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Research Article

Ultrasound-assisted extraction of phenolics from *Spirulina platensis* by different solvents: An optimization study based on simplex lattice mixture design

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Abstract: Spirulina has attracted attention in recent years because it is an important source of bioactive compounds such as phenolics. In this study, different solvents (acetone, ethanol, and distilled water) were evaluated in terms of their phenolic extraction performance from *Spirulina platensis* which is a popular functional nutraceutical. This was accomplished by performing optimization research and using the simplex lattice mixture design approach to identify the optimal solvent type or solvent mixture that may provide the highest phenolic yield. Fifteen extracts were prepared, and their total phenolic content was determined. After the data had been modeled, it was concluded that the solvent with the highest phenolic recovery performance was distilled water. Alone the water could extract more phenolics than acetone and ethanol or their mixtures at different ratios. Total phenolic contents of acetone, ethanol, and distilled water extracts were 9.26, 11.82, and 36.51 mg GAE/g samples showing that the water was the best. Having established water as the optimal solvent for *S. platensis* extraction, we investigated the influence of various pH conditions (3, 5, 7, and 9) on both conventional hot water extraction and ultrasound-assisted extraction methods. Results showed that higher pH values significantly increased total phenolic content, and ultrasound extraction yielded better results than traditional hot water extraction.

1. INTRODUCTION

Nowadays it is known that there is a close relationship between nutrition and the development of some diseases such as cancer, obesity, etc. (Ozawa *et al.*, 2021). This relationship has increased in demand for products with bioactive compounds. Among bioactive compounds, phenolics have an important position now that they have a protective mechanism in preventing various diseases by scavenging free radicals and can be used as a food antioxidant in lipid oxidation (Brewer, 2011).

Spirulina platensis, a blue-green microalga, contains several bioactive compounds such as phenolics, essential fatty acids, β-carotene, and α-tocopherol (Chopra & Bishnoi, 2007) and in recent years, researchers have focused on those compounds from *Spirulina platensis*, so it has

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attracted attention due to its antioxidant activity (Shalaby & Shanab, 2013; Wang *et al*., 2014). Moreover, it possesses antimicrobial activity against some pathogenic microorganisms in addition to their antifungal (Usharani *et al*., 2015), and therapeutic properties such as the hepatoprotective effect, neuroprotective ability, antitumor and anticancer functions (Nuhu, 2013), and antihyperglycemic activity (Gouda *et al*., 2015).

Also, it is an important source of protein, which includes between 50-70 %, and vitamins such as vitamin B12, and pro-vitamin A (Chopra & Bishnoi, 2007). Therefore, it may be incorporated into foods for fortification as a nutritional supplement. Santos *et al*. (2016) developed a chocolate-flavored shake powder containing Spirulina to meet the nutritional needs of elderly individuals. The study found that Spirulina biomass served as an effective nutritional supplement in the powdered food formulation.

Extraction method and solvent choice significantly affect Spirulina's bioavailability. Identifying the optimal solvent for maximizing phenolic yield is valuable for both consumers and the food industry. This study aimed to determine the most effective solvent for extracting phenolics from *S. platensis* using ultrasound-assisted extraction based on simplex lattice mixture design, while also examining how pH values influence the total phenolic content in the resulting extracts.

2. MATERIAL and METHODS

2.1 Material

S. platensis powder was purchased from Algbiotek Co. (İstanbul, Turkiye). Total protein, total carbohydrate and total lipid content of the provided Spirulina sample were 66.6, 16.6 and 5.0 g/100 g sample, respectively.

