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β-Carotene Isomer Composition of Sub- and Supercritical Carbon Dioxide Extracts. Antioxidant Activity Measurement

LAURA JAIME,[†] JOSÉ A. MENDIOLA,[‡] ELENA IBÁÑEZ,[‡] PEDRO J. MARTIN-ÁLVAREZ,[‡] ALEJANDRO CIFUENTES,[‡] GUILLERMO REGLERO,[†] AND FRANCISCO J. SEÑORÁNS*,[†]

Sección Departamental de Ciencias de la Alimentación, Universidad Autónoma de Madrid, Campus de Cantoblanco, 28049 Madrid, Spain, and Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain

In the present work sub- and supercritical extraction conditions using carbon dioxide were studied in order to obtain extracts with different compositions from the green microalgae *Dunaliella salina*. Different compositions of β-carotene isomers were identified in the extracts by using HPLC-DAD. Also, antioxidant activity of the extracts was measured using a TEAC assay. An experimental design was applied considering two factors, extraction pressure and temperature, in a wide range of values, trying to maximize the extraction yield. Higher yields were obtained at high pressures and low temperatures, that is, at higher CO_2 densities. Attempts were made to correlate the antioxidant activity of the extracts with their chemical composition by means of principal component analysis. A certain relationship was found between their antioxidant activity and the isomeric composition of β-carotenes. As a result, an original equation is proposed to predict the antioxidant activity of extracts from *D. salina* in terms of the ratio 9-*cis*-β-carotene/all-*trans*-β-carotene, the concentration of α-carotene, and, especially, the concentration of 9-*cis*-β-carotene.

KEYWORDS: *Dunaliella salina*; TEAC; ABTS; HPLC; antioxidant; β-carotene isomers; microalgae; supercritical

INTRODUCTION

Nowadays, there is an increasing interest in the use of antioxidants in foods since they markedly delay or prevent lipid oxidation (*1*, *2*) and, more interestingly, they have been demonstrated to have important beneficial effects on health (*3*). In this sense, there seems to be a relationship between the reactive oxygen species (ROS) and pathogenesis of many diseases and aging (*4*). Antioxidants appear to act against these negative effects by raising the levels of endogenous defense [e.g., by upregulating the expression of genes encoding the enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and lipid peroxidase] (*5*, *6*).

The potential use of antioxidants as food ingredients in the so-called functional foods requires several steps such as the extraction or purification from a natural source (in order to obtain "natural ingredients" rather than synthetic ones), preferably using environmentally clean processes, the evaluation of their potential *in vitro* activity, the chemical characterization of the extracts, and the statement of the properties of the functional food by *in vivo* assays.

Edible microalgae have long been considered a good source of energy and nutritional compounds, but lately there has been an increasing interest in their use as a potential source of compounds with biological properties (mainly antioxidants). Among these, carotenoids have widely been studied (*7*, *8*), showing through epidemiological evidence that β-carotene antioxidant activity can prevent cancer of various organs such as lungs, stomach, cervix, pancreas, colon, rectum, breast, prostate, and ovary (*9*–*12*).

Dunaliella salina is a unicellular biflagellate green alga from Chlorophyceae gender. First sighted in 1838 in saltern evaporation ponds in the south of France by Michel Felix Dunal, it was named after its discoverer by Teodoresco in 1905 (*13*). *Dunaliella* is known to accumulate carotenoids under various stress conditions. The algal cells, lacking a rigid cell wall, instead are surrounded by a thin elastic membrane. This alga can yield three major valuable products, namely, glycerol, β-carotene, and proteins. In recent years, it is mainly cultivated for carotenoid obtention. *D. salina* under ideal conditions can yield ∼400 mg of β-carotene/m^2 of cultivation area (*9*). The ability of *Dunaliella* to proliferate over practically the entire range of salinities makes its cultivation easy and economically feasible

* Corresponding author. Phone: +34 91 497 3775. Fax: +34 91 397 8255. E-mail: javier.senorans@uam.es.
[†] Universidad Autónoma de Madrid.
[‡] Instituto de Fermentaciones Industriales, CSIC.

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(*14*). Massive carotenoid accumulation during its cultivation is typically induced by reducing the growth rate by deprivation of nutrients or applying high light intensities. Thus, some strains may contain up to 10% of β-carotene including a large percentage of the 9-cis isomer (*13*, *15*).

