Title: Efficient extraction of hydrophilic and lipophilic antioxidants from microalgae with supramolecular solvents

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ABSTRACT

There is strong evidence that lipophilic and hydrophilic antioxidants interact *in vivo* through different mechanisms and that it is their mixed effects what leads to health benefits. Simultaneous extraction of both types of antioxidants from biomass using organic solvents is inefficient because extraction yields for these phytochemicals are highly dependent on the polarity of the solvent. In this work, we firstly propose the use of supramolecular solvents (SUPRAS) as an innovative approach for the simultaneous extraction of a wide polarity range of antioxidants. Different SUPRAS compositions were investigated for extraction of the total content of carotenoids and polyphenols, as representatives of lipophilic and hydrophilic antioxidants, respectively. Under optimal conditions, antioxidants were extracted from freeze-dried biomass of *Scenedesmus sp.* in an excellent rate related to their total content (up to 1 mg carotenoids/g dw and 10 mg of gallic acid equivalents, GAE/g dw). Screening based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) revealed that lutein was the major carotenoid and caffeic acid the main phenolic compound. The SUPRAS extracts showed high antioxidant activity (25.04 ± 0.73 µM ascorbic acid equivalents antioxidant capacity, AAAC/g dw and 17.02 ± 0.45 µM AAAE/g dw for ABTS and DPPH assays, respectively). SUPRAS offered advantages in terms of non-toxicity, rapidity (5 min extraction) and cost-effectiveness (room temperature, atmospheric pressure) for the recovery of antioxidant compounds from algal biomass and constitute a good alternative to the use of conventional solvents.

Keywords: supramolecular solvent (SUPRAS); microalgae; carotenoids; polyphenols; antioxidant

1. Introduction

Oxidative stress arises from the imbalance between free radical production in cells and antioxidants acting in the human defence system (i.e. enzymes, chain breaking antioxidants and transition metal binding proteins) [1]. Oxidative stress results in damage to lipids, proteins and nucleic acids, and it is now considered a main contributor to certain cancers, atherosclerosis, and inflammatory and ageing related diseases [2].

Chain breaking antioxidants are low molecular weight compounds with high reducing power that directly scavenge free radicals [1]. They are classified in two broad groups; water-soluble (hydrophilic) antioxidants, which act in the cell cytoplasm and the blood plasma, and lipid-soluble (lipophilic) antioxidants that protect cell membranes from lipid peroxidation [3]. Humans synthesize only a few chain breaking antioxidants and most of them are obtained from diet. Among dietary antioxidants, carotenoids and phenolics represent the most abundant lipid- and water-soluble antioxidants, respectively.

There is a broad consensus that antioxidants interact *in vivo* and consequently is their combination or mixture effects what leads to health benefits [4-7]. Several mechanisms have been reported for such interactions (e.g. recycling of a specific antioxidant by another one after scavenging of the free radical, use of different pathways for radical inhibition, etc.) [8], and many positive synergistic effects related to the antioxidant capacity of mixtures of hydrophilic and lipophilic compounds have been described [9,10]. So, in order to fulfil the growing demand for natural antioxidant-rich food and nutraceuticals, extraction methods for phytochemicals from biomass should be able to simultaneously extract both hydrophilic and lipophilic antioxidants in a cost-effective and eco-friendly way.

Carotenoids and phenolics, as representative of lipophilic and hydrophilic antioxidants, are usually extracted from fruits and vegetables using different organic solvents (e.g. acetone or methanol for carotenoids and methanol-water for phenolics), through procedures that require long extraction times (up to 12 h) and high energy consumption (e.g. evaporation of 60 mL of solvent per gram of sample) [11]. As a general rule, the simultaneous extraction of both types of antioxidants with organic solvents is intrinsically inefficient because the content of carotenoids and phenolics in solvent extracts will strongly depend on the polarity of the solvent. Goiris et al. (2012) reported the extraction of the phenolics and carotenoids from 32 microalgae using ethanol-water for polar antioxidants and a three-step sequential extraction with hexane, ethyl acetate and hot water for apolar compounds [12]. Both the antioxidant content and

antioxidant capacity of solvent extracts were highly dependent on the nature of the solvent. As expected, some carotenoids such as monoester and diester astaxanthin were only significantly extracted in hexane [12]. Another illustrative study isolated hydrophilic and lipophilic antioxidants from 10 *salvia* species using three-step consecutive extraction with supercritical carbon dioxide (SFE-CO_2), pressurized ethanol and pressurized water [13]. The antioxidant potential of the residues after each extraction was comparatively high, particularly after SFE-CO_2, this suggesting that considerable amount of antioxidants remained in the plant material after extraction [13]. The use of auxiliary energies such as microwaves can help to improve extraction yields for both lipophilic and hydrophilic antioxidants but optimal operation conditions are quite different for both type of antioxidants [14].