2.2. Methods

2.2.1. *Extraction of the samples*

To determine the different solvent effects on the bioactivity of the sample, three different solvents such as acetone, absolute ethanol, and distilled water were used for the extraction of *S. platensis* powder. To prepare the *S. platensis* extract, a design approach of simplex lattice mixture was followed to determine the best solvent type or solvent mixture to obtain the highest phenolic yield from the sample. For this purpose, the mixture design tabulated in Table 1 was followed to prepare the solvent system. After the preparation of solvents to be used in the extraction, 0.5 g of *S. platensis* powder was weighed, and 10 mL of related solvent system was incorporated in a screw tap test tube. After that, the sample was mixed for 1 min using a vortex. In the end, the extraction process for 15 samples was started in an ultrasonic water bath (Kudos, (Shanghai Kudos Ultrasonic Instrument Co, Ltd., Shanghai, China). The frequency and amplitude level were set up to be 53 kHz and 50%, respectively. The temperature of the water bath for classical and ultrasonic-based extraction was 60 ºC and the extraction time was 30 min. After the samples were kept for 30 min in a water bath, they were removed from the bath and cooled down to room temperature. After that, the samples were centrifuged (Hettich, Zentrifugen, Tuttlingen, Germany) at 6000 rpm for 10 min at room temperature. After the phase separation, the supernatant part was filtered by using a syringe filter (0.45 µm, 0.45 µm, Millipore, USA), and the filtered extract was used for further analysis.

2.2.2. *Determination of total phenolic content of the extract*

The total phenolic contents (TPC) of the sample extracts were determined according to the modified method described by Singleton and Rossi (1965). Briefly, 200 µL of the *S. platensis* extract and 1.8 mL of distilled water were added into a tube and homogenized with vortex for a short time after incorporation of 1 mL of Folin Ciocelteau's phenol reagent (diluted as 1:10 with distilled water). The final solution was obtained by the addition of 2 mL of Na_2CO_3 solution (2% w/v). Then the tubes were incubated for 2 h in the dark at room temperature. Following the incubation period, the absorbance of the samples was measured at 760 nm using a spectrophotometer (8453E UV-Vis, Spectroscopy System, Agilent, USA). TPCs of the

samples were calculated as mg gallic acid equivalents (GAE) per g of sample. The measurements were repeated two times with four replications.

Table 1. Components of the solvent system prepared according to simplex lattice mixture design.

	Coded values			Uncoded values (Ingredient proportions)		
Mixtures	X_1	X_2	X_3	Acetone (%)	Ethanol (%)	Water (%)
1	0.33	0.33	0.33	33	33	33
2	0	0.5	0.5	0	50	50
3	0.5	0	0.5	50	0	50
4	0	1	0	0	100	0
5	0	0	1	0	0	100
6	0	1	0	0	100	0
7	1	0	0	100	0	0
8	0	0	1	0	0	100
9	0.166	0.666	0.166	17	67	17
10	1	0	0	100	0	0
11	0.166	0.166	0.666	17	17	67
12	0.5	0.5	0	50	50	0
13	0.5	0.5	0	50	50	0
14	0.666	0.166	0.166	67	17	17
15	0.5	0	0.5	50	0	50

2.2.3. *Determination of pH effect on the phenolic yield of S. platensis*

To determine the pH effect on the phenolic yield and so, total phenolic content of *S. platensis*, four different pH values were selected (pH 3, 5, 7, and 9). The extraction solvent was distilled water, and the pH values of the extraction solvent were adjusted using the proper buffer system Na_2HPO_4 and NaH_2PO_4. To be sure that the pH values were adjusted to be correct, the final pH values were checked by a calibrated pH-meter (WTW, Germany) and the final correct and sensitive pH value was set up to the aimed pH values by using 0.1 N HCl or 0.1 N NaOH. The extraction procedure was the same as stated above for all pH values. Two different extraction processes were followed for the phenolic recovery classical hot water bath and ultrasonic water bath. For the classical water bath extraction process, the temperature of the water was 60 ºC and the time was set to be 30 min with the shaking at 50 rpm. For the ultrasonic water bath system, the frequency and amplitude were set to 53 kHz and 50%. The temperature and time for the extraction were 60 ºC and 30 min, respectively. After the extraction process, they were removed from the bath and cooled down to room temperature. After that, the samples were centrifuged (Hettich, Zentrifugen, Tuttlingen, Germany) at 6000 rpm for 10 min at room temperature. After the phase separation, the supernatant part was filtered by using a syringe filter (0.45 µm, Millipore, USA), and the filtered extract was used for total phenolic content analysis.

2.2.4. *Experimental design and optimization*

In the present study, the simplex lattice mixture design (SLMD) was used to evaluate the effect of different solvent types acetone (A), ethanol (B), and distilled water (C) on the total phenolic content of the *S. platensis*. Component proportions in the solvent blends were expressed as fractions of the mixture with a sum (A+B+C) of one. These three factors; namely, acetone, ethanol, and distilled water (processing components), levels, and experimental design in terms of coded and uncoded as 15 combinations are presented in Table 1.

