Compressed CO₂ technologies for the recovery of carotenoid-enriched extracts from *Dunaliella salina* with potential neuroprotective activity

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ABSTRACT

Natural carotenoids from microalgae have raised a huge interest for their potential health benefits. Among microalgae species with high carotenoid content, *Dunaliella salina* has been highlighted since it is able to accumulate relatively high amounts of β -carotene and other carotenoids of industrial interest when grown under specific conditions. In the present contribution, extractions based on carbon dioxide under sub- and supercritical conditions have been optimized to improve the recovery of carotenoids and extracts purity from *D. salina*. An experimental design was employed to investigate the effect of pressure and temperature variations ranging from 250 to 400 bar and from 15 to 45 °C, respectively. The chemical characterization of the carotenoids extracts was carried out by HPLC-DAD. Moreover, inhibition of the acetylcholinesterase activity of all the extracts was measured by using a recently developed *in-vitro* fluorescence methodology. High carotenoid yield and purity were obtained at 302-313 bar and 45 °C. Nine carotenoids were identified and three other compounds were recognized as carotenoids and quantified. Acetylcholinesterase activity inhibition could be satisfactorily explained by a partial least-square model (63% explained variance in cross-validation) built considering the chemical composition of the different extracts. The model indicates a positive effect of lutein, 15-*cis*- β -carotene and the negative effect of zeaxanthin, cryptoxanthin and the ratio 9-*cis*- β -carotene/all-*trans*- β -carotene and 9-*cis*- β -carotene/total carotenoids in the inhibition of acetylcholinesterase enzyme.

KEYWORDS:

β -carotene; carotenoids; compressed CO₂ extractions; *Dunaliella salina*; *in-vitro* fluorescence AChE methodology.

INTRODUCTION

At present, there is an increasing demand for natural bioactive compounds able to provide health benefits. In this regard, microalgae are promising microorganisms that can play a key role in the bio-based economy, since they may serve as a continuous and reliable source of safe bioactive natural products. *Dunaliella salina* is a green microalga able to accumulate relatively high amounts of β -carotene when grown under specific conditions. β -carotene in *D. salina* can constitute over 80% of the total carotenoid content¹; in most microalgae, there is not a major component representing more than 70% of total carotenoids². Although this compound is the main terpenoid in this microalga species, other carotenoids related to its biosynthetic pathway could also be of industrial interest. Carotenoids have health-promoting properties such as anti-cancer, antioxidant, or anti-diabetic activities³. Furthermore, when compressed fluids technologies using green solvents are used for their extraction, these compounds are compliant with processes and regulations implemented in food, cosmetic, and pharmaceutical sectors⁴.

As every aspect related to sustainability increases their importance and relevance nowadays, the development of new green extraction protocols to obtain bioactives from natural sources is a must. Compressed fluids can provide interesting advantages in the search for processes adapted to the recovery of valuable natural compounds⁴. The use of greener solvents in combination with greener techniques has several benefits involving less time, a reduction in the employed energy and produced wastes⁵. Among them, supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) are the most popular and widely used⁴. Although the use of compressed fluids has previously been described for the extraction of carotenoids from *D. salina*⁶⁻⁹, the particular extraction conditions have to be tuned according to the existing biomass and its specific carotenoid composition. Previously published works dealing with the use of SFE have focused on

the antioxidant and/or antimicrobial effect of the extracts^{10, 11}. However, up to now, the potential of *D. salina* extracts rich in carotenoids to inhibit acetylcholinesterase (AChE) activity has not been explored so far.

Alzheimer's disease is one of the main neurodegenerative disorders causing dementia. This disease has a multifactorial origin and, despite its importance, there is no effective clinical treatment for this chronic issue. Current treatments are just focused on the alleviation of symptoms. For this reason, the prevention of the development of this disease is of utmost importance. In this regard, dietary components have been highlighted as potential effective agents towards the prevention of Alzheimer's disease, among other neurodegenerative diseases¹². In particular, terpenoids with a wide array of different chemical structures, including carotenoids, have been demonstrated to retain a good preventive potential against neurodegeneration^{13,14}, although their molecular mechanisms of action have not comprehensively been described so far. One of the possible targets through which the bioactivity of these components may be exerted is the inhibition of acetylcholinesterase (AChE) activity, as a significant reduction of acetylcholine levels in the hippocampus and cortex of the brain of Alzheimer's disease patients has been confirmed¹⁵. As a result, AChE inhibitors may not only produce an increase in acetylcholine levels in the brain but also reduce and prevent the formation of β -amyloid plaques, protecting neurons from neurodegeneration¹⁶. For this reason, the study of the potential of *D. salina* carotenoids to inhibit the activity of this enzyme is interesting. However, up to now, this activity has not been thoroughly studied.

One of the possible reasons for this lack of studies is the fact that there was not an effective methodology for determining this activity in colored extracts, such as those containing carotenoids. The new *in-vitro* AChE fluorescent methodology developed by Sánchez-Martínez *et al.*¹⁷ overcomes this problem. In this new methodology, 5,5'-dithio-bis (2-

nitrobenzoic acid) (DNTB) reagent is replaced by 4-(amino-sulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F). The thiols originated in the hydrolysis reaction between AChE and their acetylthiocholine iodide (ATCI) substrate react with ABD-F producing a fluorescent complex¹⁸. Furthermore, the presence of carotenoids has been described in the human brain¹⁹, which means that they could cross the blood-brain barrier (BBB), and therefore they could be active against neurodegenerative diseases.

