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Optimisation of Ultrasound-Assisted Extraction of Protein from *Spirulina platensis* Using RSM

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Abstract

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The protein extraction from the blue-green microalgae *Spirulina platensis* was carried out using ultrasound-assisted extraction and response surface methodology (RSM) was used to optimise extraction conditions. Extraction yield, total phenolic content, antioxidant activity and *in vitro* protein digestibility of protein extracts were determined. A three factors Box-Behnken design (BBD) of experiments was employed at pH values of 7, 8 and 9; temperatures of 25, 35, and 45°C; and for durations of 60, 90 and 120 minutes. Based on the RSM analysis, optimum extraction conditions (temperature 45°C, pH 7.46 and time 120 min) were obtained for extraction yield (29.05%), total phenolic content (3.52 mg caffeic acid equivalent/g dw), antioxidant activity (11.32 mg Trolox equivalent/g dw) and *in vitro* protein digestibility (99.36%). We report the first evaluation of the *in vitro* protein digestibility of *Spirulina platensis* and find it to be over 90%. This value is higher than the *in vitro* protein digestibility values of proteins obtained from other algae and plant species, and, in particular, is greater than that of commercial soybean protein isolate.

Keywords: antioxidant activity; bioavailability; *in vitro* protein digestibility; protein concentrate; total phenolic content

Spirulina platensis is one of the more important multicellular blue-green cyanobacteria due its chlorophyll (green) and phycocyanin (blue) pigments. It possesses the ability to carry out photosynthesis and can grow well in both seawater and fresh water (Agustini *et al.* 2015; Yücetepe & Özçelik 2016). Because of its rich pigments, it has been described as a plant; on the other hand, it has also been classified as a member of the kingdom of Bacteria due to some of its biochemical properties (Vo 2016).

Spirulina platensis is one of the more promising cyanobacteria, as it is rich in proteins, essential and nonessential amino acids, long-chain polyunsaturated fatty acids, vitamins, minerals and many phytonutrients. It harbours high protein concentrations, which can reach about 60–70% of its dry weight, depending upon the environmental conditions at which it is grown. In previous studies, protein concentrates with a protein content of at least 65% dry weight and protein isolates with a protein content of at least 90%

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dry weight were obtained from *Spirulina platensis* (Bermejo *et al.* 2008; Yücetepe & Özçelik 2016).

Antioxidants are compounds that decrease or inhibit the adverse effects of reactive oxygen species on normal physiological function in humans (Karadag *et al.* 2009). Numerous studies have shown that *Spirulina* or its protein extracts have antioxidant activity (Estrada *et al*. 2001; Bermejo *et al.* 2008; Gad *et al*. 2011; El-Tantawy 2015). Gad *et al*. (2011) evaluated the antioxidant activity and hepatoprotective effects of phycocyanin and *Spirulina platensis* protein in *in vitro* and *in vivo* studies, respectively. They demonstrated that the *in vitro* antioxidant capacity of the aqueous extract of *Spirulina platensis* showed a strong inhibitory activity against ferrozine-Fe^{2+} complex formation and an *in vivo* study showed that *Spirulina platensis* succeeded in preventing liver damage. Estrada *et al*. (2001) observed that increase in the amount of phycocyanin also caused an increase in antioxidant activity, and that therefore phycocyanin is the compound mainly responsible for the antioxidant activity of protein extracts of *Spirulina platensis*. Similarly, Bermejo *et al*. (2008) demonstrated antioxidant activity in a *Spirulina platensis* protein extract.

The aim of this study was to optimise conditions for the ultrasound-assisted extraction of *Spirulina platensis* protein with respect to extraction yield, total phenolic content, antioxidant activity and *in vitro* protein digestibility. The chemical composition of *Spirulina platensis* powder (SPP) is also described. To the best of our knowledge, ours is the first report in the literature to describe the *in vitro* protein digestibility of protein concentrates obtained from *Spirulina platensis*.

MATERIAL AND METHODS

Characterisation of SPP. SPP was obtained from a local manufacturer in Turkey (Akuatik Fisheries and Cosmetics Products Ltd., Turkey). Folin-Ciocalteu reagent was purchased from Merck KGAEA (Germany). The other chemicals used in analyses were purchased from Sigma-Aldrich Chemie GmbH (Germany).

Chemical composition. Moisture, ash, protein, and lipid content of SPP were determined according to the methods of the Association of Official Analytical Chemists. The total nitrogen content of SPP was determined using the Kjeldahl method (Firestone 1990). Carbohydrate levels were determined by subtracting the sum of the percentages of moisture, ash, protein and lipid from 100. All measurements were carried out in triplicate.