Multiple linear regression analysis approach was used in the modelling. The following second-order polynomial equation of function xi was fitted for each factor assessed at each experimental point.

$$Y = \sum_{i=1}^{3} \beta_i x_i + \sum_{\substack{i=1 \\ i<j}}^{3} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j$$
$$= \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (1)$$

where Y was the estimated mixture response; β1, β2, β3, β12, β13, and β23 were linear and interaction terms, respectively, produced for the prediction models of processing components. Predictive models were constructed to evaluate the effect of mixture components on the characterized properties of blended samples. The best-fitting models were determined using multiple linear regressions

2.2.5. *Statistical analysis*

Design-Expert version 7.0 (Stat-Ease Inc., Minneapolis, USA) and JMP version 9.0.2 (SAS Institute, Inc., Cary, USA) were used for the computational work including designation of experimental points, randomization, and fitting of the second-order polynomial models as well as optimization. Analysis of variance (ANOVA) was performed using the JMP version 5.0.1 (SAS Institute, Inc., Cary, USA).

3. RESULTS and DISCUSSION

3.1. Effect of Solvent Type on The Phenolic Yield

In this study, three different solvents acetone, ethanol, and distilled water having quite different polarities were evaluated for the extraction of the phenolic substances from *Spirulina platensis*, which is a commonly consumed and very popular microalgae in the world. To determine the best solvent system or solvent mixtures to be used for the extraction of phenolics from the structure of the algae, a simplex lattice mixture design, which presents an opportunity to show the optimum levels for the processing variables, was used. Extracts were obtained from *S. platensis* by 15 solvent systems, and the total phenolic content of the extracts was determined and tabulated in Table 2. The total phenolic contents of the prepared extracts were in the range of 8.69-36.51 mg GAE/ g sample. The highest total phenolic content was in the extract of run 5 (water) and the lowest was determined in the extract prepared according to run 10 (acetone) in the simplex lattice mixture design. The acetone showed the lowest phenolic extraction yield compared to sole ethanol and distilled water and the highest phenolic yield obtained by the extraction of *S. platensis* was recorded for distilled water (Table 2).

According to Table 2, the solvent mixture prepared by the addition of three solvents at equal proportions showed quite lower total phenolic content compared to the extract obtained by distilled water. Statistical analysis showed that all linear effects of mixture variables (acetone, ethanol, and distilled water) showed a significant effect on the total phenolic content of *S. platensis*. The constructed model for the determination of the best solvent system to obtain the maximum phenolic yield from *S. platensis* was also found to be significant (p<0.01) which means that the model selection was perfect, and the estimation could be performed effectively. The interactive effect of acetone-ethanol was insignificant (p>0.05) on the phenolic yield while the interactions between acetone-water and ethanol-water were determined to be very significant (p<0.05) on the total phenolic content of the algae sample (Table 3). The mean for the total phenolic content of all extracts was calculated to be 16.24 mg GAE/g sample while the coefficient of variation was 10.49, which shows that the recorded data range was within acceptable limits.

The calculated coefficient of determination (R^2) was found to be 0.976 showing that the constructed regression equation could be effectively used to estimate the total phenolic contents of extracts obtained from different solvents mixture prepared by acetone, ethanol, and distilled water. It was also observed that the calculated “pred R-squared of 0.948 was in reasonable agreement with the “Adj R-squared of 0.963. Also, the value of Adeq precision, which measures

the signal to ratio was determined to be quite higher (25.08) indicating an adequate signal showing the model, can be used to navigate the design space (Table 3).

Table 2. Change in total phenolic content (TPC) of *S. platensis* depending on the solvent systems.

