ± 0.02%) but a relatively high TPC (35.74 ± 0.37 mg GAE/g). SWE (extraction with water kept in the liquid state at 140 °C under pressure) roughly doubled the yield to 2.22 ± 0.12%, consistent with the wider solubilizing range of subcritical water (García-Marino et al., 2006). As the temperature of pressurized water rises above 100 °C, its dielectric constant, polarity, and viscosity decrease while its diffusivity increases, allowing compounds of a broader polarity range to be released from plant matrices (Cheng et al., 2021; Zhang et al., 2020). Soybean and its by-products have demonstrated similar characteristics, that is, the recovery of substantial amounts of isoflavones and other soluble

compounds by SWE (An et al., 2023; Barea et al., 2025; Nkurunziza et al., 2019). However, the additional mass recovered by SWE was poor in phenolics: TPC was 41.6% lower than that of HWE and the lowest of all treatments (20.86 ± 0.69 mg GAE/g), so TPC recovery rose only from 38.66 to 46.39 mg GAE/100 g meal, whereas TFC recovery increased by 87% (from 39.49 to 73.85 mg QE/100 g meal). Subcritical water therefore appears to have co-extracted flavonoid-type compounds together with a large amount of non-phenolic material that was partly retained by the resin, so that bulk recovery and phenolic enrichment moved in opposite directions.

Against this baseline, ultrasound alone produced a different pattern. SF and DF gave purified yields (1.36 ± 0.10% and 1.38 ± 0.09%) only slightly higher than HWE and TPC values comparable to or slightly lower than HWE (33.53 ± 0.29 and 35.98 ± 0.29 mg GAE/g), yet their TPC recoveries (45.50 and 49.50 mg GAE/100 g meal) were 18–28% higher than that of HWE. TF reversed the yield–enrichment trade-off observed for SWE: it increased the yield to 2.02 ± 0.08% while also giving the highest TPC (38.45 ± 0.26 mg GAE/g), so its TPC recovery (77.69 ± 0.53 mg GAE/100 g meal) was twice that of HWE and significantly higher than that of every other treatment, including SUSWE (63.09 ± 0.86 mg GAE/100 g meal). TFC followed a different ranking: DF had the highest concentration (41.79 ± 0.23 mg QE/g), whereas TF had the lowest (26.85 ± 0.76 mg QE/g), and SUSWE gave the highest TFC recovery (92.76 ± 1.07 mg QE/100 g meal), followed by SWE (73.85 ± 0.70 mg QE/100 g meal). Thus, the high TPC of TF was not simply the result of concentrating a small extract mass; triple-frequency ultrasound increased phenolic density and overall phenolic recovery simultaneously.

Frequency dependence of acoustic cavitation is a reasonable explanation (Ashokkumar, 2015). Internal and external mass-transfer resistance is reduced by ultrasound to accelerate extraction, while changes in frequency alter bubble dynamics and the mechanical effects generated in the liquid-solid system. Multi-frequency systems have produced higher or compositionally different polyphenol recoveries in other plant matrices (Chemat et al., 2017; Xu et al., 2022). The specific frequency configurations used here help explain the differential selectivity. At 60 kHz alone (SF), cavitation is dominated by small bubbles whose collapse is relatively mild, which favors surface erosion and the release of readily accessible solutes but leaves much of the matrix-bound fraction unextracted, consistent with the low yield of SF. Adding 28 kHz (DF) introduces larger, more violently collapsing bubbles (Brotchie et al., 2009); the stronger shear increased TPC and, particularly, TFC without increasing yield, suggesting enhanced release of flavonoid-type compounds from partially disrupted surface tissues. Adding 20 kHz (TF) introduces the largest bubbles

297 and the strongest microjets and shock waves. Superposing several frequencies is also expected to increase the number
298 of cavitation events and to make the acoustic field more uniform (Feng et al., 2002; Tatake & Pandit, 2002). Such a
299 field would disrupt cell walls and protein–phenolic aggregates throughout the suspension, which is consistent with the
300 simultaneous increase in yield, TPC, and phenolic recovery observed for TF. Because frequent bubble collapse also
301 generates local hot spots and radicals (Kanthale et al., 2008), the triple-frequency field may additionally have favored
302 the release or partial conversion of isoflavone aglycones, a possibility examined in Section 3.5.

303 The progression from SF to DF to TF shows selective enrichment rather than cumulative intensity. SF and DF
304 produced similar purified yields (1.36 and 1.38%, respectively), yet DF had significantly higher TPC (35.98 vs 33.53
305 mg GAE/g). TF then increased both yield and TPC. This indicates that ultrasound changed both the recovered
306 compounds' profile and their quantity. This compound-selective extraction is further supported by TFC rankings (TF
307 lowest at 26.85 mg QE/g; DF highest at 41.79 mg QE/g). The two assays gave different treatment rankings, suggesting
308 that not all phenolic subclasses were enhanced by frequency regimes in parallel. For TPC and TFC recovery, rankings
309 changed again; i.e., the highest TFC recovery (92.76 ± 1.07 mg QE/100g meal) was produced by SUSWE, while SWE
310 ranked 2nd with 73.85 ± 0.70 mg QE/100g. On the other hand, DF had the highest flavonoid content/g of purified
311 extract but had limited total recovery due to lower extract yield.

