Green compressed fluid technologies for downstream processing of *Scenedesmus obliquus* in a biorefinery approach

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Abstract

The fractionation of algae biomass in several high-value compounds that can be used as ingredients in other applications sets the basis of the algae biorefinery approach. The present study aimed at the extraction and fractionation of bioactive compounds from the microalga *Scenedesmus obliquus*, by means of applying a sequential continuous process without the manipulation of the biomass in the extraction cell. This integrated platform of compressed fluid extraction technologies of low-environmental impact was designed in order to produce increases of solvent polarity using non-toxic solvents. The process involved the following steps:(1) supercritical fluid extraction (SFE) using supercritical carbon dioxide ($ScCO_2$); (2) gas expanded liquids (GXL) using 75% ethanol and 25% $ScCO_2$ (v/v) and; (3) pressurized liquid extraction (PLE) using water. Extraction conditions were optimized using response surface methodology (RSM) and kinetic studies. Extraction yield, antioxidant activity as well as contents of total phenols, carotenoids, proteins and sugars were the studied response variables. High performance liquid chromatography coupled to evaporative light-scattering detector (HPLC-ELSD) analyses of the fractions revealed that triacylglycerols were mainly extracted by SFE. Lutein and β-carotene were the main pigments identified in the extracts by HPLC coupled to diode array and mass spectrometry detectors (HPLC-DAD-MS/MS), which were preferentially extracted in the GXL step. Polar compounds such as proteins and sugars remained predominantly in the residue. Therefore, the green downstream platform developed in this study for valorization of the microalgae biomass, is able to produce different fractions with potential application in the food, pharmaceutical and cosmetic industries.

Keywords: Carotenoid, algae, supercritical fluid extraction, pressurized liquid extraction, biorefinery, lipid.

1. Introduction

In recent years, the biorefinery concept has emerged with the aim of "developing integrated processes for the conversion of biomass into energy and a variety of products, mainly biofuels and added-value co-products, in a sustainable approach" [1]. Biorefinery implies the use of a renewable carbon source and a minimum waste generation or even the exploitation of industrial residues.

Microalgae are a promising feedstock as they are rich in a variety of valuable and bioactive compounds, such as carbohydrates, polyunsaturated lipids, proteins and pigments [2,3]. Some advantages of microalgae include that they use natural sunlight and carbon dioxide (that can be obtained from flue gas) to produce biomass, they can grow on non-arable lands (some strains grow in extreme climatological conditions) or even in wastewater treatment plants [3]. On the other hand, among the main challenges in the development of algae-based biorefinery is the continuous growth of microalgae under controlled conditions of light, temperature, nutrients supply, contamination, etc., which is a key point to maintain a homogeneous profile of the biomass. Furthermore, the economic viability of the algae biorefinery depends on the installation and operational costs of the required technologies, as well as on the products market and regulations [3,4]. Notwithstanding, microalgae are one of the most promising raw materials for a sustainable supply of commodities. Carbohydrates from microalgae can be used for bioethanol production or in the cosmetic/pharmaceutical industry, due to their bioactive properties [5], while triacylglycerides and fatty acids can be used for biodiesel production via transesterification [6]. Microalgae are also a source of

appreciated natural pigments, and some strains are currently being used for commercial production of carotenoids, for example, astaxanthin from *Haematococcus pluvialis* and β-carotene from *Dunaliella salina*. [3,6]. The interest on certain carotenoids of natural origin is caused to a great extent by their bioactive properties, as is the case of lutein, a xanthophyll present in the macula of human eye that has been related to the prevention of certain eye diseases, such as age-related macular degeneration and cataract [7-8]. The commercial production of lutein from microalgae, as an alternative to marigold flower, is currently under study [9]. *Scenedesmus obliquus* is a fresh water ubiquitous microalga rich in lutein. This species may have high potential for industrial exploitation because of its rapid growth and ease of cultivation. *S. obliquus* can alter its morphology in the presence of its natural predators, thus forming protective colonies instead of single cells [10]. The productivity of lutein and β-carotene in *S. obliquus* is favored by cultivation at a high pH, being the concentration of these carotenoids related to the antioxidant activity observed [11].

An important challenge, considering the different products that can be extracted and valorized from microalgae, is to design a suitable integrated platform able not only to efficiently extract/fractionate the target compounds but also to comply with the green chemistry principles and sustainability issues. High-pressure extractions such as supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) take advantage of the use of food-grade solvents such as CO_2, ethanol or water, have a good potential to be successfully applied to integrated processes complying with those requirements [12-15]. As an example, SFE using supercritical CO_2 ($ScCO_2$) has been reported as an interesting approach for the extraction of lipids from *Scenedesmus sp*. [16], and for the extraction of essential fatty acids from *Scenedesmus obliquus* and other microalgae [17]. Moreover, $ScCO_2$ has been employed for the extraction of lutein and

β-carotene from *Scenedesmus almeriensis* [18], although the low polarity of $ScCO_2$ caused the extraction of a low amount of lutein. The use of $ScCO_2$ with 10% ethanol as co-solvent improved the extraction of lutein from *Scenedemus sp*. two-fold compared to $ScCO_2$ without co-solvent [19]. The extraction yield of lutein was even better by increasing the amount of ethanol in the mixture up to 40 mol% [20]. When high percentages of organic solvent are used in combination with $ScCO_2$, extractions are performed in the region of gas-expanded liquids (GXLs), which possess solvent properties between those provided by SFE and PLE. GXLs could therefore be useful to increase the extraction of higher polarity components compared to $ScCO_2$ [21]. At present, some applications of GXLs in the field of microalgae extraction have already been developed, such as the use of CO_2-expanded ethanol to efficiently recover astaxanthin from *H. pluvialis* [22]. On the other hand, PLE has likewise applied to the extraction of bioactive components from microalgae, including the use of ethanol for the successful extraction of lutein (along with other carotenoids) from *Chlorella vulgaris* [23], or the employment of a mixture of ethanol/limonene for the extraction of lipids from different species [24].

Thus, the aim of this work is to propose a new integrated downstream processing of the microalga *S. obliquus,* as an approach to develop a microalgae-based biorefinery procedure based on the use of green compressed fluids. Such an approach is in line with the sequential process recently developed for *Isochrysis galbana* [25]. The developed process for *S. obliquus* relies on the use of response surface methodology (RSM) for optimization [26, 27] of each processing step, including a continuous sequential process using $ScCO_2$, CO_2-expanded ethanol, and subcritical water extraction (SWE). This development implies a reduction in the number of extraction steps, compared to the downstream platform developed for *I. galbana* [25]. Moreover, considering that *S.*

obliquus has a strong cell wall, the effect of a high-pressure treatment for cell wall disruption was studied, in order to improve the extraction efficiency. The attained fractions were chemically and functionally characterized using different chromatographic methods and in-vitro assays, respectively.

2. Materials and methods

2.1. Samples and reagents

All the HPLC-grade solvents employed were acquired from VWR (Leuven, Belgium) excepting chloroform and tetrahydrofuran (Avantor materials, Gliwice, Poland) and isooctane (Carlo Erba, Val de Reuil, France). Sea sand (0.25–0.30 mm diameter) and potassium persulfate were from Panreac (Castellar del Vallés, Spain). Butylated hydroxytoluene (BHT), formic acid (LC-MS grade), triethylamine (99.5%), sodium chloride and standards of ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), β-carotene, chlorophyll a (from *Anacystis nidulans* algae), lutein, D-methionine and Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) were obtained from Sigma-Aldrich (St Louis, MO, USA). The water used was Milli-Q water (Millipore, Billerica, MA, USA).

Freeze-dried samples of *S. obliquus* were obtained from Fitoplancton Marino S.L. (Cadiz, Spain), and stored at 4ºC until use. *S. obliquus* was grown outdoors in horizontal tubular 2000L reactors. The reactors used pure CO_2 injection to control pH in the culture by a pH controller and flowmeters. Set pH was 7.5, while natural light-dark cycles and ambient temperature were used (10-11 h of light, temperatures ranging from 10-22ºC). The reactors were inoculated with cultures grown in growth chambers with the standard conditions of Fitoplancton Marino S.L. After harvesting, cells were disrupted at high pressure (1200 bar) and freeze-dried.