As mentioned, β-carotene functional activity has been largely reported *in vivo* and *in vitro*, and specifically the 9-cis isomer has demonstrated a higher antioxidant activity due to the higher reactivity of the cis bond compared to trans (*16*). On the other hand, there is a preferential absorption of all-*trans*-β-carotene and α-carotene compared to the 9-cis β-isomer (*17*). In fact, the *in vivo* antioxidant activity of carotenoids from *D. salina* has been reported and compared to synthetic carotenes, and the results clearly demonstrated the beneficial effects of algal carotenoids (*9*). Dunaliella extracts have been tested against fibrosarcoma (*18*), hepatotoxicity (*19*, *20*), diabetes mellitus, and atherosclerosis (*21*) or even for cosmetic uses (*22*, *23*).

Sub- and supercritical CO_2 has properties of liquid-like density and high compressibility, which enable an easy control over its solvent power. It has high diffusivity, which reduces mass transfer limitations, and low surface tension, which allows penetration of pores smaller than those accessible by traditional liquid solvents. It allows extractions at low to moderate temperatures, leaves no solvent residues, and is environmentally acceptable (*24*). Besides, CO_2 provides a nonoxidizing atmosphere in extractions avoiding extracts from degradation and also provides the possibility to fractionate extracts by sequential decreases of pressure and temperature tuning its solvating power (*25*). Supercritical fluid extraction (SFE) is a well-known technique for obtaining carotenoid-rich extracts (*26*–*29*), but only one reference can be found related to the selective extraction of isomers of carotene (*30*). In this field, previous work has been done in our laboratory using pressurized solvent extraction (*31*).

In this work, selective extraction of cis–trans geometrical isomers of β-carotene by SFE is explored. The hypothesis is based on the different dissolution rate of 9-cis/all-trans isomers in CO_2 which, in fact, depends on the different physicochemical properties of both isomers. Indeed, all-trans is practically insoluble in oil and easily crystallized at low temperatures, while 9-*cis*-β-carotene is much more soluble in hydrophobic−lipophilic solvents, amorphous, and has a high melting point (*32*). These properties suggest a higher solubility of 9-cis isomer in pressurized CO_2 at certain conditions. Considering the interest of the selective enrichment in a given carotene isomer and its associated antioxidant activity, it is remarkable that no studies have been performed so far on the effect of different sub- or supercritical extraction conditions.

Therefore, the goal of the present investigation was to study the effect of a wide range of sub- and supercritical conditions on the extraction of antioxidants from *D. salina* and, specifically, on the selective extraction of different β-carotene isomers. The extracts were analyzed to determine their chemical composition, and statistical correlations were studied to establish a mathematical model able to predict the antioxidant activity of the extracts based on their isomeric composition.

EXPERIMENTAL PROCEDURES

Samples and Reagents. Microalgae samples (*D. salina*) consisted of freeze-dried microalgae from NBT Ltd. (Jerusalem, Israel) that were stored under dry and dark conditions packed in the absence of oxygen until utilization.

ABTS [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] and potassium persulfate were purchased from Sigma-Aldrich (St. Louis, MO) and the carotene isomer mixture was from Darmstad (Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and all *trans*-β-carotene was from Fluka Chemie AG (Buchs, Switzerland). All solvents, HPLC quality, were obtained from Labscan (Dublin, Ireland), except ethanol (99% purity) from Panreac Quimica (Barcelona, Spain). Milli-Q water was purified using a Milli-Q system (Millipore Corp., Billerica, MA). Carbon dioxide N48 quality was from Air-Liquide España (Madrid, Spain).

Table 1. Factor Levels (Coded and Real Values) of the Experimental Matrix Design for SFE of *D. salina* and Results Obtained for Yield (% Dry Weight/Initial Weight) and Antioxidant Activity (AA) Measured Using TEAC Assay, along with Values of Density

	factor levels						
	coded values		real values				
expt	pressure	temp	*P* (atm)	*T* (°C)	CO_2 density (g/L)	yield (%)	AA[a]
1	0	0	310	27.5	962.4	4.41	0.181
2	0	0	310	27.5	962.4	4.42	0.169
3	1	1	400	40	956.8	5.48	0.176
4	1	−1	400	15	1035.7	6.08	0.268
5	−1	1	220	40	858.1	4.12	0.093
6	−1	−1	220	15	969.6	4.31	0.115
7	1.414	0	437.3	27.5	1008.9	6.58	0.452
8	−1.414	0	182.7	27.5	890.9	4.02	0.157
9	0	1.414	310	45.2	896.2	4.38	0.253
10	0	−1.414	310	9.8	1024.4	4.52	0.259

[a] Antioxidant activity is expressed as TEAC mmol of Trolox/g of extract.