312 This pattern suggests three process outcomes: TF favored phenolic enrichment and recovery; DF favored flavonoid
313 concentration; SUSWE favored total flavonoid-equivalent recovery. This division is crucial as it provides the basis
314 for the extraction strategy selection for the desired product attribute rather than adopting a single generic measure of
315 extraction. Because these differences arose under identical ultrasound temperature and power, the next question was
316 whether a subsequent SWE step would build on, or erase, the selectivity established by each frequency configuration
317 (Section 3.2).

3.2. The effect of SWE depended on the ultrasound treatment that preceded it

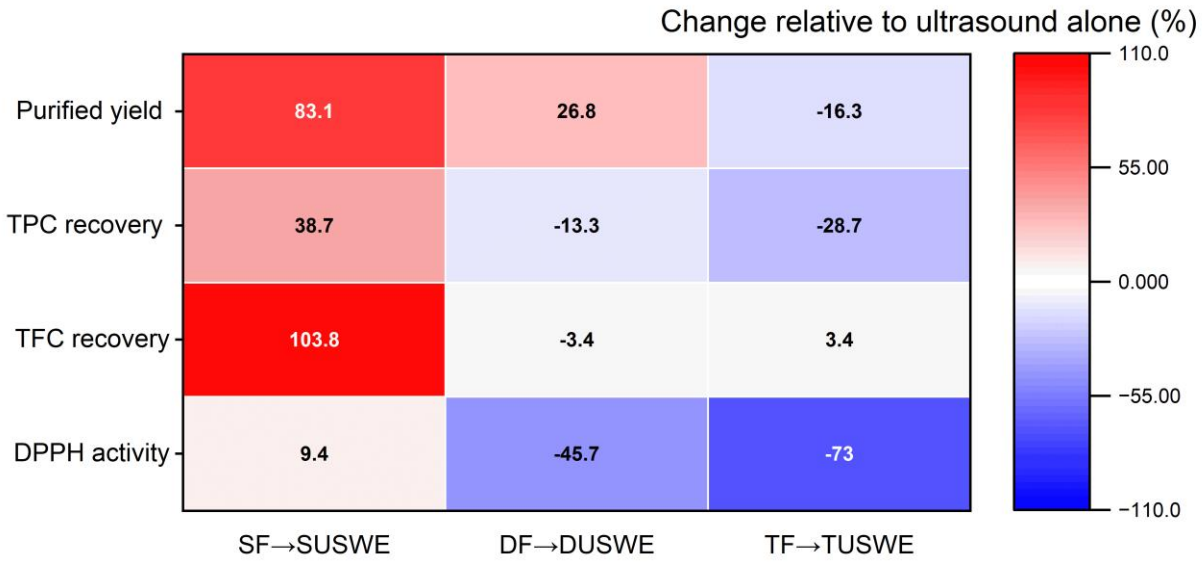


Fig. 2 *SWE contribution heatmap shows that SWE conditions produced different responses after SF, DF, and TF*

To better understand the behaviour of the second stage, paired comparison between the combined treatment and its corresponding ultrasound-only treatment is crucial, as shown in Fig. 2. SWE did not respond in the same way after SF, DF, and TF. This shows that ultrasound frequency has already shaped the state of the compound before entering SWE.

SF versus SUSWE: SWE significantly increased purified yield by 83% (1.36 → 2.49%)

Combining SWE with SF increased yield by 83% (1.36% to 2.49%), increased TPC recovery by 39% (45.50 to 63.09mg GAE/100g), and doubled TFC recovery (45.51 to 92.76 mg QE/100g meal). On the other hand, it also decreased TPC concentration (33.53 to 25.37 mg GAE/g). This indicates that a substantial number of recoverable phenolics were left by SF that SWE accessed effectively.

DF versus DUSWE: SWE increased yield by 27% (1.38 → 1.75%) but lower enrichment

In the case of DF, SWE increased yield from 1.38 to 1.75% (27%) but also decreased TPC (from 35.98 to 24.55mg GAE/g), TPC recovery (from 49.50 to 42.93 mg GAE/100g), and TFC (from 41.79 to 31.77 mg QE/g). This means the additional mass that was recovered by SWE was phenolics-depleted.

TF versus TUSWE: SWE reversed the strongest phenolic response

Accompanying TF, SWE reduced yield (from 2.02% to 1.69%), TPC by 15% (from 38.45 to 32.86 mg GAE/g), and TPC recovery by 29% (from 77.69 to 55.40 mg GAE/100g), and increased TFC from 26.85 to 33.27 mg QE/g while keeping the TFC recovery almost the same. It also caused a 73% reduction in DPPH activity (from 51.06 to 13.79%). As SWE was applied to the whole suspension, its impact can either be due to additional extraction from solids or thermal modification of already existing compounds. It improved recovery after SF, as SF released little; declined after DF; and decreased after TF, which may be due to thermal degradation or polymerization of already extracted compounds.

This pattern is consistent with reports on soybean isoflavones extracted with subcritical water, in which temperature and molecular form at the start of processing determine the conversion and extraction that lead to different maximum yields and conversion pathways of different compounds (An et al., 2023; Nkurunziza et al., 2019). This suggests that, in practical implications, sequential extraction should not be revolved around the assumption that the second step would automatically improve the first. In this case, the first stage decides how much product is already in solution and is exposed to the hydrothermal step.

