Optimization of the Extraction of Antioxidants from *Dunaliella salina* Microalga by Pressurized Liquids

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In this work, extraction of antioxidant compounds from *Dunaliella salina* microalga is optimized by combining pressurized liquid extraction (PLE) and experimental design (three-level factorial design) with three different solvents (hexane, ethanol, and water). Two main factors were considered, the extraction temperature (40, 100, and 160 °C) and the extraction time (5, 17.5, and 30 min). As response variables, the extraction yield (percent dry weight/initial weight) and the antioxidant activity of the extracts (determined using the TEAC method) were used. The parameters of the model were estimated by multiple linear regression. Results showed that the extraction temperature was the factor having the strongest influence (positive) on the two response variables. The best yields were obtained with ethanol at the higher extraction temperature and time tested. Besides, although hexane extracts provided the best antioxidant activity, ethanol extracts were also very active. The chemical characterization of ethanol extracts was carried out using HPLC-DAD, and attempts have been made to correlate their chemical composition with the antioxidant activity measured. Results pointed out that the extracts contained, besides *all-trans-β*-carotene and isomers, several different minor carotenoids that seemed to make a contribution to the antioxidant activity of the extracts.

KEYWORDS: PLE; *Dunaliella salina*; TEAC; antioxidant compounds; optimization

INTRODUCTION

Functional foods are often defined as foods that, in addition to the nutritional and energetic requirements they supply, are able to provide a beneficial physiological action for human health (*1*). Nowadays, consumers are more health conscious and, as a consequence, functional foods are in great demand over less healthy food, increasing the need of adding ingredients with nutritional value. These ingredients are preferred to have a natural origin (i.e., nonsynthetic compounds), for which plants, algae, and microalgae are possible natural sources. In this sense, several authors have pointed out microalgae as potential sources of compounds with functional nutritional properties (*2−5*). Among these types of compounds that can be obtained from microalgae, antioxidants have been the most studied substances. These compounds are important for the food industry (*6*), because they are used as food preservatives. Moreover, these compounds are currently being more widely employed because of their additional physiological benefits in human health (*7−9*).

To obtain these compounds, environmentally clean extraction techniques are necessary. Pressurized liquid extraction (PLE) is an efficient technique for extraction obtaining environmentally clean extraction procedures (*10*). PLE is based on the extraction at temperature and pressure high enough to maintain the solvent employed in the liquid state during the whole extraction procedure. As a result, faster extraction processes can be achieved compared with traditional extraction techniques, whereas higher extraction yields are obtained with a significantly lower amount of solvent (*5*). At present, PLE has been used to extract bioactive compounds from natural sources in several different studies (*10−16*).

In this work, the microalga *Dunaliella salina* is studied as a natural source of antioxidants. This organism is a unicellular biflagellate green alga that belongs to the Clorophyaceae family (*17*). This microorganism is characterized by its ability to grow in high-salinity environments (*18*). Although the production of *β*-carotene from *D. salina* has been broadly studied (*19−21*), only two references can be found about the use of PLE to extract valuable compounds from this microalga (*22, 23*), and none of them have been devoted to the optimization of the extraction of antioxidant compounds. In this sense, carotenoids act in *D. salina* as accessory pigments in the light-harvesting photosystem during photosynthesis, being also the main antioxidant compounds from this microalga (*24−28*).

The objective of the study was to optimize the extraction of antioxidants from *D. salina* microalga by means of PLE using

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three solvents (hexane, ethanol, and water) under different temperature conditions. The antioxidant activity of the extracts obtained was examined and characterized. Relationships between the carotenoid composition of the extracts and the antioxidant activity were determined.

MATERIALS AND METHODS

Samples and Chemicals. Microalga samples (*D. salina)* consisted of freeze-dried microalgae from NBT Ltd. (Jerusalem, Israel) that were stored under dry and dark conditions. The solvents used for carotenoid extraction were ethanol (Scharlaü Chemie S.A., Barcelona, Spain) and hexane (Panreac Quimica S.A., Barcelona, Spain). For HPLC analyses methyl *tert*-butyl ether (MTBE) and methanol were used (LabScan, Dublin, Ireland). Water was purified using a Milli-Q system (Millipore Corp., Billerica, MA). α- and β-carotene standards (purity minimum of 95%) and triethylamine were obtained from Sigma (Madrid, Spain) as well as potassium persulfate. The sea sand was supplied by Panreac Quimica. For the antioxidant activity analyses 2,2-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were employed (Fluka Chemie AG, Buchs, Switzerland).

Statistical Analysis. The influence of extraction temperature (temp) and extraction time (time) on the antioxidant activity and the extraction yield of *D. salina* extracts was studied using a three-level factorial design. A total of 12 experiments (9 points of the factorial design and 3 center points to consider the experimental errors) were carried out in randomized run order. By using this design, the two factors were tested at three different levels: extraction temperature at 40, 100, and 160 °C and extraction time at 5, 17.5, and 30 min. The response variables selected were extraction yield (yield, determined as percent dry weight/initial weight) and antioxidant activity (antiox) determined using the Trolox equivalent antioxidant capacity (TEAC) test. The quadratic model proposed for each response variable (Y_i) was

$$Y_i = \beta_0 + \beta_1 \text{ temp} + \beta_2 \text{ time} + \beta_{1,1} \text{ temp}^2 + \beta_{2,2} \text{ time}^2 + \beta_{1,2} \text{ temp} \times \text{time} + \text{error} \quad (1)$$

where β_0 is the intercept, β_1 and β_2 are the linear coefficients, $\beta_{1,1}$ and $\beta_{2,2}$ are the quadratic coefficients, $\beta_{1,2}$ is the interaction coefficient, and error is the error variable.