Mixtures	Acetone (%)	Ethanol (%)	Water (%)	TPC (mg GAE/g)
1	33	33	33	11.26±1.21
2	0	50	50	17.46±1.85
3	50	0	50	12.61±0.85
4	0	100	0	11.82±1.11
5	0	0	100	36.51±2.65
6	0	100	0	12.60±1.25
7	100	0	0	09.26±0.89
8	0	0	100	35.21±2.36
9	17	67	17	13.84±0.96
10	100	0	0	08.69±0.54
11	17	17	67	24.58±2.24
12	50	50	0	12.18±1.05
13	50	50	0	11.97±1.09
14	67	17	17	12.69±1.12
15	50	0	50	12.94±1.87

Figure 1 shows the relationship between the phenolic yield and solvent type. It is clearly seen from the figures that the correlation coefficient between the total phenolic content and acetone level in the solvent system was calculated to be negative (r=-0.585). Similarly, a negative correlation (r=-0.319) was determined between the total phenolic content and ethanol level in the solvent system. Only a positive and quite significant correlation (r=0.889) was observed between the phenolic yield and distilled water level in the solvent system. It is seen from Figure 1 that the total phenolic levels are determined to be high levels in the extracts obtained by the solvent system having high levels of water and low levels of acetone and ethanol. The exact change of total phenolic content of the *S. platensis* depending on the solvent type in the extraction solvent system was illustrated in Figure 2 as a 3D and contour graphic.

Figure 2 shows the increase and decreases of the total phenolic content of the sample depending on the solvent type. As we found, distilled water was the best solvent for extracting the total phenolic content from *S. platensis* compared to acetone and ethanol. Total phenolic content increased significantly (p<0.05) and tremendously towards the vertex of the water on the contour. Shalabay and Shanab (2013b) investigated the potential antioxidant activity of water and methanolic extracts of *S. platensis* and concluded that the water extract of *S. platensis* recorded higher antiradical and antioxidant activity, and they attributed this activity to the phycobiliprotein pigments. It is a water-soluble colored pigment found in cyanobacteria, and has anti-inflammatory, anti-oxidative, and anti-cancer activities (Wang *et al*; 2023). Potent antiradical activity of phycobiliprotein was reported by different researchers in the literature (Bryant 1979; Miranda *et al*., 1998; Shalaby *et al*., 2010). Our findings were in agreement with the findings of Goiris *et al*. (2012). They reported the highest phenolic yield in hot water extracts followed by the ethyl acetate fractions and hexane fractions. In addition, this high phenolic content of water extract was attributed to the polar nature of the phenolic compounds. Hajimahmoodi *et al*. (2010) also reported the highest phenolic content of some strains of microalgae in the water fraction.