150

151 **2.2. Extraction methods and instruments**

152 *2.2.1. Compressed fluids sequential continuous extraction processes*

153 Sequential extractions of *S. obliquus* at semi-pilot scale were carried out in a Spe-ed
154 Helix supercritical fluid extractor (Applied Separations, Allentown, PA, USA). This
155 equipment can be used to perform SFE, GXL and PLE operations. For each sequential
156 extraction, 10 g of freeze-dried biomass of *S. obliquus* were placed into a 300 mL
157 basket, which was inserted into the high-pressure stainless-steel (SST) extraction cell.
158 The CO_2 pneumatic pump pressurized liquid CO_2 (Carburos Metálicos, Madrid, Spain)
159 to the required set value. For the GXLs experiments, ethanol was fed at the required
160 volumetric flow rate by a liquid pump and the solvent mixture CO_2-EtOH in the feed
161 tubing was then preheated to the extraction temperature. In all experiments, CO_2 flow
162 rate of 7 L min^{-1} was used, measured using a CO_2 gas flow meter placed at the exit of
163 the extraction cell. All extracts were collected separately in plastic bottles.

164 Extractions were performed in three sequential steps, selected in increasing order of
165 polarity, using (1) supercritical CO_2 ($ScCO_2$), (2) $ScCO_2$/ethanol (GXL), and (3) pure
166 water (PLE) as solvents, respectively.

167 Step 1: SFE. A 3^2 factorial design was designed for the optimization of $ScCO_2$
168 extraction conditions, using RSM to reveal the relationship between the response
169 variables (extraction yield and total carotenoids) and the independent factors (extraction
170 pressure and temperature). Pressure was studied in the range 100-400 bar, and
171 temperature was varied in the range 40-60 ºC. Nine experiments, plus two additional
172 center points were carried out (see details in **Table 1**). A kinetic test was performed at
173 the set conditions corresponding to the center point of the experimental design (250 bar,
174 50ºC). Fractions were collected every 20 min and the percentage of extracted material

175 was calculated in order to fix the optimum extraction time of this step. The parameters
176 of the statistical model were estimated by multiple linear regression using the
177 Statgraphics Centurion XVI software (Statpoint Technologies, Warrenton, Virginia,
178 USA). The experimental design provided optimum conditions at 360 bar and 50 ºC.
179 Using these conditions, a second kinetic test was performed and the optimum extraction
180 time was finally set at 120 min.

181 **< Table 1>**

182 Step 2: GXL. This step of increased polarity with respect to the first one, was optimized
183 in two parts: firstly, a three-level and two-factors (3^2) factorial experimental design
184 using PLE was employed to select the best solvent or mixture of solvents (see *2.2.2.* for
185 an in depth description of the methods). Once selected 100% ethanol as the best solvent
186 (optimum PLE conditions according to the experimental design), the second step of the
187 sequential process was fine-tuned using carbon dioxide expanded ethanol extraction
188 (GXL). The biomass remaining after the extraction corresponding to the first step was
189 used. Pressure was set at 70 bar to minimize operational costs [25]. In addition,
190 temperature was kept at the optimum temperature used in the first step, 50ºC, in order to
191 avoid unnecessary heating or cooling of the system and thus, to minimize also
192 operational costs [25]. Three different percentages of ethanol were tested, 45%, 75%
193 and 100% and a kinetic test was performed for all of them. Optimum extraction
194 conditions were set at 75% EtOH (25% $ScCO_2$, v/v) during 150 min.

195 Step 3: PLE. The residue from the previous extractions was extracted again by PLE at
196 100 bar and 50°C for 45 min, using water as extracting solvent. As the remaining
197 compounds expected in the residue were high polarity components (mainly, sugars and
198 proteins), a low temperature was selected to avoid undesired reactions (e.g.: Maillard
199 reaction) during this extraction step.

All collected extracts were immediately covered in aluminum foil to protect them from light. Finally, the solvent (ethanol extracts) was evaporated under vacuum in a rotary evaporator (Buchi, Flawil, Switzerland), and water was removed by freeze-drying (Lyobeta, Telstar, Terrassa, Spain). SFE extracts were stored at -80ºC, while ethanol and water extracts were stored at -20ºC to prevent further reactions until analysis.

2.2.2. Pressurized liquid extraction (PLE)

Extractions of *S. obliquus* using pressurized liquid solvents at laboratory scale were performed in an accelerated solvent extraction system (ASE 200, Dionex, Sunnyvale, CA, USA). Each solvent or solvent mixtures were degassed in an ultrasound bath (to prevent oxidation during the extraction process) before they were placed in the solvent controller. Extractions were carried out in 11-mL SST extraction cells at 100 bar containing 0.5 g of microalga homogeneously mixed with 1.5 g of sea sand. Prior to each extraction, a cell heat-up step was performed for a given time, which was fixed by the system (i.e., 5 min when the extraction temperature was 50 and 100°C, and 8 min at 170°C). All extracts were covered in aluminum foil to protect them from light. The ethanol extracts were evaporated under a stream of N_2, while water was removed by freeze-drying (Lyobeta, Telstar, Terrassa, Spain).

As mentioned in section 2.2.1., a three-level factorial experimental design with two independent factors (3^2) was used. The examined factors were solvent composition (water containing 0-100% EtOH) and temperature (50-170ºC), fixing 20 min as extraction time (see **Table 2**). A quadratic model was proposed for each response variable studied: extraction yield, total phenolic content, total chlorophylls and carotenoids, and antioxidant activity. As for the optimization of SFE extraction

conditions (section 2.2.1.), multiple linear regression using the Statgraphics Centurion XVI software (Statpoint Technologies, Warrenton, Virginia, USA) was used for creation and analysis of experimental design The model adequacy was evaluated by the determination of residual standard deviation (RSD), coefficient (R^2), the and the lack-of-fit test for the model from the ANOVA table, using a confidence level of 95% ($p = 0.05$). The optimal conditions were provided by the software from the fitted model.

A 95% confidence level was also used to determine statistical differences between the experimental results obtained at optimal extraction conditions, and the predicted values. A hypothesis test, being the null hypothesis (equal results) rejected for $p < 0.05$, was applied to evaluate the accuracy between experimental and theoretical results.

< **Table 2**>

2.2.3. Benchmark extraction method

A conventional acetone extraction was performed (in triplicate) to determine the total extractable compounds in *S. obliquus*, as described elsewhere [22,25,27], as a benchmark method. Briefly, 200 mg of freeze-dried microalga were mixed with 20 mL acetone containing 0.1% (w/v) BHT for 24 h under agitation. After centrifugation, the supernatant was collected, and N_2 stream was used to remove the solvent. Dry acetone extracts were weighted and stored at −20 ºC.

2.3. Total phenols content (TPC)

TPC was estimated according to the Folin–Ciocalteu assay [28] with some modifications [29]. Briefly, 600 µL of ultrapure water were mixed with 10 µL of extract solution (typically, concentration ranging from 5 to 10 mg mL^{-1}). Then 50 µL of undiluted Folin–Ciocalteu reagent (Merck, Darmstadt, Germany) were subsequently added. After 1 min, 150 µL of 20% (w/v) Na_2CO_3 was added and the volume was

adjusted to 1 mL with water. After vortexing, the mixture was incubated for 2 h at room temperature in the darkness. Later, 300 μL of each reaction mixture were transferred to a 96-well microplate,. A microplate spectrophotometer reader Synergy HT (Bio Tek Instruments, Winooski, VT) was used to measure the absorbance at 760 nm in. A standard curve of gallic acid (0.031–2 mg mL^{-1}) was used for calibration. Data, expressed as mg gallic acid equivalents (GAE) g^{-1} extract, were presented as the average of triplicate analyses.