Carbon Dioxide Extractions. All supercritical fluid extractions were done in a Suprex PrepMaster supercritical fluid extractor (Suprex, Pittsburgh, PA). The flow rate was controlled using a needle valve as the variable restrictor. Extracts were collected in glass vessels cooled by self CO_2 expansion. Thr extraction time was fixed at 100 min: first 10 min of static extraction, followed by 90 min of dynamic extraction; 1 g of microalga was used in all extractions, mean cell size 4–12 μm (*33*).

Extractions were done following a Central Composite Rotable design considering the pressure (atm) and temperature (°C) as design factors. Experimental conditions were selected in order to test a wide range of densities, but special emphasis has been put in those conditions providing maximum densities; so pressure ranged between 182.7 and 438.3 atm and temperature between 9.8 to 45.2 °C. The different combinations provided extraction densities from 0.85 to 1.03 g/mL with most of the experiments done in the liquid-subcritical region in order to avoid carotenoids from degradation (*34*). A total of 10 experiments [4 points of the full factorial design, 4 star points (start distance = α = 1.414), and 2 center points] were carried out in randomized run order. The response variables selected were extraction yield (yield, determined as percent dry weight/initial weight) and antioxidant activity (Antiox) determined using the TEAC test. **Table 1** shows the factor levels (coded values) corresponding to the experimental matrix design and their physical values, along with the results obtained for the response variables evaluated. The quadratic model proposed for each response variable (Y_i) was

$$Y_i = \beta_0 + \beta_1 P + \beta_2 T + \beta_{1,1} P^2 + \beta_{2,2} T^2 + \beta_{1,2} PT + \text{error} \quad (1)$$

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). The effect of the each term in the model and their statistical significance, for each of the response variables, were analyzed from the standardized Pareto chart. The quadratic and the interaction terms not significantly different from zero ($P > 0.10$) 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) and the residual standard deviation (RSD) from the ANOVA table. From the new fitted model, the optimum conditions that maximize the responses were also provided by the program. Surface plots were developed using the fitted quadratic polynomial equation obtained.

Antioxidant Analysis: TEAC Assay. The antioxidant activity of supercritical extracts of *D. salina* was measured by the TEAC (Trolox equivalent antioxidant capacity) assay, performed essentially as previously described (*35*) for carotenoid standards with minor modifications. Briefly, 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 around 0.70 at 734 nm. The reaction was initiated by the addition of 10 μL of *Dunaliella* extract (dissolved in dichloromethane) to 0.990 mL of 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 (millimoles of Trolox per gram of extract); a higher value means a higher antioxidant activity. 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.

Chromatographic Conditions. All analyses of the supercritical extracts were done by HPLC-DAD following a previously published method (*36*) in an Agilent 1100 HPLC-DAD chromatograph. Chromatograms were monitored at 450 nm (4 nm bandwidth; reference wavelength at 550 nm with 50 nm bandwidth). A YMC C30 analytical column (5 μm, 250 × 4.6 mm i.d.) was used (YMC Schermbeck, Germany). 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; a separation funnel were used to separate surplus water and avoid two phases). The following gradient was used (min/% *A*): 0/93.5; 34/0; 38/93.5; 53/93.5 (flow rate, 1 mL/min; injection volume, 20 μL). All samples were dissolved in dichloromethane (5 mg/mL). Quantification was done using a pure all-trans standard and assuming the same response factor for all carotenoids overcoming in this way the impossibility to find commercial standards of all β-carotene isomers.

Statistical Analysis. For experimental design, the STATGRAPHICS Plus v.5.1 program (Statistical Graphics Corp., Manugistics Inc., MD, 2000) was used. This program permits both the creation and the analysis of experimental designs. Other statistical methods used for data analysis were principal component analysis (from standardized variables) to examine the relationship among the analyzed variables and stepwise multiple regression analysis to study the relationship between the antioxidant activity and chemical composition of extracts. The STATISTICA program for Windows, release 7.1 (Statsoft Inc., Tulsa, OK, 2005), was used for data processing.