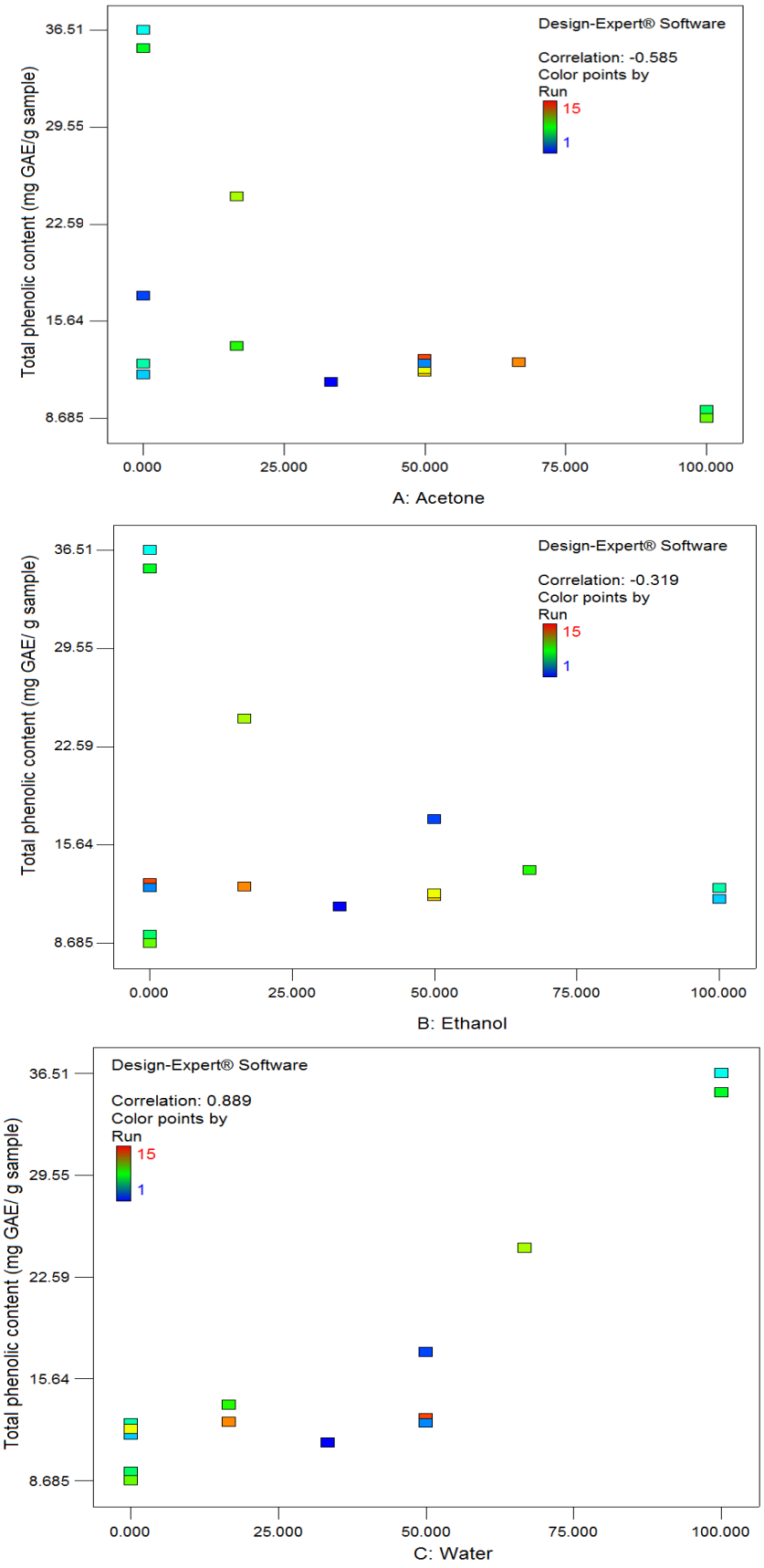

Figure 1. Relationship between the solvent type and total phenolic content.

Table 3. ANOVA table for the simplex lattice mixture design.

Source	Sum of Squares	*df*	Mean Square	*F* Value	*p*-value Prob> *F*	
Model	1056.2	5	211.24	72.77	< 0.0001	
Linear Mixture	888.4	2	444.19	153.02	< 0.0001	
AB	5.2	1	5.22	1.80	0.2126	
AC	123.5	1	123.50	42.54	0.0001	
BC	32.9	1	32.92	11.34	0.0083	
Residual	26.1	9	2.90			
Lack of Fit	24.7	4	6.18	22.10	0.0022	
Pure Error	1.4	5	0.28			
Cor Total	1082.3	14				
Std. Dev.						1.70
Mean						16.24
C.V.						10.49
R-squared						0.976
Pred R-squared						0.963
Adj R-squared						0.948
Adeq Precision						25.08

A: Acetone, B: Ethanol C: Distilled water, C.V: coefficient of variation.