SUPRAS are nanostructured liquids spontaneously formed by adding a coacervation-inducing agent to colloidal suspensions of amphiphiles [15]. The coacervation-induction agent (e.g. organic or inorganic salts, pH changes, addition of a poor solvent for the amphiphile, etc.) provokes the growth of the amphiphilic aggregates in the colloidal suspension, which finally separate as a new denser or lighter liquid phase [16]. The capability of SUPRAS to simultaneously extract compounds in a wide polarity range arises from the differential polarity regions available in the supramolecular aggregates making them up (i.e. differentiated hydrophobic and polar regions are provided by the hydrocarbon chains and head groups of the amphiphiles, respectively) [16]. The ability of SUPRAS for efficient extraction derives from the high concentration of amphiphiles they contain (0.1-1 mg μL^{-1}), and consequently the high number of binding sites available, and from the possibility of establishing different types of interactions (e.g. dispersion, dipole-dipole, hydrogen bonding, etc.) that can work in a cooperative manner [17]. Finally, the capability of SUPRAS for quick extraction emanates from their discontinuous character (i.e. they are formed by coacervate droplets), and consequently their high surface area, which facilitates solute mass transfer from sample to SUPRAS [15].

In addition to extraction properties, there are significant operational characteristics of SUPRAS that make them suitable for extraction of antioxidants. Thus, SUPRAS are obtained from energyless and eco-friendly processes compatible with green chemistry principles. On the other hand, they can be synthesized from bioamphiphiles, which makes SUPRAS extracts directly compatible with applications as nutraceuticals or cosmeceuticals [18]. A significant property of SUPRAS is that they can be tailored to preserve antioxidants [19]. SUPRAS have been already proved to efficiently extract alkaloids and polyphenols from vegetal biomass such as coffee by-products [20,21].

Microalgae, which constitute a sustainable and renewable feedstock for carotenoids and phenolic compounds, were selected as a model for vegetal biomass [12,22,23]. Among microalgae, *Scenedesmus sp*. was selected in this study because it has been reported to contain both carotenoids and phenolics [24,25] and other valuable compounds like lipids and carbohydrates [26]. This species has also proved its application as potential feedstock for biodiesel production and wastewater treatment [27,28]. The microalgae was obtained from standard conditions, no attempt was made to increase its phytochemical content by cultivation under stress conditions [29,30].

The aim of this article was to propose supramolecular solvents (SUPRAS) as extractants for the simultaneous isolation of hydrophilic and lipophilic antioxidants from microalgae. We hypothesize that SUPRAS have the potential to extract efficiently and quickly both types of antioxidants on the basis of their valuable intrinsic properties. SUPRAS composition and extraction parameters were optimized for maximum yield of total carotenoids and phenolic content. Major bioactive compounds of each class were identified by characteristic transitions in LC-MS/MS. Finally, the antioxidant activity of SUPRAS extracts was evaluated.

2. Material and methods

2.1. Chemicals

The following reagents were of analytical grade and used as received. Potassium persulfate, octanoic acid , 2,2 diphenyl-1-picrylhydrazyl (DPPH), 2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Folin-Ciocalteu reagent, sodium carbonate, aluminium chloride and standards of phenolic compounds (gallic acid, (+)-catechin hydrate) and carotenoids (astaxanthin, lutein, canthaxanthin, β-carotene) were purchased from Sigma–Aldrich (Steinheim, Germany). HPLC grade methanol, ethanol, acetonitrile, acetone, acetic acid, and hydrochloric acid were from Panreac (Barcelona, Spain). Ascorbic acid was obtained from Fluka (Germany). All the reagents and standard solutions were prepared using methanol or Milli-Q deionized water (Millipore, Bedford, MA, USA).

2.2. Microalgae biomass

Lyophilized biomass of *Scenedesmus sp*. was utilized. This strain was isolated from samples of natural algal biofilms owing to its resistance and growth potential comparing to other algae

158 species cultivated in Waris-H culture medium [31]. The species was then grown in fertilizer
159 concentrate medium (Sportsmaster WSF Spring & Summer) prepared as indicated in (Appendix
160 A) where significant growth rate of 12.2 ± 2.5 g m^{-2} d^{-1} was reached. Biomass was harvested
161 during the exponential growth phase and finally freeze-dried. The dry biomass was ground into
162 powder and stored in dark containers in dry place until use.
163
164 2.3. SUPRAS synthesis

