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ULTRASOUND-ASSISTED ENZYMATIC EXTRACTION OF ANTIOXIDATIVE PROTEIN EXTRACTS FROM *SARGASSUM VULGARE*: OPTIMIZATION OF EXTRACTION PARAMETERS USING RSM

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ABSTRACT

In this study, extraction conditions of proteins from *Sargassum vulgare* were optimized. The Box-Behnken design (BBD)-based Response Surface Methodology (RSM) was used to investigate and optimize the protein content (PC), total phenolic content (TPC), and antioxidant activity (AOA), which were affected by extraction parameters (ultrasonic probe time: 0.09-2.91 min and enzyme/substrate ratio (E/S): 0.18-1.02). The optimal extraction was achieved while applying an ultrasonic probe for 2.5 min and using an E/S of 0.90. Under this optimum conditions PC and TPC were found to be as 248.30 mg protein/g dry weight (dw) and 38.03 mg gallic acid equiavalent (GAE)/g dw, respectively. Moreover, AOA was determined to be 53.77 mg Trolox equivalent (TE)/g dw by CUPRAC and 19.88 mg TE/g dw by ABTS methods. These findings provide a good basis for future research into the potential of macroalgae protein extracts, which have a high protein content and antioxidant potential for food industry.

Keywords: *Sargassum vulgare*, macroalgae, extraction, protein extract, antioxidant activity

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ULTRASON DESTEKLİ ENZİMATİK YÖNTEMLE *SARGASSUM VULGARE*'DEN ANTİOKSİDAN PROTEİN EKSTRAKSIYONU: RSM İLE EKSTRAKSİYON PARAMETRELERİNİN OPTİMİZASYONU

ÖZ

Bu çalışmada, *Sargassum vulgare*'den proteinlerin ekstraksiyon koşulları optimize edilmiştir. Ekstraksiyon parametrelerinin (ultrases prop süresi: 0.09-2.91 dk ve enzim/substurat oranı (E/S): 0.18-1.02), protein miktarı (PM), toplam fenolik madde miktarı (TFMM) ve antioksidan aktivite (AOA) üzerine etkisini araştırmak ve optimizasyon çalışmalarını gerçekleştirmek için Box-Behnken Tasarım-Yanıt Yüzey Metodolojisi kullanılmıştır. Optimum protein ekstraksiyon koşulları, 2.5 dk ultrases prop uygulama süresi ve 0.90 E/S oranıdır. Optimum ekstraksiyon koşullarında, PM ve TFMM sırasıyla 248.30 mg protein/g kuru madde (km) ve 38.03 mg gallik asit eşdeğeri (GAE)/g km olarak bulunmuştur. Ayrıca AOA, CUPRAC yöntemi ile 53.77 mg Trolox eşdeğeri (TE)/g km ve ABTS yöntemi ile 19.88 mg TE/g km olarak belirlenmiştir. Bu bulgular, yüksek protein miktarı ve antioksidan aktivitesiye sahip makroalg protein ekstraktlarının gıda endüstrisi için potansiyelini araştıracak yeni çalışmalara bir temel oluşturabilir.

Anahtar kelimeler: *Sargassum vulgare*, makroalg, ekstraksiyon, protein ekstraktı, antioksidan aktivite

INTRODUCTION

Macroalgae are considered a viable source of protein with an essential amino acid composition, and their use for protein synthesis has several advantages over the traditional use of protein-rich plants in terms of productivity and nutritional content (Taboada, 2010; Sirbu, 2019; Bleakley and Hayes, 2017). Alternative sources and techniques for protein production are needed to meet consumer demand and the projected global protein demand (Bleakley and Hayes, 2017). Numerous health benefits of brown algae are attributed to their protein hydrolysates and bioactive peptides for the control, treatment, and risk reduction of degenerative and chronic diseases (Alvarez-Vinas, 2021).

The brown seaweed *Sargassum vulgare* is a member of the *Phaeophyceae* family, which includes many species found in both tropical and temperate waters worldwide. Shallow waters and coral reefs are the most important habitats for these algae (Karkhane et al., 2020; Mahmoud et al., 2019). It is known that the total protein content of *S. vulgare* varies greatly depending on the growth environment and ranges from a relatively low to a high content (10-15% dry weight) (Field et al., 2017). On the other hand, *S. vulgare* contains vital components such as polyphenols, carotenoids, vitamins, unsaturated fatty acids, and free amino acids (Karkhane et al., 2020). The stimulating effect of the macroalgae extract could be related to all these different substances contained in the extract (Mahmoud et al., 2019; Khan et al., 2009). *S. vulgare* is widely distributed along the Mediterranean coasts, but this species originating from Türkiye has not been studied yet.