2.4. Trolox equivalents antioxidant capacity assay

The TEAC (Trolox equivalents antioxidant capacity) value was determined using the method described by Re et al., [30] with some modifications [25]. $ABTS^{\bullet+}$ radical was produced by the reaction of 2.45 mM potassium persulfate and 7 mM ABTS in the dark at room temperature for 16 h. The $ABTS^{\bullet+}$ solution was diluted with sodium phosphate buffer 5 mM pH 7.4 to an absorbance of 0.7 (±0.02) at 734 nm. A volume of 10 μL of sample (prepared at 5 different concentrations) and 1 mL of $ABTS^{\bullet+}$ solution were mixed in a 1,5 mL reaction tube vial and 300 μL of the mixture was transferred into a 96-well microplate. The absorbance was measured every 5 min during 45 min at 734 nm in a microplate spectrophotometer reader (Synergy HT, BioTek). Results are expressed as TEAC values (mmol trolox equivalents/g sample), since trolox was used as reference standard. The values were obtained from five different concentrations of each sample tested in the assay, giving a linear response between 20 and 80% of inhibition of the radical activity. All analyses were performed in triplicate.

2.5. Carotenoids and chlorophylls determination.

2.5.1. Total carotenoids and chlorophylls determination.

A simple spectrophotometric method was used to determine the total carotenoid and total chlorophylls concentration, based on their characteristic absorbance, as described elsewhere [25]. SFE and PLE extracts were dissolved in methanol (concentrations ranging from 5 to 0.05 mg mL^{-1}). Absorbance of these solutions was recorded at two specific wavelengths, 470 nm for carotenoids and 665 nm for chlorophylls. External standard calibration curves of lutein (0.5 – 10 µg mL^{-1}) and chlorophyll a (0.5 – 7.5 µg mL^{-1}) were used to calculate the total carotenoid and chlorophyll content, respectively. Total carotenoids were expressed as mg carotenoids.g^{-1} extract and, total chlorophylls were expressed as mg chlorophyll.g^{-1} extract.

2.5.2. Chemical characterization of carotenoids and chlorophylls by HPLC-DAD-APCI-MS/MS

The profile of carotenoids and chlorophylls of *S. obliquus* extracts was determined by HPLC using a diode-array detector (DAD) connected in series to an ion trap mass spectrometer, according to the method previously described [25,31]. HPLC-DAD analyses of the extracts were performed in an Agilent 1100 series liquid chromatograph (Santa Clara, CA, USA), using a YMC-C_{30} reversed-phase column (250 mm × 4.6 mm i.d., 5 µm particle size; YMC Europe, Schermbeck, Germany) and a pre-column YMC-C_{30} (10 mm × 4 mm i.d., 5 µm). The mobile phases were mixtures of methanol–MTBE–water (90:7:3 v/v/v) (solvent A) and methanol–MTBE (10:90 v/v) (solvent B), respectively. Pigments were eluted from de column according to the following gradient: 0 min, 0 % B; 20 min, 30 % B; 35 min, 50 % B; 45 min, 80 % B; 50 min, 100% B; 60 min, 100% B; 62 min, 0% B. The injection volume was 10 µL, while the flow rate was 0.8 mL min^{-1}. Absorbance was recorded at 280, 450 and 660 nm, although spectra from 240 to 770 nm were collected using the DAD (peak width > 0.1 min (2 s), slit 4 nm).

LC ChemStation 3D Software Rev. B.04.03 (Agilent Technologies, Santa Clara, CA, USA) was used to control the instrument. Extracts were dissolved in pure phase A at an appropriate concentration (1 - 10 mg mL^{-1}) prior to HPLC analysis.

Six different concentrations of lutein in ethanol, ranging from 0.1 to 100 µg mL^{-1}, were analyzed at least by duplicate using the LC-DAD-MS instrument for the calibration curve.

The liquid chromatograph equipped with DAD detector was directly coupled to an Agilent ion trap 6320 ion trap mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) via an atmospheric pressure chemical ionization (APCI) interface. The APCI ion source was operated under positive ionization mode using the following parameters: capillary voltage, −3.5 kV; vaporizer temperature, 400°C; drying temperature, 350°C; drying gas flow rate, 5 L min^{-1}; nebulizer gas pressure, 60 psi corona current (which sets the discharge amperage for the APCI source), 4,000 nA. Full scan spectrum was acquired in the range from *m/z* 150-1,300. Automatic MS/MS analyses were also performed, fragmenting the two highest precursor ions (10,000 counts threshold; 1V Fragmentor amplitude).

2.6. Analysis of the lipid fraction (SFE and GXL extracts)

2.6.1. Total lipids analysis.

Total lipid extraction was performed following the method of Axelsson and Gentili [32], as previously described [27]. Briefly, 25 mg of sample (extracts) or initial biomass as reference (freeze-dried algae) were weighted in a 15-mL centrifuge tube. Then, 8 mL of chloroform/methanol 2:1 (v/v) was added and mixed with the sample, followed by the addition of 2 mL of NaCl 0.73% (w/v) and mixing again. Subsequently, two phases were separated in a centrifuge (Rotina 380R, Hettich, Tuttlingen, Germany) operating at

350 × *g* at room temperature during 5 min. The lower layer was collected with a glass pipette and transferred to a pre-weighed glass vial, while the upper layer was discarded. The solvent was removed using a stream of N_2 and the vial was weighted again. The total lipids were calculated by a gravimetrical determination and the results were expressed as percentage (%w/w).

2.6.2. Chemical characterization of lipid class compositions by HPLC-Evaporative Light Scattering Detection (ELSD).

Separation of lipid classes was done using the method described by Castro-Gómez et al., [33], with minor modifications [27]. The analysis was performed using an Agilent 1290 Infinity II liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a DAD detector and coupled with an Agilent 1260 evaporative light scattering detector (ELSD), which uses nitrogen as the nebulizing gas at a flow rate of 2 SLM (Standard liter per minute). Evaporator and nebulizer temperatures were set at 90°C and 50°C, respectively; led intensity was set at 50% and the gain was set at 1. Two Zorbax Rx-SIL columns were used in series (250 mm × 4.5 mm i.d. with 5-µm particle diameter each; Agilent Technologies, Santa Clara, CA, USA) and a precolumn (12.5 mm × 4.5 mm i.d.) with the same C18 packing. Before analysis, samples were dissolved in CH_2Cl_2 (5 mg mL^{-1}) and 20 µL were injected. The column temperature was set at 40°C, the autosampler temperature was kept at 8ºC. Solvent mixtures and gradient were used as described by Castro-Gómez et al. [33]. In order to identify the chromatographic peaks corresponding to pigments co-extracted with lipids, absorbance signals were collected by the DAD at 450 and 640 nm to detect the presence of carotenoids and chlorophylls.

2.7. Protein analysis of GXL and PLE extracts

Dumas method as described by Gilbert-López et al. [25] using a FlashEA 1112 nitrogen analyzer (Thermo Fisher Scientific, Waltham, MA, USA) was used for protein analysis. A calibration curve of D-methionine (1-20 mg) was used. To calculate total protein from total nitrogen, a N-to-protein conversion factor of 5.08 was used, according to the determination of the aminoacid composition of *S. obliquus* following the method of Meussen et al. [34]. Analyses were done in duplicate.

2.8. Sugar composition analysis of GXL and PLE extracts

The hydrolysis of algae extracts was performed as described previously by Gilbert-López et al. [25]. Also, the determination of neutral sugar composition was performed following the method reported by [25]. The sugar composition was determined by high performance anion exchange chromatography (HPAEC) using an ICS-3000 Ion Chromatography HPLC system equipped with a CarboPac PA-1 column (250x2 mm) in combination with a CarboPac PA guard column (25x2 mm) and a pulsed electrochemical detector in pulsed amperometric detection mode (Dionex, Sunnyvale, USA) at 20 °C. The column was equilibrated with H_2O, and a flow rate of 0.25 mL min^{-1} was used. Elution was performed as follows: 0-35 min H_2O, 35-50 min 0-40% 1 M sodium acetate in 100 mM NaOH, 50-55 min 1 M sodium acetate in 100 mM NaOH, 55-60 min 150 mM NaOH, 70-85 min H_2O. Addition of 0.5 M sodium hydroxide (0.15 mL min^{-1}) allowed monomers detection. Before analysis, samples were diluted (1:3) in water and 10 µL 0.1% (w/v) bromophenol blue in ethanol was added to 1 mL sample. Solid barium carbonate was added until a clear magenta color was obtained to adjust the pH. Subsequently the solution was filtrated using a 0.45 µm PTFE filter. Deoxy galactose was used as internal standard in the sample. Analysis was done in duplicate.