The aim of the present work was to evaluate the effect of a wide range of sub- and supercritical conditions on the extraction of carotenoids from *D. salina* and their effect in the inhibition of AChE activity, looking more specifically for differences in carotenoids bioactivity due to variations on their chemical structure. For this purpose, the extracts were chemically characterized and a mathematical model was built to predict their AChE inhibition activity.

MATERIALS AND METHODS

Sample and reagents

D. salina freeze-dried microalgae biomass was supplied by A4F (Lisbon, Portugal) and were stored in the dark at a temperature of -18°C packed in the absence of oxygen until utilization. For the sub- and supercritical fluid extraction, premier quality CO₂ was provided by Carburos Metálicos (Madrid, Spain) and sea sand by Panreac Quimica (Barcelona, Spain). HPLC-grade ethanol was purchased from LabScan (Gliwice, Poland), methyl tert-butyl ether (MTBE; 100.0%), and LC-MS grade methanol were from (VWR, Barcelona, Spain). Washed glass wool, HPLC-grade acetone (≥99,9%), HPLC-grade tetrahydrofuran, butylated hydroxytoluene (BHT), acetylcholinesterase (AChE) Type VI-S from *Electrophorus electricus*, acetylthiocholine iodide (ATCI), Trizma hydrochloride (Tris-HCl), all-*trans*-β-carotene (≥ of 97%), lutein (≥ 96%) and zeaxanthin (≥ 95%)

standards were obtained from Sigma-Aldrich (Madrid, Spain). 4-(Aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) and galantamine hydrobromide were purchased from TCI Chemicals (Tokyo, Japan). Water was purified using a Milli-Q system (Millipore Corp., Billerica, MA, USA).

Experimental design and statistical analysis

Experimental design and its statistical assessment were carried out using Statgraphics Centurion XVI software (StatPoint Technologies, Inc., Warrenton, VA, USA). The influence of extraction temperature and pressure on *D. salina* extracts was studied using a three-level factorial experimental design. A total of 12 extractions (9 points of the factorial design and 3 center points to consider the experimental error) were run in randomized order. By using this design, two factors were tested at three different levels: pressure at 250, 325, and 400 bar and temperature at 15, 30, and 45 °C. The selected response variables were extraction yield (expressed as the percentage of extract weight per initial algae weight), carotenoid yield or extraction efficiency (expressed as mg of carotenoids extracted per g of algae), and the purity of the extract (or total carotenoids content) (expressed as mg of carotenoids per 100 mg of extract).

The obtained data were optimized applying response surface methodology (RSM). The quadratic model proposed for each response variable (Y_i) follows equation 1:

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

where P stands for pressure (bar) and T for temperature (°C), the independent variables, while 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. Those parameters were estimated by multiple linear regression (MLR) and their effect on the

model and statistical significance, for each response variable, were illustrated in a Pareto chart. 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. The model also provided the optimum extraction conditions which maximized each response variable and results were graphically represented in a surface plot.

Compressed carbon dioxide extractions

Extractions were performed in a homemade compressed fluids extraction system (Figure S1, Supporting information). The CO₂ from a cylinder was cooled and then compressed by a CO₂ pump from Jasco (Tokyo, Japan). Afterward, the liquid CO₂ is preheated and pushed towards the oven, where the extraction cell containing the algae material is placed. The flow rate was set at 4.5 mL/min. The pressure was controlled adjusting the opening of two needle valves. The extraction cell was filled with a mixture of 100 mg of sample and 2 g of sand placed between two layers of glass wool. Subsequently, the extracts were collected in a liquid ethanol trap. The extract recovering system was cooled by the CO₂ expansion and protected from light by aluminum foil. The extraction time was fixed at 90 min. The extractions were carried out following a randomized run order. Each extract was transferred to a previously weighted glass vial and evaporated by a gentle nitrogen stream. Extracts containing 0.05 % (w/w) BHT were stored in the dark at -20 °C.

Conventional extraction

For the conventional solid-liquid extraction (SLE) protocol used as a benchmark method, 200 mg of microalgae were mixed with 20 mL of HPLC-grade acetone containing 0.1% (w/v) BHT, as described elsewhere²⁰. The extraction was performed in a stirred thermostatic bath (Selecta, Barcelona, Spain) operating at 500 rpm and 20 °C in the dark

for 24 h. After refrigerated centrifugation (5804 R Eppendorf, Hamburg, Germany) at 10,000 rpm and 4 °C for 10 min, the supernatant was collected, poured in a previously weighted glass vial and evaporated by using a gentle nitrogen stream. Extracts were stored in the dark at -20 °C.