The extraction of algal proteins has received less research attention than that of proteins from other plants. The traditional methods for extracting algal proteins are aqueous, acidic, and alkaline (Bleakley and Hayes, 2017). One of the novel extraction techniques is ultrasound-assisted extraction (UAE), which produces a final product with higher purity while reducing the need for downstream processing due to its fast-processing time, non-thermal properties, and minimal solvent consumption (Bleakley and Hayes, 2017). The sonicated liquid and its components are chemically excited by the violent implosion of the bubbles formed by UAE, resulting in the formation of microscopic zones of high pressure and temperature. This facilitates degradation of the target compound and disruption of the particles (Mason et al., 1996).

In this study, the UAE of proteins from *S. vulgare* collected from the Mediterranean coast of Türkiye was described. The extraction conditions were optimized using response surface methodology (RSM). The objectives of the study are (i) to establish a protocol for the extraction of *S. vulgare* protein extract (SVPE) with high protein

content; (ii) to optimize the conditions for the enzymatic UAE of proteins from *S. vulgare* in terms of protein content (PC), total phenolic content (TPC), and antioxidant activity (AOA); and (iii) to compare protocols for the extraction process with different extraction time, sonication time, and amounts of added enzyme (hemicellulose). Within our knowledge, this is the first study in the literature detailing the extraction procedures of protein extracts from *S. vulgare* from the Türkiye seas using RSM.

MATERIAL AND METHOD

Collection and preparation of algae

Sargassum vulgare was collected on the Aegean coast of Türkiye (coordinates: 40°1'35.90 "N and 26°19'49.49 "E). The collected algae samples were prepared for analysis according to the procedures of Bozdemir et al. (2022). The dried and pulverized algae, which had a 8% moisture content and a particle size of less than 500 μm, were carefully packed to protect them from light and air and stored at -20 °C for further analysis.

Chemicals

The phenol reagent Folin-Ciocalteu was purchased from Merck (Merck, Darmstadt, Germany). The hemicellulase (HSP 50000) supplied from Bakezyme. All the other chemicals and solvents were obtained from Sigma-Aldrich (Sigma-Aldrich Chemie, St. Louis, Missouri, USA). All of the solvents and chemicals used in this study were of the analytical grade.

Ultrasound-assisted enzymatic protein extraction

To extract proteins from *S. vulgare*, a combination of ultrasound pretreatment and carbohydrase addition was used as described by Bozdemir et al. (2022) based on the design of the experiment (Table 1). In brief, 0.5 g algae sample was mixed with 50 mLcitrate buffer (0.1 N, pH 4.5). The suspension sonicated with an ultrasonic probe (53 kHz and 65% amplitude) (Sonopuls HD 2200, Bandelin Electronic GmbH & Co. KG, Berlin, Germany) at ~25 ºC for the given time periods under RSM settings. Hemicellulase was then added to the mixture and kept in shaking water at 1 g-force and 35 °C for 24 h. Finally, the samples were kept in the shaking water bath at 85°C for 10 min for enzyme inactivation. At the end of the procedures, the samples were centrifuged at 4100 rpm, 4 °C, 15 min and the supernatant (*S. vulgare* protein extract) was collected, then stored in a dark place at -20 °C until further analysis.

Table 1: Actual and coded levels of independent variables for central composite design.

Independent variables for extraction of SVPE*	Codded levels				
	-α	-1	0	1	+α
X_1; Ultrasound probe time (min)	0.09	0.50	1.50	2.50	2.91
X_2; Enzyme/substrat ratio	0.18	0.30	0.60	0.90	1.02

*: SVPE: *Sargassum vulgare* protein extracts.

Determination of protein content

The protein content of *S. vulgare* extract was determined using a modified Lowry method (trichloroacetic acid (TCA)-Lowry). In this method, the proteins are separated from the samples using TCA in order to eliminate possible interfering substances (Moein et al., 2015). Using the method of Lowry et al. (1951), the protein content of the extracts was determined by using UV spectrophotometer (Scilogex, USA). A standard curve had a linear equation (R^2= 0.99) was generated using bovine serum albumin (BSA) at concentrations ranging from 0.05 to 0.1 mg/mL. The protein content was expressed as milligrams per gram of sample dry weight (dw) using bovine serum albumin as a standard.

Total phenolic content (TPC)

The TPC of the SVPE was determined according to the Folin-Ciocalteu's method (Toor and Savage, 2006) as described by Bozdemir et al (2022). The samples' absorbance was measured at 765 nm by using the UV spectrophotometer. The TPC was computed using a linear equation (R^2= 0.99) derived from a calibrated curve at five different point including 0.01 to 0.1 mg/mL, with gallic acid functioning as the standard. The results

are expressed in mg of gallic acid equivalents (GAE) per gram of dw.

Antioxidant activity (AOA)

The cupric reducing antioxidant capacity (CUPRAC) method

The CUPRAC assay was carried out in accordance with the method of Apak et al. (2005), as described in our previous study (Bozdemir et al., 2022). Using the UV spectrophotometer, the absorbance of the samples was determined at 450 nm. Trolox prepared at 5 different points ranging from 0.2 to 0.01 was utilized as a standard on the calibration curve (R^2= 0.99). The Trolox equivalent (TE) in milligrams per gram of dry weight was used to express the results.