165 SUPRAS of different compositions were produced by dissolving octanoic acid in ethanol and
166 then adding water (pH ~3) as the coacervation-inducing agent. SUPRAS spontaneously formed
167 in the mixture and separated as a top layer from the bulk solution (named as EqS, equilibrium
168 solution). The volume of the three-component synthetic solution was 50 mL, the octanoic acid
169 concentration was 5% v/v, and ethanol and water varied in the ranges 9.5-36 % v/v and 59-
170 85.5% v/v, respectively. Mixtures were vortex shaken (Vortexer, Heathrow Scientific, Vernon
171 Hills, IL, USA) for 30 s at 500 rpm and then centrifuged (Mixtasel BLT, Selecta, Cham,
172 Switzerland) for 5 min at 3,500 rpm. SUPRAS and EqS phases were separately collected and
173 stored in closed containers at room temperature until use (~25 °C, within one week).
174
175 2.4. Optimization of the extraction of antioxidants with SUPRAS

176 Extractions were done in 2 mL-microtubes Safe-Lock (Eppendorf Ibérica, Madrid, Spain) by
177 mixing the lyophilized biomass and different volumes of the SUPRAS (and corresponding EqS)
178 prepared according to the previous section. Variables were optimized by varying each factor at
179 a time and experiments were done in triplicate (results were calculated as mean ± standard
180 deviation, SD). Conditions giving the maximum yield for the total content of polyphenols and
181 carotenoids were selected as optimal. Samples were extracted by glass bead vortexing (3mm
182 glass beads, 2500 rpm, 5 min). Thereafter, mixtures were centrifuged at 10,000 rpm until
183 complete separation of the microalgae residue from the EqS (if added) and SUPRAS occurred.
184 SUPRAS extracts were collected and further diluted with methanol (if needed) before analysis.
185 The full process was conducted under dim light.

186 Optimization of the experimental conditions for extraction of antioxidants from *Scenedesmus*
187 *sp*. involved the study of three variables; (1) *SUPRAS composition,* which was carried out by
188 extracting the microalgae with SUPRAS synthesized in different ethanol:water ratios (from 9.5
189 to 36 % of ethanol) according to the procedure specified in section 2.3; (2) *SUPRAS:EqS ratio,*

which was studied in the range 30% to 100% of SUPRAS; the SUPRAS and EqS used as extractant and wetting phases, respectively; and (3) *total volume of SUPRAS* (from 0.25 to 5 mL). Total content of carotenoids and phenolics in *Scenedesmus sp*. was also calculated by extracting it with acetone and methanol, respectively, according to conventional procedures [10] but with higher organic solvent/sample ratio (100 mL per g of sample), and the values obtained were taken as reference for calculation of SUPRAS extraction yields.

2.5. Total carotenoid content and identification of major constituents

SUPRAS extracts were diluted ten times with methanol and centrifuged at 10,000 rpm to remove possible solids and obtain clear solutions. For total carotenoid content, the absorbance of the methanolic solution of the SUPRAS extract was measured at 665.2, 652.4, and 470.0 nm (Lambda 25 UV/VIS spectrophotometer, PerkinElmer Inc., Norwalk, CT, USA), which are the major absorption peaks of chlorophylls a, b and carotenoids respectively. Concentrations were calculated according to the formulas [32]:

- Chlorophyll a: Chla (mg/L) = 16.72 $A_{665.2}$ - 9.16 $A_{652.4}$
- Chlorophyll b: Chlb (mg/L) = 34.09 $A_{652.4}$ - 15.28 $A_{665.2}$
- Carotenoids: Caro (mg/L) = (1000 $A_{470.0}$ – 1.63 Chla – 104.9 Chlb) /221

LC-MS/MS(APCI+) analysis was performed for the identification of the major carotenoid constituents in the supramolecular extract. In order to simplify the identification procedure, esterified carotenoids were transformed in free carotenoids by saponification as previously described [33]. Briefly, supramolecular extracts were diluted five times with methanol, then 1 mL aliquot was mixed with 200 µL of fresh 50 mM NaOH solution prepared in methanol. The mixture was left for 3 h at room temperature in the dark for complete hydrolysis of ester bonds. Identification of carotenoids was carried out with an Agilent 1200 series reverse phase HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with an on-line degasser, a binary pump, a high performance auto-sampler and ACE 3 C18-PFP column (3 mm × 150 mm, 3 µm), connected to an Agilent Technologies 6420 Triple Quadrupole mass spectrometer operated in a positive APCI mode. The following settings were used: gas drying temperature 350 °C; drying gas flow rate 4 L min^{-1}; vaporizer temperature 400 °C; capillary voltage 4000 V; corona current 4 µA; nebulizer gas pressure 30 psi. A target list of common algae carotenoids with reported MS/MS transitions (see Table 1, Appendix B) [34,35] was recorded for tentative identification. Final solutions were filtered (0.22 µm Nylon tube filter, Corning, NY, USA) before injection

(10 μL). LC was operated at 300 μL min^{-1} flow rate and with a gradient previously reported [36]. Data were recorded and treated using Agilent MassHunter Software® (version B.07.00). Standard solutions of asthaxanthin, lutein, canthaxanthin and β-carotene prepared in acetonitrile were injected for confirmation (retention time and MS/MS spectra match) and quantitation (external calibration 0.05-1 mg L^{-1}).