The parameters of the model were estimated by multiple linear regression (MLR) using the Statgraphics Plus v. 5.1 program (Statistical Graphics Corp., Manugistics Inc., Rockville, MD, 2000). This program permits both the creation and the analysis of experimental designs. The effect of each term in the model and its statistical significance, for each of the response variables, was analyzed from the standardized Pareto chart. The quadratic and interaction terms not significantly different from zero at $P \leq 0.05$ were excluded from the model, and the mathematical model was refitted by MLR. The goodness of fit of the model was evaluated by the coefficient of determination (R^2), the residual standard deviation (RSD), and the lack of fit test for the model from the ANOVA table. The test is performed by comparing the variability of the current model residuals to the variability between observations at replicate settings of the factors. From the new fitted model, the optimum conditions, which maximize the yield and the antioxidant activity response variables, were also provided by the program. Surface plots were developed using the fitted quadratic polynomial equation obtained.

Other statistical methods used for data analysis were principal component analysis (PCA) and cluster analysis (from standardized variables) to examine the relationship among the analyzed variables and to discover natural groupings, respectively. The Statistica program for Windows, release 7.1 (Statsoft Inc., Tulsa, OK) was used for data processing.

Pressurized Liquid Extractions. PLEs of *D. salina* were performed using an accelerated solvent extractor (ASE 200, Dionex Corp., Sunnyvale, CA) equipped with a solvent controller. Three different solvents (i.e., hexane, ethanol, and water) were used to obtain extracts with different compositions. Extractions were performed at three

different extraction temperatures (40, 100, and 160 °C) and extraction times (5, 17.5, and 30 min). An extraction cell heat-up was carried out for a given time prior to any extraction, the warming-up time changed depending on the extraction temperature (i.e., 5 min when the extraction temperature was 40 and 100 °C and 8 min if the extraction temperature was 160 °C). All extractions were done using 11 mL extraction cells, containing 2.0 g of sample. When water was used for the extraction, the extraction cell was filled with sand between the sample (6.0 and 2.0 g of sand at the bottom and top, respectively) to prevent the clogging of the system.

The extraction procedure was as follows: (i) sample was loaded into the cell; (ii) cell was filled with solvent to a pressure of 1500 psi; (iii) initial heat-up time was applied; (iv) a static extraction with all system valves closed was performed; (v) the cell was rinsed (with 60% cell volume using extraction solvent); (vi) solvent was purged from the cell with N$_2$ gas; and (vii) depressurization took place. Between extractions, a rinse of the complete system was made to avoid carry-over. For solvent evaporation, a Rotavapor R-200 (from Büchi Labortechnik AG, Flawil, Switzerland) was used for the extracts obtained using organic solvents. For water extracts, a freeze-dryer (Unitop 400 SL, Virtis, Gardiner, NY) was employed.

Antioxidant Activity Determination. *TEAC Assay.* The antioxidant activity of PLE extracts from *D. salina* was measured using the TEAC assay as described by Re et al. (*29*). Namely, the ABTS$^{\bullet+}$ radical cation was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate (final concentration) after incubation at room temperature for 16 h in the dark. The ABTS$^{\bullet+}$ radical solution was diluted with ethanol to an absorbance of around 0.70 at 734 nm. The reaction was initiated by the addition of 10 μL of *Dunaliella* extract dissolved in dichloromethane at different concentrations to 0.990 mL or diluted ABTS$^{\bullet+}$. The reactive mixture was allowed to stand at room temperature for 20 min (until the reaction reached a steady state), and the absorbance was immediately recorded at 734 nm. Trolox was used as reference standard, and results were expressed as TEAC values. These values were obtained from at least three different concentrations of each extract tested in the assay giving a linear response. Moreover, all analyses were done in triplicate.

HPLC Analysis. HPLC analyses of the ethanol and hexane extracts from PLE were carried out following the procedure described for carotenoids by Breithaupt (*30*). An HPLC Agilent HP 1100 series (Agilent, Palo Alto, CA) equipped with a diode array detector was used together with a YMC (YMC, Schermbeck, Germany) C30 analytical column (5 μm, 250 × 4.6 mm i.d.) to perform the experiments. The chromatograms were recorded at 450 nm (scan from 190 to 600 nm). Two mobile phases were employed (A, methanol/water/triethylamine 90:10:0.1 v/v/v; B, MTBE/methanol/water/triethylamine 90:6:4:0.1 v/v/v/v) following the gradient elution from 93.5% A at $t = 0$ to 0% A at $t = 34$ min and then to 93.5% A at $t = 38$ min, maintaining these conditions until $t = 53$ min. Samples were injected using a manual injection port (20 μL injection volume) at concentrations of 10 mg/mL. A β-carotene calibration curve ($R^2 = 0.995$) was obtained by injecting β-carotene standard at concentrations ranging from 0.009375 to 0.15 mg/mL. All of the calibration points were injected in duplicate.

RESULTS AND DISCUSSION

Optimization of PLE Conditions. Table 1 shows the yield and antioxidant activity (response variables Y_1 to Y_6), as affected by temperature and time of extraction. As can be observed from these results (**Table 1**), water extracts provided both the worst antioxidant activity and yield, whereas organic solvents gave better results. For example, ethanol extracts seemingly showed higher yields than hexane extracts, whereas hexane extracts provided better antioxidant activities. Even though the activities provided by the ethanol extracts were lower, they can also be considered actives (in absolute values). Moreover, a rough analysis of the data showed that the antioxidant activity increased with the temperature for the three solvents (see experiments 4−6 in **Table 1**). Interestingly, the behavior of the yield was slightly different; higher yields were obtained at higher