RESULTS AND DISCUSSION

Optimization of Extraction Conditions. Table 1 shows the results obtained for the different extracts in terms of extraction yield (percent dry weight/initial weight) and antioxidant activity (measured using the TEAC assay and expressed as millimoles of Trolox per gram of extract) under the different experimental conditions tested. A column including the CO_2 density has also been given for information. As can be seen, in general the best results in terms of extraction yield were obtained working at higher densities (experiments 4 and 7) that were achieved by increasing the pressure and decreasing the temperature. As previously demonstrated by other authors (*37*, *38*), extraction of carotenoids can be favored using dense CO_2 at subcritical conditions. Although in the total extraction yield other compounds different from carotenoids have to be considered, it seems clear that carotenoids have an important contribution to the total amount of extracted material.

Analysis of the experimental design data provides information on the importance and the statistical significance of each term in the model for each response variable. Pressure is the most important term followed by the quadratic term of pressure and temperature. As expected, pressure and its quadratic term have a positive influence in the extraction yield while temperature has a negative effect, increasing the response by decreasing the temperature. Once the interaction and quadratic term of temperature of the model not significantly different from zero ($P > 0.1$, 90% confidence level) were excluded from eq 1, the mathematical model was refitted by MLR giving the equation:

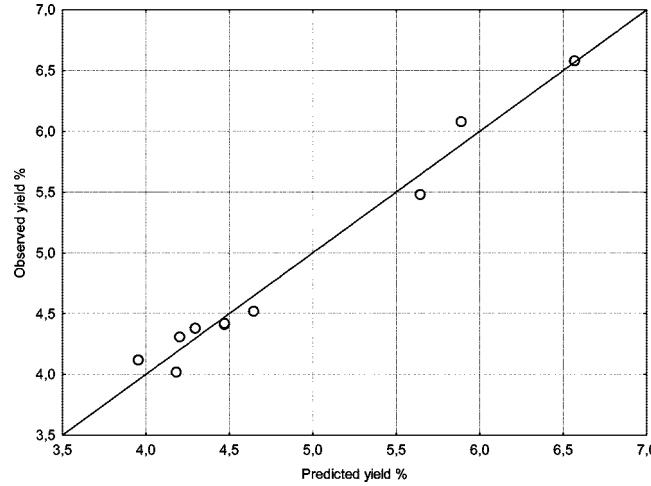

Figure 1. Plot of the observed values for yield and predicted values using eq 2 (see text for further details).

$$\text{yield}\,(\%) = 7.14462 - 0.024823P - 0.00988T + 0.0000551589P^2 \quad (2)$$

where P is in atm and T in °C. This equation explains 97.8% of the variation of the response variable ($R^2 = 0.978$), and the residual standard deviation (RSD) was equal to 0.161, which was expressed as a percentage of the mean value of the response ($100\text{RSD}/\bar{Y}$), and provides a relative error of the fit lower than 4%. Thus the model was found to be adequate enough to describe the data. The validity of this equation was calculated by using the leave-one-out cross-validation procedure (*39*, *40*). As can be seen in eq 2, an increase in extraction yield is achieved by raising the pressure and lowering the temperature. Using the statistical program, the optimum extraction conditions in terms of pressure and temperature are provided, being equal to 437 atm and 9.8 °C. The predicted value for the yield at the optimum conditions, using the fitted model shown in eq 2, was equal to 6.72%.

Figure 1 shows a representation of observed vs predicted values for yield. As can be seen, the agreement between the experimental values and those predicted by the model is very good with residues ranging from −0.16 to 0.19.

Antioxidant Activity. Analysis of the data based on the antioxidant activity as response (see **Table 1**) did not provide any acceptable model, and only 61% of the variation of the experimental data ($R^2 = 0.61$) could be explained by considering the pressure and their quadratic term in the model (eq 1). This lack of fit can be explained by considering that the antioxidant activity will depend not only on the presence or absence of certain compounds but also on their relative proportion. Although a good mathematical model is not obtained for the antioxidant activity, a certain tendency can be observed between such activity and density, which can be correlated to the different solubility of carotenoids at different pressures (*41*) as mentioned above. Therefore, it seems that a more complex model has to be considered to explain the antioxidant activity of the supercritical extracts. With the objective to chemically characterize the extracts, HPLC coupled to DAD was used to analyze and identify the carotenoids present. A typical chromatogram can be seen in **Figure 2**. Identification of each peak was done using