2.6. Total polyphenol content and identification of major constituents

The total phenolic yield in supramolecular extracts was determined by the colorimetric Folin-Ciocalteu method as previously reported [37]. First, an aliquot (100 μL) of crude supramolecular extract was oxidized with 0.1 N Folin-Ciocalteu reagent (250 μL); next, the mixture was neutralized with 0.5 mL of 20% (w/v) sodium carbonate solution and incubated in the dark at room temperature. After 90 min, the absorbance of the resulting blue colour was measured at 725 nm using the aforementioned spectrophotometer. Gallic acid was used as standard with distinct concentrations ranging from 0.008 to 1 mg mL^{-1}; the calibration curve (Abs_{725}=1.4188 TP, R^2= 0.996) served to quantify total polyphenols. Results were expressed as milligram of gallic acid equivalent (GAE) per gram dw.

The characterisation of phenolic compounds was performed by LC-MS/MS(ESI-) analysis using the aforementioned chromatograph and column. The triple quadrupole mass spectrometer was set as follows: electrospray ionization (ESI) source operating in negative mode, nebulizer 30 psi; dry gas 12 L/min; temperature 350 °C; and capillary voltage 4000 V. The separation was achieved by a mixture of two solvents: water/acetic acid (99/1, v/v) (A) and acetonitrile/methanol (50/50, v/v) (B). The mobile phase flow rate was 1.0 mL/min and the gradient was started with 5% of solvent B, increasing to 30% in 25 min, 40% in 10 min, 48% in 5 min, 70% in 10 min, 100% in 5 min, isocratic at 100 % for 5 min, resuming to 5% in 10 min and finishing the run in 12 min. A target list of polyphenols with reported MS/MS transitions was made for tentative identification only [38,39].

2.8. Antioxidant activity of SUPRAS extracts

The radical-scavenging capacities of supramolecular extracts were assessed via the ABTS radical cation ($ABTS^{\bullet+}$) and the DPPH radical ($DPPH^{\bullet}$) assays. The whole procedure was done under dim light. The $ABTS^{\bullet+}$ inhibition assay was performed according to Guedes et al. [40] with minor modifications and referred to ascorbic acid equivalent antioxidant capacity (AAAC). Briefly, a 7 mM stock solution of the free radical $ABTS^{\bullet+}$ was prepared by mixing

1/1 (v/v) ABTS solution (7 mM) and persulfate solution (2.45 mM) in methanol. The mixture was first let to react for 16 h in the dark at room temperature, then, diluted with methanol to give an absorbance of 0.7±0.05 at 734 nm. Aliquots of supramolecular extracts (100 µL) were added entirely or diluted with methanol (to give concentrations of 1, 2, 4, 6, 8 and 10 mg supramolecular extract $mL^{-1)}$ to 1.9 mL of diluted ABTS solution and the absorbance was measured after incubation overnight at room temperature. Methanol diluted SUPRAS were considered as blanks. The scavenging effect of ABTS radical was calculated using the following equation:

$$\text{Inhibition effect (IE; \%)} = [(\text{A control} - \text{A sample}) / \text{A control}] \times 100$$

where *A sample* is the absorbance of the solution of ABTS+SUPRAS extract and *A control* is the absorbance of the diluted ABTS solution. The antioxidant content of supramolecular extracts was expressed as µM AAAC per gram of dry biomass (µM AAAC.g_{DB}^{-1}) and obtained from a calibration curve (IE = 289.95 AA+ 5.1552; R^2 = 0.9846) made from ascorbic acid in methanol (12.5 – 200 µM).

The DPPH radical scavenging assay was conducted according to Assunção et al. [22] with minor modifications. Supramolecular extracts were diluted with methanol (concentration tested: 1, 2, 4, 6, 8 and 10 mg mL^{-1}) and then further 100 µL of each solution were mixed with 1.9 mL of the DPPH solution (0.06 mM in methanol). Mixtures were incubated overnight at room temperature in the dark. The absorbance was recorded at 517 nm against a blank corresponding to diluted SUPRAS prepared in the same way as the samples. Fresh ascorbic acid solutions were prepared in the same concentration range as indicated above and analysed in the same conditions as the samples (Scavenging percentage SP (%) = 427.62 AA- 15.497; R^2 = 0.996). The SP was calculated by the formula:

$$\text{Scavenging percentage (\%)} = [(A_0 - A_I) / A_0] \times 100$$

Where A_I is the absorbance of methanolic DPPH solution mixed with the supramolecular extract or the ascorbic acid standards and A_0 is the absorbance of the methanolic DPPH solution at 0.06 mM. All determinations were conducted in triplicate and results were calculated as mean ± SD.