Table 1. Experimental Matrix Design and Results Obtained for the Six Response Variables Studied (Y_1–Y_6) Depending on the Time and Temperature of Extraction

			water		hexane		ethanol	
expt	temperature (°C)	time (min)	yield (Y_1)[a]	antiox (Y_2)[b]	yield (Y_3)	antiox (Y_4)	yield (Y_5)	antiox (Y_6)
1	100	17.5	3.1	0.0127	16.65	0.968	25.105	0.275
2	100	17.5	2.8	0.0134	17.3	0.942	24.4	0.268
3	100	17.5	3.8	0.0125	17.4	1.042	26.4	0.283
4	40	5	1.25	0.0107	12.3	0.749	17.45	0.165
5	100	5	2.45	0.0114	16.65	0.832	25.25	0.26
6	160	5	7.3	0.0271	16.95	0.865	28.55	0.357
7	40	17.5	1.8	0.0115	12.25	0.827	18.45	0.201
8	100	17.5	2.8	0.0133	17	0.945	25.2	0.283
9	160	17.5	7.75	0.0325	17.65	1.118	31.35	0.411
10	40	30	2.35	0.0112	12.2	0.837	17.15	0.192
11	100	30	4.1	0.0131	16.85	0.782	26.25	0.229
12	160	30	9.2	0.0346	17.15	0.852	34.6	0.353

[a] Extraction yield obtained from dry weight/total weight expressed in percent. [b] TEAC (mmol of Trolox/g of extract).
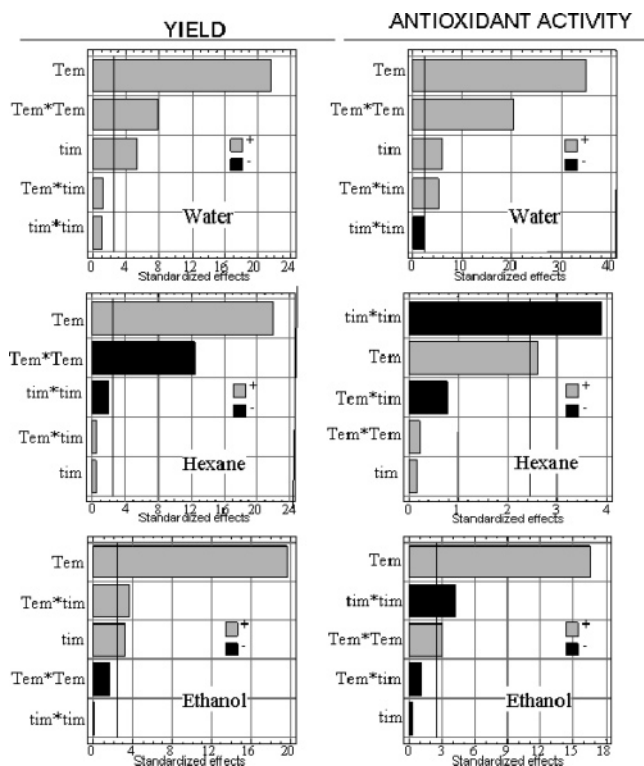

Figure 1. Standardized Pareto chart with the effect of each term in the model divided by its standard error for the six response variables of **Table 1** depending on the extraction temperature and time.

temperatures when using water and ethanol, whereas the best yields were obtained at medium extraction temperatures (100 °C) with hexane. In absolute values, the highest yield (34.6%) was obtained with ethanol at 160 °C (see experiment 12 in **Table 1**). From these results, it can be concluded that the chemical composition of *D. salina* seems to be mainly based on medium-low-polarity compounds, explaining in this way the highest yields obtained with ethanol.

Figure 1 shows the standardized Pareto charts for the different response variables evaluated, illustrating the importance and the statistical significance of each term in the model. The vertical line in the chart tests the significance of the effects at the 95% confidence level. Positive (+) and negative effects (−) in the response variables are indicated by different bar shadings. In good agreement with what has been mentioned above, it can

be deduced from **Figure 1** that the linear effect of the temperature is the most important term in the model, having a positive contribution on the six studied variables (i.e., yield and antioxidant activity determined for the three solvents, Y_1–Y_6). Besides, in three of these variables (Y_1–Y_3), the quadratic effect of the temperature is the second term in importance. Only the antioxidant activity of the hexane extracts (**Figure 1**, Y_4) showed a term more important than the temperature (the quadratic term of time). On the other hand, it can also be deduced from **Figure 1** that the effect of the extraction time is much lower than that of the temperature, being in many cases not statistically significant (see Y_3, Y_4, and Y_6 in **Figure 1**).

Once the interaction and quadratic terms of the model not significantly different from zero at $P \leq 0.05$ were excluded from eq 1, the mathematical model was refitted by MLR (**Table 2**). From these results, the following interpretations can be drawn: the models proposed for each response variable did not present lack of fit at $P \leq 0.05$; they explained >97% ($R^2 > 0.97$), except the one corresponding to the antioxidant activity of hexane extracts (Y_4); the RRSD values obtained [RRSD (%) = $100 \times RSD/\bar{Y}$] were <9%. Therefore, all estimated models, except that of the antioxidant activity of hexane extracts, were found to be adequate to describe the data.

Figure 2 shows the estimated surface plots for the mentioned response variables (Y_1–Y_6) as a function of temperature and time. They are used for predicting future responses and for optimizing the response. As can be seen in **Figure 2**, the two response variables for water (Y_1 and Y_2) follow the same behavior, that is, an increase of yield (Y_1) or antioxidant activity (Y_2) when either extraction time or extraction temperature was raised. However, when ethanol and hexane were used, a sigmoid curve was observed. For instance, although, in general, a higher yield and antioxidant activity can be obtained at the highest extraction temperature (as can be seen in Y_4, Y_5, and Y_6 of **Figure 2**), a maximum yield is obtained for hexane extracts at medium temperatures (120 °C; see Y_3 in **Figure 2**). As for the antioxidant activity of hexane and ethanol extracts, it can be seen that an increase in the extraction time gives rise to an initial increase of the antioxidant activity (see Y_4 and Y_6 in **Figure 2**), and after reaching a maximum, the antioxidant activity decreases. The main difference in the behavior of these two solvents is that this trend is more pronounced in hexane extracts than in ethanolic ones. Moreover, in both cases, the prediction for the yield confirmed the different pattern.

