

Optimization of Ultrasound-Assisted Extraction of Bioactive Compounds from Satsuma Mandarin Pulp Agro-Industrial Residue using Water and Water/Ethanol Solvent Mixtures

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Satsuma mandarins (*Citrus unshiu* Marc.) are widely consumed due to their taste and easy-to-peel thin skin. While primarily eaten fresh, market saturation has increased juice production, generating significant pulp residues, which remain rich in bioactive compounds and antioxidants like phenolic acids, flavonoids and carotenoids, making it a valuable resource for the food and pharmaceutical industries. Efficient extraction of these compounds, however, remains challenging. Ultrasound-assisted extraction (UAE) is an emerging highly efficient extraction technology that enhances bioactive compound recovery while reducing energy input and solvent use. This study optimized the UAE of bioactive compounds from mandarin pulp using either water or water/ethanol mixtures as green solvents. A Box-Behnken design within Response Surface Methodology was applied to assess the effect of three factors: a) solvent composition (water, 30% (v/v) ethanol and 60% (v/v) ethanol), b) treatment time (1, 8 and 15 min), and ultrasound amplitude (25, 50 and 75%). The analyzed responses were total polyphenolic content (TPC), total flavonoid content (TFC) and total carotenoid content (TCC). Results indicate that higher ethanol concentration, longer extraction time, and increased amplitude enhanced TPC and TFC extraction. In contrast, a lower ethanol concentration was optimal for TCC. Optimal conditions of 31% ethanol, 12 min extraction time, and 65% amplitude maximized bioactive yields. Validation experiments confirmed the model's high predictive accuracy.

1. Introduction

Mandarins are among the most widely consumed citrus fruits, with global demand increasing due to their distinctive flavor and nutritional benefits. Thus, due to their increasing popularity, they are cultivated all around the world (Jurić et al., 2023). In addition to their sensory appeal, mandarins are rich in bioactive compounds such as polyphenols, flavonoids and carotenoids, which exhibit various biological properties including anticancer, anti-inflammatory, anti-hyperlipidemic, and anti-diabetic effects (Shorbagi et al., 2022). The expansion of mandarin cultivation has led to a rise in juice production, generating significant pulp residues. While partially depleted after juicing, this byproduct remains rich in bioactive compounds. Extractable compounds from citrus pulp can be a valuable resource for the pharmaceutical and food industries, making them particularly attractive alternatives to synthetic compounds (Boninsegna et al., 2024). Nevertheless, their efficient extraction remains a challenge.

Ultrasound-assisted extraction (UAE) is an emerging highly efficient extraction technology that enables the recovery of bioactive compounds by utilization of green solvents, while operating at low energy, respectively, making final products more eco-friendly (Buvaneshwaran et al., 2022). Thus, this work aimed to utilize UAE as an emerging extraction technique for bioactive compounds from mandarin pulp by using water and water/ethanol as solvents. To maximize the efficiency of the extraction procedure, an experimental design using the Response

Surface Methodology was employed to optimize the process parameters refined using the desirability approach and validated through confirmation experiments (Chelladurai et al., 2021).

2. Materials and Methods

2.1 Plant material and chemicals

Satsuma mandarin (*Citrus unshiu* Marc.) pulp was sourced from the local mandarin juice producer Novallis, located in Opuzen, Croatia. The pulp was freeze-dried, milled using a laboratory grinder, and sieved through the 450 μm mesh stainless steel mesh.

2.2 Experimental design

A Box-Behnken design within RSM was used for experimental optimization (DesignExpert 7.0.0). Freeze-dried mandarin pulp (0.3%, w/v) was suspended in 100 mL of water, or a water/ethanol solvent mixture and UAE was conducted using UP200St-Sonotrode S26d14 equipment (Hielscher, Teltow, Germany). UAE was optimized by varying the three factors. Independent factors F1-F3 are shown in Table 1, with the following parameters: ethanol/water solvent ratio (0, 30 and 60%, v/v), time (1, 8 and 15 min) and amplitude (25, 50 and 75%). The temperature was measured during the extraction and was never above 75°C to minimize the degradation of the bioactive compounds. The extracted solution was filtered through Whatman No. 4 filter paper using vacuum filtration and the volume was adjusted to 100 mL by adding the corresponding solvent.