2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) method

The ABTS assay was performed according to Miller and Rice-Evans (1997), as described in our previous study (Bozdemir et al., 2022). The absorbance was measured at 734 nm and results were given in mg TE/g dw. Trolox prepared at 5 different points ranging from 0.2 to 0.01 was utilized as a standard on the calibration curve (R^2= 0.97).

Experimental design and statistical analysis

The extraction conditions are optimized by the application of RSM. The effects of two independent variables (ultrasonic probe time and enzyme/substrate ratio) for optimization at 5 levels (-α, -1, 0, 1. +α) were investigated using Central Composite Design (CCD). In the present study, ultrasonic probe time (0.5-2.5 min) and enzyme/substrate ratio (0.30-0.90), coded as X_1 and X_2, were chosen as independent variables. The experimental design consists of 13 (run) conditions comprising five center points, four factorial points, and four axis points.

As shown in Table 1, the parameters for extraction were standardized as coded variables. Response functions (Y) were PC (mg protein/g dw sample), TPC (mg GAE/g dw sample) and AOA (mg TE/g dw sample). Surface response with a second-order polynomial was used to determine the relationship between the independent factors and the response (Tekin et al., 2015). The mathematical model used for the bivariate central composite design is given in Equation 1.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{12} X_1 X_2 \quad (1)$$

The regression coefficients for the second-order polynomial model are as follows: β_0 represents the constant term, β_i represents a linear effect, β_{ii} represents a quadratic effect and βi_j represents an interaction effect. The fit of the model was assessed using statistical significance analysis of variance and regression coefficients. Surface responses and contour plots of the polynomial regression equations to visualize the relationship between the responses and the independent variables and optimum conditions for the target responses were obtained using the trial version of Design Expert 7.1 software (Stat-Ease, Inc., USA). The results were statistically tested at the statistical significance level p=0.05. The fit of the model was determined based on the model analysis, the coefficient of determination (R^2), and the model error. Mathematical models were created to describe the interaction effects of a single parameter and/or multiple parameters on each response studied.

Validation of model for the optimum conditions

Protein extraction was carried out using the optimum extraction conditions given by RSM, and the predicted and actual values were compared.

RESULT AND DISCUSSION

Fitting model

13 combinations of two independent variables (ultrasonic probe time and enzyme/substrate ratio) were used to determine the protein, phenolic and antioxidant content of SVPEs and the results were given in Table 2. Table 3 shows the analysis of variance and model coefficients (R^2) for each dependent variable. The *P*-value was used to calculate the significance of each coefficient. The most significant factor is the enzyme/substrate ratio (P<0.05). Previous studies showed a comparable result (Liadakis et

al., 1995; Morais et al., 2015; Yucetepe et al., 2022). In addition, the interaction effect of E/S ratio and ultrasonic probe time was significant ($P< 0.05$).

Table 2. Box–Behnken experimental design with natural and coded extraction conditions and experimentally obtained values of all investigated responses.

	Independent variables					Responses			
	Run	A: Ultrasound application time (min)		B: Enzyme/ substrate		PC (mg protein/g extract, dw)	TPC (mg GAE/g extract, dw)	CUPRAC (mg TE/g extract, dw)	ABTS (mg TE/g extract, dw)
	1	1.50	0	1.02	+α	248.53	41.57	55.01	16.98
	2	1.50	0	0.18	-α	34.92	20.79	37.60	19.90
	3	1.50	0	0.60	0	147.00	31.97	53.66	20.62
	4	2.91	+α	0.60	0	143.10	32.86	52.63	19.70
PC:	5	1.50	0	0.60	0	146.70	32.42	47.35	17.55
	6	1.50	0	0.60	0	152.50	33.06	49.90	18.94
	7	0.50	-1	0.90	1	194.11	37.62	53.20	12.60
	8	1.50	0	0.60	0	161.97	34.17	43.70	19.70
	9	2.50	1	0.30	-1	85.95	25.45	36.61	18.66
	10	2.50	1	0.90	1	258.55	39.37	53.30	17.85
	11	1.50	0	0.60	0	176.30	31.98	44.35	14.72
	12	0.50	-1	0.30	-1	100.00	23.46	32.50	20.70
	13	0.09	-α	0.60	0	157.76	31.40	40.80	20.90

Protein content, TPC: Total phenolic content, CUPRAC: Cupric reducing antioxidant capacity; ABTS: 2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt.