Characterization of carotenoids by liquid chromatography coupled to diode array detection

Qualitative and quantitative analyses of carotenoids were performed using an Agilent 1100 series liquid chromatograph (Santa Clara, CA, USA) equipped with a diode-array detector (DAD) and using a YMC-C30 reversed-phase column (250 mm × 4.6 mm i.d., 5 µm particle size; YMC Europe, Schermbeck, Germany) and a pre-column YMC-C30 (10 mm × 4 mm i.d., 5 µm particle size). A solution of methanol/MTBE/water (90:7:3 v/v/v) was used as mobile phase A, while methanol/MTBE (10:90 v/v) was employed as mobile phase B. The flow rate was 0.8 mL/min and the injection volume was 10 µL. The chromatographic separation was run according to the following gradient: 0 min, 30% B; 15 min, 40 % B; 18 min, 80% B; 23 min, 100% B; 25 min, 0% B; 28 min, 30% B; 30 min, 30% B. The samples were dissolved in HPLC-grade ethanol at an appropriate concentration (0.1-1.0 mg/mL) and filtered through 0.45 µm nylon filters. Absorption spectra were recorded from 240 to 770 nm using the DAD whereas the quantification of carotenoids was carried out at 450 nm. The instrument was controlled by LC ChemStation 3D Software Rev. B04.03 (Agilent Technologies, Santa Clara, CA, USA).

The identification of carotenoids was performed by comparison with available commercial standards, known absorption spectra, retention times, and elution orders reported in the literature.

For the calibration curves, stock solutions of zeaxanthin and lutein were prepared in ethanol at a concentration of 2 mg/mL. Then six serial dilutions were performed and injected in triplicate. For β -carotene calibration curve, the stock solution was dissolved in THF (1.5 mg/mL), and eight aliquots at different concentrations were prepared in ethanol and injected in triplicate. Since no standards of β -carotene isomers were available, the quantification of the different isomers was performed using the pure *all-trans*- β -carotene standard, assuming the response factor would be the same. Amounts of compounds assigned as carotenoids that could not be unequivocally identified were also estimated using the aforementioned calibration curves.

Acetylcholinesterase inhibitory capacity

The acetylcholinesterase (AChE) inhibitory capacity of the extracts was determined by fluorescent enzyme kinetics as developed by Sánchez-Martínez *et al.*¹⁷. Briefly, a mixture of 100 μ L extract sample diluted in water-ethanol (50:50, v/v), 100 μ L Tris-HCl buffer at pH 8 and 25 μ L of AChE diluted in buffer (0.8 U/mL) was incubated for 10 minutes. In the reference enzymatic activity assay, 100 μ L of water-ethanol (50:50, v/v) containing the same amount of BHT than samples were added in the solution assay, to take into account the possible effect of BHT in the enzymatic activity. Then, 25 μ L of 125 μ M ABD-F in the buffer and 50 μ L of 1.4 mM ATCI in water were added to each well. Excitation and emission wavelengths (389 nm and 513 nm, respectively) were recorded in a fluorescence reader (Synergy HT, BioTek Instruments, Winooski, VT, USA) every minute for 10 minutes at 37 °C.

This kinetic measurement is needed to obtain V_{mean} value. V_{mean} corresponds to enzymatic mean velocity achieved during kinetic measurement. Percentage of inhibition degree (ID%) was calculated through equation 2:

$$ID\% = \frac{V_0 - V_1}{V_0} \times 100 \quad (2)$$

201

202 where V_0 and V_1 are the V_{mean} of enzyme kinetic without and with extract sample,
 203 respectively. Galantamine hydrobromide diluted in water-ethanol (50:50, v/v) was used
 204 as inhibitor control.

205 Three independent assays of each extraction were carried out for AChE assay. Results
 206 were expressed as the concentration of extract that causes 50% inhibition (IC_{50} value), for
 207 which different measurements of ID% at seven concentrations were needed.

208

209 **Life Cycle Assessment (LCA)**

210 The two extraction processes were evaluated in terms of environmental performance
 211 according to LCA procedure using the software SimaPro 8 (Pré Consultants B.V,
 212 Amersfoort, The Netherlands) using the calculation method ILCD (International
 213 reference Life Cycle Data system)²¹, using the SimaPro option “ILCD 2011 Midpoint+
 214 V1.08 / EU27 2010, equal weighting”. To be able to do this comparison between SFE
 215 using CO₂ and SLE using acetone, the best carotenoid yield obtained in both cases was
 216 used. The functional unit (FU) to which the environmental impact categories were
 217 normalized is here defined as 1g of carotenoids. Besides, Figure 1 represents the flowchart
 218 of the steps followed to obtain *D. salina* extract by both processes, the technical system
 219 boundaries considered in the LCA analysis are inside the dotted box. A gate-to-gate
 220 approach was considered, where the upstream and downstream steps linked to the
 221 extraction period were not included. The differences in the quantity of raw material
 222 employed were estimated to have a minor impact on the overall environmental
 223 performance.

Figure 1. System boundaries considered for the LCA calculation to compare SLE and SFE to obtain *D. salina* carotenoids.

Statistical Analysis

Principal Component Analysis (PCA) of the chemical composition of the extracts was performed using XLSTAT (Addinsoft, version 2015). Partial least square regression (PLSR) model was developed using the statistical software The Unscrambler 9.7 (CAMO, Trondheim, Norway) to explain AChE IC₅₀ values as a function of the amounts of carotenoids present in the extracts. PLS model was obtained taking into account cross-validation criteria. In this sense, the model was built in iterations leaving out one extract sample in each iteration. The predicted result for the extract that was left out, was used to calculate the model error. A reiterative process was carried out until every extract sample has been excluded once; then all the prediction residuals are used to calculate the validation residual variance and the root mean square error (RMSE) of the prediction.