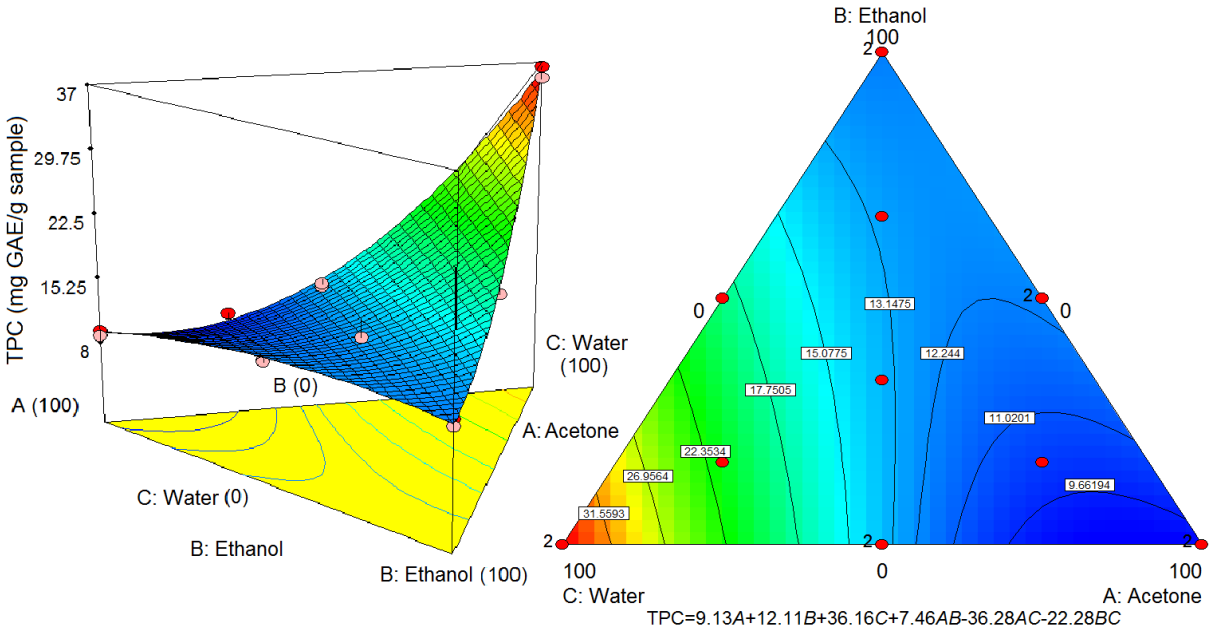

Figure 2. Change in total phenolic content of *S. platensis* depending on the solvent type.

3.2. Determination of the Best Solvent to Increase Phenolic Yield

Figure 3 illustrates the optimization results for selecting the most effective solvent for phenolic extraction from *S. platensis*. The analysis revealed that distilled water alone yielded maximum phenolic content, achieving a 36.51 mg GAE/g sample. This finding demonstrated high reliability with a desirability value of 0.987. A minimization procedure applied during optimization confirmed acetone's negative impact on phenolic yield, as shown in Figure 3. The bottom portion of the figure demonstrates that using acetone alone as an extraction solvent resulted in the lowest total phenolic content (9.13 mg GAE/g sample). High desirability values for this optimization indicate strong agreement between experimental and estimated phenolic content values.

3.3. Effect of pH on Phenolic Recovery from *S. platensis*

After the determination of the best solvent for the extraction of phenolics from *S. platensis*, *the* effect of different pH values on the recovery of total phenolic substance was investigated to show the best pH values for the high phenolic extract yield. For this purpose, four different pH values were used (pH 3, 5, 7, and 9) to see the clear effect of acidity on the extraction of phenolic substance. Also, two different extraction processes classical hot water bath and ultrasound water bath were used for the extraction. Only pure distilled water was used for all analyses as extraction solvent. Figure 4 shows the differences between the total phenolic content of *S. platensis* at four different pH values and two different extraction processes. For both extraction processes, an increase in pH value increased the total phenolic content of the samples showing that the yield was affected clearly by the acidity of the solvent system (p<0.05). The total phenolic content of the extract obtained by the distilled water at pH 3 by classical hot water extraction system was calculated to be 5.78 mg GAE/g sample while the total phenolic content was found to be 47.22 mg GAE/g sample for the extract obtained by distilled water at pH 9.