Table 3: Analysis of variance (ANOVA) of the fitted second-order polynomial model

Source	Sum of squares	DF	Mean square	F-value	p-value
Protein content					
Model	42216.30	5	8443.26	33.17	<0.0001*
Linear	110.02	1	110.02	0.43	0.5319
$ß_1$	40440.67	1	40440.67	158.88	<0.0001*
$ß_2$					
Quadratic	0.19	1	0.19	7.384E-004	0.9791
$ß_{11}$	122.03	1	122.03	0.48	0.5110
$ß_{22}$					
Interaction $ß_{12}$	1540.00	1	1540.00	6.05	0.0435*
Residual	1781.70	7	254.53	-	-
Lack of fit	1157.67	3	385.89	2.47	0.2011
Pure error	624.03	4	156.01	-	-
Cor total	43998.00	12	-	-	-
R^2=0.96; C.V.(%)=10.33					

Table 3: Analysis of variance (ANOVA) of the fitted second-order polynomial model

Source	Sum of squares	DF	Mean square	F-value	p-value
Total phenolic content					
Model	421.90	5	84.38	145.74	<0.0001*
Linear					
$ß_1$	4.20	1	4.20	7.25	0.0310*
$ß_2$	412.70	1	412.70	712.82	<0.0001*
Quadratic					
$ß_{11}$	0.80	1	0.80	1.38	0.2784
$ß_{22}$	4.60	1	4.60	7.95	0.0258*
Residual	4.05	7	0.58	-	-
Lack of fit	0.63	3	0.21	0.24	0.8622
Pure error	3.43	4	0.86	-	-
Cor total	425.95	12	-	-	-
R^2=0.9905; C.V.(%)= 2.38					

Table 3 (cont.): Analysis of variance (ANOVA) of the fitted second-order polynomial model.

Source	Sum of squares	DF	Mean square	F-value	p-value
Antioxidant activity (CUPRAC)					
Model	556.11	5	111.22	6.36	0.0154*
Linear					
$ß_1$	54.83	1	54.83	3.13	0.1199
$ß_2$	476.72	1	476.72	27.25	0.0012*
Quadratic					
$ß_{11}$	10.10	1	10.10	0.58	0.4721
$ß_{22}$	13.07	1	13.07	0.75	0.4160
Interaction					
$ß_{12}$	4.04	1	4.04	0.23	0.6454
Residual	122.44	7	17.49	-	-
Lack of fit	54.74	3	18.25	1.08	0.4532
Pure error	67.70	4	16.93	-	-
Cor total	678.55	12			
R^2=0.82; C.V.(%)=9.05					
Antioxidant activity (ABTS)					
Model	36.17	5	7.23	1.47	0.3096
Linear					
$ß_1$	0.29	1	0.29	0.059	0.8144
$ß_2$	17.59	1	17.59	3.58	0.1005
Quadratic					
$ß_{11}$	2.22	1	2.22	0.45	0.5228
$ß_{22}$	2.22	1	2.22	0.45	0.5235
Interaction					
$ß_{12}$	13.18	1	13.18	2.68	0.1457
Residual	34.43	7	4.92	-	-
Lack of fit	13.33	3	4.44	0.84	0.5378
Pure error	21.10	4	5.28	-	-
Cor total	70.60	12			
R^2=0.51; C.V.(%)=12.12					

*significant at $P \leq 0.05$, β_1: Ultrasound probe application time (sec), β_2: Enzyme/substrate ratio.

The R^2 values were 0.96, 0.99, 0.82, and 0.51 for PC, TPC, CUPRAC, and ABTS, respectively (Table 3). Apart from ABTS (<0.80), TPC, CUPRAC, and ABTS had high R^2 values for the models. The model's fit is indicated by the high R^2 values (Moorthy et al., 2015). A low coefficient of variation (CV) in the model indicates that the evaluated systems are highly reproducible. Similarly, PC (CV=10.33%), TPC (CV=2.38%), CUPRAC (CV=9.05%) and ABTS (CV=12.12%) showed low variation in their mean scores (Table 3). The lack of fit has no significance for PC and all AOA methods measured (P>0.05, Table 3). The results indicate that the PC, TPC, and AOA models (by CUPRAC method) can be utilized to optimize the parameters for protein extraction from *S. vulgare* (P<0.05). Statistically significant linear effects of E/S on PC, TPC, and CUPRAC were observed (P<0.05, Table 3).

Protein content

RSM was used to determine the best method for extracting proteins from *S. vulgare* and to investigate optimum extraction conditions. Two different factors that were investigated in relation to protein content are the ultrasonic probe time and the enzyme/substrate ratio. The effect of these factors on protein content is shown in Figure 1. According to the results, the protein content of *S. vulgare* was determined between 34.92-258.55 mg protein/g dw (3.50% and 25.86%) depending on the extraction parameters. Similar to the current study, the PC of some *Sargassum* spp. in the study by Bonilla Loaiza (2022) and Perumal et al. (2019) ranged from 4.13% to 15.42%. On the other hand, the PC of some *Gracilaria* species ranged from 5.6% to 30.2% (Chan and Matanjun, 2017; Rodrigues et al, 2015; Gressler et al, 2010). De Melo et al. (2021) found that the highest protein content was between 22.93 and 21.27% in dw for *C. corneus*, followed by *U. fasciata* between 17.97 and 11.42% in dw and *S. vulgare* between 14.02 and 10.32% in dw. Vasquez et al. (2018) reported that the protein content obtained by enzyme-assisted extraction was 7.39% g protein in dried sample for *M. pyrifera* and 6.35% g protein in dried sample for *C. chamissoi*. Our results were in agreement with those of these authors, although we obtained protein contents in a wider range depending on the extraction parameters.