Table 3 shows the optimum extraction temperature and time provided by the statistical program for each solvent and the predicted value for the response variables obtained using the fitted model of **Table 2**. As can be seen, in almost all cases, the maximum temperature tested provided the best results. Because of experimental constraints, higher temperatures could not be investigated. Only for hexane extraction yield were optimum values achieved at medium temperatures and times, in good agreement with the results mentioned above. On the other hand, optimum antioxidant activities were obtained for hexane and ethanol at medium extraction times, possibly indicating some kind of antioxidant degradation during longer extraction at the selected temperature.

Although better antioxidant activities were obtained from the hexane extracts, it is important to consider that ethanol provides higher yields (double the yield achieved with hexane); this, along with the fact that ethanol extracts also show important antioxidant activities and that it is considered to be safe (GRAS), led to the selection of ethanol as solvent to obtain *D. salina* extracts with adequate antioxidant activity and suitable for the food

Table 2. Regression Coefficients, for Unscaled Factors, and Statistics of the Fitting Obtained Using MLR[a]

	water		hexane		ethanol	
terms of the model	yield (Y_1)	antiox (Y_2)	yield (Y_3)	antiox (Y_4)	yield (Y_5)	antiox (Y_6)
constant	1.7613	0.02137	6.62778	0.54844	15.6282	0.124084
temp	−0.04579**	−0.000346**	−0.16528**	0.001172*	0.0781**	0.0001472**
time	0.062**	−0.0001029*		0.0034867	−0.121667*	0.0079573
temp × temp	0.000491**	0.00000237**	−0.000618**			0.00000708*
time × time				−0.000986**		−0.0002304**
temp × time		0.00000232**			0.002117**	
R^2	0.986	0.994	0.985	0.777	0.979	0.976
RSD	0.36	0.00085	0.29	0.047	0.91	0.014
P	0.87	0.08	—[b]	0.29	0.44	0.09
RRSD (%)	8.8	5.0	1.8	5.2	3.6	5.1

[a] R^2, determination coefficient; RSD, residual standard deviation; P, P value of the lack of fit test for the model; RRSD, the residual standard deviation expressed as a percentage of the mean value of the response. *, regression coefficient significantly different at $P < 0.05$; ** , regression coefficient significantly different at $P < 0.01$. [b] Data not provided by the statistical program (Statgraphics Plus 5.1).
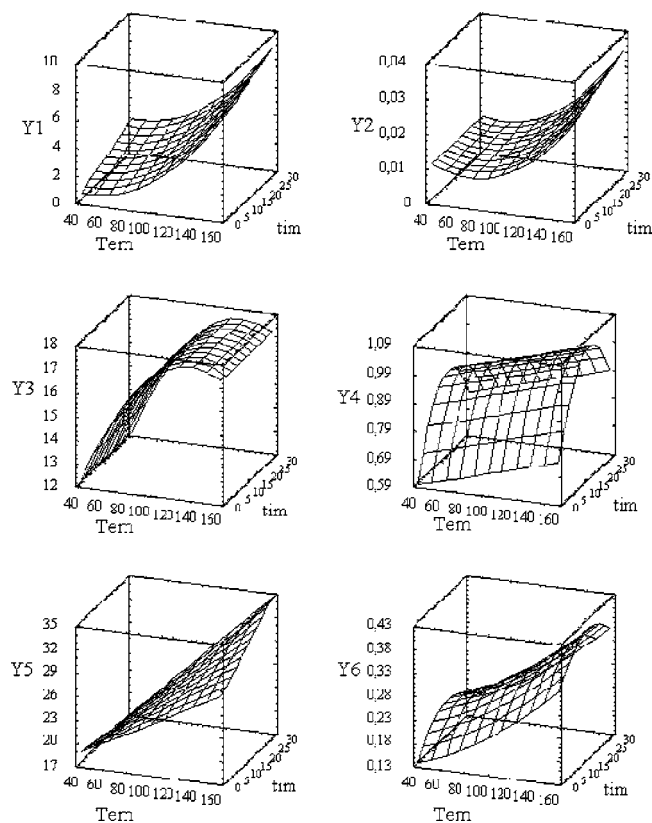

Figure 2. Surface plots of the six response variables Y_1–Y_6 of **Table 1** depending on the extraction temperature and time. Response variables: Y_1, yield with water; Y_2, antioxidant activity with water; Y_3, yield with hexane; Y_4, antioxidant activity with hexane; Y_5, yield with ethanol; Y_6, antioxidant activity with ethanol.

Table 3. Optimum Conditions (Maximum Antioxidant Activity and Maximum Yield), Provided by the Statistical Program, Predicted Value for the Response Variables (Using the Fitted Model of **Table 2**), and Experimental Values, When Data Are Available

		optimum conditions		predicted values		
		temperature (°C)	time (min)	value	95% CI[a]	exptl value
water	yield (%)	160	30	8.86	8.42, 9.30	9.2
	antiox	160	30	0.035	0.033, 0.037	0.034613
hexane	yield (%)	133.7	19.2	17.68	16.80, 18.56	
	antiox	160	17.7	1.04	0.99, 1.09	1.118[b]
ethanol	yield (%)	160	30	34.63	32.90, 36.36	34.6
	antiox	160	17.3	0.40	0.38, 0.42	0.411[a]

[a] CI, confidence interval. [b] Results obtained for an extraction time equal to 17.5 min.

industry. Moreover, the lower antioxidant activity could be overcome by using higher amounts of extracts. It is important also to consider that the stability of ethanol extracts was reasonably good, and when one of these extracts was left unprotected from light and air at room temperature for 2 days, the chromatogram obtained after this time was not significantly different form the initial analysis, indicating that no degradation of the different compounds present in the extract took place.

