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Application of Box-Behnken Design and Desirability Function for Green Prospection of Bioactive Compounds from *Isochrysis galbana*

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Abstract: A microalga, *Isochrysis galbana*, was chosen in this study for its potent natural antioxidant composition. A broad bioactive compounds spectrum such as carotenoids, fatty acid polyunsaturated (PUFA), and antioxidant activity are described with numerous functional properties. However, most of the optimization of extraction use toxic solvents or consume a lot of it becoming an environmental concern. In this research, a Box-Behnken design with desirability function was used to prospect the bioactive composition by supercritical fluid extraction (SFE) after performing the kinetics curve to obtain the optimal extraction time minimizing operational costs in the process. The parameters studied were: pressure (20–40 MPa), temperature (40–60 °C), and co-solvent (0–8% ethanol) with a CO_2 flow rate of 7.2 g/min for 120 min. The response variables evaluated in *I. galbana* were extraction yield, carotenoids content and recovery, total phenols, antioxidant activity (TEAC method, trolox equivalents antioxidant capacity method), and fatty acid profile and content. In general, improvement in all variables was observed using an increase in ethanol concentration used as a co-solvent (8% v/v ethanol) high pressure (40 MPa), and moderately high temperature (50 °C). The fatty acids profile was rich in polyunsaturated fatty acid (PUFA) primarily linoleic acid (C18:2) and linolenic acid (C18:3). Therefore, *I. galbana* extracts obtained by supercritical fluid extraction showed relevant functional ingredients for use in food and nutraceutical industries.

Keywords: microalgae; fucoxanthin; fatty acids; antioxidant; supercritical CO_2 extraction; co-solvent.

1. Introduction

Lately, the interest in studying the food applications of microalgae has increased significantly due to high nutritional value and a vast variety of novel metabolites with numerous innovative food applications. These could be considered such as nutraceuticals, food supplement, functional ingredients, or functional foods. Continually, new interpretations, and dynamic situation in the food sector provoke that these applications are situated between ordinary food and medical drugs [1]. In addition, huge interest has emerged among consumers and the nutrition industry in novel products that can promote good health, improve the state of wellbeing, and decrease the risk of diseases [1,2]. Diabetes, cardiovascular diseases, obesity, hypertension, cancer, or depression are some examples of diseases where these novel products combined with healthy lifestyle could modulate them [1,2]. Although microalgae have been used for centuries as a source of nutrition [3],

many species such as *Arthrospira maxima*, *A. platensis*, *Chlorella vulgaris*, *Haematococcus pluvialis*, *Isochrysis galbana*, *Scenedesmus* sp., *Porphyridium cruentum*, or *Phaeodactylum tricornutum* have been recently included for their composition [4–6]. Proteins, amino acids, polysaccharides, pigments, carotenoids (β-carotene or astaxantina), vitamins, and fatty acids between other are compounds described for theirs beneficial health effects such as: anti-inflammatory, protection of UV radiation, immune system, arthritis, Alzheimer's disease, or cancer or applications in pharmaceutical or cosmetics industries (natural colorants or anti-aging products) [1,4,5,7].

This current research particularly focused on *Isochrysis galbana* (Phylum: Haptophyta). This marine microalga has served in the aquaculture industry as a feed of bivalves, fish larvae, crustaceans, and mollusks for years [8]. Recently, this microalga has also been of interest in adjusting the composition of significant biomolecules, such as polysaccharides, fatty acid, carotenoids, vitamin, and sterols which are bioactive compounds that elicit positive nutrition in human foods or animal feed. Further, these molecules have demonstrated their therapeutic potential against several diseases like cancer, diabetes, cardiovascular and infectious diseases, among others [9,10]. *I. galbana* has also been described as a significant source of vitamin A and E, folic acid, nicotinic acid, pantothenic acid, biotin, thiamin, riboflavin, pyridoxine, cobalamin, chlorophyll (a and c), fucoxanthin, and diadinoxanthin [11].

I. galbana harbors specific carotenoid fucoxanthin and has high content of bioactive compounds including polyunsaturated fatty acids (PUFA) profile such as docosahexaenoic acid (DHA). Fucoxanthin is also present in brown seaweed or several diatoms, such as *Phaeodactylum tricornutum*, and its activity as an anti-inflammatory, antioxidant, and anticancer has been demonstrated in several studies [12]. Additionally, fucoxanthin is also able to modulate certain genes implicated in the cell metabolism, a property seemingly essential for good health [13,14]. In addition, the presence of PUFA is beneficial because they contribute to the production of prostaglandins, and/or thromboxanes which are biologically active substances that play important roles in the reduction of cholesterol and triglycerides in the blood as well as prevention of cardiovascular diseases, atherosclerosis, skin diseases, and arthritis [15,16]. PUFAs should be included in the daily diet because they cannot be synthesized by humans or animals per se [16].

For the extraction of bioactive compounds, an ideal extraction method should result in a rapid quantitative recovery of the target without degradation, and the removal of the solvent post-extraction should be easy and rapid. Many bioactive natural products are thermolabile and can degrade while using traditional extraction methods. For these reasons, supercritical fluids extraction (SFE) through carbon dioxide has been demonstrated as an effective method for the extraction of bioactive compounds [9,17]. The primary advantages of this green extraction are: (i) the possibility to change the density of the fluid through controlled pressure and/or temperature accompanied by modified extraction solubility, (ii) use of green solvent generally recognized as safe (GRAS), and (iii) lower extraction times with enhanced extraction yield [9,18].

Therefore, the aim of this study was to extract and investigate the total carotenoids (fucoxanthin represented as main total carotenoids by spectrophotometry method) present in *I. galbana* using the supercritical fluid extraction technique through carbon dioxide. Parameters of temperature (40–60 °C), pressure (20–40 MPa), and percentage of co-solvent (0–8% ethanol) were controlled based in other previous studies [9]. The extraction yield, total carotenoids content and its recovery, total phenols, antioxidant activity (TEAC, trolox equivalents antioxidant capacity), and fatty acid profile of the obtained extracts were determined. The kinetic study was also performed to control the optimal extraction time in the prospection of total carotenoids from *I. galbana*. Finally, the optimal parameters are recommended for time and extraction conditions of total carotenoids and other compounds present in *I. galbana*, which have significant potential in functional and biotechnological applications minimizing operational costs in the process, for example, the volume of ethanol.

2. Materials and Methods

2.1. Chemicals and Samples

The microalga *I. galbana* was selected for this research. Mexican Company "Microalgas Oleas de México S.A." (Guadalajara, Mexico) kindly donated the dry biomass for this research. The microalga was cultured under a controlled condition and harvested in its exponential growth phase. The biomass was dried under the freeze-dry system such as Labconco FreeZone 2.5 L Benchtop Dry System (Labconco, Kansas City, MO, USA), packed in vacuum sealing plastic bags, and stored at 4 ± 2 °C in darkness until use. The chemical materials used for supercritical fluid extraction (SFE) were carbon dioxide (99% purity), purchased from Indura Group Air Products (Santiago, Chile) and ethanol (99.5%), from Merck (Darmstadt, Germany). Other chemicals such as ultrapure water, fucoxanthin standard, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, ≥ 97%), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS ≥ 99%), and Folin–Ciocalteu phenol reagent were purchased from Sigma-Aldrich (Santiago, Chile). Chromatographic grade ethyl acetate, water, acetonitrile, methanol, and n-hexane were purchased from Sigma-Aldrich (Santiago, Chile). For fatty acid identification and quantification, a standard fatty acid methyl ester (FAME) mix, C4-C24, supplied by Supelco Analytical (Bellefonte, PA, USA) was used, and tripentadecanoin > 99% (Nu-Check Pre, Inc., Elysian, MN, USA) was used as the internal standard.