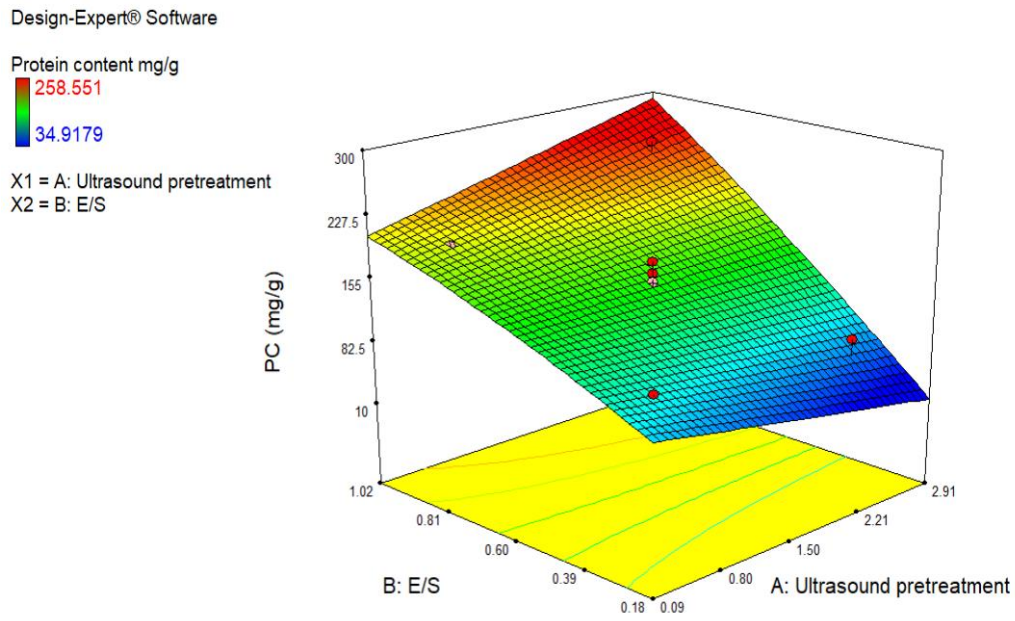

Figure 1. 3D contour plot response surface for the effect of cross-interaction between ratio of enzyme/substrate ratio and ultrasonic probe pretreatment on protein content.

The response equation (Table 4) demonstrates that two independent factors, ultrasonic probe time (β_1) and enzyme/substrat ratio (β_2) had a positive effect on protein content. The quadratic effect of enzyme/substrat ratio had a negative effect on protein content, whereas the interaction between ultrasonic probe time and enzyme/substrat ratio, as well as the quadratic effect of ultrasonic probe time, had a positive effect on protein content. Increasing the enzyme/substrate ratio and the application time of the ultrasonic probe led to an increase in protein content during extraction (Fig. 1). As shown in Table 2, the maximum PC content was 258.55 mg/g under the defined extraction conditions (ultrasonic probe application time of 2.5 seconds, E/S of 0.90). Table 3 shows that the linear effect of E/S on PC showed statistically significance (P<0.05). This could be due to the fact that the enzyme utilized breaks down the cell wall of the algae and releases more protein into the solvent. Similarly, Joubert and Fleurence (2008) investigated the effects of specific enzymes, including xylanase and cellulase, and the concentration of the enzymes on the PC of *P. palmata* and found that PC increased proportionally to the amount of enzyme. Similar results were reported by Harnedy and FitzGerald (2013), who used polysaccharidase to disrupt the cell wall increased the efficiency of protein extraction from macroalgae. In addition, Suwal et al. (2019) showed that protein content increased by 17% when the enzyme cellulase was utilized in the extraction process. They also reported that the extraction yield for *P. palmata* increased from 9 to 37% when a cell wall-dissolving enzyme was used (Suwal et al., 2019).

Table 4: Estimated coefficients of the fitted second-order polynomial model for all response variables.