Correlations between Antioxidant Activity and Extract Composition. The compounds present are shown in **Table 4**. To go further with the characterization of the antioxidant activity of the ethanol extracts, HPLC analyses were performed to identify the compounds present in these extracts, presumably

carotenoids. It is already well-known the high carotenoid content, in particular β-carotene, that can be found in *D. salina* (*19*−*21*). A representative chromatogram of the carotenoid profile found in the ethanol PLE extract is given in **Figure 3A**. As can be seen, the method employed allowed the separation of 11 carotenoids in <26 min in the ethanol extracts. Some of these carotenoids were characterized using standards, whereas the rest were assigned on the basis of their specific elution and UV−vis spectra (*30*−*32*). For comparison, the composition of hexane extracts (at the optimum extraction conditions, that is, 160 °C and 17.5 min) is also presented (**Figure 3B**). Results showed a less complex composition, α-carotene and *all-trans*- and 9-*cis*-β-carotene being the main compounds found.

In the following, possible correlations between the different amounts of carotenoids and β-carotene isomers and the antioxidant activities of the ethanol extracts are investigated. Therefore, quantification of the carotenoids in the extracts was carried out. To overcome the limitation imposed by the lack of commercial standards for some carotenoids, *all-trans*-β-carotene equivalent was used to quantify the rest of the carotenoids observed (limit of quantification of 0.002 mg per 100 mg of extract). **Table 4** shows the quantities found for each compound as well as several relationships between them (namely, total amount of β-carotene isomers, ratio of 9-*cis* to *all-trans*, and total amount of carotenoids detected). As can be observed, the higher amount of carotenoids detected corresponded to the β-carotene isomers, the more abundant being the *all-trans*- and 9-*cis*-β-carotene (carotenoids 9 and 11, respectively). As for the correlation between antioxidant activity and carotenes, some references can be found regarding the different antioxidant

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Table 4. Carotenoid Contents Determined in the Ethanol Extracts (Extraction Conditions as in **Table 1**)

expt	total amount of β-carotene isomers (mg/100 mg of extract)	total amount of carotenoids (mg/100 mg of extract)	% β-carotene isomers/total carotenoids	9-*cis*/all-*trans*	carotenoid[a] 1	2	3	4	5	6	7	8	9	10	11
1	1.090	1.924	56.61	2.143	0.083	0.019	0.055	0.125	0.353	0.129	0.072	0.095	0.257	0.187	0.550
2	1.183	2.063	57.31	1.859	0.093	0.019	0.055	0.128	0.357	0.157	0.071	0.102	0.310	0.193	0.577
3	1.204	2.153	55.91	2.357	0.051	0.062	0.062	0.150	0.418	0.118	0.090	0.112	0.253	0.241	0.597
4	1.517	1.999	75.89	1.409	0.168	nd	nd	0.029	0.099	0.150	0.070	0.086	0.469	0.301	0.661
5	1.119	1.969	56.82	1.495	0.081	0.016	0.047	0.120	0.360	0.163	0.063	0.082	0.349	0.167	0.521
6	2.118	3.830	55.31	1.564	0.008	0.082	0.123	0.354	0.794	0.144	0.207	0.184	0.602	0.391	0.942
7	1.408	1.903	74.00	1.332	0.181	nd	0.007	0.026	0.093	0.149	0.058	0.083	0.458	0.258	0.610
8	0.978	1.820	53.73	1.791	0.094	0.047	0.050	0.114	0.345	0.140	0.052	0.082	0.273	0.136	0.489
9	2.241	4.160	53.89	1.474	0.026	0.067	0.156	0.411	0.858	0.165	0.236	0.207	0.664	0.392	0.978
10	1.028	1.312	78.32	1.518	0.132	nd	nd	0.013	0.069	0.078	0.044	0.062	0.279	0.264	0.423
11	1.615	2.603	62.04	2.172	0.057	0.041	0.064	0.148	0.408	0.149	0.120	0.129	0.374	0.300	0.812
12	2.250	3.926	57.32	1.441	0.012	0.052	0.127	0.354	0.753	0.157	0.221	0.195	0.656	0.454	0.945
H[b]	25.07	29.50	84.98	0.37	nd	nd	nd	nd	nd	2.477	nd	nd	22.775	nd	2.292