The slope of the regression curve between real and predicted Y variables (m) we also used to evaluate the predictive ability of the model.

RESULTS AND DISCUSSION

Extraction of carotenoids from *Dunaliella salina* microalgae

The optimization of compressed CO₂ extraction conditions was performed using an experimental design. Sub- and supercritical CO₂ extractions have already been successfully applied by several authors to extract carotenoids from *D. salina*^{6-9, 11}. It makes sense that most authors choose CO₂ as extraction solvent since it is inert, non-toxic, has a low cost, is abundant and easy to recover and, furthermore, products containing extracts obtained with food grade CO₂ are safe in terms of human health²². That is why CO₂ is considered a GRAS (generally recognized as a safe) solvent. Different pressure (100-500 bar) and temperature (40-60 °C) conditions above the CO₂ supercritical point have previously been studied to optimize the extraction process and to suggest predictive models explaining the attainable yield of carotenoids as a function of these two variables^{6, 8}. Besides, in an early study developed in our laboratory¹¹, the effect of pressure and temperature on the antioxidant activity of the extracts was evaluated.

However, to cover a wider range of conditions (and thus, a wide range of carbon dioxide physico-chemical properties), in the present contribution sub- and supercritical CO₂ process conditions were tested and optimized to improve the extraction yield, the extraction efficiency (carotenoids yield) and purity (total amount of carotenoids). Moreover, it has to be considered that the specific strain and growing conditions used will significantly influence both the carotenoid content inside the cells as well as the extraction process. Therefore, ad-hoc optimization processes are highly desired to obtain optimum results. To do that, pressures between 250 and 400 bar, and temperatures ranging from 15

to 45 °C have been investigated. All the response variables studied including the AChE inhibitory activity measured for all the obtained extracts are summarized in Table 1.

Table 1. Factor levels of the experimental design and results obtained for yield, efficiency, purity, and AChE inhibition depending on extraction pressure and temperature employed.

Exp.	Pressure (bar)	Temperature (°C)	Extraction yield (%)	Efficiency Carotenoid yield (mg/g of alga)	Purity Total carotenoids (mg/100 mg of extract)	AChE IC ₅₀ (µg/mL)
1	325	30	11.96	56.04	46.86	74.07
2	325	15	7.34	26.77	36.47	48.31
3	325	30	10.22	52.40	51.27	62.54
4	400	15	7.80	18.15	23.27	74.21
5	325	30	11.43	51.69	45.22	50.12
6	400	30	12.11	28.23	23.31	48.31
7	250	45	12.70	57.82	45.53	37.84
8	250	30	9.18	35.46	38.63	41.85
9	400	45	12.99	51.91	39.96	47.49
10	325	45	10.54	41.89	39.74	18.85
11	250	15	7.41	26.52	35.79	56.20
12	325	30	12.75	54.24	42.54	45.88
SLE	1	20	24.52	45.04	18.37	99.39

SLE: solid-liquid extraction

As can be seen, extraction yields ranged from 7.34 to 12.99 %, which can be considered quite high compared to a maximum of 6.60% reported in previous studies^{9, 11} were obtained. A model equation that explains 79.5% of the variation of extraction yield (Equation 3), indicates that the linear effect of temperature is the most important term in the model, with a positive significant correlation (Figure S2, Supporting information).

$$Y_y = 0.88667 - 0.01096P + 0.56856T + 0.00003P^2 - 0.00002PT - 0.00682T^2 \quad (3)$$

where, Y_y stands for extraction yield, P for pressure (in bar), and T for temperature (in °C).

The non-significant terms from the model were excluded and the mathematical model was refitted giving a new equation (Equation 4) with higher deviation ($R^2 = 62.0\%$) but with more physical credibility of the results.

$$Y_y = 5.976 + 0.152T \quad (4)$$

Other previously studied models¹¹, instead, reported that pressure was the most relevant parameter and stated that the extract yield positively correlated with pressure and negatively with temperature. A higher CO₂ density is, in fact, responsible for an improved solvation power towards carotenoids and other lipids, which are supposed to be the main constituents of the extracted material from *D. salina*. As shown in Table 1, in the present work, extractions carried out using liquid CO₂ (those at 15 °C) did not show a remarkably high yield (<8 %) which is probably related with differences in the initial biomass compared to other published reports. As significant differences in chemical composition and even cell morphology could be due to the specific growing conditions and the particular strain grown²³⁻²⁵, it is necessary to carefully optimize the extraction conditions in each case to maximize the recovery of carotenoids. Furthermore, it has been reported that at constant pressure the solubility of β -carotene decreases with increasing density in the supercritical region^{26,27}; this fact is confirmed in the present work, as can be observed comparing experiments 4, 6 and 9 in terms of total carotenoids. Indeed, density, despite enhancing the solvation power of CO₂, correlates negatively with its diffusivity.