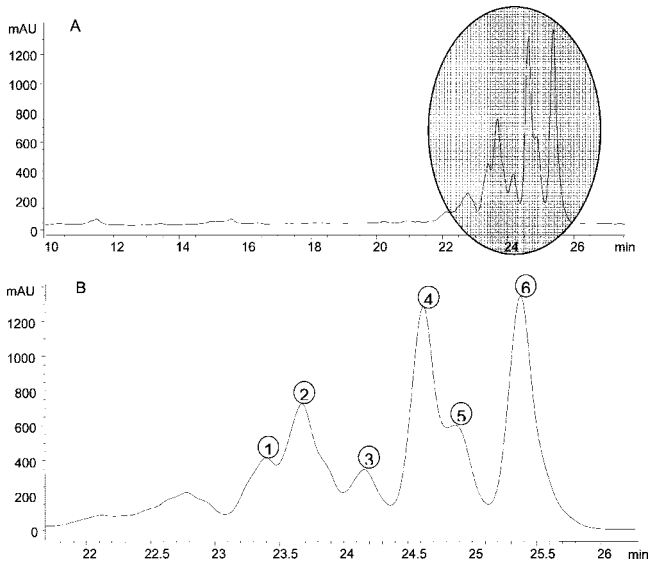

Figure 2. Chromatogram of sample 7 acquired at 450 nm (**A**) and zoom of the β-carotene isomer zone, 21–27 min (**B**). Peak identification: 1, ϵ-carotene; 2, α-carotene; 3, 13-*cis*-β-carotene; 4, all-*trans*-β-carotene; 5, 15-*cis*-β-carotene; 6, 9-*cis*-β-carotene.

the carotene mixture (α and β) and the all-*trans*-β-carotene standard; the rest of the peaks were identified comparing both UV–vis spectra and elution order based on data previously published by other authors (*36*, *42*, *43*).

The approximate carotene composition of *Dunaliella* supercritical extracts was determined using a calibration curve that was obtained by injecting all-*trans*-β-carotene standards and assuming the same response factor for all of the carotenoids. **Table 2** shows the concentration found for each compound as well as several relationships between them. As can be observed, carotenoid constitutes only a small percentage [1–7% (w/w)] of the total composition, other compounds of interest being present. Taking in account the solubilities of other components of *Dunaliella*, the rest of the extract should be composed mainly of glycerol (*44*, *45*) and lipids (*46*).

In order to study possible relations between the antioxidant activity (AA) and the chemical composition of the extracts, principal component analysis was applied. Four principal components were obtained that explained 89.9% of the total variance of the data. The first principal component, which explained 60% of the total variance, correlated positively with AA (0.79), total amount of carotenes (0.99), α-carotene (0.94), 13-cis isomer (0.98), all-trans isomer (0.97), 15-cis isomer (0.98), 9-cis isomer (0.99), concentration of other carotenoids (0.98), and the total amount of β-carotene isomers (0.99). The second principal component, which explained 20.9% of the total variance, correlated negatively with the extraction pressure (-0.79) and density (-0.89) and positively with the ratio α-carotene/β-carotene (0.75). On the basis of these results, it seems that there is an important correlation between the antioxidant activity and the concentration of several carotenoids, thus confirming our previous statement. Therefore, considering the loadings of the variables in the first principal component, the extracts that provide high antioxidant activities are those having high concentrations of carotenes (total), α-carotene, and β-isomers and also high concentration of other nonidentified carotenoids. This result corroborates the fact already mentioned in the introduction about the influence of different β-carotene isomers in the final antioxidant activity and the importance of using natural sources of carotenoids as antioxidants (e.g., *D. salina* microalga) with a complex carotenoid composition.

As an attempt to obtain a model able to predict the antioxidant activity of supercritical extracts based on their chemical composition, forward stepwise MLR was used, considering as independent (predictor) variables the different carotenoid concentrations and relations shown in **Table 2**. Values of 2 and 1.9 were used for *F*-statistics to enter and remove variables, respectively. Three variables, in decreasing order of importance, were found to predict the mentioned activity, namely, 9-*cis*-β-carotene, 9-cis/all-trans ratio, and α-carotene. The estimated model was

$$\text{TEAC} = 0.332 + 0.3925[\text{9-}cis\text{-}\beta\text{-carotene}] - 0.1923[\text{9-cis}]/[\text{all-trans}] - 0.3069[\alpha\text{-carotene}] \quad (3)$$

where concentrations are given in milligrams of caretonoid per 100 mg of extract. All of the coefficients are significantly different from zero ($P > 0.10$). The model provided values of 0.82 for the coefficient of determination (R^2) and 0.05 for the standard error of estimate (RSD). The predicted value of eq 3 is limited to this specific extract, because *Dunaliella* composition can change with culture conditions and so can the extracts, even if the extraction is done using the same conditions.