[a] Peak assignment: carotenoids 1–5 and 7, not identified; carotenoid 6, α-carotene; carotenoid 8, 13-*cis*-β-carotene; carotenoid 9, *all-trans*-β-carotene; carotenoid 10, 15-*cis*-β-carotene; carotenoid 11, 9-*cis*-β-carotene. [b] Hexane extract obtained at 160 °C and 17.5 min.
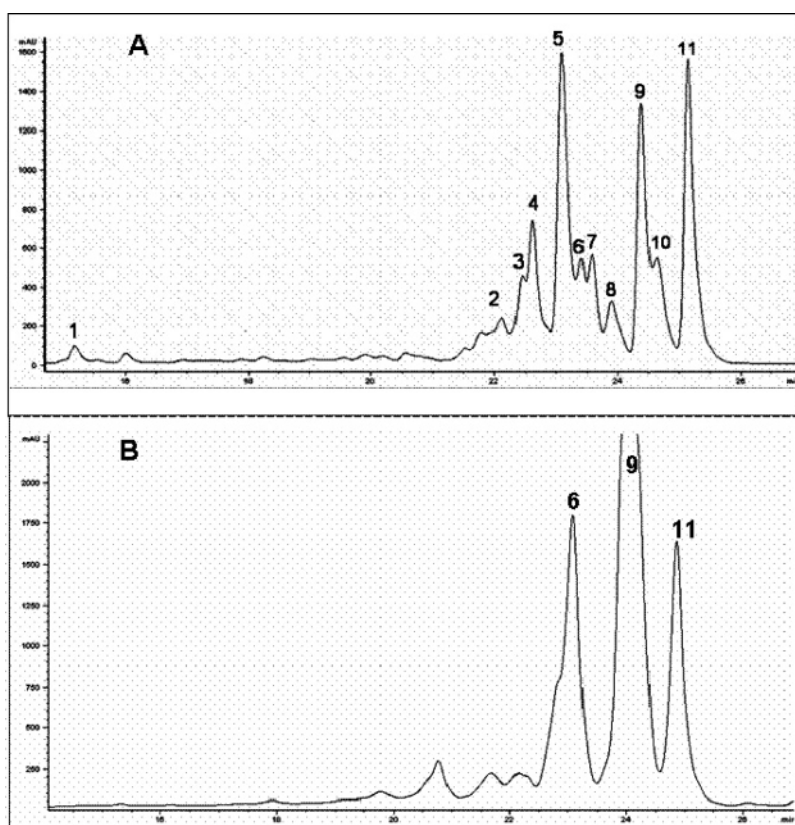

Figure 3. HPLC chromatograms (450 nm) of an ethanol extract obtained at 160 °C and 5 min (**A**) and of a hexane extract obtained at 160 °C and 17.5 min (**B**). Peak assignment: 6, α-carotene; 8, 13-*cis*-β-carotene; 9, *all-trans*-β-carotene; 10, 15-*cis*-β-carotene; 11, 9-*cis*-β-carotene; 1–5 and 7, not identified.

activities of the different β-catorene isomers (*33*, *34*). Although the highest in vitro antioxidant activity has been described for the 9-*cis* isomer (*33*), it has also been reported that the 9-*cis* isomer was not detected in human serum as was the *all-trans*-β-carotene (*35*). By comparing data between ethanol and hexane extracts, it can be seen that the amount of carotenoids (mainly *all-trans*- and 9-*cis*-β-carotene) extracted with hexane is >7 times higher; however, the antioxidant activity observed does not follow the same trend, being only twice higher than the one obtained for ethanol extracts. Therefore, it can be inferred that the presence of other carotenoids (more polar) in the ethanol extracts can have an important influence on the antioxidant activity, thus being "valuable" compounds for the extract.

PCA was applied to examine the relationships between the antioxidant activity of ethanol extracts (antiox3), the temperature of extraction, and the carotene concentrations. Three principal components were obtained, and these explained 95.3% of the total variance of the data. The first principal component, which explained 73% of the total variance, correlated negatively with peak 3 (−0.98), peak 4 (−0.99), peak 5 (−0.98), peak 7 (−0.98), peak 8 (−0.98), total amount of carotenoids (−0.98), temperature (−0.94), antiox3 (−0.91), peak 11 (9-*cis*-β-carotene, −0.91), total amount of β-carotene isomers (−0.89), and peak 2 (−0.84) and positively with peak 1 (0.86); this means there is a positive relationship between antioxidant activity and carotenoids corresponding to peaks 2−5, 7, and 8 (13-*cis*-β-
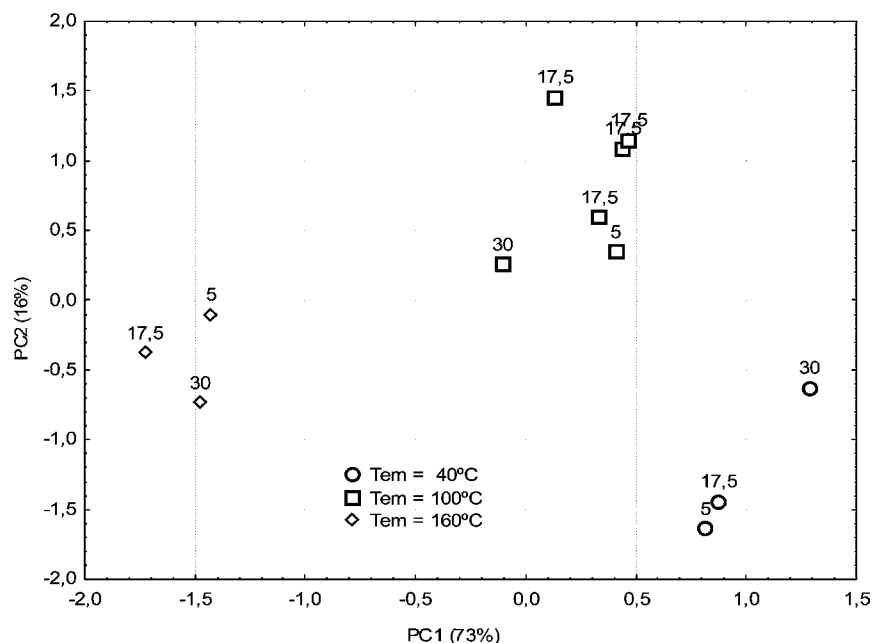
Figure 4. Plot of the 12 extraction experiments on the plane defined by two principal components. The experiments are labeled according to the extraction temperature and time factors.

carotene), peak 11 (9-*cis*-*β*-carotene), the total amount of carotenoids, the total amount of the *β*-carotene isomers, and the extraction temperature and implies an increase of antioxidant activity of the extract by increasing the concentration of the mentioned compounds, which is directly related to an increase of the extraction temperature. On the other hand, there is a negative relationship between antioxidant activity and carotenoid 1 (peak 1). The second principal component, which explained only 16.1% of the total variance, correlated positively with the ratio 9-*cis*-/*all*-*trans*-*β*-carotene (0.81). In **Figure 4**, the 12 experiments are plotted on the plane defined by these two principal components. It is possible to observe that the experiments are adequately grouped according to the temperature of extraction. The experiments at high temperature (160 °C) are on the left side of the figure, with low values of principal component 1 (PC1), whereas those corresponding to low temperatures (40 °C) are on the right side, that is, higher values of PC1. Therefore, considering the loadings of the variables in the PC1, the extracts at high temperatures (160 °C) had higher values of compounds corresponding to peaks 3, 4, 7, 8, 11, and 2 and of the variables total amount of carotenoids, antioxidant activity, and total amount of *β*-carotene isomers than those obtained at lower temperatures and lower values of compound corresponding to peak 1.