Extraction efficiency

A model was computed on the basis of the carotenoid yields (Table 1), expressed as mg of carotenoids per g of extracted alga. The non-significant terms of the initial model (quadratic temperature and the interaction between temperature and pressure) were excluded and the following equation was found (Equation 5):

$$Y_E = -167.258 + 1.203P + 0.891T - 0.002P^2 \quad (5)$$

where, Y_E stands for carotenoid extraction efficiency, P for pressure (bar), and T for temperature ($^{\circ}\text{C}$). The model presents an R^2 of 69.1% and predicts the optimum extraction conditions as 312.6 bar and 45.0 $^{\circ}\text{C}$, with a predicted value of 60.8 mg of carotenoids/g alga. RSD% calculated was 9.1%. The corresponding estimated response surface is shown in Figure 2A. As for the total extraction yield, linear temperature correlated positively and represents the only significant term ($p < 0.01$), however the quadratic pressure term seems to have a negative trend ($p < 0.1$).

319

320 **Figure 2.** Pareto chart representation (left) and estimated response surface (right) of A) efficiency (mg/g alga) and B) purity (mg/100mg extract)

321 as a function of pressure and temperature.

Despite not being the most influential factor, pressure shows an interesting behavior: up to 313 bar the extraction of carotenoids is fostered by the increasing CO₂ density, then the trend is inverted because the solvation power cannot compensate for the decrease in diffusivity. The same phenomenon can be observed in the models developed by Pour Hosseini *et al.*⁸ and Macías-Sánchez *et al.*⁶. Furthermore, analyzing the response surface outlined by Pour Hosseini, it is possible to notice that in our supercritical working ranges of pressure and temperature, no significant impact of pressure can be expected. Thus, the results gathered in the present contribution are in agreement with the literature and a plausible explanation of their behavior has been found in the physico-chemical properties of CO₂. Additionally, the efficiency of an extraction method does not depend entirely on the solvation power of the employed solvent. For instance, the sample composition can affect the outcome of the efficiency; in this sense, microalgae cultivation conditions directly affect the available amount of carotenoids and other molecules present in the biomass that can contribute to saturate the solvent or to interact with the compounds of interest, thus, preventing the mass transfer during the extraction process²⁸.

Comparisons of the results achieved under sub- and supercritical conditions with those obtained by conventional extraction using acetone as solvent (Table 1) reveal that only subcritical conditions (15 °C extractions) are less advantageous in terms of efficiency. Furthermore, SFE allows for a reduction of the extraction time from 24 h to 90 min.

Recovery has been estimated in this work as the percentage of total carotenoids extracted with respect to those extractable using a conventional extraction procedure (assumed to be 100% of carotenoids). As can be seen in Table 2, the predicted values in the present work (135% recovery) are much better than those previously reported in the literature.

345 **Table 2.** Summary of studies on *D. salina* using scCO₂ for the extraction of carotenoids.

Co-solvent	Optimum extraction conditions			Carotenoids recovery (%)	<u>Purity</u>	Reference
	Pressure (bar)	Temperature (°C)	Time (min)		Total carotenoids (mg/100mg extract)	
-	437	27.5	10+90	n.r.	7.2	[10]
Ethanol 5% [mol/mol]	400	60.0	180	35	n.r.	[6]
-	300	60.0	180	54	n.r.	[5]
-	400	55.0	90	47	n.r.	[7]
Ethanol 5% [w/w]	200	45.0	180	n.r.	46.0	[8]
-	313	45.0	90	135	-	Predicted values in this work
-	309	45.0	90	-	49.5	Predicted values in this work

346 n.r.: not reported; extraction time: static + dynamic

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Extraction purity

Another proposed model was designed considering the amount of carotenoids quantified per 100 mg of extract (Table 1), which can be considered a measurement of the extract purity. Extract purity is related to some applications in which the absence of other interfering or non-desired components in the extracts is highly valued, independently if the original biomass is completely exhausted or not. The resulting equation after removing the non-significant terms of the initial model (quadratic temperature and the interaction between temperature and pressure) was (Equation 6):

$$Y_P = -116.126 + 0.997P + 0.33T - 0.0016P^2 \quad (6)$$

where Y_P stands for extract purity, P stands for pressure (bar) and T for temperature ($^{\circ}\text{C}$). The model is able to explain 72.7% of the variation of purity and the RSD% was calculated as 5.3. The predicted optimum extraction conditions were 302.3 bar and 45.0 $^{\circ}\text{C}$, providing a predicted value of 49.5 mg of carotenoids/100 mg extract. As illustrated in Figure 2B, the only significant factor is pressure. In particular, the extract purity correlates negatively with linear and quadratic pressure terms ($p < 0.05$), while seems to have a positive trend with linear temperature ($p < 0.1$).

In addition to the lower environmental impact of the compressed fluid technology employed, purity values obtained were always above the ones achieved using conventional extractions (Table 1). Once again, the predicted purity value estimated in this work (49.5 mg carotenoids/100 mg extract) is higher than the best one obtained previously reported⁹ (46 mg carotenoids/100mg extract) (Table 2). Furthermore, our extracts present the advantage of being obtained avoiding the use of ethanol as a

cosolvent, producing a solvent-free extract potentially ready to be used, increasing the sustainability of the whole process.