Regression coefficient	PC (mg protein/g extract, dw)	TPC (mg GAE/g extract, dw)	CUPRAC (mg TE/g extract, dw)	ABTS (mg TE/g extract, dw)
β_0	156.89	32.72	47.79	18.30
Linear				
β_1	3.71	0.72	2.62	0.19
β_2	71.10	7.18	7.72	-1.48
Quadratic				
β_{11}	0.16	-0.34	-1.21	0.57
β_{22}	-4.19	-0.81	-1.37	-0.56
Interaction				
β_{12}	19.62	-0.062	-1.01	1.82

β_1: Ultrasound pretreatment, β_2: Enzyme/substrate

Protein content was generally sensitive to especially proteolytic enzyme treatment. In an ideal reaction, enzyme and substrate react continuously until the central equilibrium was reached. According to Ramakrishnan et al. (2013), there was a clear correlation between increasing enzyme concentration and protein yield, with the highest enzyme concentration resulting in 76.30% of protein extracted. The amount of enzyme used and the duration of extraction had a positive relationship that improved protein extraction (Bozdemir et al., 2022). When the E/S value was above 1, the protein content was basically increased (Fig. 1). Once it approached 2.5, the protein content increased by a considerable percentage more than without enzyme treatment (Bozdemir et al., 2022). Thus, the ratio of E/S 1.60 in our study seems to be the ideal value for extraction. This result is in line with previous findings by Joubert and Fleurence (2008) and Suwal et al. (2019). A similar result was obtained by Bozdemir et al. (2022), who showed that the optimal E/S ratio for protein extraction from *G. dura* was higher than 1.50. These results indicate

that protein content was significantly affected by the two variables. The appropriate range of the variables was found using the previously discussed one-factor test, which provided significant support for the RSM.

TPC and AOA

According to our results, the TPC of the protein extracts ranged from 20.79 to 41.57 mg GAE/g dw, depending on the extraction conditions listed in Table 1. Similarly, Nursid et al. (2020) determined the TPC value of 23.37 mg GAE/g for *G. salicornia*, 24.97 mg GAE/g for *Laurencia sp.*, and 24.38 mg GAE/g for *G. latifolium*. On the other hand, Khaled et al. (2012) determined the TPC of *S. vulgare* as 12.71±0.03 mg GAE/g and as 10.55 mg GAE/g for *P. pavonica*. In addition, Arguelles et al. (2019) reported that the TPC of *S. vulgare* was 10.55 mg GAE/g. Prasedya et al. (2021) investigated the TPC of ethanolic extracts of some *Sargassum* spp. and reported TPC of 66.13 mg GAE/g for *S. cristaefolium*, 39.83 mg GAE/g for *S. aquifolium*, 38.93 mg GAE/g for *S. polycystum* and 52.90 mg GAE/g for *S. crassifolium*.

The lowest TPC was determined for an ultrasonic probe time of 1.50 and an E/S ratio of 0.18 (Table 2). The TPC increased under experimental conditions with an ultrasonic probe time of about 1.50 and an E/S of about 1.02 (Figure 2). The breakdown of phenolic compounds in response to extended exposure to ambient conditions may be responsible for the statistically significant (P<0.05) decreased in TPC with time (Thoo et al., 2010).

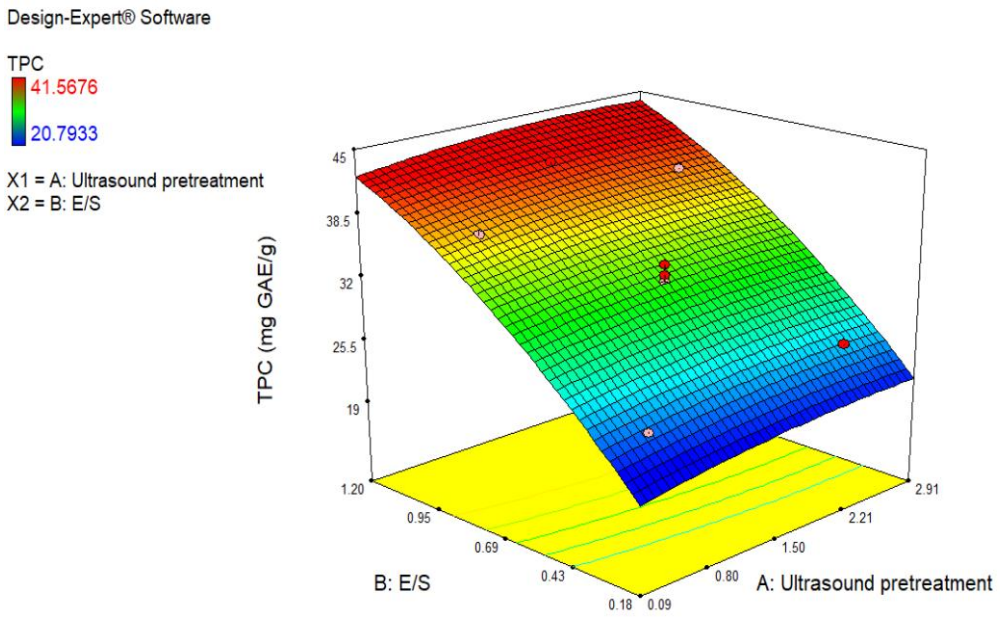

Figure 2. 3D contour plot response surface for the effect of cross-interaction between ratio of enzyme/substrate ratio and ultrasonic probe pretreatment on total phenolic content.