Fatty acid profile. The fatty acid composition of SPP oil was determined according to the method of Krienitz and Wirth (Krienitz & Wirth 2006) using a gas chromatograph (GC 7820A; Agilent Technologies, Inc., USA) equipped with a capillary column (30 mm × 0.25 mm i.d., × 0.25 µm; Agilent 112-8837) and a flame ionisation detector (FID). Results were expressed as percentage of weight. All measurements were carried out in triplicate.

Sugar profile. Sugar extraction was performed according to the ultra performance liquid chromatography (UPLC) method described in Muir *et al*. (2009). A Shimadzu LC-10A apparatus (Shimadzu, Japan) equipped with a RID-20A refractive index detector (RID) was used to determine the sugar profile. Reversed-phase chromatography was performed with a 250 mm × 4.6 mm inert sustain NH_2 column packed with 5-µm particles (Teknokroma, Spain) at 40°C. The mobile phase was $CH_3CN : H_2O$ (85 : 15, v/v) at a flow rate of 1 ml/minute. The injection volume was 20 µl. The chromatograms were recorded at 300 nm. All measurements were carried out in triplicate.

Experimental design and statistical analysis. RSM was used for optimisation of three extraction parameters (temperature, pH, and time) on three levels. BBD was applied to the experimental data. An experimental design including 12 factorial points and five central points was employed.

In this work, independent variables (temperature 25–45°C, pH 7–9, and time 60–120 min) were used in the experimental design. Extraction parameters were normalised as coded variables. Variables were coded according to the Equation (1):

$$X = (x_i - x_0)/\Delta x \quad (1)$$

where: x_i – corresponding actual value; x_0 – actual value in the centre of the domain; Δx – increment of x_i corresponding to a variation of one unit in X

The response functions (Y) were extraction yield (%), total phenolic content, antioxidant activity and *in vitro* protein digestibility (%). The response variables were fitted to a second-order polynomial model to obtain the regression coefficients (β). The generalised second-order polynomial model used in the response surface analysis is as follows:

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$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon \quad (2)$$

where: β_0 – constant term; β_i – linear effects; β_{ii} – quadratic effects; β_{ij} – interaction effects; ε – random error term that represents the variability of the response

To evaluate model adequacy, regression coefficients and statistical significance, analysis of variance (ANOVA) was used. To visualise the relationships between the responses and the independent variables, surface response and contour plots of the fitted polynomial regression equations, optimal conditions for the targeted responses were generated using the trial version of Design Expert 7.1 software (Stat-Ease, Inc., USA). The results were statistically tested at a significance level of $P = 0.05$. The adequacy of the model was determined using model analysis, the coefficient of determination (R^2) and lack of fit testing. A mathematical model was established to describe the influence of a single process parameter and/or the interaction of multiple parameters on each investigated response.

Preparation of protein concentrates from SPP. SPP protein was extracted using the method described by Benelhadj *et al.* (2016) with some modifications. Firstly, samples were defatted using the method of Stone *et al.* (2015). After the defatting process, 1 g of sample was weighed into beakers followed by the addition of 15 ml of distilled water. The beakers were mixed using a magnetic stirrer at room temperature for 60 minutes. Then, the pH of samples was adjusted by adding 0.1 M HCl and 0.1 M NaOH. The samples were transferred into an ultrasound device (VWR Ultrasonic cleaner; VWR International, USA), and ultrasound extraction took place at 4 ± 1°C for 60 minutes. Samples were kept in a shaking water bath (Classic C76; New Brunswick Scientific, USA) at 135 g, at the respective temperatures and for the respective lengths of time. After centrifugation at 4000 *g*, 4°C for 30 min, the supernatant was taken and the pH was adjusted to 3.0 which is the isoelectic point of Spirulina platensis (Benelhadj *et al.* 2016). Samples were centrifuged at 4000 *g*, 4°C for 30 min and the supernatant was discarded. Finally, sediments were freeze-dried and *Spirulina platensis* protein concentrates (SPPCs) were stored at 4°C until analysis.

Determination of protein content of SPPCs. The total nitrogen was determined according to the Kjeldahl method (Firestone 1990). The protein content of protein concentrates was calculated after multiplication by a conversion factor of 6.25. The extraction yields (% w/w) of protein concentrates were calculated as follows:

Extraction yield = [(content of SPPC after extraction × × protein content of SPPC after extraction)/ (content of SPP before extraction × protein content of SPP before extraction)] × 100 (3)

Total phenolic content (TPC). The TPC of the protein concentrates was determined according to the Folin-Ciocalteu method (Ling 2014) and was calculated from a calibration curve using caffeic acid as standard. The results are expressed as milligrams caffeic acid equivalent per g dry weight (mg CAE/g dw).