Likewise, the relationship between the antioxidant activity and some compounds' concentration can also be estimated from the dendrogram obtained by cluster analysis of standardized variables. The correlation coefficient (r) was taken as a measure of similarity among two variables, and Ward's method was used as a linkage rule. In the dendrogram obtained, two groups were observed, one of them formed by antiox3 (ethanol extract's antioxidant activity) and almost every analyzed variable that has a positive correlation with antiox3, and another group containing peak 1, the relationship between the amount of *β*-carotene isomers and the total amount of carotenoids (Isom/cartot), and the ratio 9-*cis*/*all*-*trans*-*β*-carotene. Strong correlation between antiox3 and peak 3 ($r = 0.947$), peak 4 ($r = 0.947$), and peak 5 ($r = 0.957$) and the extraction temperature ($r = 0.952$) was observed. Thus, these results do not corroborate that the antioxidant activity of the ethanol extracts depended on the

different 9-*cis*/*all*-*trans*-*β*-carotene proportions ($r = 0.0$). Moreover, it seems that other minor carotenoids (3−5, not identified in this work) can also have some influence on the antioxidant activity of the investigated extracts. This result can have important implications, taking into account that up to now *β*-carotene has been the main compound investigated (and produced) from *D. salina* microalga. On the other hand, results obtained in the present work support the idea of the importance of using *D. salina* extracts as a source of antioxidants because its complex composition provides stronger antioxidant activity than that achieved with synthetic *all*-*trans*-*β*-carotene (*28*). In this work, it has been demonstrated that the antioxidant activity can be associated with a mixture of carotenoids including some natural *β*-carotene isomers and other unidentified carotenoids.

In conclusion, in this work PLE has been proven to be capable of extracting antioxidant compounds from *D. salina*. To optimize the extraction process, an experimental design was developed. Different extraction conditions were investigated for the three solvents tested (water, hexane, and ethanol). Ethanol extracts were selected as more suitable because they gave the best results in terms of yield (twice the yield achieved with hexane) and solvent safety (GRAS), providing important antioxidant activities (half of the antioxidant activity of hexane extracts). Moreover, the chemical characterization of the extracts was performed, and new correlations between carotenoids and antioxidant activity of extracts were observed, suggesting that ethanol extracts are more complex, having in their composition different carotenoids that can have a positive influence on the antioxidant activity.

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LITERATURE CITED

(1) Goldberg, I. *Functional Foods. Designer Foods, Pharmafood, Nutraceuticals*; Chapman and Hall: London, U.K., 1996.

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(2) Dufosse, L.; Galaupa, P.; Yaronb, A.; Malis Aradb, S.; Blanc, P.; Chidambara Murthyd, K. N.; Ravishankar, G. A. Micro-organisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? *Trends Food Sci. Technol.* **2005**, *16*, 389−406.

(3) Mahajan, G.; Kamat, M. γ-Linoleic acid production from *Spirulina platensis. Appl. Microbiol. Biotechnol.* **1995**, *43*, 466−469.

(4) Otles, S.; Pire, R. Fatty acid composition of *Chlorella* and *Spirulina* microalgae species. *J. AOAC Int.* **2001**, *84*, 1708−1714.

(5) Herrero, M.; Cifuentes, A.; Ibáñez, E. Sub- and supercritical fluid extraction of functional ingredients from different natural sources: plants, food-by-products, algae and microalgae: a review. *Food Chem.* **2006**, *98*, 136−148.

(6) Madhavi, D. L.; Singhai, R. S.; Kulkarni, P. R. In *Food Antioxidants*; Madhavi, D. L., Deshpande, S. S., Salunkhe, D. K., Eds.; Dekker: New York, 1996; p 159.

(7) Aruoma, O. I. Free radicals, oxidative stress and antioxidants in human health and disease. *J. Am. Oil Chem. Soc.* **1998**, *75*, 199−212.

(8) Van den Berg, H.; Faulks, R.; Granado, H. F.; Hirschberg, J.; Olmedilla, B.; Sandmann, G.; Southon, S.; Stahl, W. The potential for the improvement of carotenoid levels in foods and the likely systemic effects, *J. Sci. Food Agric.* **2000**, *80*, 880−912.

(9) Herrero, M.; Ibáñez, E.; Cifuentes, A. Analysis of natural antioxidants by capillary electromigration methods. *J. Sep. Sci.* **2005**, *28*, 883−897.

(10) Luque de Castro, M. D.; Jimenez-Carmona, M. M.; Fernández-Pérez, V. Towards more rational techniques for the isolation of valuable essential oils from plants. *Trends Anal. Chem.* **1999**, *19*, 708−716.

(11) Basile, A.; Jiménez-Carmona, M. M.; Clifford, A. A. Extraction of rosemary by superheated water. *J. Agric. Food Chem.* **1998**, *46*, 5205−5209.