2.3 Determination of total polyphenolic, flavonoid and carotenoid content in mandarin pulp

The extracts were analyzed for three responses: total polyphenolic content (TPC), total flavonoid content (TFC) and total carotenoid content (TCC). The modified Folin Ciocalteu's spectrophotometric method (Singleton et al., 1999) was used to determine TPC with gallic acid as the standard. TFC was determined using the modified spectrophotometric method of Ivanova et al. (Ivanova et al., 2010) at 360 nm with quercetin as the standard. TCC was measured based on a modified spectrophotometric method according to Bandić et al. (2023a) with β -carotene as the standard. Results were expressed as mg of standard equivalents per g of freeze-dried mandarin pulp.

2.4 HPLC determination of narirutin and hesperidin

Extraction of flavonoids was conducted according to Wang et al. (2008) with minor modifications. An extract was evaporated to dryness, resolved in 1.5 mL of methanol/dimethylsulfoxide (1:1, v/v), and sonicated in an ultrasonic bath (Elma S 10H Elmasonic, Elma Schmidbauer, Germany) for 15 minutes at room temperature. After sonification, the samples were centrifuged at 9000 rpm for 10 min at 4 °C. The supernatant was filtered through 0.45 μm LLG-RC syringe filters (LLG GmbH, Grevenbroich, Germany) before use. Analysis was performed in triplicate and carried out using an Agilent 1260 Infinity II System (Agilent, Germany), equipped with an autosampler, quaternary pump, column thermostat and DAD detector. Separation of flavonoids was conducted on an Agilent Poroshell 120 SB-C18 150 \times 4.6 mm 4 μm (Agilent, Palo Alto, CA, USA) at 40 °C and 0.8 mL/min. The injected volume was 20 μL . The mobile phase consisted of (A) aqueous 2 % formic acid and (B) methanol, with a gradient elution as follows: 0 min, 10 % B; 10 min 20 %B; 20 min, 30 % B;30 min 40 % B; 35 min 40 % B; 42 min, 50 % B; 52 min, 90 % B; 53 min 10% B; 60 min 10 % B. Flavonoids were quantified at a wavelength of 280 nm.

3. Results and Discussion

In this study, Box-Behnken/RSM was used to optimize UAE by evaluating three independent factors (F): a) solvent composition (water or water/ethanol solvent mixture), b) extraction time, and c) ultrasound amplitude. Three responses (R) were analyzed: a) TPC, b) TFC and c) TCC (Table 1). UAE is widely recognized as an emerging green technology for the efficient extraction methods of bioactive compounds (Shen et al., 2023). UAE enables the efficient extraction of targeted bioactive components through the cavitation effect, which enhances mass transfer, resulting in reduced extraction time, and improved yields and quality of the extracted compounds. Furthermore, UAE has been reported to preserve the bioactivity and functionality of the extracts (Dzah et al, 2020). Pretreatment of samples, including grinding and particle size reduction, has been found to enhance extraction efficiency by increasing the surface area available for mass transfer. Ultrasonic treatment additionally causes the rupture of cell walls, aiding content release and further improving mass transfer (Shen et al., 2023). In this study, freeze-dried pulp was ground and sieved to ensure uniform particle size distribution. A high solvent/dried pulp ratio was selected based on preliminary trials, which indicated that this condition maximized extraction yields. High solvent/material ratios ameliorate and accelerate mass transfer, enhancing the diffusion of bioactive compounds into the solution until equilibrium is approached (Xu et al., 2015).