Environmental performance of extractions

There are several metrics to compare the environmental performance and impacts of chemical processes. In fact, Chemat *et al.*²⁹ developed a metric particularly designed for extraction processes. This green metric has been recently applied to the environmental impacts of compressed fluids extraction *vs* conventional extraction process to obtain antioxidants from rosemary³⁰. Even though this and other green metrics are easy to be applied, none of them are considered as standard and universal as LCA, since it provides quantifiable results with a holistic view on the environmental impacts. Moreover, it is based on the international standards ISO14040 and ISO14044. Therefore, in the present study, the environmental performance of both studied extraction processes was compared in terms of LCA.

Previously, Kyriakopoulou *et al.*³¹ have demonstrated that environmental impacts of obtaining carotenoids from *D. salina* are lower than using carrots. In their work, they only compared organic solvents and vegetable oil as extraction agents. Later, Espada *et al.*³² investigated the effect of supercritical CO₂ extraction on the environmental impact of carotenoid extraction. The novelty of our work relies on the improved results found for carotenoid efficiency and yield that has been optimized using Response Surface Methodology which allowed to use lower temperature with an optimal use of CO₂. Moreover, in the present work, the calculation method has been done following the ILCD method, which joins efforts towards harmonization done by the European Commission²¹.

The comparison regarding the environmental performance of supercritical CO₂ vs a classic SLE using acetone to obtain 1g of carotenoids from *D. salina* (Functional Unit: 1g of carotenoids) is shown in Figure 3. Taking into account that the aim of this LCA calculation method is the comparison of processes, products or components, the values are not relevant by themselves but rather they represent a comparison. Therefore, results are expressed in normalized values, as impact millipoints (mPt), in the Y axis. The environmental impacts in the categories included for the two analyzed extraction processes (SFE using CO₂ and SLE using acetone) are specified in the figure. As can be seen, the SFE process produces a 39% of the impacts found for SLE using the ILCD calculation method.

Figure 3. Impact assessment comparison of 1 g of *D. salina* carotenoids (FU) by SFE vs SLE. Normalized values with respect to SLE. Impact values are expressed in millipoints (mPt) to homogenize their values as stated in ILCD calculation method.

Chemical characterization of CO₂ extracts from *D. salina*

Figure 4 shows an example of an HPLC chromatogram obtained for a *D. salina* extract. Thanks to the information provided by their typical absorption spectra, twelve peaks were assigned as carotenoids. Injection of pure standards was carried out to confirm the presence of lutein, zeaxanthin, and *all-trans*- β -carotene (peaks 2, 3, and 9, respectively). By comparison with the absorption data, elution order and retention times reported in the literature^{10, 11, 33}, it was possible to identify peaks 6, 7, 8, and 11 as 13-*cis*- β -carotene, *all-trans*- α -carotene, 9-*cis*- α -carotene, and 9-*cis*- β -carotene, respectively. It was possible to identify peak 10 as 15-*cis*- β -carotene although partially co-eluted with *all-trans*- β -carotene, as previously reported¹¹. This compound has two absorption maxima at 444 and 470 nm that differ from those of *all-trans*- β -carotene (452 and 478 nm). Moreover, peak 5 was tentatively identified as cryptoxanthin. The capability of *D. salina* to produce cryptoxanthin has also been reported³⁴. Peaks 1 and 4 were not identified but, being less retained than cryptoxanthin within the C30 column, they should be polar carotenoids and might contain at least a hydroxyl group. Therefore, in the present work, these latter components have been included for the estimation of total xanthophylls instead of total carotenes. Another remarkable piece of information emerging from the HPLC analysis is the absence of peaks in the first 10 min of the chromatogram acquired at 665 nm (data not shown) which reflects the outstanding low content of chlorophylls in the CO₂ extracts.

Figure 4. HPLC chromatogram of carotenoid extract from *D. salina* acquired at 450 nm. SFE conditions: 325 bar; 30 °C. The sample was injected at a concentration of 0.1 mg/mL. Peak assignment: 2, lutein; 3, zeaxanthin; 5, cryptoxanthin; 6, 13-*cis*- β -carotene; 7, *all-trans*- α -carotene; 8, 9-*cis*- α -carotene; 9, *all-trans*- β -carotene; 10, 15-*cis*- β -carotene; 11, 9-*cis*- β -carotene; 1, 4, and 12, not identified carotenoids.

The quantitative composition of *D. salina* extracts was estimated using external standard calibrations using the available commercial compounds (Table S1, Supporting information). Lutein, zeaxanthin, and *all-trans*- β -carotene were quantified by interpolation in the calibration curves created with their respective commercial standards. Amounts of unidentified peak 1 were estimated using the lutein calibration curve, while the zeaxanthin curve was employed for cryptoxanthin and unidentified peak 4 quantifications. The rest of the compounds identified as carotenes, including unidentified peak 12, were quantified using the *all-trans*- β -carotene calibration curve, assuming a similar response factor. The quantitative results related to the identified compounds are reported in Table 3. As can be seen in the table, *all-trans*- β -carotene is the main

carotenoid of *D. salina* regardless of the extraction conditions. On the other hand, carotenoids represent only from 1.8 to 5.8% (w/w) of the total composition of the alga whereas the rest of the extract should be other relatively non-polar components such as glycerol and other lipids ³⁵.