Identification of phenolic acids in protein concentrates. For identification of phenolic acids in protein concentrates, a Shimadzu LC-10A apparatus equipped with a SPD-M10A photodiode array detector (PDA) was used for analytical UPLC separations. Reversed-phase chromatography was performed with 250 × 4.6 mm Kromasil 100 C-18 column packed with 5-µm particles (Teknokroma, Barcelona, Spain), fitted with a security guard C18 ODS (4 × 3.0 mm i.d). Gradient was formed with He-degassed solvent. Solvent A was H_2O containing 0.1% formic acid, and solvent B was MeCN subjected to different elution conditions. Separation was accomplished by starting with 5% A solvent for 2 min at a pressure of 115 bar, followed by a linear gradient for 10 min from 5% B to 95% A and a final linear gradient from 20% to 95% A in 5 minutes. The flow rate was 0.5 ml/min, and the operating temperature was 40°C. The injection volume was 10 µl. The chromatograms were recorded at 286 nm.

Antioxidant activity determined by CUPRAC. The cupric reducing antioxidant capacity assay was performed according to the method of Apak *et al.* (2004). Results were expressed as milligrams Trolox equivalent per gram of dry weight (mg TE/g dw).

Antioxidant activity determined by DPPH radical scavenging assay. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was carried out according to the method of Kumaran and Karunakaran (2006). Trolox was used as a reference compound and results were expressed as milligram Trolox equivalent per gram of dry weight (mg TE/g dw).

In vitro protein digestibility. Digestion of the samples was performed according to the procedure described by Świeca *et al.* (2013). A saliva solution was prepared by dissolving 0.19 g KH_2PO_4, 2.38 g Na_2HPO_4, and 8 g NaCl in 1 l of distilled water. The pH of the mixture was adjusted to 6.75 and α-amylase was added to the saliva solution. Pepsin

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enzyme solution was prepared in 0.03 M NaCl, pH 1.2 for gastric digestion. Intestinal digestion was simulated using an intestinal solution containing 0.3 g of bile extract in 35 ml, 0.1 mol/l $NaHCO_3$, and 0.05 g of pancreatin. The samples were submitted to simulated gastrointestinal digestion as follows: The samples and 15 ml of the prepared salivary juice were mixed for 10 min at 37°C. The pH values of the samples were adjusted to 1.2 using 5 M HCl; afterwards, 15 ml of the prepared gastric juice was added. The samples were incubated in a shaking water bath for 60 min at 37°C. Subsequently, the pH values of the samples were adjusted to 6 with 0.1 M of $NaHCO_3$ and then, pancreatin and 15 ml of a mixture of bile extract were added. The pH values of the samples were adjusted to 7 with 1 M NaOH and 5 ml of 120 mM KCl, and 5 ml of 120 mM NaCl were added to each sample. Subsequently, the samples were incubated in a shaking water bath for 120 min at 37°C in the dark. Finally, each of the samples were used for determination of *in vitro* protein digestibility (PD%). Protein concentrations of the samples were estimated using the Bradford method (Bradford 1976) and bovine serum albumin was used as a protein standard. The *in vitro* protein digestibility of SPPCs was calculated on the basis of total soluble protein content and the content of protein determined after digestion *in vitro*.

$$PD\% = 100\% - [(P_r/P_t) \times 100\%] \quad (4)$$

where; P_t – total protein content; P_r – content of proteins after *in vitro* digestion

RESULTS AND DISCUSSION

Characterisation of SPP. Analysis of the composition of SPP showed that it contained 65.6 ± 0.12% protein, 14.2 ± 0.62% lipid, 10.7% carbohydrate, and 9.5 ± 0.02% ash in dry weight (Table 1). The proximate composition for SPP was similar to those reported in previous works conducted by Rafiiqul *et al*. (2005), Agustini *et al*. (2015) and Madkour *et al*. (2012). Agustini *et al*. (2015) reported values in the range of 7.78, 67.18, 2.64, 11.74, and 10.66%, respectively, for moisture, protein, lipid, carbohydrate and ash in dried *Spirulina platensis*. Similar to our study, Rafiikul et al. (2005) found the protein content of *Spirulina* sp. to be 59.16 ± 0.22% in wet weight.

In our study, the amount of lipid in SPP was determined to be 14.2 ± 0.62%. The fat content of *Spirulina platensis* was found to be 11.4, 4.3, 6–9, and 10.9% by Chaiklahan *et al*. (2008), Tang *et al*. (2011), Seo *et al*. (2013), and El Shimi *et al*. (2015), respectively. These differences in lipid content probably result from differences in parameters that affect growth conditions like temperature, pH, dissolved oxygen, salinity and nutrient limitation. Moreover, lipid extraction yield can change depending on the type and nature of solvent, particle size of lipid or oil, solvent-to-sample ratio, temperature and time of extraction (Chaiklahana *et al.* 2008).