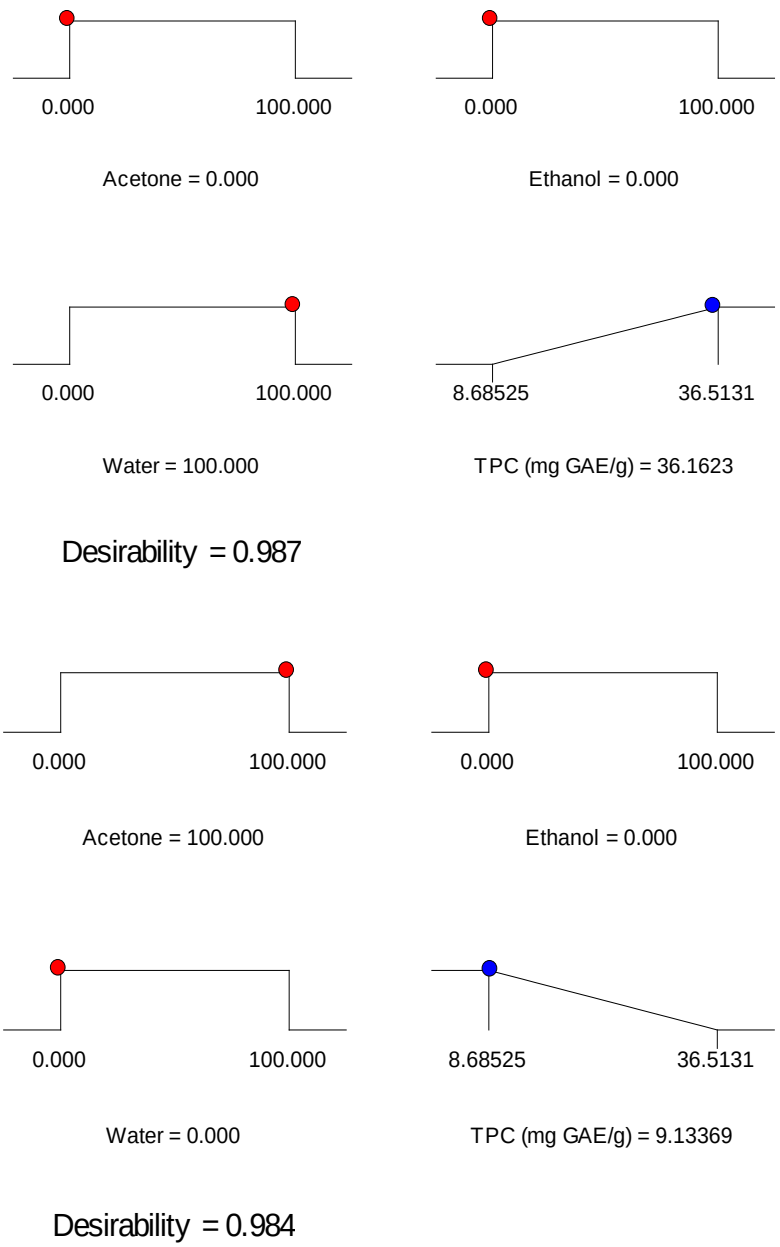

Figure 3. Best solvent type depending on the optimization procedure.

The increase was observed to be significant (p<0.05). For the ultrasound-assisted extraction, total phenolic content also increased with the increase in pH value. The highest total phenolic content was determined to be 48.69 mg GAE/g sample for pH 9 while the lowest total phenolic content was 7.00 mg GAE/g sample for pH 3 for the samples exposed to ultrasound-assisted extraction.

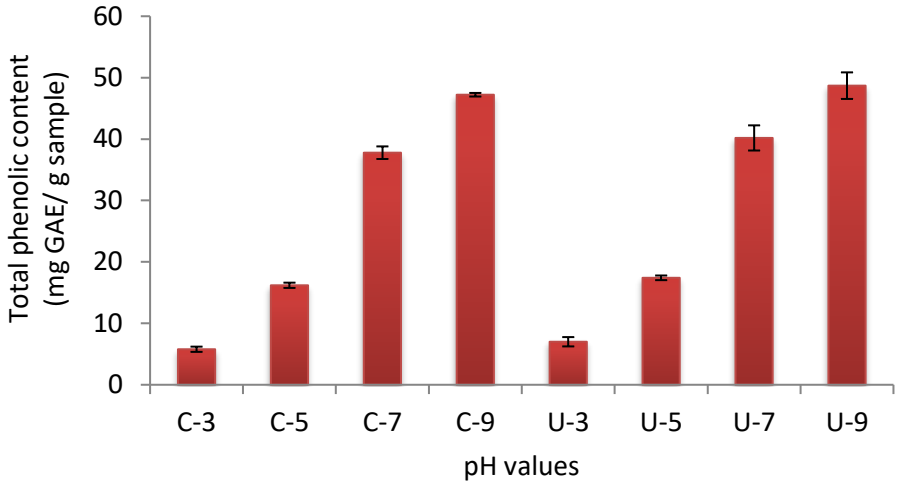

Figure 4. Effect of extraction type and pH value on total phenolic content of *S. platensis.*

The ultrasound creates bubbles in the solution because of compression and expansion (Carreira-Casais *et al*., 2021). When the formed bubbles burst due to the pressure difference, the explosion creates the microjet, an increase in temperature and pressure (Chemat *et al*., 2017). When this occurs near the plant cell wall, the cell wall is destroyed and thus the target compounds pass into the solvent, thereby increasing the yield of the target substance (Goswami *et al*., 2024). In this study, the total phenolic content of *S. platensis* using ultrasound-assisted extraction is significantly higher than the classical hot water extraction value (p<0.05).