375

376 ## 3. Results and discussion

377 ### 3.1. Optimization of unit operations

378 *3.1.1. Optimization of SFE of S. obliquus*

379 Carbon dioxide is a GRAS (generally recognized as safe) solvent, and thus SFE using
380 $ScCO_2$ is considered a green process for the extraction of non-polar compounds from
381 natural sources, such as algae [35]. Therefore, the objective of the first step of the
382 sequential process was maximizing the extraction of the less polar fraction of *S.*
383 *obliquus* biomass [25]. Supercritical CO_2 extraction conditions were optimized using a
384 3^2 factorial design of experiments comprising 9 different experiments plus 2 additional
385 replicates in the center point (**Table 1**). Extraction time was selected after performing a
386 kinetic test at both central conditions of the experimental design (250 bar, 50ºC) and
387 optimum conditions (360 bar, 50ºC) measuring the percentage of extractable material *vs*
388 extraction time (data not shown). An extraction time of 120 min was selected as the
389 most appropriate since after that time the amount of extracted material did not
390 significantly increase. **Table 1** shows the experimental design employed, together with
391 results of the different response variables measured, namely extraction yield, total
392 carotenoids and total chlorophylls contents. Extraction yields ranged from 0.41 to
393 1.15% while carotenoids content reached values as high as 48.39 mg g^{-1} extract at a
394 pressure of 400 bar and low extraction temperature (40°C).

395

396 **Table 1**. Experimental design results corresponding to the SFE of *Scenedesmus obliquus*.

Experiment #	Temperature, ºC	Pressure, bar	Extraction yield, %	Total carotenoids, mg g^{-1} extract	Total chlorophylls, mg g^{-1} extract
S01	40	100	0.41	2.32	0.26
S02	50	100	0.50	7.39	1.91
S03	60	100	0.63	2.62	0.55
S04	40	250	0.68	8.78	3.62

S05-07 [a]	50	250	0.93 ± 0.05	41.46 ± 4.87	15.68 ± 0.26
S08	60	250	1.11	38.62	9.93
S09	40	400	1.15	48.39	14.47
S10	50	400	0.97	35.85	11.03
S11	60	400	0.80	32.48	6.63

[a] Average values of center points of experimental design (n=3).

An ANOVA was performed for each of the responses (data not shown), and the statistical model was fitted and optimized. A desirability function to maximize all the response variables was selected giving to all responses the same importance (the weight factor and the impact were set at 1.0 and 3.0, respectively). The resulting surface response plot is shown in **Figure 1 a)**. This function provided an optimum of 362.9 bar and 47.6ºC to maximize the extraction yield, carotenoids and chlorophyll content. The desirability was equal to 0.872, while the values predicted by the model at the optimum extraction conditions were 1.00% for extraction yield, a carotenoids content of 43.72 mg carotenoids g^{-1} extract and 14.62 mg chlorophylls g^{-1} extract for total chlorophylls. Experiments at the optimum conditions (rounded to 360 bar and 50ºC) provided experimental values close to the predicted values by the statistical model (data not shown), and thus they were selected as the first step of the sequential process under development.

3.1.2. Optimization of pressurized liquid extraction of S. obliquus

PLE was investigated for the subsequent steps of the sequential process, in order to develop a downstream platform for the fractionation of *S. obliquus*, starting from the less polar compounds and ending with the extraction of the most polar ones. A 3^2 factorial experimental design was employed for PLE optimization. The experimental factors were %EtOH in the solvent mixture (0, 50, 100%) and extraction temperature (50, 110, 170 ºC). An in-depth characterization of the obtained extracts was performed, covering compounds from a wide-array of polarities, including carotenoids, phenolic

421 compounds, sugars and proteins. Experimental results of all studied variables are
422 detailed in **Table 2**. After performing the ANOVA for each of the responses (data not
423 shown), the statistical model was fitted and optimized. Extraction yield was positively
424 influenced by the temperature and the mixture water:ethanol (50:50) yielded higher
425 amounts of extract than the pure solvents (see **Table 2**). Therefore, the model proposed
426 170ºC and 43% EtOH as the optimum conditions to maximize the extraction yield. On
427 the other hand, the response variables total phenols content, antioxidant activity
428 (TEAC), total carotenoids and total chlorophylls, were mainly influenced by the
429 composition of the solvent, obtaining better results in pure ethanol extracts at low
430 temperature (**Table 2**). Sugar and protein contents, as expected, were favored by the
431 increase of water in the solvent mixture. However, while protein content increased with
432 the temperature, sugar content decreased.

433 **Table 2**. Experimental design for PLE of *Scenedesmus obliquus*.

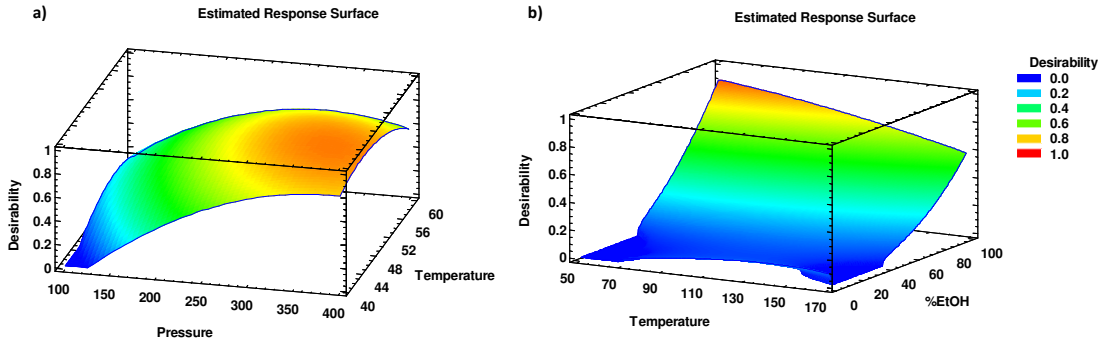
Experiment #	Temperature, ºC	%EtOH[a]	Extraction yield, %	TPC, mg GAE g^{-1} extract	TEAC, mmol Trolox eq g^{-1} extract	Total carotenoids, mg g^{-1} extract	Total chlorophylls, mg g^{-1} extract	Total protein, %	Total sugars, %
P01	50	0	6.31	6.89	0.11	3.63	6.25	35.74	14.59
P02	50	50	4.86	15.57	0.36	10.62	21.45	20.50	18.75
P03	50	100	4.83	59.25	0.94	75.48	124.10	7.50	8.46
P04	110	0	4.83	10.74	0.19	3.18	5.27	36.53	17.12
P05-08[b]	110	50	32.12 ± 3.50	15.45 ± 1.21	0.36 ± 0.02	14.60 ± 1.16	22.42 ± 1.14	37.02 ± 2.49	13.56 ± 0.47
P09	110	100	14.85	49.41	1.60	50.61	113.69	8.65	10.90
P10	170	0	42.52	31.94	0.71	0.45	0.66	51.84	14.62
P11	170	50	78.04	19.95	0.33	7.73	13.71	48.37	9.50
P12	170	100	23.76	36.64	0.80	35.63	84.22	17.37	8.44
Optimized desirability for PLE: 0.903. Optimal conditions: 50 ºC and 100% EtOH									
Predicted	50	100	-	60.20	1.18	70.14	123.03	-	-
Optimum	50	100	5.76 ± 0.26	62.45 ± 1.68	1.07 ± 0.12	72.66 ± 9.60	118.58 ± 16.47	7.50 ± 0.81	9.40[d]
PW[c]	50	0	6.68 ± 0.79	-	-	-	-	21.32 ± 0.34	21.69 ± 0.37