3.3. Antioxidant response tracked phenolic quality more closely than extract yield

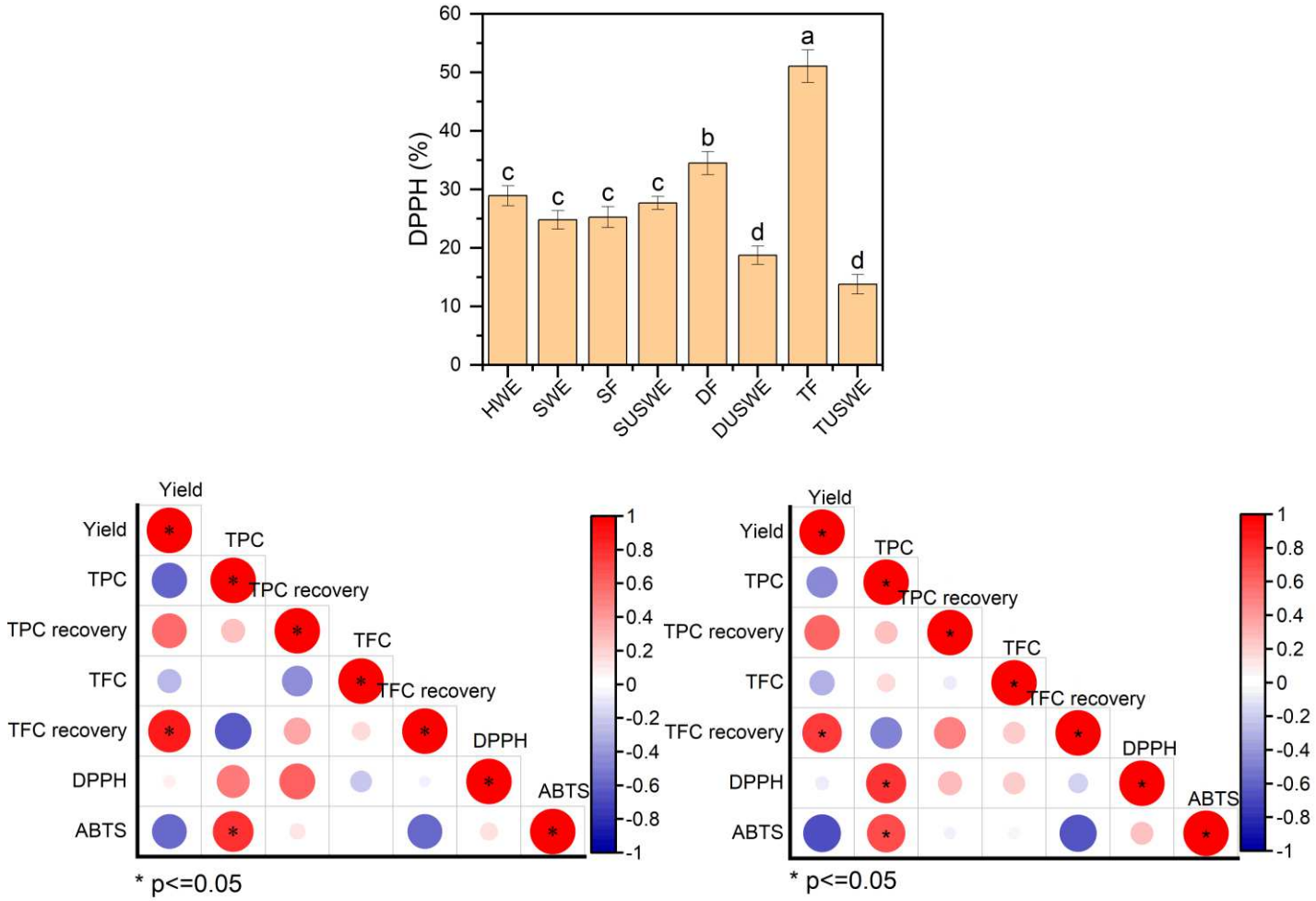


Fig. 3 *(a) DPPH radical-scavenging activity provided a clear functional separation among the treatments; Pearson (b) and Spearman (c) correlation matrices between phenolic indices and antioxidant activities of purified soybean-meal extracts. *p < 0.05*

Table 2. Antioxidant activity and selected co-recovered components in purified soybean-meal extracts

Treatment	DPPH (%)	ABTS (%)	Tannin (mg/g)	Phytic acid (mg/g)
HWE	28.93 ± 0.97^{c}	91.75 ± 2.71^{a}	27.70 ± 0.29^{c}	23.78 ± 0.30^{c}
SWE	24.81 ± 0.80^{c}	83.42 ± 1.90^{b}	29.61 ± 0.30^{b}	27.25 ± 0.30^{a}
SF	25.28 ± 0.80^{c}	90.54 ± 3.30^{ab}	29.41 ± 0.47^{b}	23.59 ± 0.33^{c}
DF	34.50 ± 0.20^{b}	90.93 ± 2.69^{a}	30.89 ± 0.31^{a}	26.90 ± 0.20^{ab}
TF	51.06 ± 0.50^{a}	91.30 ± 2.05^{a}	31.27 ± 0.32^{a}	19.39 ± 0.46^{e}
SUSWE	27.66 ± 1.00^{c}	88.75 ± 2.03^{ab}	29.10 ± 0.30^{b}	27.54 ± 0.25^{a}
DUSWE	18.74 ± 0.80^{d}	90.15 ± 3.05^{ab}	31.42 ± 0.30^{a}	26.33 ± 0.38^{b}