Table 1. Proximate analysis of *Spirulina platensis* powder (SPP) (%)

Composition (w/w)	Dried basis	Wet basis
Moisture	–	5.4 ± 0.26
Protein	65.6 ± 0.12	62.1 ± 0.12
Lipid	14.2 ± 0.62	13.4 ± 0.62
Ash	9.5 ± 0.02	9.0 ± 0.02
Carbohydrate[a]	10.7	10.1

[a]by difference; values are mean ± SD of three determinations

According to the results of the GC analysis, the fatty acids of SPP were mainly palmitic acid (56%) and linoleic acid (19.63%), linolenic acid (17.07%), oleic acid (2.98%), palmitoleic acid (2.78%), and stearic acid (1.49%). Similarly, Herrero *et al*. (2007) detected three main fatty acids in *Spirulina*: γ-linolenic, palmitic and linoleic acids. Retention times and the proportions of different fatty acids as percentage in SPP are given at Table 2.

The sugar profile of SPP, which was determined using UPLC, consisted of four sugars. The major component was rhamnose, which comprised 56% of total sugars; while glucose, galactose, mannose and unknown sugars comprised 22, 8, 5, and 9%, respectively. Chaiklahan *et al*. (2013) determined the sugar composition of crude polysaccharides from *Spirulina platensis* as rhamnose, ribose, xylose, mannose, galactose, glucose, and unknown sugars, which represented around 53.7, 10.0, 4.4, 2.1, 5.6, 14.5, and 11.1% of the total, respectively. Similarly, Majdoub *et al*. (2009) also found that rhamnose was the major polysaccharide (49.7%) in *Spirulina* extract.

Model fitting. Response were evaluated as functions of linear, quadratic and interaction terms of the independent variables including temperature, pH and time using BBD and are shown in Table 3. Analysis of variance of the fitted second-order polynomial model and coefficients of determination (R^2)

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Table 2. Fatty acid profile and relative content of *Spirulina platensis* powder (SPP)

Retaining time for GC (min)	Acid	Molecular formula	Relative content (%)
7.804	palmitic	$CH_3(CH_2)_{14}COOH$	56.04
10.022	linoleic	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	19.63
10.552	linolenic	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	17.07
9.341	oleic	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	2.98
8.108	palmitoleic	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$	2.78
8.955	stearic	$CH_3(CH_2)_{16}COOH$	1.50

for each dependent variable are shown in Table 4. The R^2 values were 0.91, 0.84, 0.91, 0.85, and 0.72 for extraction yield (%), TPC, CUPRAC, DPPH, and PD%, respectively. Jouki *et al*. (2014) explained that R^2 should be at least 0.80 for a good fitness of a response model.

While the R^2 value for PD% was only 0.72, values were particularly high for the other polynomial models (> 0.80), indicating suitability for representing the real relationships between variables. Moreover, low coefficients of variation (CV) for extraction yield 5.26%, PD% 3.04%, and DPPH 6.57% (Table 4) suggested good reproducibility of the investigated systems, since CV defines the dispersion of the data and small values indicate low variation in the mean value. However, TPC (CV = 28.71%) and CUPRAC (CV = 13.14%) exhibited particularly high variation in their mean values.

A significant lack of fit indicates that a model fails to represent the data in the experimental domain at points that are not included in the regression. *P* of the lack of fit for all variables were > 0.05, meaning that all models accurately predicted the related responses (Table 4). The results suggest that the models used

Table 3. Box-Behnken experimental design with natural and coded extraction conditions and experimentally obtained values of all investigated responses

Run	Independent variables							Responses			
	pH		temperature (°C)		time (min)		yield (%)	TPC (mg CAE/g dw)	CUPRAC (mg TE/g dw)	DPPH (mg TE/g dw)	PD (%)
1	0	8	0	35	0	90	27.778	1.57	10.89	8.10	90.59
2	1	9	0	35	1	120	28.352	2.10	11.03	8.41	93.25
3	0	8	0	35	0	90	27.894	2.61	8.12	8.28	97.25
4	1	9	−1	25	0	90	20.132	1.60	10.89	10.01	93.45
5	−1	7	0	35	−1	60	22.064	4.17	7.02	6.83	93.3
6	0	8	−1	25	1	120	25.325	1.20	8.55	7.31	87.45
7	0	8	0	35	0	90	28.776	2.52	6.80	6.71	89.10
8	−1	7	0	35	1	120	24.514	2.23	10.45	6.81	97.75
9	0	8	1	45	1	120	29.955	4.16	7.67	8.38	97.31
10	0	8	−1	25	−1	60	25.254	4.07	8.48	7.12	92.41
11	1	9	0	35	−1	60	25.782	5.18	6.88	7.07	94.41
12	0	8	1	45	−1	60	28.887	4.84	13.3	7.49	87.75
13	−1	7	−1	25	0	90	20.03	2.46	8.40	7.53	92.41
14	0	8	0	35	0	90	27.603	2.42	8.11	8.67	92.31
15	−1	7	1	45	0	90	25.138	2.03	7.77	7.42	96.43
16	1	9	1	45	0	90	26.203	2.09	6.75	6.89	97.81
17	0	8	0	35	0	90	25.772	1.02	2.30	8.32	88.33