434 [a] Proportion of ethanol in ethanol/water mixtures; [b] Average values of center points of experimental design (n=4); [c] PLE with water after PLE
435 at optimum conditions (50 ºC, 100%EtOH); [d] Not enough amount of sample to do replicate analysis.
436

437

Multiple response optimization was performed to maximize all studied variables except sugar and proteins, considering all of them equally important (the weight factor and the impact were set at 1.0 and 3.0, respectively); since sugar and protein contents followed a completely different trend than antioxidant activity, they were not taken into account for the optimization. The optimum conditions proposed by the model were 160.4ºC and 100% EtOH, with an overall desirability value of 0.547. This result was a consequence of the different behavior of the extraction yield compared to the rest of responses. In this regard it has to be considered that solubility increases with the temperatureas well as that polar compounds, such as proteins and sugars, are better extracted using more polar solvents, while phenolic compounds and carotenoids (which are related to the antioxidant activity) are preferentially extracted using 100% ethanol. Therefore, the extraction yield is increased when using a mixture of water and ethanol because the extraction of compounds of different polarities is favored. However, when using the pure solvents, selectivity towards a group of analytes is achieved. In order to improve the desirability function (and thus the prediction of the mathematical model) related to the antioxidant activity, the extraction yield was excluded and the other selected response variables were maximized. As a result, the adjusted model proposed optimum extraction conditions at 50ºC and 100% EtOH, with an overall desirability value of 0.903. These results are in agreement with those previously observed for *P. tricornutum* microalgae [27]. Surface response plot of the adjusted model is depicted in **Figure 1b)**. Predicted values by the model at the optimum extraction conditions were 60.20 mg GAE g^{-1} extract for TPC, 1.18 mmol Trolox eq g^{-1} extract for TEAC, 70.14 mg g^{-1} extract for total carotenoids and 123.03 mg g^{-1} extract total chlorophylls. Triplicate extractions at the optimum conditions (100% EtOH and 50 ºC) provided experimental values not statistically different ($p = 0.05$) to those predicted by the model (**Table 2**).

463 Therefore, these optimum conditions were tested as second step of the sequential
464 process.
465 Finally, PLE using water was performed with the residue of PLE extraction using 100%
466 EtOH at 50ºC to confirm the possibility of extracting sugars and proteins. The analysis
467 of these water extracts (PW), shown in **Table 2**, revealed that around 20% of sugars and
468 20% proteins could be extracted from the residue. These residues were obtained after
469 PLE extraction of antioxidant phenols and pigments using 100% EtOH.
470



471

472 **Figure 1.** Surface response plot for a) SFE and b) PLE experimental designs.

473

474 **3.2. Sequential continuous extraction process of *S. obliquus* (Steps 1-3)**

475 From the results discussed above, a three-step sequential continuous extraction
476 procedure was proposed: 1) SFE using $ScCO_2$, 2) GXL using EtOH/$ScCO_2$ and 3) PLE
477 using water.
478 PLE using 100% EtOH and 50ºC was evaluated as a second step at semi-pilot scale, but
479 even working with a 45 min long extraction time, that is 2.25-fold the extraction time
480 evaluated at laboratory scale, the yield only increased from 6.7% (20 min, lab-scale) to
481 8.4% (45 min, semi-pilot scale). The use of several cycles to improve the yield was also
482 evaluated, although as the number of cycles increased, the consumption of ethanol

483 increased too. The yield was 14.84 g of extract/ 100 g algae after 3 static cycles, which
484 consumed 1 L of ethanol.
485 In order to reduce the usage rate of EtOH, gas-expanded liquid conditions were also
486 explored, since GXLs allow working at lower pressures than those of SFE and using
487 smaller volumes of solvents compared to PLE. This approach has been already
488 successfully applied to the extraction of astaxanthin from *H. pluvialis* [22] and recently
489 it has been integrated in a sequential process for the fractionation of *I. galbana* [25].
490 Thus, a kinetic test was proposed in the second step of the downstream platform, as
491 performed in the SFE step (see section *3.1.1.*), using $ScCO_2$/EtOH mixtures. Two
492 different percentages of ethanol in CO_2, 45% and 75% levels were tested to study the
493 possible advantages offered by this intermediate process. A pressure of 70 bar was
494 selected, which is lower than the CO_2 critical pressure (73.8 bar). Temperature was
495 fixed at the optimum value of the first step (50°C) in order to minimize energy
496 consumption due to heating or cooling of the system. Using 150 min of extraction time,
497 a yield of 15.5% was obtained using GXL with 75% ethanol, which is similar to the
498 14.8% obtained by PLE (3x45 min). However, the second approach consumed an
499 average of 457 mL of EtOH, which is significantly lower than the solvent consumed by
500 PLE (1 L). Therefore, GXL with 75% EtOH at 50ºC during 150 min were the selected
501 conditions for the second step of the sequential process.
502 Step 3 was performed under PLE conditions using water, which implies an increasing
503 order of polarity. At this point, different bioactive polar compounds were expected such
504 as proteins and carbohydrates. Moreover, the final objective was to extract all the
505 valuable components contained in the microalgae biomass attaining different fractions
506 and minimizing the leftovers [25]. The extraction values selected included a pressure of
507 100 bar and a temperature of 50°C during 45 min. These values were maintained

relatively low in order to avoid degradation of compounds and undesired reactions occurring at higher temperatures (e.g.: Maillard reaction).

The scheme of the overall extraction process is depicted in **Figure 2**.

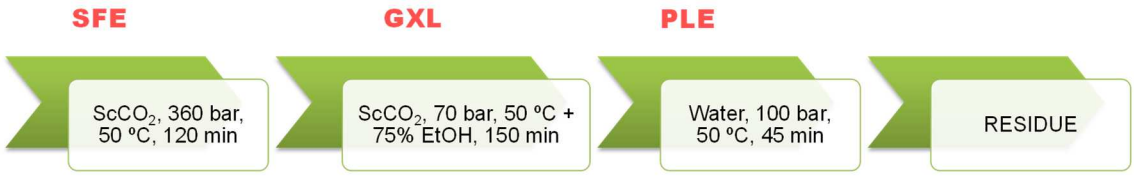


Figure 2. Downstream process proposed for *Scenedesmus obliquus*.

3.3. Chemical characterization of the extracts obtained in the Steps 1-3.

3.3.1. Chemical characterization of carotenoids and chlorophylls by HPLC-MS/MS.

Extracts produced in the 3 steps of the sequential extraction procedure were analyzed by HPLC-DAD-MS/MS to collect information about their particular carotenoids and chlorophylls composition. A tentative identification of different carotenoids was obtained combining the information provided by the two detectors (DAD and MS) and data found in literature. Confirmation of the identity of the main peaks was carried out by using commercial standards. Detailed information about characteristic UV–vis spectra, protonated ions and the main fragments obtained by MS for the different pigments detected is given in **Table 3.** Chromatographic profiles are shown in **Figure 3**.