2.2. Supercritical Fluid Extraction

The extractions were carried out using a Speed Helix supercritical extractor (Applied Separation, Allentown, PA, USA) as per the scheme presented in Figure 1. For each extraction, 2 g of freeze-dried biomass of *I. galbana* was used, previously ground and sieved using a standard sieve of 35 mesh of the Tyler series (particle size ≤ 0.354 mm), along with polypropylene wool and glass beads (Φ = 1 mm), which was then inserted into stain-steel extraction cell of 24 mL.

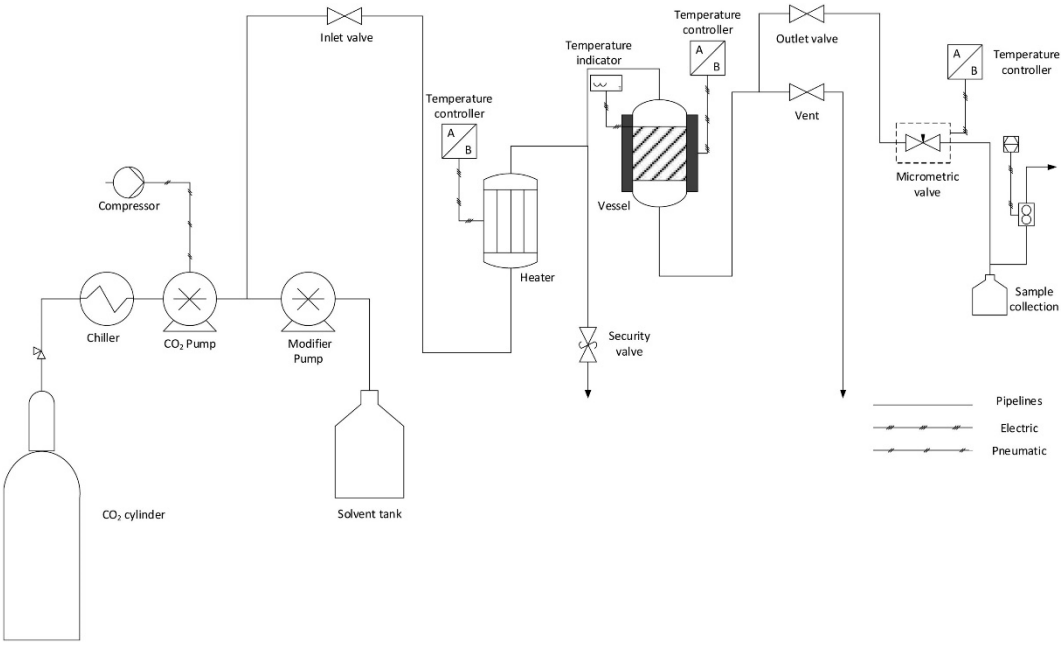


Figure 1. Diagram of the supercritical fluid extraction (SFE) equipment (Applied Separations, Speed, Allentown, PA, USA). The main parts are: CO_2 cylinder; CO_2 pump; compressor; modifier pump; solvent tank; inlet valve; heater; extraction vessel and oven vessel; micrometric valve; sample collection.

In all cases, a flow rate of 7.2 g/min was maintained for CO_2 and each extraction was carried out for 120 min. Extraction conditions of the microalga were selected based on preliminary kinetics assays with *I. galbana* and were set for 150 min to ensure the complete removal of bioactive compounds.

The resulting extracts were collected in vials and the residual ethanol was evaporated under an N_2 gas stream by Flexivap Work Station (Model 109A YH-1, Glas-Col, Terre Haute, IN, USA) for calculating extraction yield. Then, dried extracts were stored at −20 °C and protected from light until further analysis.

2.3. Experimental Design

A Box-Behnken design was implemented in random run order (Table 1), generating 15 experimental conditions tested (Table 2). As per this design, three factors were evaluated at three different experimental levels of temperature (40–60 °C), pressure (20–40 MPa), and percentage of ethanol as a co-solvent (0–8% v/v). The effects of the factors on different responses, including extraction yield (Y), total carotenoids content (TCC) and its recovery (TC recovery), total phenolic content (TPC), antioxidant activity (TEAC assay), and fatty acids profile (FAMEs) were studied. The experimental design and data analysis were carried out using response surface methodology (RSM) with Statgraphics Centurion XVI®(StatPoint Technologies, Inc., Warrenton, VA, USA) software.

In a design that involves three factors X1, X2, and X3, the mathematical relationship of the response with these factors is approximated by the quadratic polynomial equation named second degree Equation (1) described below:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y = estimate response; β0 = constant; β1, β2, and β3 = linear coefficients; β12, β13, and β23 = interaction coefficients between the three factors; β11, β22, and β33 = quadratic coefficients. Multiple regression analysis is done to obtain the coefficients and the equation can be used to predict the response.

The effects of the independent factors on the response variables in the separation process were assessed using pure error, considering a confidence level of 95% for all the variables. The effect of each factor and its statistical significance, for each of the response variables, were analyzed using ANOVA and standardized Pareto chart. The response surfaces of the respective mathematical models were also obtained, and their significance was accepted at $p \leq 0.05$. A multiple response optimization was carried out through the combination of experimental factors, to maximize the desirability function for the responses in the extracts. The desirability function method was applied to generate optimum conditions having some specific desirability value (close to =1 indicate that the setting achieves favorable results for all responses). All variables were obtained with the same equal weight = 1 (maximizing response).

2.4. Kinetic Study

A kinetic curve was plotted between the optimal extraction time versus the accumulated extract and total carotenoids content. The kinetic study was performed at the central point of the experimental design (30 MPa, 50 ºC, and 4% co-solvent ethanol, v/v) as described by Gilbert-López et al. [19]. Each sample was analyzed for 4–6 min in the first 1 h and then 15 min for a total of 150 min. In this assay, the extraction yield (Y, %) and total carotenoids content (TCC, mg/g biomass) were calculated at each point of the curve. This assay was performed in duplicate with a total of 34 points per sample.