Treatment	DPPH (%)	ABTS (%)	Tannin (mg/g)	Phytic acid (mg/g)
TUSWE	13.79 ± 0.26^{d}	91.56 ± 2.48^{a}	29.28 ± 0.30^{b}	22.65 ± 0.14^{d}

Values are mean ± SD (n = 3). Different letters within a column indicate significant differences according to Tukey's test ($p < 0.05$).

Because the practical value of a phenolic fraction ultimately lies in its function, DPPH and ABTS radical-scavenging activities were measured next to test whether the differences in phenolic yield and composition described above translate into differences in antioxidant capacity. As shown in Fig. 3(a) and Table 2, the DPPH ranking followed phenolic quality rather than extract mass: TF had the highest activity (51.06 ± 0.50%) followed by DF (34.50± 0.20%) and other being in a lower group (24.8-28.9%).

Even though the most purified powder was produced by SUSWE, its DPPH activity was only 27.66%. TF had a lower yield but showed higher DPPH activity (51.06%), which is approximately twice that of SUSWE. The contrast between TF and TUSWE was especially intriguing, as DPPH activity fell from 51.06 to 13.79% (73% loss), whereas TPC only dropped by 15%. This indicates that functional loss is far greater than can be accounted for by phenolic content loss, meaning SWE did not simply decrease the phenolic quantity, but the composition of the antioxidant pool was changed by it.

Based on aglycone vs. glycoside form and hydroxylation patterns, soybean isoflavones have different radical-scavenging capacities, which explains why TPC measurements are different from DPPH activity (Kim et al., 2022; Lee et al., 2005). SWE had an aglycone-weighted isoflavone but also showed weak DPPH activity (24.81%) (LC-MS data, section 3.5). This means aglycone enrichment is not the only factor involved in maximizing antioxidant activity. While TF has combination of different factors like favorable isoflavone shift, highest overall phenolic concentration and strongest DPPH activity which suggests various phenolic factors contribute to antioxidant performance.

ABTS activity differed little among treatments (88.75–91.75%), except for SWE (83.42 ± 1.90%). ABTS values of about 91% suggest saturation of the assay. DPPH distinguished treatments better than ABTS, most likely because assay conditions favor compounds abundant in these extracts.

To verify whether antioxidant capacity was linked to phenolic enrichment rather than to extract mass, correlation analysis was performed on the treatment means (n = 8) (Fig. 3b and 3c). DPPH activity was positively associated with

TPC (Spearman ρ = 0.79, p = 0.021; Pearson r = 0.53, p = 0.17) and with TPC recovery (r = 0.63, p = 0.093) but showed no relationship with purified extract yield (r = 0.09, p = 0.83), TFC (r = −0.23, p = 0.59), or TFC recovery (r = −0.07, p = 0.88). ABTS activity also correlated with TPC (r = 0.81, p = 0.016), although its narrow range limits its discriminating power. These results indicate that the enhancement of antioxidant capacity was associated with the enrichment of Folin-reactive phenolics rather than with extracted yield or flavonoid-equivalent content. The weaker linear correlation between TPC and DPPH arises mainly from TUSWE, which retained a moderate TPC (32.86 mg GAE/g) but lost most of its DPPH activity (13.79%), confirming that phenolic composition, and not only phenolic quantity, governs activity. The molecular basis of this compositional effect was therefore examined by UPLC-MS/MS (Section 3.5).

3.4. Frequency architecture also altered co-recovery of phytic acid and tannin

Because phytic acid and tannins are antinutritional compounds that can co-purify with phenolics, and tannins also respond in the Folin assay, their contents were determined to assess the purity of the purified fractions. Phytic acid ranged from 19.39 ± 0.46 mg/g to 27.54 ± 0.25 mg/g across all treatments, with TF having the lowest (19.39 ± 0.46 mg/g) and SUSWE having the highest (27.54 ± 0.25 mg/g). Since purification was the same for all treatments, TF's lower value suggests that ultrasound frequency affected compound availability for resin adsorption (Zhou & Erdman, 1995).

Varying trends of tannin were observed across treatments. TF, DF, and DUSWE showed the highest tannin (31 mg/g approx.) and HWE showed the lowest. The untreated soybean had 4.96 ± 0.05 mg/g tannin content, while purified extracts had much higher content, which reflects the general concentration of phenolics during extraction, resin purification, and freeze-drying rather than selective tannin enrichment.