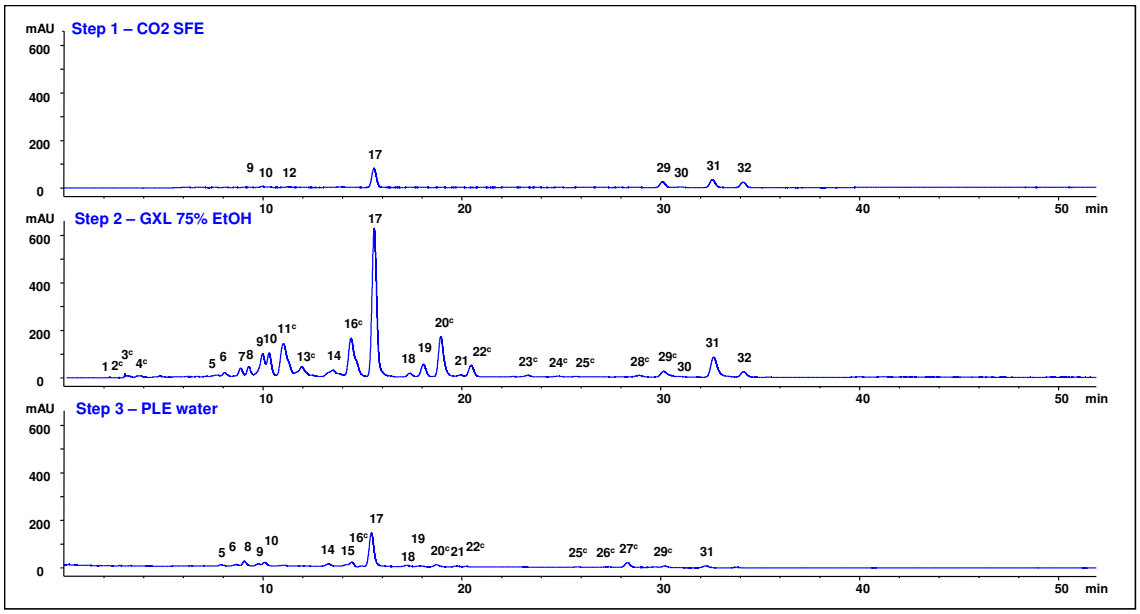


Figure 3. HPLC-DAD profile (λ=450 nm) of pigments extracted from *Scenedesmus obliquus* in the different steps of the sequential process. Chlorophylls are marked by a "c" superscript, while the other chromatographic peaks correspond to carotenoids (see **Table 3**).

$ScCO_2$ extracted mainly carotenoids from *S. obliquus*. The most important peaks in the chromatogram, could be tentatively assigned due to their UV and MS/MS spectra as lutein (peak 17, t_R=15.6 min) and β-carotene (peak 31, t_R=32.7 min) [36]. Protonated ions of both carotenoids were identified (*m/z* 569.4 $[M+H]^+$ for lutein and *m/z* 537.9 $[M+H]^+$ for β-carotene), as well as some fragment ions of lutein produced by the sequential loss of water molecules (e.g. *m/z* 551.6 $[M+H-H_2O]^+$, *m/z* 533.6 $[M+H-2H_2O]^+$). Finally, the identification of lutein and β-carotene was confirmed by using commercial standards. Other minor carotenoids such as violaxanthin (peak 9, t_R=10.3 min) and luteoxanthin (peak 12, t_R=11.3 min) were tentatively identified. Both compounds presented the protonated $[M+H]^+$ ion at *m/z* 601.5 as well as a fragment ion resulting from the loss of a water molecule (*m/z* 583.5 $[M+H-H_2O]^+$). MS/MS analysis of violaxanthin also showed a fragment corresponding to the loss of a second water molecule (*m/z* 565.6, $[M+H-2H_2O]^+$) and a fragment corresponding to the loss of toluene (92 Da) from the dehydrated molecule (*m/z* 491.5, $[M+H-18-92]^+$) [37].

Luteoxanthin showed the loss of toluene directly from the protonated molecule (*m/z* 509.4 $[M+H-92]^+$) [36,38]. Besides carotenoids, pheophytin a (peak 29, t_R=30.1 min) was also tentatively identified in the extract in agreement with its $[M+H]^+$ ion (m/z 872) and its characteristic MS/MS fragment at *m/z* 594.0, corresponding to the loss of the phytyl group $[M+H-C_{20}H_{39}]^+$ [25,39].

Table 3. Pigments detected in *Scenedesmus obliquus* sequential extracts. (see Figure 3 for chromatograms)

Peak	Extract	Identification	t_R, min	λmax, nm	$[M+H]^+$, m/z	Fragments
1	GXL	Not identified	2.30	450, 687	-	-
2	GXL	Chlorophyll-type	3.07	467, 602, 653	577.8	503.5, 313.3, 265.2
3	GXL	Chlorophyll-type	3.82	426, 577, 615, 663	675.7	658.6, 397.3, 317.3, 261.1
4	GXL	Chlorophyll-type	4.84	431, 610, 661	592.4	573.5, 499.4, 351.3
5	GXL, PLE	Chlorophyll-type	7.68	432, 455, 657	585.6	567.9, 494.1
6	GXL, PLE	cis-Carotenoid	8.08	327, 408, 430, 456	603.1	583.5, 569.5
7	GXL	Neoxanthin	8.88	416, 439, 469	601.8	583.5, 565.5, 491.4
8	GXL, PLE	Coeluting carotenoids	9.29	422, 446, 472	583.7	565.4, 549.5, 491.4
9	SFE, GXL, PLE	Carotenoid	9.99	417, 440, 466	601.7	583.6, 565.5, 509.7, 491.4
10	SFE, GXL, PLE	Violaxanthin	10.30	413, 436, 464	601.5	583.5, 565.6, 491.5
11	GXL	Hydroxychlorophyll b	11.03	464, 599, 648	924.6	645.7, 614.6, 595.5, 539.5, 525.1
12	SFE	Luteoxanthin	11.26	399, 423, 446	601.5	583.5, 509.4
13	GXL	Chlorophyll-type	11.95	455, 588, 635	968.6	921.5, 809.7, 689.2, 643.3, 616.2
14	GXL, PLE	cis-Carotenoid	13.52	330, 417sh, 439, 464	569.8	551.4 , 533.5, 515.2, 495.4
15	PLE	cis-Carotenoid	14.22	331, 437, 463	551.5	533.4, 505.1, 495.4, 266.8
16	GXL, PLE	Hydroxychlorophyll a	14.43	430, 616, 663	910.7	892.5, 631.8, 614.0
17	SFE, GXL, PLE	Lutein	15.59	420, 443, 471	569.4	551.6, 533.6, 515.6, 429.6
18	GXL,	Zeaxanthin	17.38	425, 448, 474	569.9	551.5, 515.5

	PLE					
19	GXL, PLE	Carotenoid	18.06	415, 438, 467	569.5	551.8, 533.6, 515.3
20	GXL, PLE	Chlorophyll a	18.94	430, 617, 664	893.9	615.9, 583.3, 555.4
21	GXL, PLE	Carotenoid	19.93	419, 438, 468	553.0	533.4, 495.4
22	GXL, PLE	Chlorophyll a'	20.47	431, 617, 664	894.1	615.7
23	GXL	Chlorophyll-type	23.32	467, 650	731.5	569.3
24	GXL	Pheophytin a O-allomer	24.83	405, 502, 532, 610, 666	888.0	609.7, 591.4, 550.5
25	GXL, PLE	Chlorophyll-type	25.72	434, 666	-	-
26	PLE	Chlorophyll-type	27.29	400, 661	903.7	625.7
27	PLE	Pheophythin b	28.32	435, 600, 653	885.7	858.0, 607.8
28	GXL	Chlorophyll-type	28.89	427, 670, 703	731.5	675.4, 569.3, 527.3
29	SFE, GXL, PLE	Pheophytin a	30.15	409, 505, 535, 608, 666	872.0	683.9, 594.0, 533.5
30	SFE, GXL	Pheophytin a'	30.99	408, 506, 536, 607, 666	-	-
31	SFE, GXL, PLE	β-Carotene	32.66	276, 451, 477	537.9	-
32	SFE, GXL	Carotenoid	34.15	445, 472	-	-