The AOA of the protein extracts ranged from 32.50 to 55.01 mg TE/g dw (by CUPRAC method) and from 12.60 to 20.90 mg TE/g dw (by ABTS method), as shown in Table 2. Yuan et al. (2018) reported the highest AOA with the ABTS assay for *L. nigrecens* at 0.95±0.01 mg TEAC/g dry sample. Kumar et al. (2020) reported the AOA of some algae species using the FRAP method as 8.21 mg TE/g for *S. wightii*, 6.90 mg TE/g for *U. rigida*, and 1.06 mg TE/g for *G. edulis*. According to Nursid et al. (2020), the season, the location, the time of harvest and the type of algae can have an influence on the

fluctuations in polyphenol concentration and antioxidant activity.

According to the results, the linear effect of the E/S ratio on TPC and AOA (by CUPRAC method) of the extracts was significant, and the quadratic effect of the E/S ratio on TPC was significant (P<0.05, see Table 3). The AOA of the extracts increased with increasing enzyme amounts, similar to TPC, as the phenols have high antioxidant activity. These results were compatible with the studies of Barclay and Vinqvist (2003) and Ferruzzi and Green (2006). In the TPC assay, the effect of E/S was significant (P<0.05), while ultrasonic probe time had no significant effect (P<0.05, Table 3). The response equation shows that two independent factors, ultrasonic application time (β_1) and enzyme/substrat ratio (β_2) had a substantial effect onTPC, but all other interaction and quadratic terms are negative (Table 4). Since phenolics are covalently bound to proteins, there was a strong effect of the E/S ratio on TPC, similar to PC (Acosta-Estrada et al., 2014). There was statistical significance in the TPC overall model (P<0.05, Table 3). As with CUPRAC assay, the effect of E/S was significant (P<0.05), while ultrasonic probe time had no significant effect (P<0.05, Table 3, Figure 3). In addition, the model for CUPRAC was statistically significant (P<0.05, Table 3). ABTS was not significantly different from the linear effects of the individual variables examined (P>0.05, Table 3, Figure 4). According to response equation of CUPRAC, only ultrasonic application time (β_1) and enzyme/substrat ratio (β_2) showed a positive effect. On the other hand, linear and quadratic effect of ultrasonic application time (β_1) had a positive effect on the ABTS (Table 4).

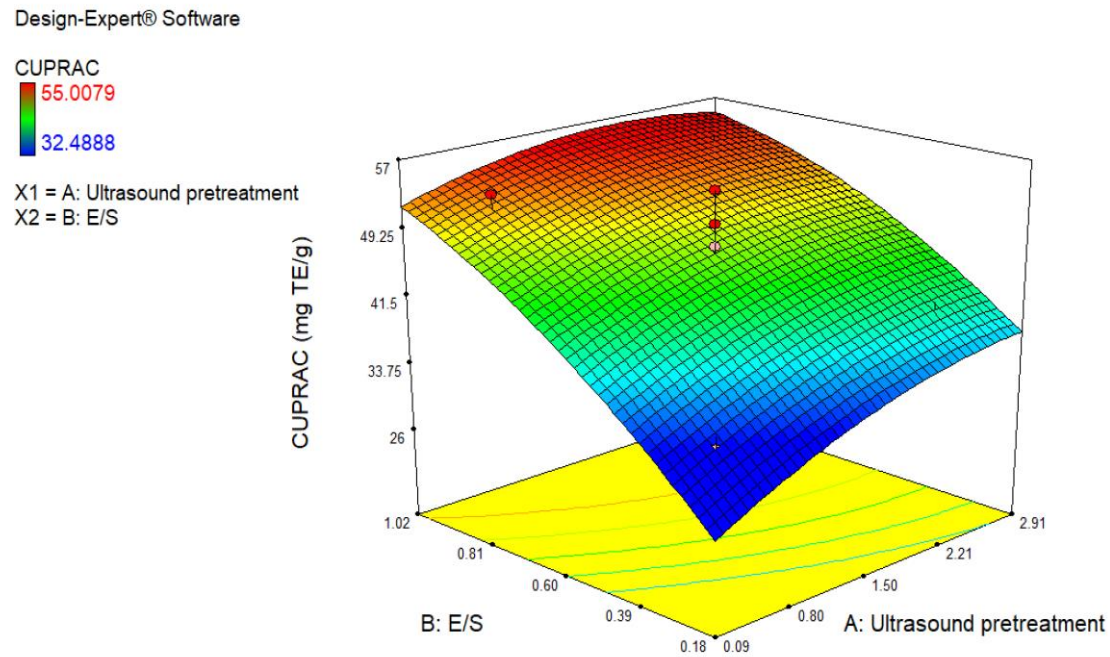

Figure 3. 3D contour plot response surface for the effect of cross-interaction between ratio of enzyme/substrate ratio and ultrasonic probe pretreatment on antioxidant activity by CUPRAC.