3.5. UPLC-MS/MS profiling: treatment-dependent isoflavone selectivity

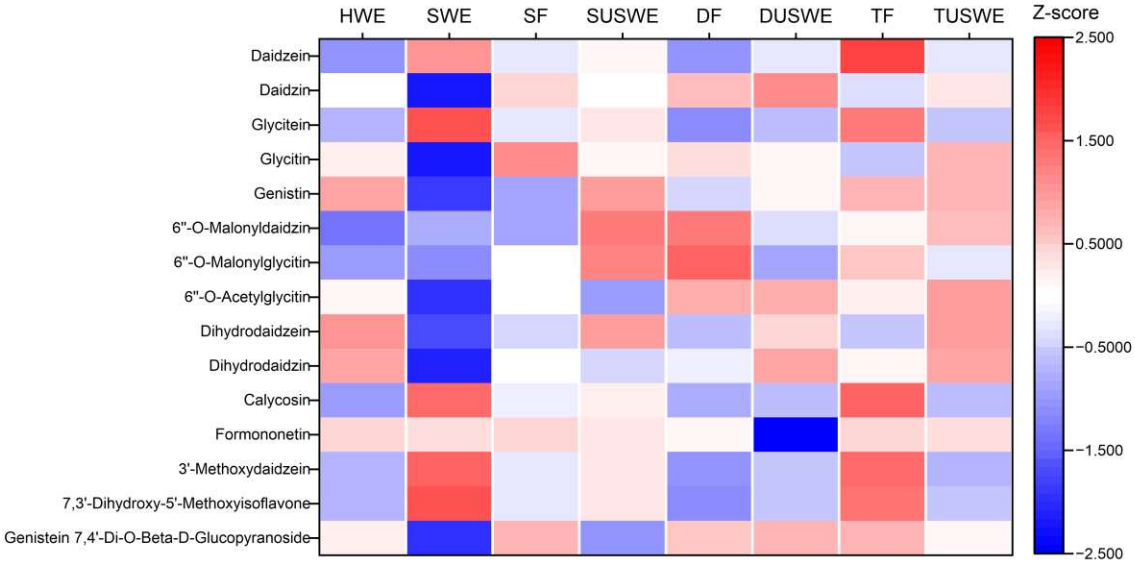


Fig. 4 *Isoflavone Z-score heatmap showing treatment selectivity by major isoflavone pairs*

Because TPC and TFC are group assays that cannot distinguish individual compounds, UPLC-ESI-QTRAP-MS/MS profiling was used to determine whether the treatments altered phenolic composition at the molecular level, with particular attention to the aglycone/glycoside balance of the isoflavones.

UPLC-MS/MS showed a total of 364 compounds detected across all the treatments. It showed that treatments had compound-specific enrichment and depletion instead of uniform phenolic profile changes. Analytical repeatability was good (median CV 5.6%; 73.4% of features CV $\leq$ 10%).

Individual compounds responded differently to frequency configuration, explaining why TPC, TFC, and antioxidant assays ranked treatments differently. The major isoflavone pairs (daidzein/daidzin, glycitein/glycitin) showed the clearest treatment selectivity (Fig. 4).