3. Results and discussion

3.1. Optimization of SUPRAS extraction of antioxidants from *Scenedesmus sp.*

Supramolecular solvents were synthesized from octanoic acid, ethanol and water [41]. Octanoic acid forms a colloidal suspension of inverted micelles in ethanol and separates as a new liquid phase (SUPRAS) under addition of water, which is a poor solvent for octanoic acid. These type of SUPRAS have been described as highly packed inverted hexagonal phases with the carboxylic groups surrounding internal aqueous pools and the hydrocarbon chains dispersed in the organic solvent and arranged in outer layers (see Figure 1A) [42]. The SUPRAS is immiscible with a hydro-organic equilibrium solution (EqS) that contains the amphiphile at a low critical aggregation concentration. The EqS is often used for extraction in combination with the SUPRAS phase with the aim of wetting the sample.

SUPRAS components were selected on the basis of their suitability for further industrial applications (food authorized ingredients) and potential to simultaneously maximize hydrogen bonding, polar and dispersion interactions as main binding forces expected to drive the extraction of carotenoids and polyphenols. Additionally, octanoic acid, with a saturated hydrocarbon chain, has been previously proved to protect carotenoids against lipid peroxidation [19].

Both the composition and size of the aqueous cavities of octanoic acid-based SUPRAS can be tailored as a function of the environment for their formation (i.e. the proportion of ethanol in the synthetic solution). Thus, as it is shown in Figure 1B (right), SUPRAS with progressively lower concentration of octanoic acid and higher concentration of water and ethanol will be obtained as the proportion of ethanol in the synthetic solution increases (the ethanol percentages tested correspond to those in which SUPRAS is formed). On the other hand, the size of the SUPRAS aqueous cavities increases as the percentage of ethanol in the synthetic solution does (Figure 1B, left) and these vacuoles can size-exclude polar macromolecules (e.g. polysaccharides) [42]. In addition, proteins are expected to remain in the microalgae residue due to the formation of macromolecular complexes with octanoic acid.

Optimization of the experimental conditions for extraction of antioxidants from *Scenedesmus sp*. was carried out by studying the influence of SUPRAS composition and SUPRAS:EqS ratio and volume on the recovery of carotenoids and polyphenols (see section 2.4). In all the

experiments the amount of microalgae kept constant (i.e. 10 mg of dry weight corresponding to around 100 mg of wet weight). The content of carotenoids and polyphenols, calculated by extraction with acetone and methanol respectively [10], were 1.38 ± 0.05 mg of carotenoids/g dw and 8.6 ± 0.9 mg of gallic acid equivalents (GAE)/g dw. These values were taken as reference for calculation of extraction yields using SUPRAS as the solvent.

Figure 2A and B show the influence of SUPRAS composition on the extraction yield for polyphenols (expressed as GAE) and carotenoids, at a SUPRAS:EqS ratio of 30:70 (i.e. 0.3 mL of SUPRAS and 0.7 mL of wetting EqS). These figures clearly show that the amount of polyphenols and carotenoids extracted by SUPRAS decreased and increased, respectively, as the percentage of ethanol in the synthetic solution increased. Under these experimental conditions, the maximum content of carotenoids and polyphenols extracted by SUPRAS was 0.58 ± 0.06 mg of carotenoids/g dw and 10.68 ± 1.1 mg GAE/g dw. This means that SUPRAS was able to improve the yield for polyphenols obtained with methanol, however, the yield for carotenoids was quite low (only around 42% of the content extracted by acetone).

The different behaviour of polyphenols and carotenoids as a function of SUPRAS composition could be a consequence of both the relative proportion of octanoic acid-ethanol-water in the SUPRAS (see Figure 1B, right) and the specific environment where they are expected to be solubilized. Thus, the length of the hydrocarbon chain of carotenoids such as lutein (i.e. the most frequently carotenoid found in *Scenedesmus* [43]) closely resembles the length of the hydrocarbon chain of two octanoic acid molecules that form the SUPRAS inner hydrophobic layers. The Lutein molecule ends contains also hydroxyl groups that can interact with the carboxylic groups of octanoic acid oriented towards the water vacuoles of SUPRAS (see schematic in Figure 1). Because of the relatively large size of polar groups in lutein, it is probable that SUPRAS with the highest vacuoles (i.e. those synthesized from the highest ethanol concentration in the synthetic solution, see Figure 1B, left) give the best environment for lutein solubilisation, this providing the highest extraction yields (Figure 2B).

On the contrary, polyphenols are expected to be solubilized near the polar groups of the amphiphile in order to establish hydrogen bonds, polar and dispersion interactions (see schematic in Figure 1A). So, SUPRAS containing a high number of amphiphiles (e.g. those synthesized from low ethanol content, Figure 1B, right) are expected to give the highest extraction efficiencies for these compounds (see Figure 2A).