2.5. Extracts Analysis

2.5.1. Total Carotenoids Content (TCC)

Total carotenoids were determined in *I. galbana* biomass using a fucoxanthin standard (Sigma-Aldrich, 0–50 ppm) on a UV–Vis spectrophotometer (Shimadzu UV–1280, Kyoto, Japan). The absorption spectrum of total carotenoids was assessed in the maximum absorption wavelength

selected of fucoxanthin because it is the main pigment on total carotenoids described in *I. galbana* [19,20]. This wavelength was 447.4 nm and Equation (2) was used to determine the total carotenoids content.

$$\mathrm{TCC} = \frac{(\mathrm{A447.4} \times 8.66 \times \ \mathrm{DF} \ \times \mathrm{V})}{M_{biomass}} \quad (2)$$

where TCC = total carotenoids content in mg/g biomass, A447.4 = the absorbance of the sample at λ max, 8.66 = the specific slope of the standard curve, DF = dilution factor of solvent, V = the solvent volume used in mL, and finally, Mbiomass is the mass of *I. galbana* in mg. Then, each ethanolic extraction after SFE of *I. galbana* biomass was measured and the final concentration of total carotenoids was calculated. The determination was carried out in triplicate (n = 3).

2.5.2. Total Carotenoids Recovery

The effect of operating conditions on total carotenoids extraction was expressed in terms of recovery, which was calculated on the basis of the initial mass of each compound as per Equation (3):

$$\text{Recovery } (\%) = \left(\frac{\mathrm{Wc}}{\mathrm{Wt}}\right) \times 100 \quad (3)$$

where WC = mass of the compound extracted (mg); Wt = theoretical mass of the compound from a conventional extraction (mg). The total carotenoids were extracted by a conventional method using methanol for 24 h in a shaker incubator at 300 rpm at 30 °C, providing an average extracted of 24.4 ± 2.2 mg/g of total carotenoids.

2.5.3. Total Phenol Content (TPC)

Estimation of TPC was based on the 96-well microplate Folin–Ciocalteu method described by Ainsworth and Gillespie [21]. A total of 20 µL of the diluted extract (2.0 mg/mL) were mixed with 100 µL of 10% (v/v) Folin–Ciocalteu reagent and shaken. The mixture was left resting for 5 min. then 75 µL of sodium carbonate solution (700 mM) was added and was again shaken for 1 min. After 60 min at room temperature, the absorbance was measured at 765 nm on a microplate reader (BioTek Synergy HTX multi-mode reader, software Gen 5 2.0, Winooski, VT, USA). The absorbance of the same reaction with methanol, instead of the extract or standard, was subtracted from the absorbance of the reaction with the sample. For calibration, gallic acid dilutions (0–2 mg/mL) were used as standards. The results were expressed as gallic acid equivalents (GAE)/g biomass and were presented as the average of three measurements.

2.5.4. Determination of Antioxidant Activity

The Trolox equivalents antioxidant capacity (TEAC) value was determined using the method described by Re et al. [22], with a few modifications [23]. 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS•+) radical was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate in the dark at room temperature for 16 h. The aqueous ABTS•+ solution was diluted with 5 mM sodium phosphate buffer at pH 7.4 to an absorbance of 0.7 (± 0.02) at 734 nm. Then, 20 µL of sample and 180 µL of ABTS•+ solution was added in a 96-well microplate reader of a spectrophotometer. The absorbance was measured at 734 nm within 10 min of the reaction. 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was used as reference standard and results were expressed as TEAC values (mmol Trolox equivalents (TE)/g biomass). All analyses were done in triplicate (n = 3).

2.5.5. Extraction of Fatty Acid

The extraction of fatty acid methyl esters (FAMEs) was performed as per the direct acid catalysis method described by Lamers et al. [24] with a few modifications. Briefly, the reaction mixture containing

10 mg of SFE extract, 10 ppm of internal standard, and 3 mL of 5% (v/v) H_2SO_4 solution in methanol was incubated at 80 °C for 1 h with continuous agitation. Then, the flasks were washed with hexane and Milli-Q water until the pH of the water after washing was neutral. The mixture was separated into two layers by centrifugation (360 ×g, 10 min). The upper oil layer (FAMEs diluted in hexane) was separated and washed with Milli-Q water for further analysis and quantification by gas chromatography.

2.5.6. Analysis of Fatty Acids

In order to analyze the fatty acid composition, a gas chromatograph (Shimadzu 2010, Kyoto, Japan) equipped with a flame ionization detector (FID) and a split/splitless injector was used. In all the cases, samples (1 µL) were injected into a capillary column (RESTEK; 30 m, 0.32 mm i.d., 0.25 µm film thickness). The injector temperature was maintained at 250 °C in the split mode with a split ratio of 20:1 and nitrogen was used as the carrier gas at a constant flow rate of 1.25 mL/min. The oven temperature was maintained at 80 °C for 5 min, increased to 165 °C at the rate of 4 °C/min and maintained for 2 min, and further increased to 180 °C at the rate of 2 °C/min and maintained for 5 min. It was further heated at a rate of 2 °C/min to 200 °C for 2 min, then at a rate of 4 °C/min to 230 °C and maintained for 2 min, and finally maintained at that temperature for 2 min, reaching 250 °C at 2 °C/min. The detector temperature was 280 °C. Individual FAMEs were identified by comparing their retention times with those of mixed FAME standards (FAME Mix C4-C24, Supelco Analytical) and quantified by comparing their peak area with those of mixed FAME standards and an internal standard (tripentadecanoin ~10 ppm/sample, Nu-Check Pre, Inc., Elysian, MN, USA).

3. Results and Discussion

3.1. Specific Kinetics and Selection of Box-Behnken Design of Supercritical Fluid Extraction from Isochrysis galbana

Optimum extraction time was set by analyzing the kinetics of the extraction carried out by SFE. Figure 2 shows the evolution of the performance and the TC accumulated vs. extraction time of 150 min. The condition was marked as a central point of the experimental design chosen (30 MPa, 50 °C, 4% v/v of co-solvent ethanol). Each extract was recovered at the established time point as described in the material and methods section and the percentage of the extractable was calculated. The yield extraction curve reached a plateau at 60 min and total carotenoids between 100 and 120 min.

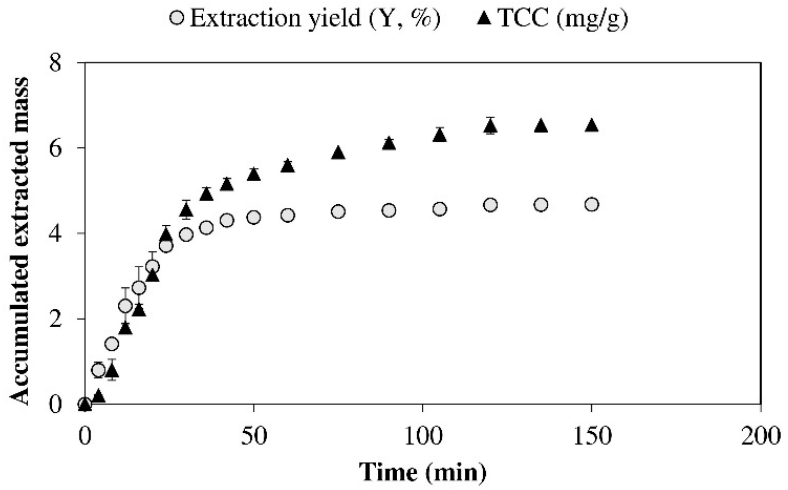


Figure 2. Kinetics of improving extraction yield (Y, %) and total carotenoids content (TCC) from *I. galbana* during 150 min. The working conditions were at the central point: 50 ºC, 30 MPa, 4% co-solvent (ethanol, v/v), and a CO_2 flow rate of 7.2 g/min.