(12) Herrero, M.; Simó, C.; Ibáñez, E.; Cifuentes, A. Capillary electrophoresis−mass spectrometry of *Spirulina platensis* proteins obtained by pressurized liquid extraction. *Electrophoresis* **2005**, *26*, 4215−4224.

(13) Herrero, M.; Arráez-Román, D.; Segura, A.; Kenndler, E.; Gius, B.; Raggi, M. A.; Ibáñez, E.; Cifuentes, A. Pressurized liquid extraction−capillary electrophoresis−mass spectrometry for the analysis of polar antioxidants in rosemary extracts. *J. Chromatogr. A* **2005**, *1084*, 54−62.

(14) Herrero, M.; Ibáñez, E.; Señoráns, F. J.; Cifuentes, A. Accelerated solvent extracts from *Spirulina platensis* microalga: determination of their antioxidant activity and analysis by micellar electrokinetic chromatography. *J. Chromatogr. A* **2004**, *1047*, 195−203.

(15) Ibáñez, E.; Kuvátová, A.; Señoráns, F. J.; Cavero, S.; Reglero, G.; Hawthorne, S. B. Subcritical water extraction of antioxidant compounds from rosemary plants. *J. Agric. Food Chem.* **2003**, *51*, 375−382.

(16) Kuvátová, A.; Lagadec, A. J. M.; Miller, D. J.; Hawthorne, S. B. Selective extraction of oxygenates from savory and peppermint using subcritical water. *Flavour Fragrance J.* **2001**, *16*, 64−73.

(17) Borowitzka, M. A.; Borowitzka, L. J. *Dunaliella*. In *Micro-algal Biotechnology*; Borowitzka, M. A., Borowitzka, L. J., Eds.; Cambridge University Press: Cambridge, U.K., 1988; pp 27−58.

(18) Ben Amotz, A. Industrial production of microalgal cell-mass and secondary products−major industrial species. In *Handbook Microalgal Culture: Biotechnology and Applied Phycology*; Richmond, A., Eds.; Blackwell Science: Oxford, U.K., 2004; pp 273−280.

(19) García-González, M.; Moreno, J.; Manzano, J. C.; Florencio, F. J.; Guerrero, M. G. Production of *Dunaliella salina* biomass rich in 9-*cis*-β-carotene and lutein in a closed tubular photoreactor. *J. Biotechnol.* **2005**, *115*, 81−90.

(20) Ben-Amotz, A. Production of β-carotene from *Dunaliella*. In *Chemicals from Microalga*; Cohen, Z., Ed.; Taylor and Francis: London, U.K., 1999; pp 196−204.

(21) Oren, A. A hundred years of *Dunaliella* research: 1905−2005. *Saline Syst.* **2005**, 1.

(22) Denery, J. R.; Dragull, K.; Tang, C. S.; Li, Q. X. Pressurized fluid extraction of carotenoids from *Haematococcus pluvialis* and *Dunaliella salina* and kavalactones from *Piper methysticum. Anal. Chim. Acta* **2004**, *501*, 175−181.

(23) Herrero, M.; Ibáñez, E.; Cifuentes, A.; Señoráns, F. J.; Reglero, G.; Santoyo, S. *Dunaliella salina* pressurized liquid extracts as potential antimicrobials. *J. Food Prot.* **2006**, in press.

(24) Jin, E.; Melis, A. Microalgal biotechnology: carotenoid production by the green alga *Dunaliella salina. Biotechnol. Bioprocess Eng.* **2003**, *8*, 331−337.

(25) Stahl, W.; Sies, H. Antioxidant activity of carotenoids. *Mol. Aspects Med.* **2003**, *24*, 345−351.

(26) Woodall, A. A.; Britton, G.; Jackson, M. J. Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: relationship between carotenoid structure and protective ability. *Biochim. Biophys. Acta* **1997**, *1336*, 575−586.

(27) Viljanena, K.; Sundberga, S.; Ohshimab, T.; Heinonena, M. Carotenoids as antioxidants to prevent photooxidation. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 353−359.

(28) Chidambara Murthy, K. N.; Vanitha, A.; Rajesha, J.; Mahadeva Swamy, M.; Sowmya, P. R.; Ravishankar, G. A. *In vivo* antioxidant activity of carotenoids from *Dunaliella salina*, a green microalga. *Life Sci.* **2005**, *76*, 1381−1390.

(29) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying and improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(30) Breithaupt, D. E. Simultaneous HPLC determination of carotenoids used as food coloring additives: applicability of accelerated solvent extraction. *Food Chem.* **2004**, *86*, 449−456.

(31) Strohschein, S.; Pursch, M.; Händel, H.; Albert, K. Structure elucidation of β-carotene isomers by HPLC-NMR coupling using a C_{30} bonded phase. *Fresenius' J. Anal. Chem.* **1997**, *357*, 498−502.

(32) Bonoli, M.; Commissati, I.; Fossati, A.; Tateo, F. A simplified method for the HPLC resolution of α-carotene and β-carotene (*trans* and *cis*) isomers. *Anal. Bioanal. Chem.* **2002**, *372*, 401−403.

(33) Levin, G.; Mokady, S. Antioxidant activity of 9-cis compared to all-trans β-carotene in vitro. *Free Radical Biol. Med.* **1994**, *17*, 77−82.

(34) Gamlieli-Bonshtein, I.; Korin, E.; Cohen, S. Selective separation of cis−trans geometrical isomers of β-carotene via CO_2 supercritical fluid extraction. *Biotechnol. Bioeng.* **2002**, *80*, 169−174.

(35) Stahl, W.; Schwarz, W.; Sies, H. Human serum concentrations of all-trans β- and α-carotene but not 9-cis β-carotene increase upon ingestion of a natural isomer mixture obtained from *Dunaliella salina* (Betatene). *J. Nutr.* **1993**, *123*, 183−191.