Considering that SUPRAS synthesized from 36% ethanol were able to extract more than 90% of the polyphenols solubilized in acetone, and that they gave the best yields for carotenoid extraction, they were selected as a compromise for further optimization experiments. The composition of the resulting SUPRAS phase from this synthesis mixture was as follows: 45:32:23 for amphiphile:ethanol:water v/v/v.

The influence of the SUPRAS:EqS ratio on the extraction yields obtained for polyphenols and carotenoids was investigated from 30 to 100% SUPRAS in order to know if the EqS was a suitable wetting solution. The results showed in figure 2 C and D clearly show that SUPRAS was a better extractant for both types of antioxidants than the mixture SUPRAS:EqS and that it allowed to solubilize up to 10.3 ± 0.3 mg GAE/g dw and 1.04 ± 0.07 mg carotenoids/g dw. These yields kept constant as the SUPRAS volume decreased up to about 0.5 mL for extraction of 10 mg of dw microalga. Lower volumes caused a decrease in recoveries of both carotenoids as a result of incomplete sample dispersion.

The SUPRAS technology shows great promise for scaling up because of its simplicity, no need of auxiliary energies, high temperature or time-consuming consecutive extractions and evaporation steps. For industrial application, we should keep the same sample to SUPRAS ratio and we should scale up the process with suitable technology for mixing and for phase separation at high volumes/amounts. It is also worth mentioning that taking into account the high water content in the SUPRAS synthesis mixture (up to 59% v/v) these solvents have a great potential for the direct extraction of wet samples without compromising the extraction yields [20], which could be advantageous to simplify the procedure.

3.2. Total carotenoid content and identification of main constituents.

Under optimal experimental conditions (room temperature, vortexing for 5 min), SUPRAS extraction provided maximum yields of total carotenoids of 1.07 ± 0.07 mg/g dw of *Scenedesmus sp*. This value was in line with those previously reported for *Scenedesmus obliquus* (0.44±0.06 mg/g dw) [43] and *Scenedesmus sp*. (1.11 mg/g dw g^{-1}) [44] cultivated under standard culture conditions. When stress conditions for the enhancement of carotenoids production were applied (e.g. light, nutrient starvation), the carotenoid content increased (i.e. up to 0.69 % dw for *Scenedesmus almeriensis* [45], and between 0.61-2.08 % dw [46] and 34.2 ± 3.8 mg/g dw [29] for *Scenedesmus sp*.), which has fostered the use of different strains of this microalgae for commercial use.

Analysis of SUPRAS extracts by LC-MS/MS revealed lutein as the main carotenoid (1.1±0.1 mg/g) followed by two less abundant peaks, asthaxanthin (6.6 ± 2.0 μg/g) and an unidentified carotenoid (see Table 1). These results are in accordance with the total carotenoid content found for SUPRAS extracts and with previous observations of Aburai et al. [29], who revealed that *Scenedesmus* algal cells accumulated astaxanthin, lutein, and canthaxanthin as the main xanthophylls under stress light conditions. Other authors also reported lutein as the main carotenoid in *Scenedesmus obliquus* [40], *Scenedesmus protuberans* [47] and *Scenedesmus obliquus* CNW-N [25]. Lutein content under favoured cultivation conditions varied from 2.17 ± 0.10 mg/g dw to 2.58 ± 0.08 mg/g dw in *Scenedesmus almeriensis* depending on the extraction technique, specie and growing conditions used [45].

3.3. Total phenolic content and identification of main constituents.

Under optimal conditions the total phenolic (TP) content of SUPRAS extracts reached a value of 10.3 ± 0.3 mg GAE/g dw. These yields are comparatively close to those reported for *Scenedesmus rubescens* (TP = 10.24 ± 1.06 mg GAE/g dw and 11.34 ± 0.88 mg GAE/g dw for the intracellular water and ethyl acetate extracts, respectively) [48]. Lower TP values have been reported for *Scenedesmus obliquus* (1.94 ± 0.16 mg GAE/g dw) in ethanol:water extracts [12], and five *Scenedesmus* strains (from 0.7 to 3.5 mg GAE/g dw) in methanol:water extracts [30]. The phenolic content of *Scenedesmus quadricauda* was increased by cultivation under irradiation with UV-light (up to 22 mg GAE/g dw) [49], that indicating that *Scenedemus* can be a valuable source for both carotenoids and polyphenols.

SUPRAS extracts were further analysed by LC-ESI(-)-MS/MS. The following phenolic compounds were tentatively identified based on the MRM transition data reported in the literature: simple phenols (phloroglucinol), simple phenolic acids (caffeic and sinapic acids and a compound that could be either p-hydroxybenzoic acid or salicylic acid (since they share the same MS transitions), a phenolic aldehyde (vanillin), a phenolic diterpene (carnosic acid) and a isoflavone (fomononetin). The most intense peak was assigned to caffeic acid (relative abundance 35%) (see Table 1).