For consolidating the maximum in all the variables studied, an optimal extraction time of 120 min was set for each SFE condition as after this time there was no increase in the amount of extracted material. In particular, the maximum value of total carotenoids recollected was approximately 99.80% (~ 6.53 mg/g) and the extraction yield was 99.8% (~ 4.67%, w/w) at 120 min.

On the other hand, Box-Behnken statistical design was selected for this study because previous works demonstrated that models 2 K did not represent adequately the bioactive compounds extraction process (results not shown). Respect to the percentage of co-solvent, we also tested with less range (0%-4% v/v) and all variables content was improved under 4% ethanol. For this reason, we expand to 8% of the volume of ethanol in order to minimize the cost process under the Box-Behnken design. Table 1 shows the results of the ANOVA and Regression coefficients for each variable. Here, R-Square statistic indicated that the quadratic model was well adjusted in against lineal or second order. It means the variability in all studied variables. In the case of the adjusted R-square statistic were slightly smaller than R-squared and in the same way lack-of-fit determined that the selected model was adequate to describe the observed data. This test was performed by comparing the variability of the current model residuals to the variability between observations at replicate values of the independent variable. Since the *p*-value for lack-of-fit in the ANOVA table was greater or equal to 0.05, the model appears to be adequate for the observed data (except FAME data that was close to 0.05).

Table 1. Regression coefficients (values of variables are specified in their original units, extraction yields (Y), total carotenoids content (TCC), TC recovery, total phenol content (TPC), Trolox equivalents antioxidant capacity (TEAC) antioxidant method), fatty acid methyl ester (FAMEs), and statistics for the fit obtained by multiple linear regression.

Terms of the Model	Y		TCC		TC recovery		TPC		TEAC		FAMEs	
	Estimated	*p*-value	Estimated	*p*-value	Estimated	*p*-value	Estimated	*p*-value	Estimated	*p*-value	Estimated	*p*-value
constant	51.47		35.73		146.47		–619.40		–2.85		–17.72	
A:P	–0.09	0.05 *	0.11	0.18	0.44	0.18	2.38	0.32	–0.0007	0.02*	0.07	0.15
B:T	–1.44	0.14	–1.73	0.67	–7.10	0.67	17.03	0.19	0.124	0.81	0.44	0.43
C:Co-solvent	–1.23	0.0012 *	–2.27	0.009*	–9.32	0.009 *	–21.94	0.07	0.0364	0.05 *	–0.63	0.01 *
AA	0.00002	0.83	–0.00021	0.31	–0.00086	0.31	–0.0045	0.05 *	$-3.33\ E^{-7}$	0.91	–0.0001	0.06
AB	0.0019	0.051	0.0004	0.83	0.0016	0.84	0.0030	0.87	0.00003	0.29	0.0004	0.64
AC	0.0001	0.96	0.0049	0.33	0.020	0.33	0.0775	0.12	–0.00001	0.86	0.0002	0.91
BB	0.0078	0.37	0.014	0.48	0.058	0.48	–0.2061	0.29	–0.00136	0.01 *	–0.0064	0.17
BC	0.045	0.06	0.037	0.45	0.151	0.45	0.2357	0.59	0.00069	0.36	0.032	0.16
CC	–0.025	0.63	0.033	0.79	0.136	0.79	–0.7877	0.50	–0.00677	0.01 *	–0.061	0.10
Lack-of-Fit		0.13		0.29		0.30		0.10		0.05		0.04
Statistics for the goodness of fit of the model												
R^2	0.930		0.825		0.825		0.806		0.918		0.874	
Adjusted R^2	0.804		0.510		0.510		0.457		0.770		0.647	
RSD	1.506		3.565		14.616		33.068		0.054		1,567	
P	0.336		0.905		0.905		0.356		0.871		0.251	
C.V.	0.639		0.647		0.647		0.676		0.529		0.558	

Note: R^2—determination coefficient, adjusted R^2, RSD—residual standard deviation, *p*-value of the lack-of-fit test for the model; C.V.—coefficient of variation; * significant coefficients of the model.

3.2. *Effects of Different Parameters on the Extraction Yield and Total Carotenoids Content and Recovery*

The experimental conditions and results of the Box-Behnken design for the extraction conditions for *I. galbana* by SFE for 120 min are listed in Table 2. The range of extraction yield (Y) was 1.09–12.82% (w/w) and that of total carotenoids content (TCC) was 1.34–19.01 mg/g.

In this table, the total carotenoids recovery is also mentioned, which was estimated as the percentage of the total carotenoids extracted in each sample to the total carotenoids extracted in a conventional methanol extraction, which was assumed to be 100% (24.40 ±2.24 mg/g) with respect to dry biomass. The recovery of total carotenoids reached 5.50% under pure CO_2 and 77.93% when CO_2 modified with 8% (v/v) ethanol was used. The conditions were at 20 MPa/50 °C and at 300 MPa/60 °C, respectively.

Letters in the experiment column are the acronyms of the tested variables: Pressure (P) and Temperature (T). Values are represented as a mean standard deviation. It was SD ≤ 5% (n = 3 analytical measurement). All values were calculated per gram of initial biomass.

Table 2. Extraction yields (Y), total carotenoids content (TCC), total carotenoids recovery (TC recovery), total phenol content (TPC), TEAC antioxidant method, and fatty acid methyl ester (FAMEs) by SFE from freeze-dried *Isochrysis galbana* using Box-Behnken experimental design. The general parameters were biomass loading = 2.0 g, CO_2 flow rate = 7.4 g/min, extraction time = 120 min.