TPC – total phenolic content; CUPRAC – cupric reducing antioxidant capacity; DPPH – 1,1-diphenyl-2-picrylhydrazyl; PD – protein digestibility

Table 4. Analysis of variance (ANOVA) of the fitted second-order polynomial model

Source	Sum of squares	DF	Mean square	*F*	*P*	Sum of squares	DF	Mean square	*F*	*P*	Sum of squares	DF	Mean square	*F*	*P*
	yield					total phenolic content					CUPRAC				
Model	123.01	9	13.67	7.41	0.0075*	21.77	9	2.42	3.96	0.0415*	86.74	9	9.64	7.85	0.0064*
Linear															
X_1	47.24	1	47.24	25.6	0.0015*	1.8	1	1.8	2.96	0.1292	11.26	1	11.26	9.16	0.01922*
X_2	9.5	1	9.5	5.15	0.0535*	0.001013	1	0.001013	0.001659	0.9687	17.91	1	17.91	14.58	0.0066*
X_3	4.74	1	4.74	2.57	0.1529	9.18	1	9.18	15.04	0.0061	2.74	1	2.74	2.23	0.1791
Quadratic															
X_1X_1	6.6	1	6.6	3.58	0.1005	0.03	1	0.030	0.05	0.8302	0.027	1	0.027	0.022	0.8857
X_2X_2	49.74	1	49.74	26.95	0.0013*	0.019	1	0.019	0.032	0.8638	5.66	1	5.66	4.61	0.0690
X_3X_3	4.62	1	4.62	2.51	0.1575	8.94	1	8.94	14.65	0.0065*	43.33	1	43.33	35.27	0.0006*
Interaction															
X_1X_2	0.23	1	0.23	0.12	0.7342	0.21	1	0.21	0.35	0.5745	6.97	1	6.97	5.67	0.0487*
X_1X_3	0.25	1	0.25	0.14	0.7237	1.19	1	1.19	1.95	0.2056	0.23	1	0.23	0.18	0.6811
X_2X_3	0.0036	1	0.0036	0.001951	0.9660	0.33	1	0.33	0.54	0.4857	1.225E-00	1	1.225E	9.972E-0	0.9757
Residual	12.92	7	1.85	–	–	4.27	7	0.61	–	–	8.60	7	1.23	–	–
Lack of fit	8.07	3	2.69	2.22	0.2285	2.31	3	0.77	1.57	0.3278	6.25	3	2.08	3.55	0.1265
Pure error	4.85	4	1.21	–	–	1.96	4	0.49	–	–	2.35	4	0.59	–	–
Cor total	135.92	16	–	–	–	26.04	16	–	–	–	95.34	16			
	R^2 = 0.9050; CV (%) = 5.26					R^2 = 0.8359; CV (%) = 28.71					R^2 = 0.9098; CV (%) =13.14				
	DPPH					*in vitro* protein digestibility									
Model	10.53	9	1.17	4.54	0.0294*	142.75	9	15.86	1.98	0.1898					
Linear															
X_1	0.54	1	0.54	2.10	0.1909	23.05	1	23.05	2.88	0.1336					
X_2	0.067	1	0.067	0.26	0.6269	0.12	1	0.12	0.015	0.9069					
X_3	1.02	1	1.02	3.94	0.0877*	7.78	1	7.78	0.97	0.3571					
Quadratic															
X_1X_1	4.112E-0030.033	1	4.112E-0030.033	0.016	0.9031	0.003981	1	0.003981	0.0004972	0.9828					
X_2X_2	2.33	1	2.33	0.13	0.7305	50.94	1	50.94	6.36	0.0397*					
X_3X_3	–	1	–	9.03	0.0198*	0.42	1	0.42	0.053	0.8249					
Interaction															
X_1X_2	0.74	1	0.74	2.87	2.87	0.029	1	0.029	0.003609	0.9538					
X_1X_3	0.10	1	0.10	0.40	0.40	52.71	1	52.71	6.58	0.0372*					
X_2X_3	5.64	1	5.64	21.87	21.87	7.87	1	7.87	0.98	0.3546					
Residual	1.81	7	0.26	–	–	56.05	7	8.01	–	–					
Lack of fit	0.35	3	0.12	0.32	0.8098	5.7	3	1.90	0.15	0.9239					
Pure error	1.45	4	0.36	–	–	50.35	4	12.59	–	–					
Cor total	12.33	16				198.8	16								
	R^2 = 0.8536; CV (%) = 6.57					R^2 = 0.7181; CV (%) = 3.04									