As can be seen in the eq 3, the concentration of 9-*cis*-β-carotene is contributing positively while concentration of α-carotene contributes negatively, that is, decreasing the TEAC value when increasing their content in the composition of the extracts. Different behavior can be deduced from the influence of the ratio 9-cis/all-trans since it contributes negatively to the equation, meaning that lower ratio 9-cis/all-trans provides higher antioxidant activity. Considering the global equation, that is, the contribution of the different factors, a synergistic behavior can be observed between 9-*cis*- and all-*trans*-β-carotene since the maximum antioxidant activity is obtained with a high

Table 2. Carotenoid Composition (Expressed as mg of Carotenoid/100 mg of Extract) of *D. salina* Supercritical Extracts Obtained at the Different Extraction Conditions Tested

			β-carotene							
expt	total carotenoids[a]	α-carotene	13-cis	all-trans	15-cis	9-cis	total β	other carotenoids[b]	9-cis/all-trans ratio	α/β ratio
1	3.223	0.429	0.183	0.486	0.282	0.624	1.575	1.219	1.284	0.273
2	3.086	0.354	0.159	0.518	0.210	0.656	1.543	1.189	1.266	0.229
3	1.250	0.158	0.059	0.221	0.080	0.227	0.587	0.505	1.026	0.269
4	2.702	0.340	0.133	0.468	0.175	0.487	1.264	1.099	1.039	0.269
5	1.176	0.134	0.065	0.132	0.085	0.210	0.491	0.552	1.594	0.273
6	1.987	0.256	0.134	0.301	0.172	0.391	0.998	0.733	1.297	0.256
7	7.199	0.912	0.419	1.244	0.591	1.497	3.751	2.536	1.204	0.243
8	4.251	0.838	0.220	0.650	0.314	0.750	1.934	1.480	1.154	0.433
9	4.651	0.690	0.221	1.048	0.327	0.888	2.485	1.477	0.848	0.278
10	1.614	0.211	0.079	0.335	0.111	0.334	0.860	0.543	0.997	0.245

[a] Measured as total chromatogram area. [b] Including ϵ-carotene and zeaxanthin and other nonidentified carotenoids.

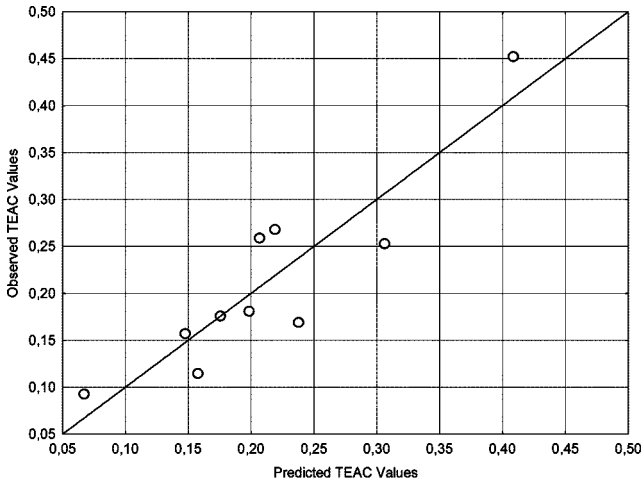

Figure 3. Plot of the observed values for antioxidant activity (TEAC) and the predicted values using the estimated equation from stepwise MLR (see text for further details).

concentration of the 9-cis isomer (up to a maximum) and a high concentration of the all-trans isomer (to minimize the second factor). It is important to emphasize the influence of the two main β-carotene isomers, 9-cis and all-trans, in the total antioxidant activity observed. Even if antioxidant activity of the pure 9-cis isomer has been found to be higher than all-trans (*16*), in the present work, the contribution of both isomers in a synergistic way has been demonstrated.

Figure 3 shows the observed values for TEAC and the predicted values obtained using the previous regression equation. As can be seen, the fit for the predictions of TEAC can be considered appropriate, corroborating the usefulness of our approach.

Therefore, in the present work the importance of using sub- and supercritical CO_2 to obtain extracts of *D. salina* with high antioxidant activity has been demonstrated. The strong influence of the supercritical extraction conditions in both the β-carotene isomer composition of the extracts and the antioxidant activity has also been shown. Statistical analysis of all the data, considering yield, antioxidant activity, and relative concentration of carotenoids in the different extracts suggested an important relationship among antioxidant activity and β-carotene isomeric relation, 9-cis/all-trans. The present study is posed as an optimization of the green extraction of antioxidants from *D. salina* that could be used as food ingredients.

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