Run	P (MPa)	T (°C)	Co-solvent (%)	Y (%, w/w)	TCC (mg/g)	TC Recovery (%, w/w)	TPC (mg GAE/g)	TEAC (mmol TE/g)	FAMEs (mg/g)
1	30	40	8	5.71 ± 0.24	14.09 ± 0.55	57.75 ± 2.30	93.33 ± 3.52	$0.11 \pm 5.3 \times 10^{-3}$	3.41 ± 0.13
2	40	40	4	6.16 ± 0.16	9.66 ± 0.32	39.61 ± 1.45	50.93 ± 2.01	$0.28 \pm 1.3 \times 10^{-2}$	5.56 ± 0.16
3	40	60	4	10.25 ± 0.49	6.22 ± 0.29	25.49 ± 1.10	22.89 ± 1.02	$0.33 \pm 1.4 \times 10^{-2}$	5.41 ± 0.21
4	40	50	0	2.28 ± 0.11	4.05 ± 0.16	16.61 ± 0.74	5.98 ± 0.23	$0.22 \pm 1.1 \times 10^{-2}$	1.18 ± 0.04
5	20	50	0	1.09 ± 0.03	1.34 ± 0.06	5.50 ± 0.21	5.71 ± 0.20	$0.15 \pm 7.5 \times 10^{-3}$	0.47 ± 0.02
6	30	50	4	5.79 ± 0.21	7.02 ± 0.33	28.78 ± 1.35	109.31 ± 3.56	$0.31 \pm 1.4 \times 10^{-2}$	7.57 ± 0.26
7	40	50	8	8.78 ± 0.32	15.33 ± 0.72	62.85 ± 2.68	157.16 ± 3.66	$0.31 \pm 1.5 \times 10^{-2}$	7.19 ± 0.33
8	30	50	4	5.19 ± 0.24	10.83 ± 0.51	44.38 ± 1.89	94.40 ± 4.02	$0.40 \pm 1.8 \times 10^{-2}$	7.63 ± 0.29
9	30	50	4	4.36 ± 0.20	6.00 ± 0.28	24.57 ± 1.02	120.77 ± 4.02	$0.33 \pm 1.5 \times 10^{-2}$	6.87 ± 0.32
10	30	60	8	12.82 ± 0.06	19.01 ± 0.92	77.93 ± 2.87	76.06 ± 3.52	$0.20 \pm 9.0 \times 10^{-3}$	8.82 ± 0.38
11	30	60	0	1.64 ± 0.08	2.74 ± 0.18	11.24 ± 0.42	37.71 ± 1.44	$0.04 \pm 1.0 \times 10^{-3}$	2.86 ± 0.12
12	20	40	4	5.73 ± 0.12	9.11 ± 0.45	37.33 ± 1.63	67.87 ± 3.21	$0.15 \pm 6.2 \times 10^{-3}$	3.50 ± 0.13
13	20	50	8	7.43 ± 0.35	4.85 ± 0.23	19.87 ± 0.75	32.90 ± 1.20	$0.26 \pm 1.2 \times 10^{-2}$	6.09 ± 0.28
14	30	40	0	1.80 ± 0.08	3.72 ± 0.17	15.25 ± 0.65	92.69 ± 3.54	$0.06 \pm 2.1 \times 10^{-3}$	2.63 ± 0.11
15	20	60	4	2.15 ± 0.10	4.10 ± 0.20	16.80 ± 0.82	28.04 ± 1.32	$0.07 \pm 2.9 \times 10^{-3}$	1.78 ± 0.07

The obtained mathematical models that maximize the yield (4), TCC (5), and TC recovery (6) were studied. The significant variables in the study, and which responded to the combined relationships between process variables, are given as follows:

$$Y = 51.46 - 0.093 \cdot P - 1.23 \cdot \text{Co-solvent} + 0.000018 \cdot P^2 + 0.0019 \cdot P \cdot T + 0.0001 \cdot P \cdot \text{Co-solvent} + 0.045 \cdot T \cdot \text{Co-solvent} - 0.025 \cdot \text{Co-solvent}^2 \quad (4)$$

$$TCC = 35.73 - 2.27 \cdot \text{Co-solvent} + 0.0049 \cdot P \cdot \text{Co-solvent} + 0.037 \cdot T \cdot \text{Co-solvent} + 0.033 \cdot \text{Co-solvent}^2 \quad (5)$$

$$\text{TC recovery} = 146.47 - 9.32 \cdot \text{Co-solvent} + 0.020 \cdot P \cdot \text{Co-solvent} + 0.15 \cdot T \cdot \text{Co-solvent} + 0.14 \cdot \text{Co-solvent}^2 \quad (6)$$

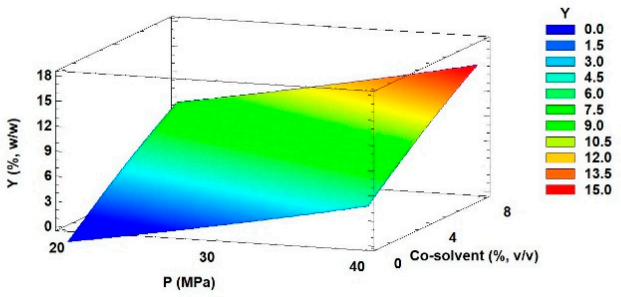
Table 1 further provides more information on the obtained statistical results by presenting the estimated regression coefficients of all the factors and interactions for each response variable.

In the case of the variables TCC content and TC recovery (Equations (4) and (5)), only the co-solvent factor was found significant in the extraction process. For the response variable Y (Equation (3)), the pressure factor was added to the co-solvent factor, as both were relevant from of *I. galbana* using SFE. In addition, Figure 3A to 3C show the response surfaces based on the selection of factors for the best recoveries of Y, TCC, and TC recovery, respectively. As is evident, higher Y, TCC, and TC recovery were obtained with an increase in co-solvent percentage.

This corroborates with the outcomes described by Gilbert-López et al. [19]. This study demonstrated that sequential steps using various pressurized green solvents have improved selectivity for bioactive compound recovery from *Isochrysis galbana*. The assays performed by SFE under pure CO_2 improved the yield of triacylglycerides but not carotenoids (optimal condition was 30 MPa/50 °C, achieving 16.2 ± 0.3 mg carotenoids/g extract and 5% (w/w) of yield). Conversely, the best possible recovery of fucoxanthin was observed by increasing the ethanol co-solvent to 45% (v/v), denominated as carbon dioxide expanded ethanol (CXE) extraction under low pressures [19]. This recovery of carotenoids is in accordance with a study by Conde et al. [25] using ethanol as a modifier and allowed higher extraction yields, particularly of fucoxanthin.

The yield also improved when the pressure was increased to 40 MPa. Several reports confirm that high pressure and temperature result in better yield for the same extraction duration [25,26]. A clear benefit of the increased pressure with co-solvent was the enhanced solvent density of CO_2. Nevertheless, the temperature was not a significant parameter in the results of our statistical analyses, but in general, the best response was observed under higher values of this factor such as 60 °C

(Figure 3). In this way, the "optimal values" delivered by the software for statistical analysis were 15.15%, 18.14 mg/g, and 74.36% w/w of Y, TCC, and TC recovery, respectively, under 40 MPa, ~60 °C and ~8% co-solvent. These experiences, in a certain way corresponded to the extraction conditions used in the experiment where the variables were improved (30 MPa, 60 °C, and 8.0% co-solvent) obtaining a lower value of 12.82% in extraction yield. However, TCC values and TC recovery of 19.01 mg/g and 77.93% w/w, respectively, were slightly higher than those predicted by the software.



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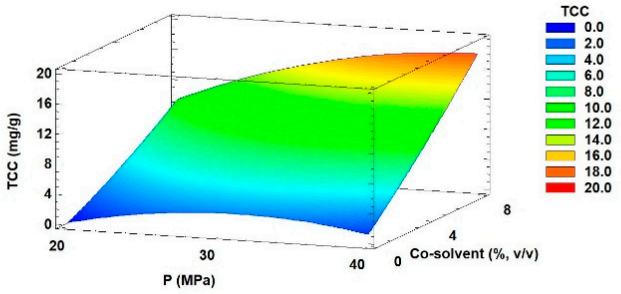

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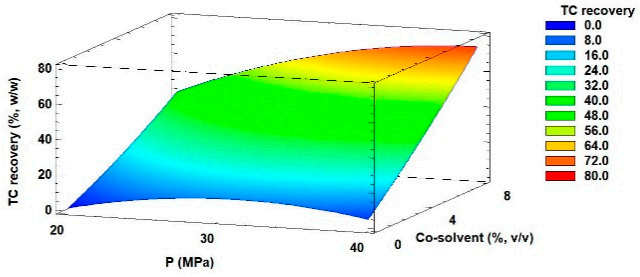

C

Figure 3. Response surface plot showing combined effects of pressure and co-solvent on (**A**) extraction yield (Y), (**B**) total carotenoids content (TCC), and (**C**) recovery (TC recovery) from *I. galbana*. All extractions were done at 60 °C temperature.