*significant at $P \leq 0.05$; X_1 – temperature (°C); X_2 – pH; X_3 – time (min)

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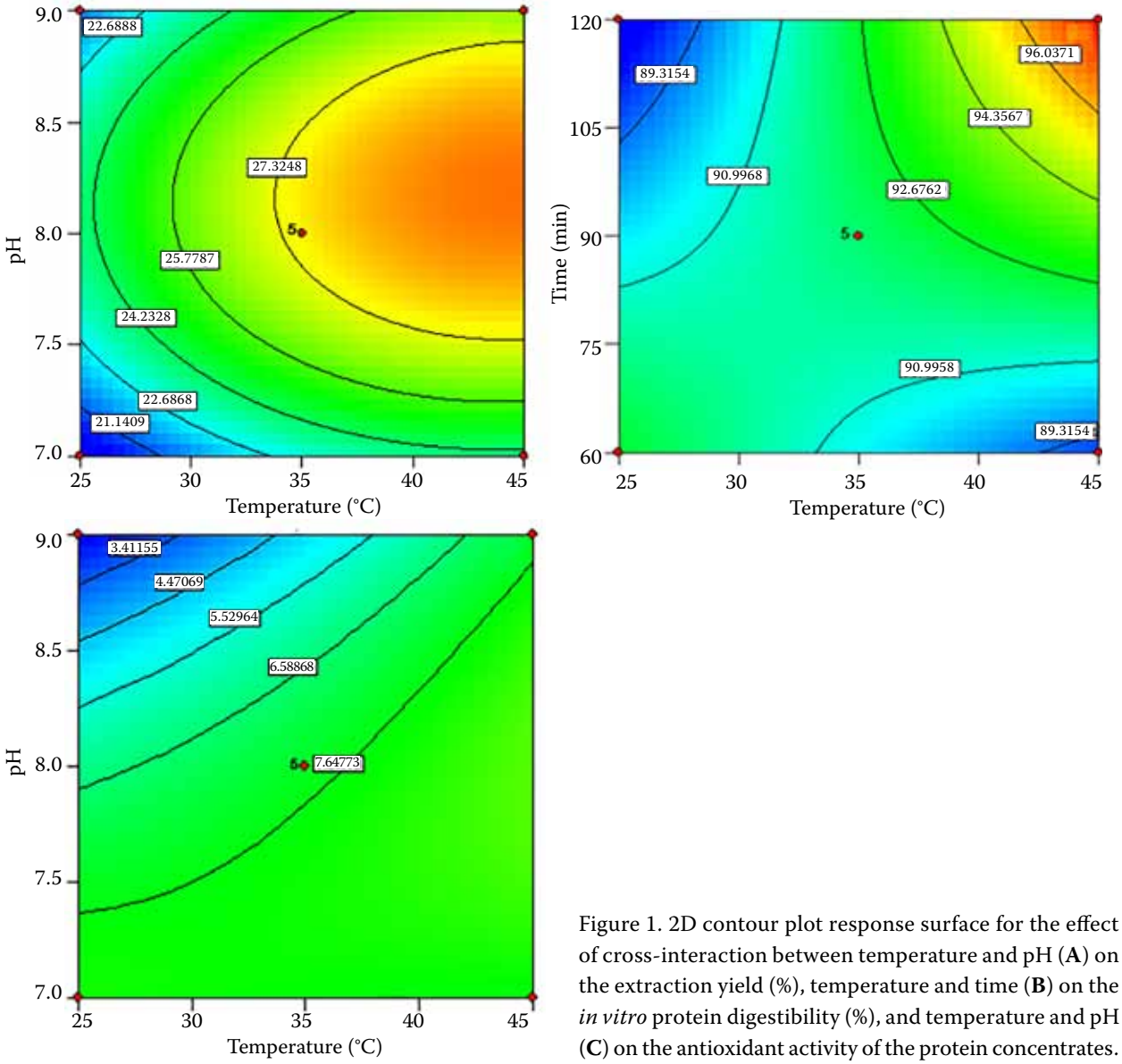

Figure 1. 2D contour plot response surface for the effect of cross-interaction between temperature and pH (**A**) on the extraction yield (%), temperature and time (**B**) on the *in vitro* protein digestibility (%), and temperature and pH (**C**) on the antioxidant activity of the protein concentrates.

in this study can be used for the optimisation of the conditions used in protein extraction from *Spirulina platensis*. The models allowed the prediction of the effects of extraction parameters on the yield of protein extraction from *Spirulina platensis* and on the total phenolic content and antioxidant activity of the protein concentrates.