Multivariate statistical tools, including PCA, have been used to study relationships between the chemical composition and the compressed CO₂ extraction conditions, as previously reported for yerba mate leaves ³⁶. The relationship between carotenoids levels obtained in the different extraction conditions can be seen in the PCA plot illustrated in Figure 5, which retains 73.84% of the original variance. As shown in that figure, PC1 reveals a clear separation between extraction conditions that provide CO₂ densities higher than 0.98 g/mL (on the left), from the ones that provide densities lower than 0.96 g/mL (on the right). Moreover, all carotenoids have positive loadings on PC1 which, in general terms, correlates lower CO₂ densities with a high carotenoid extraction purity. On the other hand, PC2 sorts by types of extracted carotenoids. Specifically, this PC reveals that xanthophylls and 9-*cis*-carotenes follow a quite similar extraction pattern, while *all-trans*-carotenes, 15-*cis*- and 13-*cis*-β-carotene follow a different one, being the latter group more extracted at higher temperatures (extracts obtained at 45 °C: 7, 9, 10).

467 **Table 3.** Carotenoid content determined in the SFE extracts from *D. salina* obtained at the different extraction conditions tested.

Exp.	Carotenoids (mg/100 mg of extract)									9- <i>cis</i> - β -carotene/ all- <i>trans</i> - β -carotene	9- <i>cis</i> - β -carotene/ total carotenoids
	Lutein	Zeaxanthin	Cryptoxanthin	13- <i>cis</i> - β - carotene	all- <i>trans</i> - α - carotene	9- <i>cis</i> - α - carotene	all- <i>trans</i> - β - carotene	15- <i>cis</i> - β - carotene	9- <i>cis</i> - β - carotene		
1	10.24	1.17	1.99	1.15	4.31	0.90	13.47	0.93	9.07	0.67	0.19
2	4.54	0.32	1.63	1.54	4.10	0.61	14.42	0.91	5.45	0.38	0.15
3	12.02	0.83	1.53	1.33	4.75	1.15	14.02	1.39	11.45	0.82	0.22
4	3.14	0.22	1.08	0.83	1.82	0.69	6.35	0.68	6.20	0.98	0.27
5	9.61	0.70	1.30	1.24	4.43	0.84	15.46	1.13	8.12	0.53	0.18
6	4.14	0.37	1.18	1.12	2.23	0.47	6.01	1.23	4.20	0.70	0.18
7	11.13	0.71	1.16	1.56	4.13	0.97	12.47	1.44	9.34	0.75	0.21
8	4.50	0.38	1.07	1.75	4.49	0.84	14.29	1.39	7.63	0.53	0.20
9	8.78	0.66	1.25	1.75	4.03	0.66	13.78	1.10	5.54	0.40	0.14
10	9.46	0.61	1.16	1.41	3.89	0.69	12.57	1.29	6.33	0.50	0.16
11	4.91	0.33	1.62	1.35	3.54	0.79	11.35	1.09	7.46	0.66	0.21
12	9.68	0.59	1.35	1.29	4.20	0.80	13.83	0.92	7.59	0.55	0.18
SLE	4.81	0.76	0.92	0.20	1.49	0.34	4.17	0.75	2.90	0.69	0.16

468 SLE: solid-liquid extraction

Figure 5. PCA plot with data of carotenoids levels obtained in the different extraction conditions

Modeling AChE inhibition from quantitative data

As previously mentioned, microalgal carotenoids have not been previously studied regarding their AChE inhibition potential. The twelve analyzed extracts were able to inhibit the activity of AChE enzyme (Table 1) which can be related to positive effects towards the prevention of neurodegeneration. *D. salina* exhibited promising inhibitory properties against AChE, with IC_{50} values ranging from 75 to 18 $\mu\text{g/mL}$. As results are expressed as IC_{50} value, the lowest the value, the highest the inhibition activity achieved. The activities found were also higher than the activity provided by the extract obtained using a conventional extraction procedure (SLE, Table 1).

Control results from the synthetic inhibitor galantamine hydrobromide (0.40 ± 0.01 $\mu\text{g/mL}$) were lower than *D. salina* results as expected from this specific drug. Nevertheless, synthetic inhibitors are reported to have more adverse effects ³⁷ and therefore, *D. salina* could be pointed out as a biomass of great interest to produce neuroprotective natural extracts that could be subsequently used in the functional food industry. In addition, it would be expected that *D. salina* extracts could present a double effect against Alzheimer's disease, namely, antioxidant activity and inhibition of AChE, combating two known factors of this multifactorial disease.