In general, the values achieved in this study are better than other reports of specific carotenoids extraction from natural sources. For example, the microalgae *Phaeodactylum tricornutum* or *Odontella aurita* [27,28] gave higher yield than those by brown seaweeds including *Laminaria japonica*, *Eisenia*

bicyclis, and *Undaria pinnatifida* [27,29,30]. Kim et al. [27] reported several methods and solvents to maximize fucoxanthin extraction from *P. tricornutum*, achieving the best yield of 16.51 mg/g under pressurized liquid extraction (PLE) at 10.3 MPa, 100 °C, 100% ethanol, and static time of 30 min. The content yielded was similar to that by maceration extraction (15.71 mg/g). Likewise, *O. aurita* is another microalga and a natural producer of fucoxanthin as described by Xia et al. [28]. In their study, besides optimizing culture conditions for improving this carotenoid, they attempted to determine the optimal conditions for fucoxanthin extraction using five conventional solvents. The best yield of fucoxanthin among the five tested solvents was obtained with methanol (16.18 mg/mg), followed by ethanol (15.83 mg/g) and acetone (13.93 mg/g). Generally, conventional methods are applied to extract bioactive compounds (especially from carotenoids family) using organic solvents [31] achieving better extraction yield although they are often required large volumes, making the method expensive and environmentally unfriendly [32]. For instance, Kim et al. [20] used different solvents and extraction time to improve the fucoxanthin recovery and were able to obtain 20.87 mg/g of fucoxanthin from dry biomass of marine microalga *I. aff. galbana* (CCMP1324) using acetone. These results are better than total carotenoids rich in fucoxanthin extracted by SFE in our study, which may be due to an increase in solvent density and swelling of the matrix because of co-solvent addition.

3.3. Total Phenolic Content and Antioxidant Response in Isochrysis galbana by Supercritical Fluid Extraction

Table 2 presents the results of total phenolic content (TPC) and TEAC method as an antioxidant response. The estimated response surfaces are described in Figure 4A based on the parameters for TPC, which was complemented with a summarized second-order polynomial Equation (7) as a mathematical model to find the optimum conditions that maximize TPC as follows:

$$\text{TPC} = -619.40 + 2.38 \cdot \text{P} - 0.0045 \cdot \text{P}^2 + 0.0029\text{P} \cdot \text{T} + 0.077 \cdot \text{P} \cdot \text{Co-solvent} \quad (7)$$

This quadratic interaction of the pressure factor was significant for this response variable (TPC) as is evident in Table 1. The optimal condition was determined to be 34.8 MPa, 48 °C, and 8% co-solvent with an optimal yield of 133.9 mg GAE/g biomass. These results are better than those reported in other studies using *I. galbana* as natural biomass rich in bioactive compounds through conventional methods of extraction [33,34]. For example, Widowati et al. [33] and Foo et al. [35] reported yields from *I. galbana* clone Tahiti, and *I. galbana* to be nearly 17.79 mg GAE/g and 12.24 mg GAE/g, respectively. Many reports compare SFE with conventional processes because they have advantages and disadvantages in both cases. In particular, fluids under supercritical condition reducing process time and enhancing the extraction yield. Parameters as temperature or pressure ease selectivity, effective penetrating the biomass and better mass transfer between phases versus conventional methods [18,36].

The results mentioned in Table 2 indicate enhanced TPC content with an increase in the percentage of co-solvent (8% under optimal condition). In algae, the use of co-solvent such as ethanol increases the polarity of SFE and with it, the phenolic compounds, antioxidant activity, and fucoxanthin recovery are expected to increase [25,37,38]. *Sargassum muticum* is a brown alga rich in fucoxanthin, fatty acids and phenolic compounds described by Conde et al. [25]. They demonstrated that an increase in the ethanol concentration strongly improved variables such as total yield, radical scavenging capacity, and the fucoxanthin extraction yield. However, due to the lower process selectivity, the phenolic content using maximum co-solvent (10% ethanol) was moderately higher than with pure CO_2. Other reports of TPC extraction from conventional methods include studies by Goiris et al. [39] on *Isochrysis* ISO-T and *Isochrysis* sp. giving yields of 2.67 and 4.57 mg GAE/g, respectively, which are relatively low. In a study performed by Li et al. [40], 23 microalgae were evaluated using sequential organic solvent extraction and it was observed that *Nostoc ellipsosporum* CCAP 1453/17 had a TPC of 60.35 ± 2.27 mg GAE/g. The antioxidants, such as fucoxanthin and phenolic compound in *Isochrysis* sp., have been prescribed for the prevention of the age-related changes in the central nervous system as they scavenge free radicals and reactive oxygen species (ROS) [41].

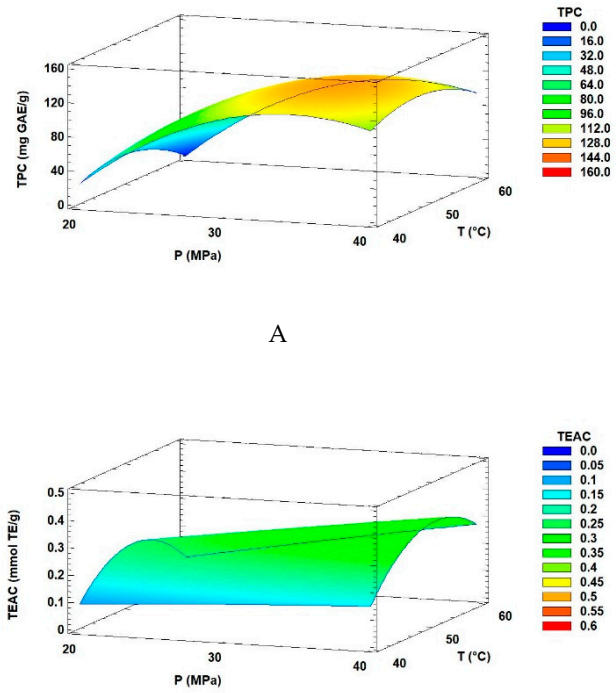

Figure 4. Response surface plot showing combined effects of pressure and temperature on (**A**) phenol content (TPC) and (**B**) TEAC method from *I. galbana*. All extractions were done with 8% co-solvent (ethanol, v/v).