Regarding the ANOVA for the Response Surface Quadratic Model, for TPC, the F-value of 29.50 indicated a highly significant model ($p < 0.0001$). The Lack of Fit test yielded an F-value of 6.07, which was not significant, suggesting that the model adequately fits the experimental data. Regarding TFC, the model was significant, with an F-value of 32.51 ($p < 0.0001$). However, the Lack of Fit was also significant (F-value of 59.53), suggesting potential limitations in predicting TFC across all regions of the design space. Nevertheless, validation experiments confirmed the model's predictive capability. Regarding TCC, the model was highly significant (F-value = 114.51, $p < 0.0001$), while the Lack of Fit was not significant (F-value = 2.70), indicating a good fit relative to the pure error.

Experimental results (Table 1) showed that TPC varied from 42.60 to 110.08 mg GAE/g while TFC ranged from 4.04 to 14.04 mg/g of dry pulp. In comparison, Xi et al. (2013) reported TPC values ranging from 15.98 to 22.26 mg GAE/g d.w. of pulp in Chinese wild mandarin (*Citrus reticulata* Balnco) depending on genotype. Furthermore, Chen et al. (2020), who analyzed 27 local citrus cultivars in China, reported significantly lower TPC values in the range from 2.65 to 10.45 mg/g of dry pulp, although, their TFC values ranged from 1.89 to 16.42 mg/g, which was within the range observed in this study. Additionally, Levaj et al. (2009) reported a total flavonoid value of 12.63 mg/g in Satsuma mandarin pulp, closely aligning with the present findings.

Table 1. Experimental design summary for factors and corresponding responses

Std	F1 (Ethanol, %)	F2 (Time, min)	F3 (Amplitude, %)	R1 (TPC, mg GAE/g)	R2 (TFC, mg QE/g)	R3 (TCC, mg β C/g)
2	60.00	1.00	50.00	65.39	8.89	0.28
17	30.00	8.00	50.00	101.52	13.57	1.80
11	30.00	1.00	75.00	70.52	8.97	1.90
15	30.00	8.00	50.00	105.28	13.53	1.71
12	30.00	15.00	75.00	110.08	14.04	1.98
13	30.00	8.00	50.00	109.78	13.17	1.77
8	60.00	8.00	75.00	100.00	13.65	0.29
5	0.00	8.00	25.00	62.24	4.04	0.64
1	0.00	1.00	50.00	42.60	4.26	0.80
3	0.00	15.00	50.00	58.33	4.27	1.09
16	30.00	8.00	50.00	103.56	13.41	1.89
10	30.00	15.00	25.00	102.83	13.67	1.97
14	30.00	8.00	50.00	105.12	13.64	1.80
7	0.00	8.00	75.00	70.04	4.91	1.17
6	60.00	8.00	25.00	99.37	13.34	0.30
4	60.00	15.00	50.00	97.10	14.03	0.44
9	30.00	1.00	25.00	59.84	7.73	1.23

Figure 1 presents the 3D surface response plots for both TPC and TFC, illustrating the influence of ethanol concentration, extraction time, and ultrasound amplitude on bioactive compound recovery. A strong correlation between TPC and TFC was observed ($r_{TPC/TFC} = 0.94$), indicating that both classes of compounds respond similarly to extraction conditions. Optimal extraction was achieved with ethanol concentrations in the range of 30-40%, with prolonged extraction times (approximately 13 min) further enhancing yields. Regarding ultrasound amplitude, a 50% amplitude was sufficient to maximize extraction efficiency for both TPC and TFC.

These findings align with previous studies employing UAE and RSM to optimize ethanol concentration for the extraction of antioxidant compounds. For instance, Xu et al. (2015) reported that the antioxidant activity of *Jatropha integerrima* flower extracts increased significantly when ethanol concentrations ranged between 10 and 50% (v/v), while a decline in activity was observed between 50 and 90% ethanol (Xu et al., 2015). Given the well-established correlation between polyphenolic/flavonoid content and antioxidant activity, the trends observed in this study are consistent with previous research. This highlights ethanol concentration as a key parameter in optimizing the extraction of antioxidants from plant-based materials.