Previous studies suggest that phenolic profiles in microalgae strongly depend on the microalgae species, culture conditions and solvent used for the extraction. Among the few reports related to the study of microalgae polyphenols profiling, López et al. [50] reported ten phenolic compounds in the methanolic extracts of *Dunaliella tertiolecta*, among which gentisic acid, catechin, epicatechin and chlorogenic acid were predominant. Zakaria et al. [51] found that

caffeic acid was the abundant phenolic acid in subcritical water extracts of *Chlorella sp.* In another study performed on *Arthrospira platensis*, Da Silva et al. [52] discerned catechin, vanillic, gallic and syringic acids as the major phenolics in the high pressure/temperature extracts. Kovácik et al. [49] detected nine benzoic acid derivatives (gallic–salicylic acid) and three cinnamic acid derivatives (caffeic–p-coumaric acid) in methanolic extracts of *Scenedesmus quadricauda* exposed to UV-A and UV-C illumination.

3.4. Antioxidant properties of SUPRAS extracts

The profile screening of SUPRAS extracts showed the presence of a variety of antioxidant molecules ranging from polar or moderate polar as phenols to strongly lipophilic compounds as carotenoids. These compounds have been previously demonstrated to be closely related to the radical scavenging ability of microalgal biomass [12,52]. In this study, both ABTS and DPPH, classified as mixed mode electron and hydrogen atom transfer (ET/HAT) assays [53], were used to assess the potential of supramolecular extracts to scavenge free radicals. Results were expressed in ascorbic acid equivalents antioxidant capacity (AAAC), which are almost equal to Trolox equivalents antioxidant capacity (TEAC) for the ABTS assay according to Apak et al .[54].

Maximum inhibition capacity for ABTS free radicals was found at 8-10 mg SUPRAS mL^{-1} with a value of 80.65 ± 2.20 %, which correspond to a concentration of 25.04 ± 0.73 µM AAAE/g dw. Goiris et al. [9] reported values from aqueous ethanolic extracts of *Scenedesmus obliquus* (5.87 ± 0.28 µmol Trolox/ g dw).

With regard to DPPH assay, the antioxidant activity of supramolecular extracts reached a maximum of 58 % at 10 mg of SUPRAS mL^{-1} with a value of 17.02 ± 0.45 µM AAAE/g dw. This result fall within the range reported by Aremu et al. [30] for five strains of *Scenedesmus* with different culture ages (3.1-69.9 % DPPH radical scavenging). In the same way, as demonstrated recently by Morowvat and Ghasemi [48] *Scenedesmus rubescens* extracts exhibited potent DPPH radicals quencher achieving a maximum of 14.04 ± 0.88 µmol Trolox g^{-1} dw.

The measured antioxidant activity agrees with the antioxidant-rich composition of supramolecular extracts since notable amounts of carotenoids and polyphenols were detected.

4. Conclusions

An easily prepared, green and cost-effective SUPRAS enabling high recovery of antioxidants from microalgal biomass (*Scenedesmus sp*) was developed and optimized. SUPRAS made up from octanoic acid, ethanol, and water at synthesis proportions of 5:36:59 % v/v/v, respectively were employed for extraction. The optimal SUPRAS yielded significant amounts of carotenoids (1.04 mg/g, mainly lutein) and polyphenols (10.29 mg/g, about 50% phenolic acids). SUPRAS extracts exhibited strong antioxidant activity against ABTS and DPPH radicals (80.65 and 58%, respectively) demonstrating that are abundant sources of antioxidants, especially polyphenols. These results show the potential of SUPRAS as an attractive tool toward sustainable, economic and functional valorisation of microalgae bioactives for clean purposes.

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Conflict of interest

The authors declare no conflict of interest.

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Figure captions

Figure 1: General scheme for (A) SUPRAS synthesis (left) and microextraction of antioxidants from the microalgae sample (right) and B) schematic picture of the increase of the aqueous vacuole size in SUPRAS with the increase of the ethanol percentage in SUPRAS (left) and SUPRAS composition (%, v/v octanoic acid, ethanol and water) as a function of the ratio ethanol/water (%, v/v) in the synthetic solution (right).