In the second step (GXL extraction), lutein and β-carotene were again the main compounds present in the extracts. Violaxanthin, together with other minor carotenoids previously described in S. *obliquus* [40], was also found in GXL extracts and at higher concentration (higher area of the chromatographic peak) than in the SFE extract. Peak 7 (t_R=8.9 min) was tentatively identified as neoxanthin, taking into account the retention time and elution order in a chromatographic method with similar mobile phases [37] and its MS/MS pattern, which is similar to the fragmentation obtained for its isomer violaxanthin. Peak 9 (t_R=10.0 min) corresponded to an isomer of violaxanthin and neoxanthin, but it could not be clearly assigned. Peak 6 (t_R=8.1 min) presented an absorption maximum at 327 nm, indicative of a *cis*- isomer [36], although no conclusive identification could be attained. Peak 8 (t_R=9.3 min) showed a distorted UV-Vis spectrum similar to the characteristic spectrum of carotenoids, which can be indicative

566 of a coelution of several carotenoids. Zeaxanthin (peak 18, t_R=17.4 min) was identified
567 by its protonated ion at *m/z* 569.9, as well as by the presence of some fragment ions
568 produced by the sequential loss of water molecules (e.g. *m/z* 551.6 $[M+H-H_2O]^+$);
569 ultimately, it was confirmed by a commercial standard.
570 Several minor peaks in the chromatogram presented the characteristic absorbance
571 spectrum of chlorophylls, and therefore, those that could not be identified were assigned
572 as chlorophyll-type (**Table 3**). Chlorophyll a (peak 20, t_R=18.9 min) and its epimer
573 chlorophyll a' (peak 22, t_R=20.5 min, *m/z* 894, $[M+H]^+$) showed the loss of the phythyl
574 group ($C_{20}H_{39}$, *m/z* 278) [25, 41] and showed the same fragment ion at *m/z* 615.7, which
575 corresponds to the chlorophyllides a and a', respectively. The identification of
576 chlorophyll a in the extract was confirmed using a commercial standard, and,
577 consequently, peak 22 was assigned to chlorophyll a' [25]. The same elution order was
578 considered for pheophytins a and a' (peaks 29 and 30). Pheophytin a O-allomer was
579 tentatively identified by its protonated ion (*m/z* 888.0) and the loss of the phythyl group
580 in MS/MS analysis to obtain a fragment at *m/z* 609.7 [42]. Peak 16 (t_R=14.4 min) was
581 assigned as hydroxychlorophyll a by its UV-Vis spectrum and its protonated ion (*m/z*
582 910.7) [43]. In the same way, peak 11 (t_R=11.0 min) and peak 27 (t_R=28.3 min) were
583 tentatively identified as hydroxychlorophyll b and pheophytin b, respectively [43].
584 Despite that these two chlorophyll b derivatives present in GXL and PLE extracts,
585 respectively, chlorophyll b was not detected in any of the sequential extracts.
586
587 *3.3.2. Quantification of lutein in the different extracts obtained (Step 1-3).*
588 Lutein, the most intense peak (number 17) in the chromatograms at 450 nm (**Figure 3**),
589 was quantified in all the extracts. Quantification of lutein was performed by using an

590 external calibration curve made-up of different dilutions of a commercial standard (as
591 mentioned in section *2.5.2.*).
592 In general, the concentration of lutein in the second extraction step is higher compared
593 to the first step, although the amount of chlorophylls (marked with a superscript in
594 **Figure 3**) is also higher. **Table 4** shows the quantification of lutein obtained after each
595 step of the sequential integrated process. The highest content of lutein was found in the
596 GXL extract: 2.17 ± 0.10 mg g^{-1} d.w., which corresponds to the 64% of the amount of
597 lutein obtained with acetone conventional solid-liquid extraction (3.36 ± 0.09 mg g^{-1}
598 d.w.) from a high-pressure homogenized sample of *S. obliquus* (taken as a reference),
599 and higher than the concentration of lutein obtained with conventional acetone
600 extraction of the same undisrupted biomass (0.90 ± 0.01 mg g^{-1} d.w.). These results
601 demonstrate the potential of GXL for the extraction of carotenoids, considering that the
602 concentration of lutein was 10-fold higher than the concentration of lutein extracted
603 using SFE (0.02 mg g^{-1} d.w.). In a previous work reported by Macías-Sanchez et al., the
604 concentration of lutein attainable from *S. almeriensis* by using $scCO_2$ was 0.047 mg g^{-1}
605 d.w., even increasing the extraction time up to 300 min. This is only 2% of the amount
606 of lutein obtained by their reference extraction method (2.33 mg g^{-1} d.w.) [18].
607
608 *3.3.3. Lipids profile of SFE and GXL extracts (Steps 1 and 2).*
609 A chromatographic method was employed for the analysis of the sequential extracts that
610 allowed the separation of lipid classes, and also a further separation of polar lipids (e.g.
611 phospholipids) in the same run [33]. Chromatograms obtained for steps 1 and 2 are
612 shown in **Figure 4**, where differences between lipid profiles of each extraction step are
613 clearly evident. Lipids elute by an increasing order of polarity, so triacylglycerides
614 (TAG) eluted in the first part of the analysis, up to 22 min. Medium polar lipids as

mono- (MAG) and diacylglycerides (DAG) eluted between 20 and 25 min, together with free fatty acids (FFA) and pigments (carotenoids, chlorophylls and their derivatives); finally, polar lipids eluted from 25 min onwards [25]. In the first step of the sequential process, corresponding to $ScCO_2$ extraction, TAGs were mainly extracted, while polar lipids are not detected at all, as was previously observed for the microalgae *I. galbana* [25]. In the second step (GXL), medium polar compounds and polar lipids were extracted. Lipid extraction was not performed in the last extract of the sequential process, as they were not expected to be present in water extracts.

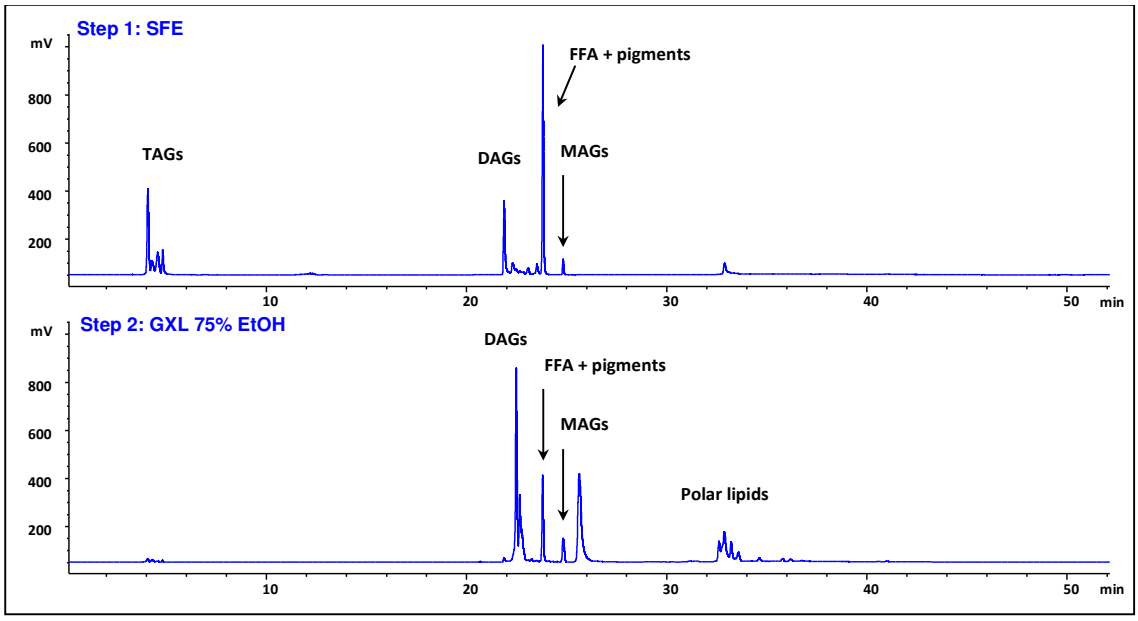


Figure 4. HPLC-ELSD lipid profiles of the SFE and GXL extraction steps of the downstream processing of *S. obliquus*. DAGs is diacylglycerides, MAGs is monoacylglycerides and FFA is free fatty acids.