The TCC ranged from 0.28 to 1.97 mg β C/g (Table 1). For comparison, Maslov Bandić et al. (2023b) reported 0.54 mg β -carotene/g in the dry pulp residue of Satsuma mandarin. The correlation between TCC/TPC and TCC/TFC was moderate with $r_{TCC/TPC} = 0.42$ and $r_{TCC/TFC} = 0.32$. Figure 3 presents the 3D surface response plots, revealing that, unlike TPC and TFC, lower ethanol concentrations favor carotenoid extraction. Specifically, 30% ethanol provided an optimal medium for carotenoid recovery while maintaining compound integrity. This trend may be attributed to the ability of ethanol-water mixtures to create a stable environment for carotenoid solubilization and emulsification. The efficiency of carotenoid extraction can also be influenced by residual juice

present in the pulp. If the pulp is not fully exhausted during juice extraction, the remaining carotenoids present in the juice sac, a known reservoir of these compounds, may enhance overall recovery. Furthermore, as observed in Figure 3, higher ultrasound amplitude and longer extraction times positively influenced TCC yield. The combination of ethanol percentage, UAE duration, and amplitude underscores the complexity of carotenoid extraction. Considering that mandarin pulp is the residue of juice extraction and that the natural solvent in juice is water, we can conclude that lower concentrations of organic solvents are necessary to stabilize, emulsify and solubilize carotenoids.

As shown in Table 2, the experimental results under optimal extraction conditions closely matched the predicted values, with relatively small errors observed. Among the three responses, TPC exhibited the lowest deviation from the predicted values, while TFC and TCC showed slightly higher variations.

The optimal UAE conditions identified through the desirability approach involved using 31%, v/v ethanol, a 12-minute extraction time, and 65% ultrasound amplitude. These parameters maximize the yields of TPC, TFC and TCC while ensuring efficient extraction with minimal organic solvent use.

The developed models provide a reliable predictive framework for optimizing the UAE process, offering a valuable tool for enhancing the extraction efficiency of bioactive compounds from mandarin pulp using environmentally friendly solvents.

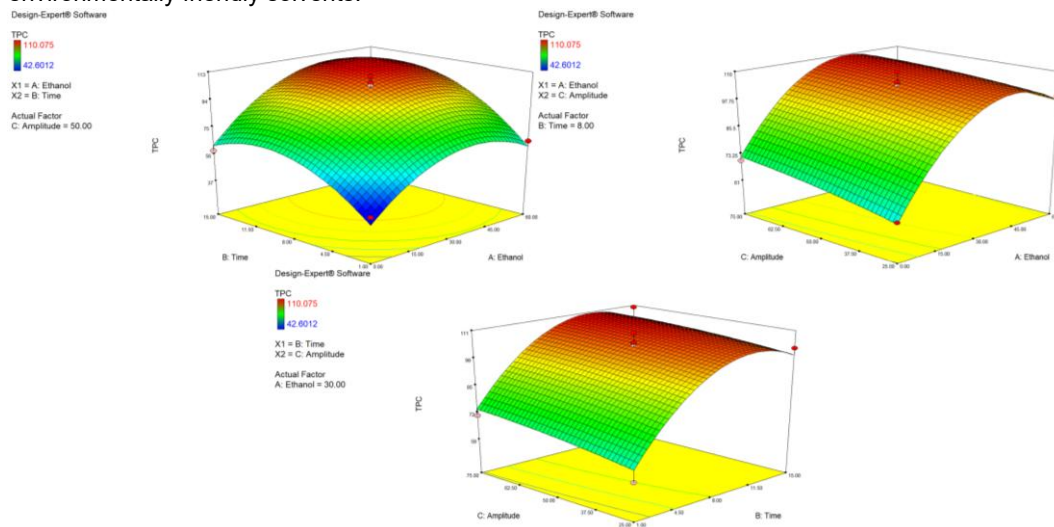


Figure 1. The 3D surface response plots of TPC as a relation of time/solvent, amplitude/solvent and amplitude/time