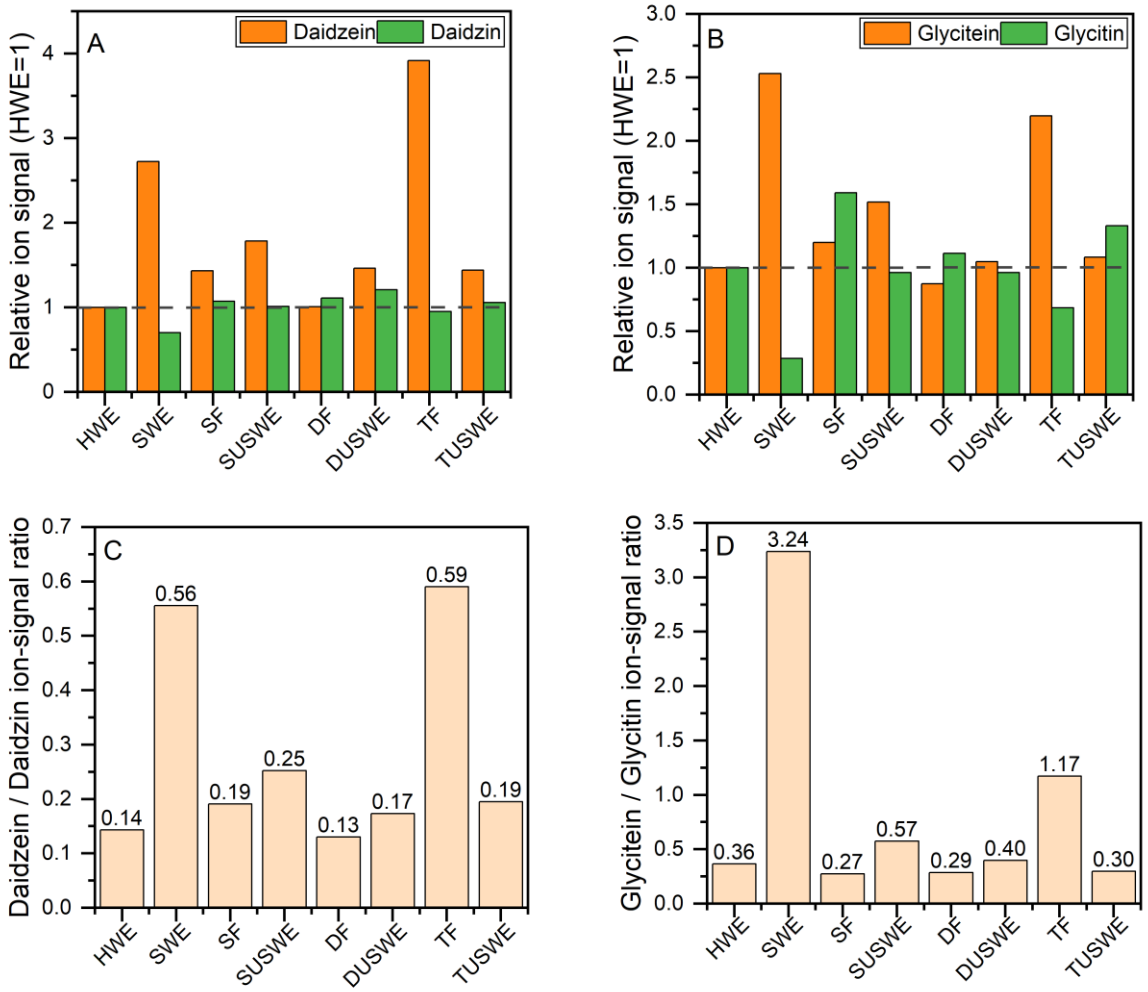


Fig. 5 *Shows Aglycone/glycoside comparisons: (a) Relative ion signal (HWE=1) of Daidzein & Daidzin, (b) Relative ion signal (HWE=1) of Glycitein & Glycitin, and ratio plots: (c) Daidzein/ Daidzin ion-signal ratio, (d) Glycitein/Glycitin ion-signal ratio across all treatments*

3.5.1. Triple-frequency ultrasound strongly favored the daidzein signal relative to daidzin

Daidzein ion signal was approximately 3.9-fold higher in TF than HWE (Fig. 5). Daidzin signal did not increase proportionally, remaining near HWE levels across treatments. The daidzein/daidzin ion-signal ratio shifted from 0.143 (HWE) to 0.590 (TF). The shift from DF (0.130) to TF (0.590) occurred despite identical bulk temperature and power, suggesting that ultrasound frequency configuration (not thermal hydrolysis) drove aglycone enrichment. Two mechanisms may contribute. First, the intense microjets and shock waves generated at 20 and 28 kHz can disrupt the

protein-rich matrix with which the less polar aglycones are associated, preferentially releasing them; second, local hot spots and radicals produced by frequent bubble collapse may promote partial cleavage of glycosidic bonds. Distinguishing between these possibilities will require targeted quantification with authentic standards.

SWE produced a similarly high ratio (0.555) through a different mechanism: hydrothermal conversion of conjugated isoflavones at 140°C (An et al., 2023; Barea et al., 2025; Nkurunziza et al., 2019). Thus, TF and SWE achieved similar aglycone/glycoside distributions through acoustic selectivity versus thermal conversion, respectively.

3.5.2. Glycitein/glycitin showed an even stronger hydrothermal shift

The most visible treatment response was shown by glycitein/glycitin. With a ratio of 3.237, SWE was the dominant ratio, and this reflected a strong glycitein signal and glycitin reduction. This was consistent with the thermodependency of soybean isoflavone conjugates. Glycosidic and malonylated forms could be altered by heating, while the conversion kinetics and solubility are affected by subcritical water. A ratio of 1.173 was realized by TF in the absence of SWE exposure, which further proves that ultrasound frequency affected extraction selectivity.

3.5.3. SWE did not preserve the distinctive isoflavone balance generated by TF

Ratios of 0.590 and 1.173 were respectively realized by daidzein/daidzin and glycitein/glycitin, and they were both produced by TF. The ratios declined to 0.195 and 0.297, respectively, in TUSWE. The isoflavone balance established by TF alone was lost largely due to the sequential treatment, paralleling the loss of phenolic recovery (77.69 → 55.40 mg GAE/100g) and DPPH activity (51.06 → 13.79%).

Contrast with SF: SF entered SWE with lower phenolic recovery (45.50 mg GAE/100g), and SUSWE increased recovery to 63.09 mg GAE/100g. A large residual range was left by the SF's less selective first extraction, and this could be improved by SWE. The first extraction limited SWE improvement. After TF, a larger aglycone-rich fraction was already in the solution and was exposed to 140 °C. This may explain TF isoflavone loss. It could be because of thermal loss of already existing aglycones or dilution by newly extracted glycosides from the pre-treated matrix.

3.6. Molecular profiles help explain the divergence between TPC and TFC

The divergences between treatments, with TF having the highest TPC but the lowest TFC and DF having the highest TFC, are explained by LC-MS data. Each compound reacted to ultrasound frequency in different ways. Some were enriched while others were reduced under the same treatment, which means these treatments changed phenolic composition even when colorimetric assays shifted in opposite directions.

TPC and TFC are complementary assays that demonstrate different sub-populations instead of the actual stock of compound quantities. TF mostly enhanced Folin- reactive phenolics and only selectively increased isoflavone aglycones (LC-MS data; section 3.5) and produced a lower flavonoid response. DF, on the other hand, produced the strongest TFC signal but had weaker DPPH activity than TF. So, compound-level profiling is more revealing than broad classification of phenolics to understand the selectivity of the treatment.