3.3.4. Protein and sugar content and antioxidant activity of GXL and PLE extracts (Steps 2 and 3).

Total protein of the GXL and PLE extracts (step 2 and 3) was analyzed by the Dumas method, according to [25]. The amount of protein found in GXL extracts was 9.7 ± 1.4

634 mg g^{-1} d.w., while 5.6 ± 1.9 mg g^{-1} d.w. was the amount of protein in PLE extracts.
635 These values represent, respectively, 1.96% and 1.14% of the total protein present in the
636 initial biomass (**Table 4**). Therefore, the proposed method is not efficient extracting
637 proteins, which remain in the residue after extraction. Thus, this residue can be
638 considered an enriched alga source of proteins.
639 Sugar composition of the extracts is shown in Figure S1 (Electronic Supplementary
640 Material). Galactose and glucose were, by this order, the main components of the
641 polysaccharides extracted in both GXL and PLE extracts. The ratio galactose/ glucose
642 (in %mol) in GXL extracts was around 7-fold higher than in PLE extracts (10.9 vs 1.6).
643 The concentration of sugars extracted was 7.5 ± 0.3 mg g^{-1} d.w. in GXL extracts, while
644 it was 5.2 ± 2.2 mg g^{-1} d.w. in PLE extracts (**Table 4**). A different composition of
645 sugars in ethanol and water extracts can be expected [25]. Since ethanol is commonly
646 used to precipitate polymeric sugars, monomers or oligomers may be preferably present
647 in ethanol extracts, while oligomeric and polymeric sugars can be expected in water
648 extracts. In any case, the amount of sugars extracted from the biomass is very low,
649 3.97% and 2.75% for GXL and PLE extracts, respectively. Sugars, thus, mainly remain
650 in the residue.
651 Results corresponding to the antioxidant capacity assay are also shown in **Table 4**. As
652 can be seen, ethanol extracts of *S. obliquus* showed an antioxidant activity 6-fold higher
653 than that of water extracts. This observation can be related to the content of lipids and
654 carotenoids present in GXL extracts. For instance, the ratio between the concentration
655 of lutein in ethanol extracts and its concentration in the subsequent water extracts is 16.
656 A similar observation was reported for *I. galbana*, where the ethanol extracts enriched
657 in the xanthophyll fucoxanthin showed higher antioxidant activity than the subsequent
658 water extracts [25].

Table 4. Comparison among sequential extracts from HPH-biomass and non-homogenized biomass. Recoveries are in between parenthesis.

Extract	Yield%	Lipids%	Lutein[a], mg g^{-1} d.w.	Protein%	Sugars%	TEAC, mmol g^{-1} extract
Reference values	-	*13.83*	*3.36*	*49.39*	*18.83*	-
Freeze-dried algae (cells not disrupted)						
S (SFE)	1.09 ± 0.07	1.07 [b] (**7.75%**)	0.02[b] (**0.62%**)	-	-	-
S (GXL 75%)	9.53 ± 1.46	9.17 ± 1.44 (**66.31%**)	2.17 ± 0.10 (**64.70%**)	0.97 ± 0.14 (**1.96%**)	0.75 ± 0.03 (**3.97%**)	0.639 ± 0.055
S (PLE Water)	4.40 ± 1.15	-	0.13 ± 0.03 (**3.98%**)	0.56 ± 0.19 (**1.14%**)	0.52 ± 0.22 (**2.75%**)	0.100 ± 0.008
Total	15.02%	10.24 (**74.06%**)	2.32 (**68.99%**)	1.53 (**3.10%**)	1.27 (**6.72%**)	
High-pressure homogenized (cell disruption) and freeze-dried algae						
S* (SFE)	4.19 ± 0.71	4.04 ± 0.36 (**29.21%**)	0.16 ± 0.06 (**4.66%**)	-	-	-
S* (GXL 75%)	13.35 ± 1.65	9.97 ± 0.53 (**72.07%**)	3.36 ± 0.11 (**100.52%**)	1.89 ± 0.21 (**3.83%**)	1.05 ± 0.37 (**5.60%**)	0.464 ± 0.037
S* (PLE Water)	10.04 ± 0.49	-	0.045 ± 0.009 (**1.33%**)	1.98 ± 0.12 (**4.01%**)	1.67 ± 0.32 (**8.88%**)	0.236 ± 0.010
Total	27.58%	14.01 (**101.27%**)	3.56 (**106.50%**)	3.87 (**7.84%**)	2.72 (**14.48%**)	

[a] Recoveries calculated using acetone maceration results as reference for total lutein ; [b] Not enough amount of sample to do replicate analysis.
* HPH biomass

3.4. Evaluation of high-pressure homogenization (HPH) for enhanced recovery of bioactives from *S. obliquus*.

As can be observed in **Table 4**, extraction yield was particularly low in the first step, corresponding to SFE. Therefore, in order to improve the extraction efficiency, not only in the first step, but in all the steps of the sequential process, high-pressure homogenization (HPH) was evaluated for cell disruption, as it was previously reported as an efficient treatment for lipid extraction of *Scenedesmus sp*. [44]. Microalgae were exposed to high pressure (*ca*. 1200 bar) after harvesting, and then quickly depressurized, causing cell disruption. The highest improvement in the extraction yield was obtained in the first step of the sequential process (SFE), where it is 4-fold increased (**Table 4**). This resulted in an increment in the extraction of lipids.

Recoveries of different compounds of interest were calculated, defined as the percentage of compound extracted from the initial dry biomass. Total lipids, total sugars and total protein reference values were calculated from the initial biomass using the same methods as for the extracts (detailed in section 2); acetone conventional solid-liquid extraction was used as reference value for total lutein [22,25]. Recoveries obtained from homogenized (HPH) biomass improved in all cases compared to those obtained from the non-HPH biomass except for lutein in the PLE water fraction (**Table 4**). This means that lutein is extracted more efficiently in the previous steps, SFE and GXL, when HPH biomass is used. Moreover, the better yield obtained in all steps of the downstream platform led to the recovery of 100% lutein present in the initial biomass. Without HPH treatment of the biomass, 68.99% of lutein was extracted. The remaining by-product, as mentioned before in section *3.3.4.*, is composed mainly of protein and sugars (see Figure S2, Electronic Supplementary Material). In future work, this residue

686 can be further processed to separate proteins from sugars, in a more complete
687 biorefinery process.
688
689 **Conclusion**
690 In the present work, an integrated downstream processing of the freshwater microalga *S.*
691 *obliquus,* based on the use of green compressed fluids, is proposed as an approach to
692 develop a microalgae-based biorefinery procedure. The developed process comprises
693 the sequential continuous extraction with $ScCO_2$, CO_2-expanded ethanol (GXL), and
694 subcritical water extraction, without the manipulation of the biomass in the extraction
695 cell. Thus, the raw material of extraction in second and third steps was the residue of the
696 previous extraction step. As observed recently for the marine microalga *I. galbana* [25],
697 the extraction process was selective according to the polarity of the solvent/mixture of
698 solvents used. $ScCO_2$ extracts were rich in triacylglycerides and showed less carotenoid
699 and chlorophyll content than GXL extracts. The main identified pigments were lutein
700 and β-carotene, of which the first one is more valuable. Lutein was mainly extracted by
701 GXL. PLE using water contained mainly proteins and sugars, although the recovery of
702 these nutrients from the biomass was very low and therefore, these compounds
703 preferentially remained in the residue. Further studies should be carried out to determine
704 more in depth the composition of the obtained extracts and their relationships with the
705 antioxidant activity. Finally, high-pressure homogenization revealed as a useful tool for
706 cell wall disruption, helping to recover completely non polar compounds such as lutein
707 from the biomass. Further studies should be made to achieve a better fractionation of the
708 polar residue, thus separating proteins from sugars.
709
710 **Supporting information**

711 Sugar composition of the extracts in %mol (Fig S1); Recoveries of total lipids, lutein,
712 total sugars and total proteins from the microalga *Scenedesmus obliquus*. Comparison
713 between freeze-dried biomass and high-pressure homogenized biomass (Fig S2).
714
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