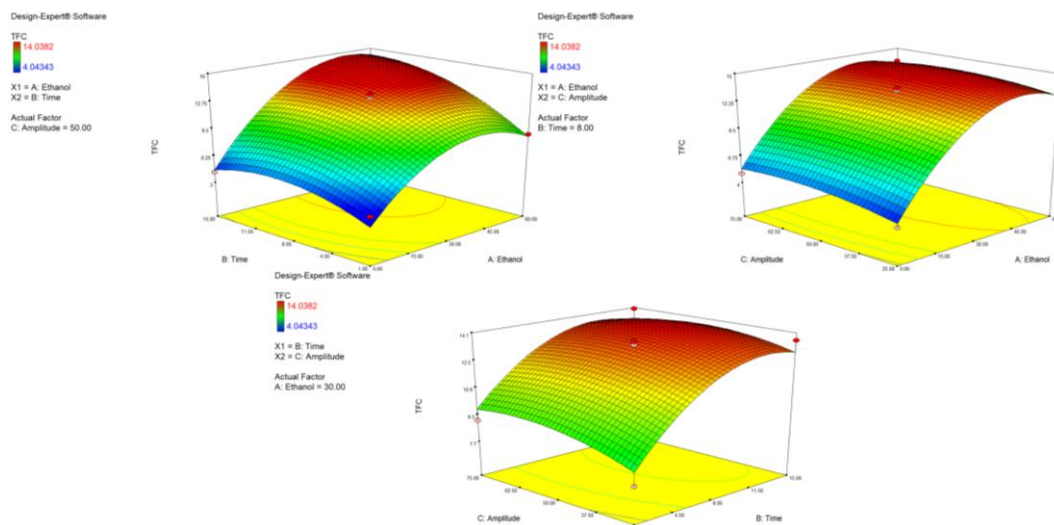


Figure 2. The 3D surface response plots of TFC as a relation of time/solvent, amplitude/solvent and amplitude/time

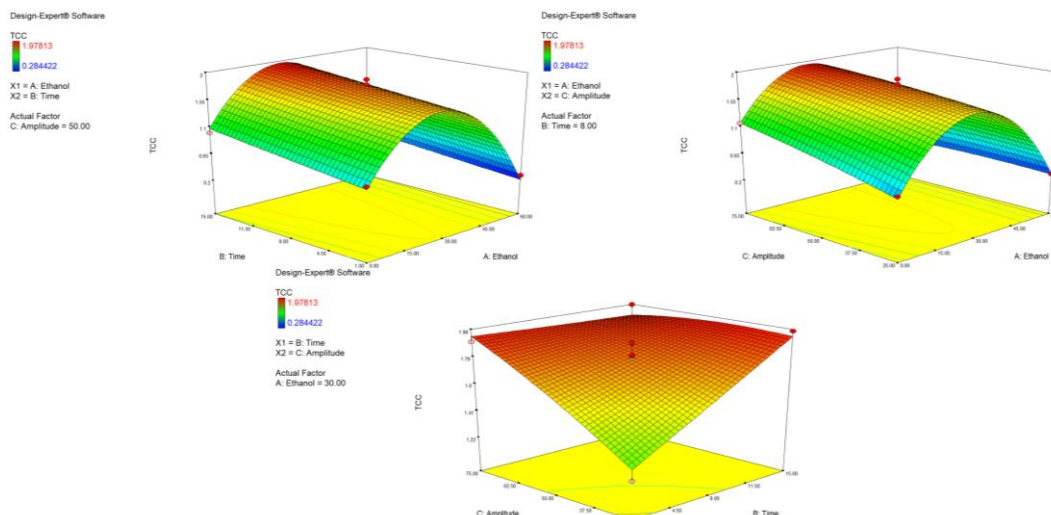


Figure 3. The 3D surface response plots of TCC as a relation of time/solvent, amplitude/solvent and amplitude/time