In terms of AChE inhibition as a single response variable, no acceptable model was achieved. This lack of fit can be justified taking into consideration the different quantitative data obtained in terms of carotenoids and the relative proportion of some of them on the different extracts (Table 3). Nevertheless, learning about the role of the different components in acetylcholinesterase activity inhibition opens a new field of study. For this reason, attempts to build a model that clearly correlates and orders carotenoids in relation to AChE inhibition were carried out. A satisfactory model relating some quantified carotenes and xanthophylls was built using PLSR type 1 (Eq. 7).

$$\text{AChE IC}_{50} = 5.38 - 1.45 \text{ lutein} + 2.39 \text{ zeaxanthin} + 6.01 \text{ cryptoxanthin} - 5.77 \text{ 15-}i\text{cis-}\beta\text{-carotene} + 4.38 (9\text{-}i\text{cis-}\beta\text{-carotene/all-}i\text{trans-}\beta\text{-carotene}) + 4.77 (9\text{-}i\text{cis-}\beta\text{-carotene/total carotenoids}) \quad (7)$$

As can be seen in Figure 6, a reasonably good prediction of AChE inhibition was achieved for all the extracts. In this plot, measured and predicted data are compared, explaining 0.792 of the variance (62.9% by cross-validation) and showing an RMSE for the prediction of 9.729. PLS regression coefficients show a relevant positive correlation of

lutein and 15-*cis*- β -carotene with AChE inhibition, as they promote lower values of IC₅₀ and a negative effect of zeaxanthin and cryptoxanthin. In addition, it can be observed that extracts obtained at higher temperatures (extracts 7, 9, 10) are the ones showing lower AChE IC₅₀ values. As observed in the PCA plot from Figure 5, these extracts contain a high amount of 15-*cis*- β -carotene and low amounts of zeaxanthin and cryptoxanthin. A special mention should be done about the ratios 9-*cis*- β -carotene/all-*trans*- β -carotene and 9-*cis*- β -carotene/total carotenoids. In this case, the proportion of all-*trans*- β -carotene or total carotenoid content exerted a positive effect in the inhibition of acetylcholinesterase enzyme when related to 9-*cis*- β -carotene. The use of ratios between different carotenoids has been considered in diverse bioactivity studies previously reported^{3, 38}. For instance, the ratio between carotenes and xanthophylls has been stated to be different in terms of anti-angiogenic activity and beauty-enhancing effects³. On the other hand, the ratio 9-*cis*- β -carotene/all-*trans*- β -carotene has shown different values of antioxidant activity due to the higher reactivity of the *cis* bond compared to the *trans* configuration³⁸. It is not surprising, therefore, that the proportions between two carotenes, or a carotene and the total amount of carotenoids present have crucial importance in determining the anticholinesterase activity of the *D. salina* extracts. Furthermore, it is quite evident the key role of the ratio 9-*cis*- β -carotene/all-*trans*- β -carotene against Alzheimer's disease, since on the one hand, high values promote antioxidant activity, whereas on the other hand, low values improve AChE inhibition.

528

529 **Figure 6.** Plot showing predicted versus experimental IC_{50} AChE values. Each point corresponds to an analyzed extract.

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CONCLUSIONS

A process based on the use of compressed carbon dioxide extraction has been optimized to obtain extracts enriched in carotenoids showing potential neuroprotective activity. The developed process takes advantage of the enhanced sustainability of compressed fluids, particularly sub- and supercritical CO₂, for the attainment of natural bioactive compounds from interesting biomasses, such as microalgae. Based on the obtained data, two mathematical models were designed; the most appropriate operating conditions to obtain the highest carotenoid yield were set at 312.6 bar and 45.0 °C (60.8 mg of carotenoids/g algae). On the other hand, the optimum conditions to maximize the extract purity were statistically predicted at 302.3 bar and 45.0°C (49.5 mg of carotenoids/100 mg extract). A comparison with a conventional solvent extraction proved that the investigated green method is by far better in terms of both efficiency and purity. Moreover, twelve compounds were recognized as carotenoids and quantified, while nine of them were identified. In addition, *D. salina* extracts exhibited promising inhibitory properties against AChE, with IC₅₀ values ranging from 75 to 18 µg/mL. These data along with the recognized antioxidant power of *D. salina* carotenoids suggest that *D. salina* extracts obtained with compressed CO₂ can be used as a natural, safe and sustainable alternative for future nutraceuticals or functional foods to prevent multifactorial neurological pathologies such as Alzheimer's disease.

Supporting Information

Electronic supporting information includes: schematic representation of the system used for compressed carbon dioxide extractions; Pareto chart and estimated response surface of extraction yield; Carotenoid calibration curve parameters obtained by HPLC-UV/Vis.

556

557 **Conflicts of interest**

558 There are no conflicts to declare.

559

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571

572 **Abbreviations**

573 ABD-F: 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole

574 AChE: acetylcholinesterase

575 ATCI: acetylthiocholine iodide

576 BBB: blood-brain barrier

577 BHT: butylated hydroxytoluene

578 DAD: diode-array detector

579 DNTB: 5,5'-dithio-bis (2-nitrobenzoic acid)

580 EV%: percentage of variance explained by the model

581 FU: functional unit
582 ILCD: international reference life cycle data system
583 LCA: life cycle assessment
584 MLR: multiple linear regression
585 MTBE: methyl tert-butyl ether
586 NQ: not quantifiable
587 n.r.: not reported
588 PLSR: partial least square regression
589 RMSE: root-mean-square error
590 RSM: response surface methodology
591 SFE: supercritical fluid extraction
592 SLE: solid-liquid extraction
593 Tris-HCl: Trizma hydrochloride
594
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717 The use of sub- and supercritical CO₂ is a green extraction procedure to increase
 718 sustainability towards the extraction of natural compounds from *Dunaliella salina*
 719 microalga with interesting neuroprotective activity.