The use of ABTS in the radical scavenging assay is a popular indirect method for determining the antioxidant capacity of bioactive compounds [42,43]. The antioxidant response was determined by TEAC method. The mathematic model can be described as the second-order polynomial Equation (8):

$$\begin{aligned} \text{TEAC} = &-2.85 - 0.00074\cdot\text{P} + 0.036\cdot\text{Co-solvent} - 3.33\text{E}^{-7}\cdot\text{P}^2 + 0.000032\cdot\text{P}\cdot\text{T} - \\ &0.000012\cdot\text{P}\cdot\text{Co-solvent} - 0.0014\cdot\text{T}^2 + 0.00069\cdot\text{T}\cdot\text{Co-solvent} - 0.0068\cdot\text{Co-solvent}^2 \end{aligned} \quad (8)$$

Here, several factors important in the TEAC determination such as pressure, presence of co-solvent, and quadratic interaction of temperature and co-solvent in SFE extracts of *I. galbana*. Figure 4B shows the estimated response surface and the obtained TEAC values are described in Table 2. The values were between 0.04 and 0.40 mmol TE/g at SFE condition of 30 MPa, 60 ºC, and 0% co-solvent and 40 MPa, 60 °C, and 4% co-solvent, respectively. The optimal value was 0.42 mmol TE/g at 40 MPa, 51.6 °C, and 4.9% co-solvent. Likewise, a study by Reyes et al. [44] demonstrated a similar influence of the factors on the TEAC response from *H. pluvialis*.

Particularly, our results indicate the influence of the solvent on the yield. Other authors have also described the possibility of using different combinations of ethanol + water as co-solvent for green extraction of bioactive compounds from a diverse natural source [45–47]. The inclusion of water as co-solvent has been described as advantageous because it influences a fast and quantitative recovery of the phenolic compounds, high anthocyanin concentration, and high antioxidant capacity [46,48]. Moreover, a reduced percentage of ethanol minimizes the cost and impact on the environment [48].

The antioxidant activity in this study was higher than that reported by other conventional methods. For example, Goiris et al. [39] described *Tetraselmis* sp. with the best antioxidant capacity among 23 microalgae at 69.4 µmol TE/g. *I. galbana* is rich in antioxidant activity because of the presence of carotenoids. The number of double and allenic bonds and the presence of an acetyl functional groups in fucoxanthin are also responsible for the higher antioxidant activities [42,49]. Fucoxanthin

and its derived metabolites display antioxidant activities comparable to that of α-tocopherol [43]. Nevertheless, very limited information is available about the phenolic contents and antioxidant activity using SFE in *I. galbana*.

3.4. Measurement of Fatty Acid Composition

The fatty acid profiles were also identified along with FAME content (mg FAME/g) because they can be used in different biotechnological applications. The FAME content per gram of biomass is listed in Table 2. The range of FAME content was from 0.47 to 8.82 mg/g and Run 10 (30 MPa, 60 °C, and 8% co-solvent) gave the best results of 8.82 mg/g followed by the central point of the Box-Behnken design with values in the range of 6.87–7.63 mg/g. A summarized polynomial equation was obtained to establish a mathematical model, reach optimum operating conditions, and maximize FAMEs contents from *I. galbana* by SFE. Equation (9) and data in Table 1 indicate the relevance of the co-solvent factor ($p < 0.05$) in the FAME quantification under these SFE conditions.

$$\text{FAME} = -39.50\,5 - 0.30{\cdot}\text{Cosolvent} + 0.0002{\cdot}\text{P}{\cdot}\text{Cosolvent} + 0.03{\cdot}\text{T}{\cdot}\text{Cosolvent} - 0.10{\cdot}\text{Cosolvent}^2 \quad (9)$$

The optimal value suggested for the response work surface was 8.84 mg/g under the experimental condition of 33.3 MPa, 57 °C, and 7.9% co-solvent, nearly similar to that observed in our study. These results are presented in Figure 5 (response surface graphic) where at medium pressure, with high temperature and co-solvent, FAME content increased in *I. galbana* although the co-solvent was the only significant factor in the process. Extractions under increased pressures gave the best results as the solubility of triglycerides could be improved due to an increase in the solvent density.

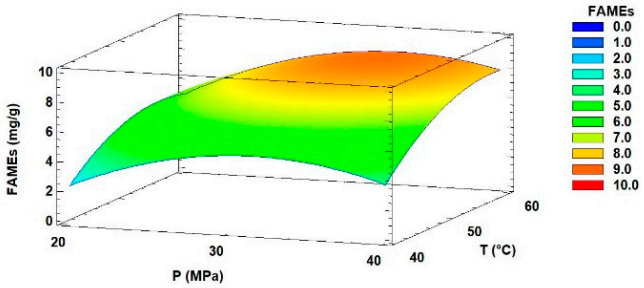

Figure 5. Response surface plot showing combined effects of pressure and temperature on FAMEs content from *I. galbana*. All extractions were done with 8% co-solvent (ethanol, v/v).

Table 3 presents the profile of primary fatty acids derived from SFE extracts of *I. galbana*. The profile (measured as % area) consists of polyunsaturated fatty acids (PUFAs) in the range of 25.4–95.6%, MUFAs (monounsaturated fatty acids) in the range of 0.0–47.7%, and saturated fatty acids (SFAs) in the range of 4.4–42.1% of total fatty acids. In general, the fatty acid profile was highlighted by linoleic acid (C18:2) followed by linolenic acid (C18:3). Myristic acid (C14:0) and palmitic acid (C16:0) were the main SFAs. The highest recoveries of MUFAs and PUFAs were found under two conditions at 20 MPa, 40 ºC, and 4% co-solvent (Run 12) and 30 MPa, 50 ºC, and 4% ethanol (Run 9), at nearly 47.7 and 95.6%, respectively. SFE is considered an appropriate method for the extraction of fatty acids and lipids or for compounds of low polarity from microalgae because of its non-polar property [50]. It is also a solvent selective for neutral lipids such as triglycerides but does not solubilize phospholipids [51]. Our results corroborate with several reports about the extraction of polar compounds, which could be enhanced by adding of polar co-solvents, such as ethanol [52,53].

Table 3. Fatty acid composition and content of SFE extracts from freeze-dried *Isochrysis galbana* used in this study (% area of total FAME and mg FAME/g). The general parameters were biomass loading = 2.0 g, CO_2 flow rate = 7.4 g/min, extraction time = 120 min (SD ≤ 5%; n = 3).