3.7. SEM and FTIR provide complementary characterization of the purified fractions

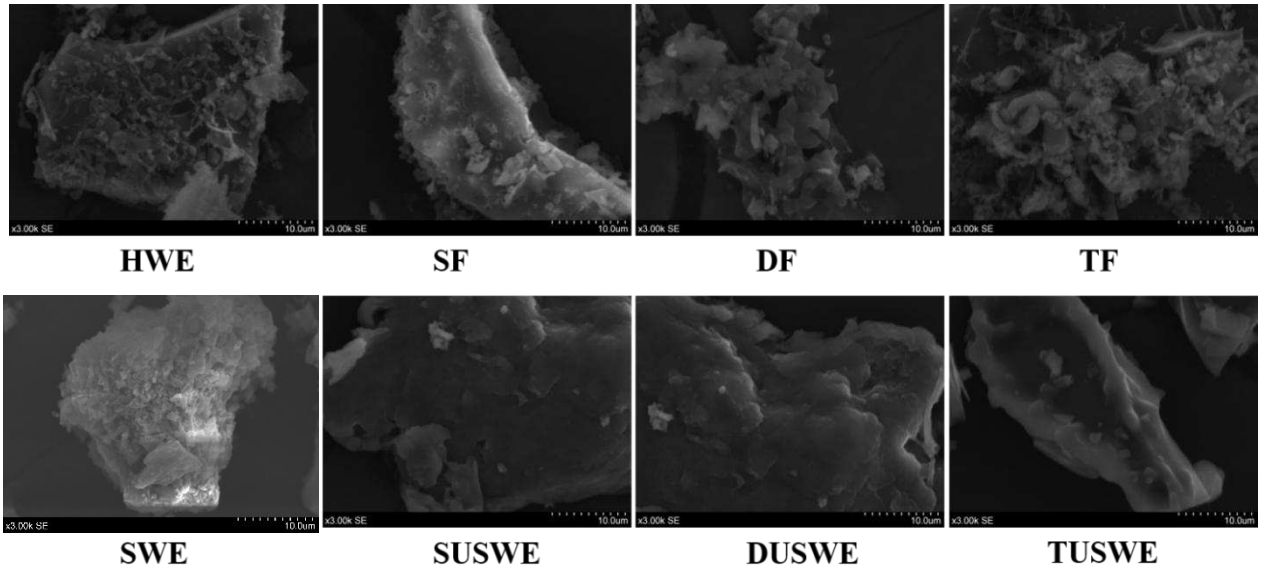


Fig. 6 *Scanning electron microscopy (SEM) images of the purified freeze-dried powders obtained by HWE, SWE, SF, DF, TF, and sequential ultrasound–SWE treatments, showing differences in fragmentation, aggregation, surface roughness, and sheet-like structure.*

Finally, SEM and FTIR were used to examine whether the compositional differences were also reflected in the morphology and functional groups of the purified powders. The SEM images clearly depicted morphological variations among purified freeze-dried powders: there were differences observed in the fragmentation, aggregation,

roughness, and the sheet-like structure across HWE, SWE, SF, DF, TF, and sequential treatments. These variations align with the compositional diversity established by different extraction routes.