Extraction yield. The experimental results showed that the extraction yield ranged from 20.03% to 29.96% and are presented in Table 3. Temperature, pH and quadratic effect of pH exhibited significant effects on the extraction yield ($P < 0.05$), whilst time had no significant effect ($P > 0.05$). Estimated coefficients of the fitted second-order polynomial model are shown for all response variables in Table 5. The variation of the extraction yield with temperature and pH at constant time (90 min) is presented in Figure 1A. The graph plot revealed that the extraction yield increased when approaching the central point. The maximum extraction yield was obtained under the experimental conditions of a temperature of > 35°C and pH 8–8.5 (Figure 1A). When the temperature reached 45°C, extraction yield was approximately 30%. A similar trend has also been reported for the extraction of phycocyanin from *Spirulina platensis* in a study conducted by Hadiyanto and Suttrisnorhadi (2016). Moreover, in the study of Sarada *et al.* (1999), phycocyanin yield increased with increasing temperature until it reached an optimum level. While the solubility of proteins increases with increasing temperatures, temperatures greater than 45°C lead to a reduction in protein yield due to protein

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denaturation and a consequent decrease in protein solubility. Moreover, strong alkali conditions also cause a reduction in protein extraction yield because of protein denaturation (Lv *et al*. 2011).

Total phenolic content and antioxidant activity. The regression equation together with significant coefficients is presented in Table 5. The TPC of protein concentrates varied from 1.02 ± 0.09 mg CAE/g dw to 5.18 ± 0.09 mg CAE/g dw (Table 3). In the study of Wu *et al.* (2005), the phenolic content of *Spirulina* was determined as 6.86 ± 0.58 mg tannic acid equivalent/g of algal powder. Pagnussatt *et al.* (2014) found the phenolic content of *Spirulina* sp. strain LEB-18 to be 0.7 mg gallic acid equivalent/g dw.

Temperature and pH had no significant effect ($P > 0.05$), whereas the quadratic effect of time had significant effects on TPC ($P < 0.05$). The decrease in TPC over time was statistically significant ($P = 0.0061$) and can be explained by the degradation of phenolic compounds in response to prolonged exposure to ambient conditions (Thoo *et al*. 2010). Thoo *et al*. (2010) revealed that an extraction protocol of excessive duration resulted in an increased diffusion of antioxidants from the mengkudu (*Morinda citrifolia*) fruit.

The phenolic compositions of SPPCs were detected for all extraction conditions. Twenty phenolic acids were found in SPPCs, namely, 3-4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, caffeic acid, caffeine, catechin, chlorogenic acid, ellagic acid, epicatechin, ethyl-3-4-dihydroxybenzoat, ferulic acid, fumaric acid, gallic acid, *p*-coumaric acid, quercetin, rutin, sinapic acid, syringic acid, *t*-cinnamic acid, vanillic acid, and vanillin. Caffeic acid, caffeine, ferulic, and syringic acid were the major phenolic compounds found in *Spirulina platensis*. In previous studies, gallic, caffeic, salicylic, *t*-cinnamic and chlorogenic acids were also found in *Spirulina platensis* (Souza e*t al.* 2011; Pagnussatt *et al.* 2014).

In terms of antioxidant activity, the CUPRAC and DPPH assays gave values ranging between 2.30 to 13.30 mg TE/g dw and 6.71–10.01 mg TE/g dw, respectively. Estimated coefficients of the fitted second-order polynomial model are shown for all response variables in Table 5. According to the results of the CUPRAC assay, the effect of temperature and pH was significant ($P = 0.019$ and $P = 0.007$), whereas time had no significant effect ($P = 0.179$). The overall model for CUPRAC was statistically significant ($P = 0.006$). None of the linear effects of the parameters examined elicited significant differences in DPPH ($P > 0.05$), whereas the overall model for DPPH was statistically significant ($P = 0.029$). The lowest antioxidant activity was determined for a temperature of 25°C, pH 9 for 90 min, whereas the highest antioxidant activity was obtained with a temperature of 45°C, pH 8 for 60 min according to the results of the CUPRAC assay. The antioxidant activity increased under experimental conditions characterised by a temperature of about > 35°C and pH of about < 8.00. In the study of Wu *et al*. (2005), the antioxidant activity of *Spirulina* was determined as 19.39 ± 1.06 μmol of ascorbic acid equivalent/g *Spirulina* extract.

In vitro protein digestibility. The interaction effect of temperature and time and the quadratic effect of time for *in vitro* protein digestibility were significant ($P = 0.0372$ and 0.0397), whereas temperature, pH and time had no significant effect ($P > 0.05$). However, the overall model for *in vitro* protein digestibility was not statistically significant ($P = 0.1898$). Estimated coefficients of the fitted second-order polynomial model are shown for all response variables in Table 5. The maximum protein digestibility was obtained under the experimental conditions of a temperature of > 40°C and a time of approximately 105 min (Figure 1B).