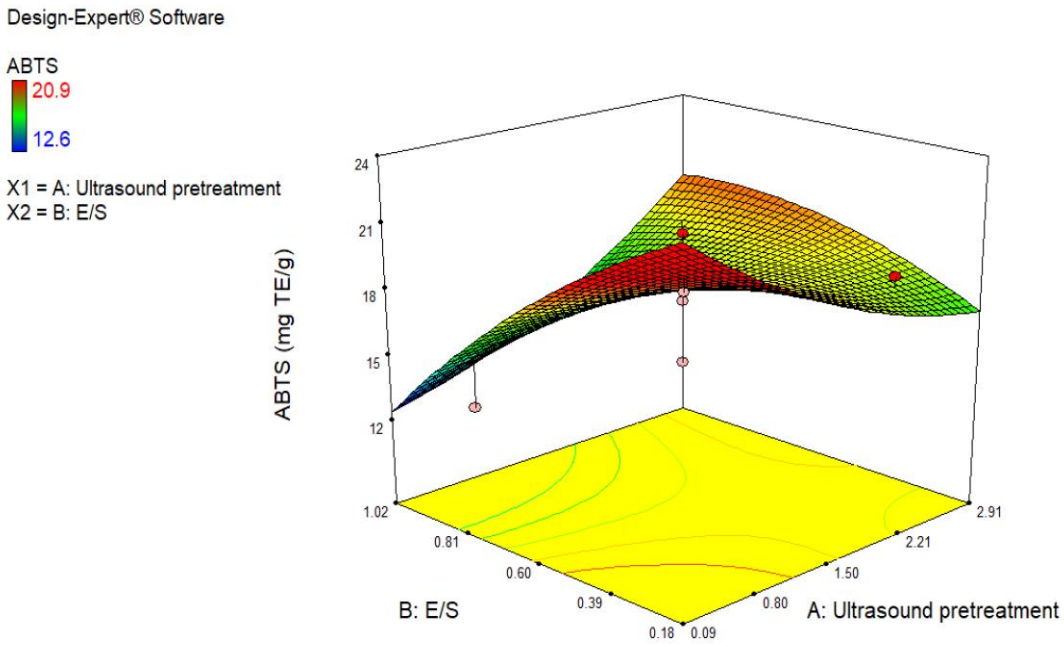

Figure 4. 3D contour plot response surface for the effect of cross-interaction between ratio of enzyme/substrate ratio and ultrasonic probe pretreatment on antioxidant activity by ABTS.

Optimization and verification

Macroalgae are high-protein alternative protein sources but their complex cell wall limits protein extraction. Therefore, the aim of optimization process is to obtain macroalgal proteins with higher yield. In order to determine the ideal level of the independent variables and obtain the highest values for PC, TPC, and AOA, optimization processes were performed. Under the optimum conditions (ultrasonic probe time of 2.5 min and E/S of 0.90), the predicted PC value was 247.29 mg/g dw, while the predicted TPC and AOA values (by CUPRAC and ABTS methods) were 39.41 mg GAE/g dw, 54.55 mg TE/g dw, and 18.83 mg TE/g dw, respectively, corresponding to a "desirability" of 0.89. The AOA by CUPRAC (53.77 mg TE/g dw), PC (248.30 mg protein/g dw), TPC (38.03 mg GAE/g dw) and ABTS (19.88 mg TE/g dw) showed no statistically significant difference from the mean and predicted values of the experiment at the 5% significance level. The limitations of optimization include the need to test different enzymes due to the structure's complexity.

CONCLUSION

According to the results of the study, RSM was successfully applied to determine the optimum extraction conditions for the brown macroalgae *S. vulgare*. The optimum conditions for extraction were as follows: an ultrasonic probe time of 2.5 minutes and an E/S ratio of 0.90. It was found that the factor that had the greatest effect on PC and AOA was the E/S ratio, and the effect of ultrasonic probe time was also significant. The R^2 values above 0.80 obtained for PC, TPC, and CUPRAC indicated that the extraction model applied was appropriate. Compared to other algal sources in the literature, the protein extract obtained under optimal conditions showed higher antioxidant activity as well as phenolic content. Consequently, further studies may provide the utilization of the protein-rich *S. vulgare* as a potential protein source in the protein development of new products and as a viable food ingredient.

CONFLICT OF INTEREST

Authors declare that they have no known conflict of interest.

CONTRIBUTIONS

H. Dinç is responsible for carrying out all experiments. E. Şensu is responsible for some experiments, interpretation of the results, writing original draft preparation. Ü. Altuntaş is responsible for data analysis, interpretation of the results, writing original draft preparation. E. Ş. Okudan is responsible for collection of macroalgae samples. B. Özçelik is responsible for providing research infrastructure and interpretation of the results. A. Yücetepe is responsible for planning of the study, some experiments, data analysis, interpretation of the results, reviewing, and coordination.

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