Table 2. The total polyphenolic content, total flavonoid content and total carotenoid content extracted from the freeze-dried mandarin pulp by using optimal conditions (31% ethanol, 12 min extraction time, and 65% amplitude, with maximum desirability of 0.983) with final equations in terms of actual factors

	TPC (mg GAE/g)	TFC (mg QE/g)	TCC (mg β C/g)
Predicted	110.08	14.04	1.90
Experimental	111.14 \pm 1.22	13.42 \pm 0.59	1.84 \pm 0.08
Error%	0.31	4.42	3.16
Final Equation in Terms of Actual Factors			
TPC	$+ 13.72385 + 1.90658 \times \text{Ethanol} + 7.92110 \times \text{Time} + 0.41687 \times \text{Amplitude} + 0.019026 \times \text{Ethanol} \times \text{Time} - 2.38830\text{E-}003 \times \text{Ethanol} \times \text{Amplitude} - 4.91094\text{E-}003 \times \text{Time} \times \text{Amplitude} - 0.023389 \times \text{Ethanol}^2 - 0.37032 \times \text{Time}^2 - 1.74096\text{E-}003 \times \text{Amplitude}^2$		
TFC	$- 1.51251 + 0.35267 \times \text{Ethanol} + 0.73537 \times \text{Time} + 0.12856 \times \text{Amplitude} + 6.12164\text{E-}003 \times \text{Ethanol} \times \text{Time} - 1.82385\text{E-}004 \times \text{Ethanol} \times \text{Amplitude} - 1.23771\text{E-}003 \times \text{Time} \times \text{Amplitude} - 4.29013\text{E-}003 \times \text{Ethanol}^2 - 0.035545 \times \text{Time}^2 - 9.92214\text{E-}004 \times \text{Amplitude}^2$		
TCC	$- 0.32835 + 0.077483 \times \text{Ethanol} + 0.069260 \times \text{Time} + 0.023728 \times \text{Amplitude} - 6.51673\text{E-}005 \times \text{Ethanol} \times \text{Time} - 1.77962\text{E-}004 \times \text{Ethanol} \times \text{Amplitude} - 9.39103\text{E-}004 \times \text{Time} \times \text{Amplitude} - 1.29420\text{E-}003 \times \text{Ethanol}^2 + 5.91563\text{E-}005 \times \text{Time}^2 - 4.90559\text{E-}005 \times \text{Amplitude}^2$		

HPLC analysis of the optimal extracts revealed narirutin and hesperidin yields of 101.81 \pm 0.67 mg/100 g and 1156.42 \pm 0.34 mg/100 g, respectively. Literature on flavonoid extraction from Satsuma mandarin pulp is limited, but Levaj et al. (2009) reported significantly lower values of 34.35 mg/100 g d.w. for narirutin and 55.97 mg/100 g d.w. for hesperidin. In an extensive study on 27 local citrus cultivars in China, hesperidin was identified as the dominant flavanone, with concentrations ranging from 2.73 to 415.59 mg/100 g d.w. in mandarin pulps (Chen et al., 2020). More recently, Maslov Bandić et al. (2023) reported narirutin levels between 489 to 918 mg/100 g and hesperidin levels between 1589 to 2476 mg/100 g across five Satsuma mandarin varieties. Variability in flavonoid content across studies can be attributed not only to differences in mandarin variety, environmental conditions, and cultivation locations but also to factors such as the level of pulp exhaustion during juicing, extraction techniques, and solvent composition. These findings highlight the importance of optimizing extraction parameters to maximize flavonoid recovery from mandarin pulp.

3. Conclusions

This study demonstrates that UAE is a promising and efficient method for extracting polyphenols, flavonoids and carotenoids from freeze-dried mandarin pulp. The synergy between acoustic cavitation and an optimized

water-to-ethanol solvent ratio enhanced matrix disruption, solubilization, stabilization and bioactive compound release, resulting in high extraction yields. These findings support the potential of UAE as a sustainable and effective approach for maximizing the recovery of valuable bioactive compounds from citrus processing byproducts.

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