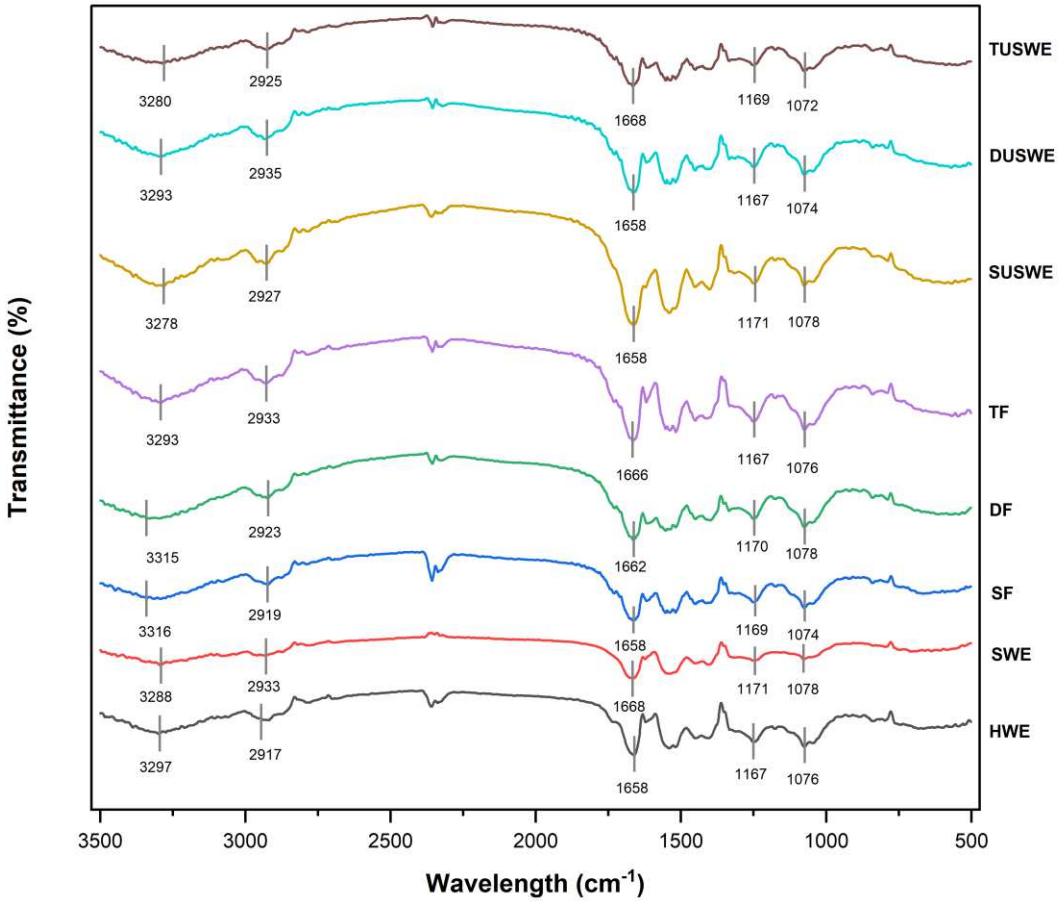


Fig. 7 Fourier-transform infrared (FTIR) spectra of the purified freeze-dried extracts across all treatments

Across all extracts, FTIR spectra shared major absorption regions (broad band ~3294–3325 cm^{-1}; prominent peaks at 1664–1668, 1527–1552, 1248–1250, 1066–1070 cm^{-1}). Compositional variations were indicated by peak positions and modest intensity, but UPLC-MS/MS profiling provided greater molecular specificity for assigning treatment differences.

3.8. Integrated interpretation: the first extraction step determines the value of the second

Efficiency of sequential extraction depends on the compounds recovered by the first step and exposed to hydrothermal extraction. Less recovery of phenolics by SF (45.50 mg GAE/100g) was compensated by SWE (63.09 mg GAE/100g) in the combined treatment. In the case of DF, SWE added mass but also lowered phenolic enrichment (TPC 35.98 → 24.55 mg GAE/g; recovery 49.50 → 42.93 mg GAE/100g). While TF, in contrast, produced high phenolic density

(38.45 mg GAE/g) and recovery (77.69 mg GAE/100g), SWE addition reduced TPC recovery (77.69 → 55.40 mg GAE/100g) and shifted isoflavone composition away from TF balance.

For product development, process selection depends on the desired endpoint. TF prioritizes phenolic concentration, recovery, and DPPH activity. DF prioritizes flavonoid-equivalent concentration. SUSWE maximizes total flavonoid recovery from starting meal.

4. CONCLUSION

This study demonstrates that ultrasound frequency configuration is not merely a means of intensifying extraction but a lever that determines both the phenolic composition of purified soybean meal extracts and the value of a subsequent SWE step, confirming the hypothesis that the first extraction step sets the chemical state on which the second acts. Among the ultrasound-only treatments, TF (20/28/60 kHz) gave the best overall phenolic performance, doubling TPC recovery relative to HWE (77.69 vs. 38.66 mg GAE/100 g meal) and increasing DPPH activity by 76.5% (51.06% vs. 28.93%), whereas DF gave the highest TFC (41.79 mg QE/g) and SUSWE gave the highest purified extract yield (2.49%) and TFC recovery (92.76 mg QE/100 g meal). At the molecular level, TF raised the daidzein/daidzin ion-signal ratio from 0.143 to 0.590 and the glycitein/glycitin ratio from 0.365 to 1.173 at only 45 °C, showing that frequency configuration, rather than bulk heating, can redirect the aglycone/glycoside balance. The effect of SWE depended on this initial state: it increased purified yield by 83% and doubled TFC recovery after SF, added mainly phenolic-poor mass after DF, and, after TF, reduced TPC recovery by 28.7% and DPPH activity by 73.0%. UPLC-MS/MS showed that in TUSWE the daidzein/daidzin and glycitein/glycitin ratios fell to 0.195 and 0.297, respectively, indicating that the aglycone-rich fraction already released by TF was degraded or diluted by newly extracted glycosides during the 140 °C step; this loss of the TF-specific isoflavone balance parallels, and helps explain, the loss of phenolic recovery and antioxidant activity. For process design, TF is recommended when a phenolic-rich, DPPH-active ingredient is the target, DF when flavonoid concentration is prioritized, and SUSWE when total flavonoid recovery or purified yield is the goal; sequential extraction should be designed around what the first stage has already released rather than on the assumption that greater process intensity gives a better product. Beyond soybean meal, this principle that the first step determines the value of the second provides a transferable framework for designing two-step green extraction of active components from other oilseed by-products and supports their high-value utilization.

Declaration of Competing Interest

The authors declare no competing interests.

Contributions

S.R. wrote the original draft, curated the data, performed the formal analysis, performed the investigation, developed the methodology, and reviewed and edited the manuscript; X.W. and A.R. performed the investigation and reviewed and edited the manuscript; J.W., C. Y., K.A., and M. reviewed and edited the manuscript; K.H. performed the validation; Y.D., H.Z. conceptualized the study, curated the data, acquired funding, developed the methodology, administered the project, supervised the study, performed the validation, reviewed and edited the manuscript. All authors reviewed the manuscript.

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