Fatty Acid	Common Name	Run														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
C14:0	Myristic acid	20.0 ± 0.5	n.d.	n.d.	22.8 ± 1.02	4.9 ± 0.21	n.d.	n.d.	3.7 ±0.2	n.d.	24.2 ±1.2	n.d.	0.2 ± 0.01	n.d.	n.d.	n.d.
C16:0	Palmitic acid	n.d.	4.9 ± 0.2	13.8 ± 0.5	9.1 ± 0.40	6.6 ± 0.28	5.6 ± 0.1	n.d.	7.1 ±0.3	n.d.	n.d.	n.d.	7.8 ± 0.3	8.5 ±0.38	11.0 ±0.4	9.7 ± 0.4
C16:1	Palmitoleic acid	9.8 ± 0.3	n.d.	n.d.	n.d.	n.d.	9.0 ± 0.3	8.9 ±0.3	14.9 ±0.5	n.d.	30.1 ±1.3	3.0 ±0.1	n.d.	n.d.	7.4 ±0.3	n.d.
C18:0	Stearic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.4 ±0.06	n.d.	2.4 ±0.05	3.1 ±0.1	1.7 ± 0.08	1.4 ±0.06	1.3 ±0.04	1.4 ± 0.07
C18:1	Oleic acid	n.d.	n.d.	n.d.	n.d.	2.2 ± 0.09	0.6 ± 0.02	n.d.	2.3 ±0.10	n.d.	n.d.	n.d.	47.7 ±1.7	36.7 ±1.5	37.9 ±1.7	36.2 ± 1.5
C18:2	Linoleic acid	39.1 ± 1.7	43.5 ± 1.9	6.0 ± 0.3	2.7 ± 0.11	35.0 ± 1.42	48.1 ± 1.7	41.9 ± 1.8	40.7 ±1.8	53.6±1.68	43.3 ±1.8	88.1 ±3.5	26.1 ±1.2	17.9 ±0.7	18.9 ±0.7	18.2 ± 0.8
C18:3	Linolenic acid	26.2 ± 1.2	36.5 ± 1.7	54.1 ± 2.5	48.4 ± 1.89	28.7 ± 1.25	25.5 ± 1.2	24.9± 1.0	n.d.	42.0 ±1.89	n.d.	0.9 ±0.04	4.0 ±0.1	19.6 ±0.8	2.9 ±0.1	n.d.
C20:0	Eicosanoic acid	n.d.	n.d.	5.8 ± 0.2	3.5 ± 0.17	3.9 ± 0.15	1.6 ± 0.04	2.9±0.05	1.5 ±0.06	n.d.	n.d.	4.1 ±0.2	10.2 ±0.5	12.6 ±0.5	15.3 ±0.7	22.6 ± 1.1
C21:0	Methyl heneicosanoate	n.d.	n.d.	n.d.	n.d.	10.8 ± 0.42	n.d.	9.2±0.3	8.8 ±0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C22:2	Cis-13,16-docosadienoic acid methyl ester	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.2 ±0.1	3.4 ±0.1	3.6 ± 0.1	8.2 ± 0.3
C20:3	Cis-11-14-17-eicosatrienoic acid methyl ester	5.1 ± 0.2	8.5 ± 0.3	11.5 ± 0.5	7.8 ± 0.3	6.0 ± 0.3	6.2 ± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Others		n.d.	6.5 ± 0.3	8.8 ± 0.4	5.8 ± 0.2	1.9 ± 0.08	3.4 ± 0.1	12.2± 0.6	18.6 ±0.8	4.4 ±0.1	n.d.	n.d.	n.d.	n.d.	1.8 ±0.0	3.8 ± 0.1
Σ SFAs	Total saturated fatty acids	20.0	6.7	23.3	35.4	26.2	7.2	24.3	42.1	4.4	26.6	7.1	20.0	22.5	29.3	37.5
Σ MUFAs	Total monounsaturated fatty acids	9.8	4.8	5.2	5.8	4.1	13.0	8.9	17.2	n.d.	30.1	3.0	47.7	36.7	45.3	36.2
Σ PUFAs	Total polyunsaturated fatty acids	70.3	88.5	71.6	58.8	69.7	79.8	66.8	40.7	95.6	43.3	89.9	32.4	40.9	25.4	26.4

Note: SFE = supercritical fluids extraction; FAME = fatty acid methyl ester; n.d. = no detection. (SD ≤ 5% ; n = 3).

Machmudah et al. [54] reported that an increase in the extraction pressure causes intensification in carbon dioxide density and, consequently, an increase in the solvation power for fatty acids. Our results show that under low pressures, more saturated fatty acids are extracted and when the pressure is increased, the proportion of unsaturated fatty acids increases in the extracted phase, an observation also supported by Cheung [55]. In general, this indicated that the triglycerides containing the more unsaturated fatty acids could be soluble at higher densities of CO_2 due to increase pressure factor. However, the combined effects of pressure and temperature on the overall solubility of PUFA can vary because it depends on their chain length and there seems to be a compromise between supercritical fluid density and vapor pressure of the solute concerned [55].

3.5. Desirability Function

The desirability to maximize all studied variables has been summarized in Figure 6. This procedure helped in determining the combination of experimental factors that involve simultaneous optimization of several response variables and was selected for meeting these goals and giving equal importance to all responses. The maximum 'desirability' predicted by the software was obtained in Run 7 with parameters of 40 MPa, 50° C, and 8% co-solvent. The results were obtained as the extraction yield of 8.78%, TCC of 15.33 mg/g, TC recovery of 62.85%, TPC of 157.16 mg GAE/g, TEAC of 0.31 mmol TE/g, and FAME of 7.19 mg/g. The optimum desirability was obtained at 38.4 MPa, 56.74 °C, and 8% co-solvent where the optimal results were extraction yield of 12.82%, TCC of 16.96 mg/g, TC recovery of 69.53%, TPC of 114.57 mg GAE/g, TEAC of 0.32 mmol TE/g, and FAME of 8.31 mg/g. Finally, the optimization desirability was determined to be 0.86 and the experiments under the optimum conditions provided values close to those predicted by the statistical model.

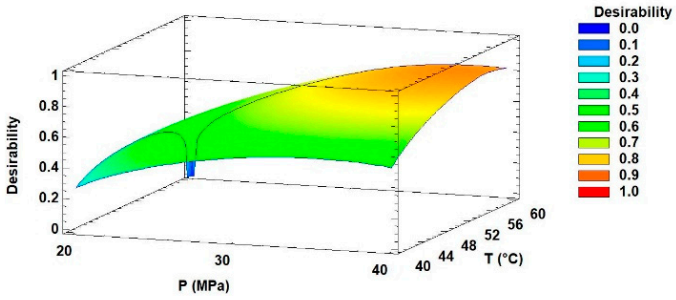

Figure 6. Surface of the desirability function in terms of pressure, temperature and maximum co-solvent (8% ethanol, v/v) obtained for maximizing extraction yield (Y), total carotenoids content (TCC) and recovery (TC recovery), phenol content (TPC), TEAC method, and FAMEs content from *I. galbana.*

4. Conclusions

The present study focused on the prospecting of bioactive compounds present in the microalgae *I. galbana* by SFE for potential use in food, pharmaceuticals, and cosmetics. Several variables were studied including extraction yield, total carotenoids content and recovery, total phenols, antioxidant activity (TEAC method), and fatty acid content and profile. In general, the addition of ethanol as co-solvent significantly increased the efficiency of SFE extraction for all variables, and to a lesser extent, TEAC as antioxidant measurement. Another parameter to enhance the bioactive compound extraction from *I. galbana* was pressure, although it was not a significant variable except in the TEAC method. Particularly, linoleic acid (C18:2) followed by linolenic acid (C18:3) was highlighted in the profile that was improved under higher pressure, thus indicating that as pressure increased, solubility of triglycerides containing the more unsaturated fatty acids increased at higher densities. Concurrently, desirability function that aided in the operational condition closer to the optimum was found in Run 7 (40 MPa, 50 °C, and 8% co-solvent, desirability predicted 0.79). To better explore the potential of *I. galbana*, new and improved supercritical extraction with a higher percentage of ethanol could be developed in spite of it could increase the operational costs in the process. In conclusion, this

study provides functional information to optimize extraction of bioactive compounds from *I. galbana* using SFE because of its rich content of bioactive compounds and antioxidant activity with increasing demand in food, pharmaceuticals, and cosmetics market.

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Conflicts of Interest: There is no conflict of interests.

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