pH affects the amount and type of phenolic substances to be extracted, especially anthocyanins are more stable in acidified organic solvents compared to non-acidified ones (Ekici *et al*., 2014). On the other hand, some phenolics may dissolve better in neutral or basic media (Honda *et al*., 2019). The results obtained in this study indicate that phenolic substances in Spirulina are sensitive to pH changes. Phenolics in spirulina lose their activity when the ambient conditions are acidic, while they maintain their activity under neutral and basic conditions.

Guldas *et al*. (2020) investigated the antioxidant and anti-diabetic effects of *S. platensis*. The researchers determined the phenolic substance composition of Spirulina and found that the highest amounts were acacetin (35.37 µg/100 g), pinocembrin (27.23 µg/100 g) followed by sakuranetin (0.78 µg/100 g), luteolin (0.68 µg/100 g), respectively. Acacetin, a flavonoid found in more than 200 plants, has pharmacological activities such as antimicrobial activity, anti-inflammatory effect, and antiproliferative activity, and can be used in the treatment of some neurological diseases such as Alzheimer's disease (Singh *et al*., 2020). Acacetin has been reported to retain 16-36% of its initial amount of activity after 24 hours at pH 1-7, and more than 85% of its activity at pH 9-13 (Han *et al*., 2021). It could be, therefore, better soluble in basic media. Pinocembrin, another flavonoid compound found in high amounts in spirulina, also has pharmacological activity such as antimicrobial, antioxidant, anticancer, and anti-inflammatory activity (Elbatreek *et al*., 2023) and has a low basic character (Shen *et al*., 2022). When solubility in three different pH media (pH 1.2, 6.8, 7.4) was compared, the best solubility was found at 7.4 pH (17.15 µg/mL, 27.49 µg/mL, 50.34 µg/mL).

Another factor for the basic character of phenolics in spirulina may be picocyanin. Picocyanin is a water-soluble pigment-protein complex that produces a blue color solution when dissolved in water (Li *et al*., 2020) and exhibits antioxidant activity (Gabr *et al*., 2020). Li *et al*. (2020)

optimized the extraction of picocyanin and investigated the stability of picocyanin at pH 6-8. According to the results of the study, pH 7.5 was the value with the highest picocyanin yield, but at the end of 4 days, the highest stability was determined at pH 6.0 and 6.5 (Li *et al*., 2020). Vali Aftari *et al*. (2015) found that pH and extraction time affect the purity and amount of phycocyanin. In their study, the highest amount of phycocyanin was obtained at pH 7 (pH range 5-8). Our findings are consistent with these previous studies.

Spirulina is an important source in terms of total phenolic matter content. The amount of total phenolic substances extracted from spirulina with water at the optimum point was higher than those of blackberries (fresh), mulberries (lyophilised powder) (Haminiuk *et al*., 2012) but lower than those of defatted mango and tamarind (seed) (Soong *et al*., 2004). The phenolic composition of the extract with water in this study should be determined by further studies.

4. CONCLUSION

It was concluded that the total phenolic yield of the *S. platensis* was significantly affected by the solvent type. The simplex lattice mixture design approach which is an efficient optimization technique showed that distilled water is an effective solvent to extract the phenolic substance from *S. platensis* compared to acetone and ethanol. The constructed regression model showed that the total phenolic content of *S. platensis* could be estimated depending on the solvent level in the solvent mixture due to quite high coefficient of determination. The effect of pH on the recovery of phenolic substance was determined to be significant and increment in the pH values which means low acidity increased the total phenolic content of the extract in both the extraction process and the total phenolic content of extract obtained by ultrasound water bath was determined to be slightly higher compared to classical hot water bath extraction process. According to the results, it could be suggested that *S. platensis* has important bioactivity, it is recommended to use distilled water in the extraction process since the number of phenolic substances to be extracted is higher compared to ethanol and methanol used in this study.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Safa Karaman: Conception, Materials, Data collection and processing, Analysis and Interpretation. **Göktürk Öztürk**: Methodology, Analysis and Interpretation and Writing, original draft preparation, editing.

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