After pancreatin digestion, protein contents and PD% of SPPCs ranged from 1.64 to 9.76 mg/g dw and from 87.45 to 97.81%. The highest PD% of SPPC was determined as 97.81% in the 7th run under extraction conditions of a temperature of 45°C, pH 9 for 90 min, and the lowest protein digestibility of 87.45% was obtained in the 9th run with extraction conditions of a temperature of 25°C, pH 8 for 120 minutes. As mentioned above, there is no information in the literature about the protein digestibility of *Spirulina platensis*; therefore, examples from studies about other algae and plant species are given. PD% values of *Hypnea charoides*, *Hypnea japonica*, and *Ulva lactuca* protein concentrates ranged from 85.7 to 88.9% in the study of Wong and Cheung (2000). In a study conducted by Świeca *et al*. (2013), the protein concentrations of gastrointestinally digested breads enriched with onion skin were found to be between 2.88 ± 0.13 and 8.53 ± 0.37 mg/g, and PD% was found to range from 55.00 ± 3.89% to 78.35 ± 2.85% in dry weight. In another study, the PD% of amaranth grain and protein content of digested extracts were determined as 76.03 ± 1.35–83.58 ± 2.24% and 2.42 ± 0.26–3.30 ± 0.07 g/100 g dw, respectively (Hejazi *et al.* 2016). The PD% values of pinto bean protein

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Table 5. Estimated coefficients of the fitted second-order polynomial model for all response variables

Regression coefficient	Yield (%)	TPC (mg CAE/g dw)	CUPRAC (mg TE/g)	DPPH (mg TE/g)	PD (%)
β_0	27.56	2.03	7.43	7.32	91.52
Linear					
β_1	2.43	0.47	1.19	–0.26	1.70
β_2	1.09	0.011	–1.50	–0.091	–0.12
β_3	0.77	–1.07	–0.59	0.36	0.99
Cross product					
β_{12}	0.24	0.23	1.32	–0.43	0.085
β_{13}	0.25	0.55	0.24	0.16	3.63
β_{23}	0.030	–0.29	–0.018	–1.19	–1.40
Quadratic					
β_{11}	–1.25	0.085	0.080	0.031	0.031
β_{22}	–3.44	–0.068	–1.16	0.089	3.48
β_{33}	1.05	1.46	3.21	0.74	–0.32

TPC – total phenolic content; CUPRAC – cupric reducing antioxidant capacity; DPPH – 1,1-diphenyl-2-picrylhydrazyl; PD – protein digestibility Y = Equation (2)

isolate and soybean protein isolate were found to be 71.3 and 85.2%, respectively (Tan *et al.* 2014). Similar to our study, PD% for amaranth grain reached up to 97.8% (Hejazi *et al.* 2016). Wong and Cheung (2000) indicated that the PD% of seaweed and plant proteins differed according to seasonal variations and the content of anti-nutritional factors like phenolic compounds and polysaccharides. Oxidized phenolic compounds found in protein extracts can interact with proteins and inhibit the activity of proteolytic enzymes (Wong & Cheung 2000).

Optimisation and verification. The adequacy of the models for predicting the optimum response values was tested by performing *Spirulina platensis* protein extraction using the optimised conditions determined using RSM (temperature 45°C, pH 7.46, and time 120 min). Predicted and mean experimental values for the extraction yield (29.05 and 30.06 ± 0.85%), total phenolic content (3.52 mg CAE/g dw and 3.45 ± 0.65 mg CAE/g dw), antioxidant activity (11.32 mg TE/g dw and 11.06 ± 0.03) and *in vitro* protein digestibility (99.36 and 98.15 ± 0.49%) indicated that the experimental values were very close to the predicted values and were not statistically different at the 5% significance level. These results of analysis indicate that the experimental values are in good agreement with the predicted ones, and also suggest that the models of total phenolic content, antioxidant activity and extraction yield are satisfactory and accurate.

CONCLUSIONS

Protein extraction from *Spirulina platensis* was successfully optimised using RSM. The optimum extraction conditions were 45°C, pH 7.46 and 120 minutes. Protein concentrates extracted from *Spirulina platensis* exhibited similar total phenolic contents and antioxidant activities as other algal species described in the literature. *In vitro* protein digestibility of *Spirulina platensis* was evaluated for the first time in this study and was found to be over 90%. This value is higher than the *in vitro* protein digestibility values of proteins obtained from other algal and plant species. As mentioned above, *in vitro* protein digestibility of other algal and plant source proteins ranged from 55% to 88%, and the *in vitro* protein digestibility of soybean protein isolate as a commercial protein product was about 85%. Therefore, *Spirulina platensis* protein concentrate, a cheap and novel source of protein with high protein digestibility, could be used as an additive to improve the antioxidant property and increase the protein content of food products.

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