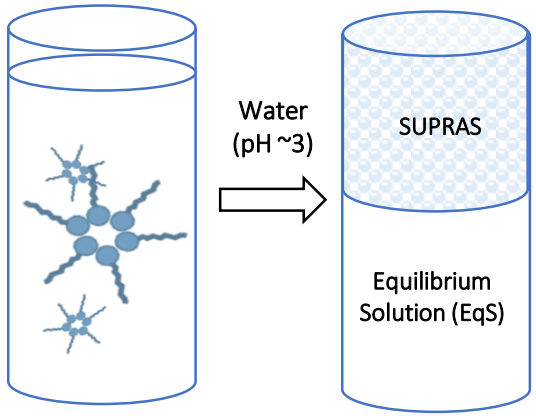
Figure 2: Optimization of SUPRAS extraction for maximum extraction yield of carotenoid and polyphenols under A and B) different ethanol percentages for SUPRAS synthesis and C) and D) different ratios SUPRAS: equilibrium solution

Highlights

- SUPRAS simultaneously extracted polar and apolar antioxidants from *Scenedesmus sp*.
- Carotenoids (1 mg/g dw) and polyphenols (10 mg GAE/g dw) were extracted.
- SUPRAS extracts showed high antioxidant capacity (ABTS and DPPH).
- SUPRAS were made up of food authorized ingredients.
- Simple extraction conditions: 5 min, room temperature and atmospheric pressure.

A

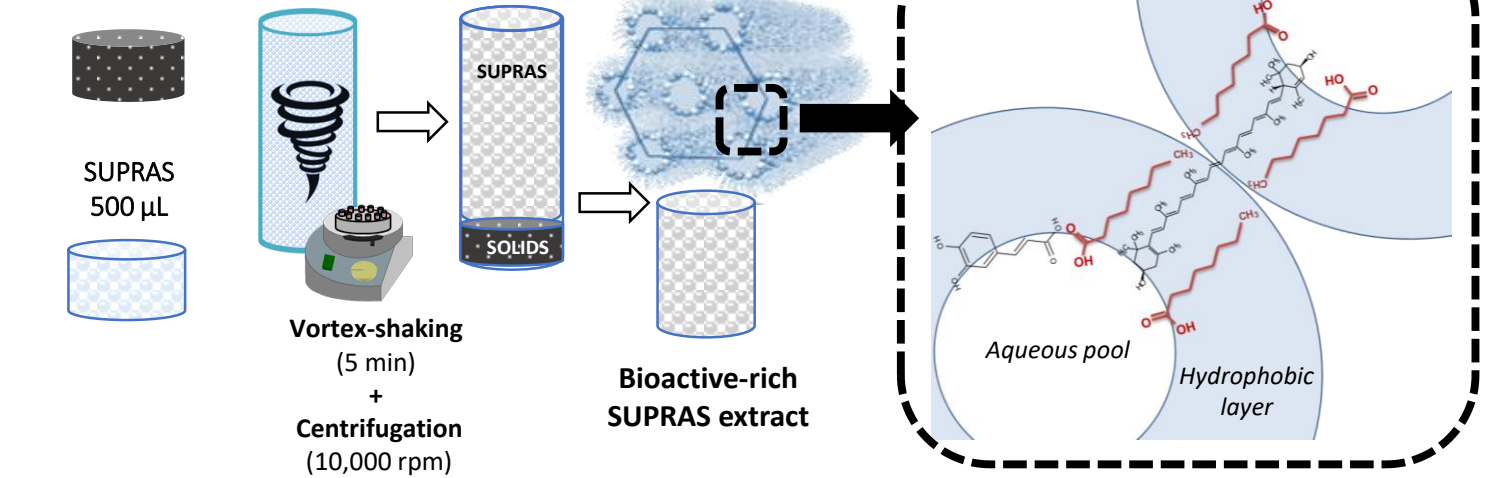
SUPRAS SYNTHESIS



Colloidal solution of octanoic acid, inverse micelles in ethanol

*__Optimal synthesis conditions__:
5% v/v octanoic acid, 36 % v/v ethanol, 59% v/v acidified water

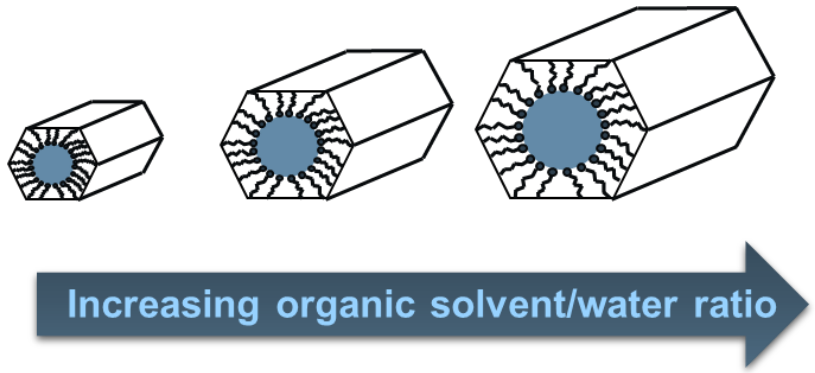
SUPRAS EXTRACTION OF ANTIOXIDANTS

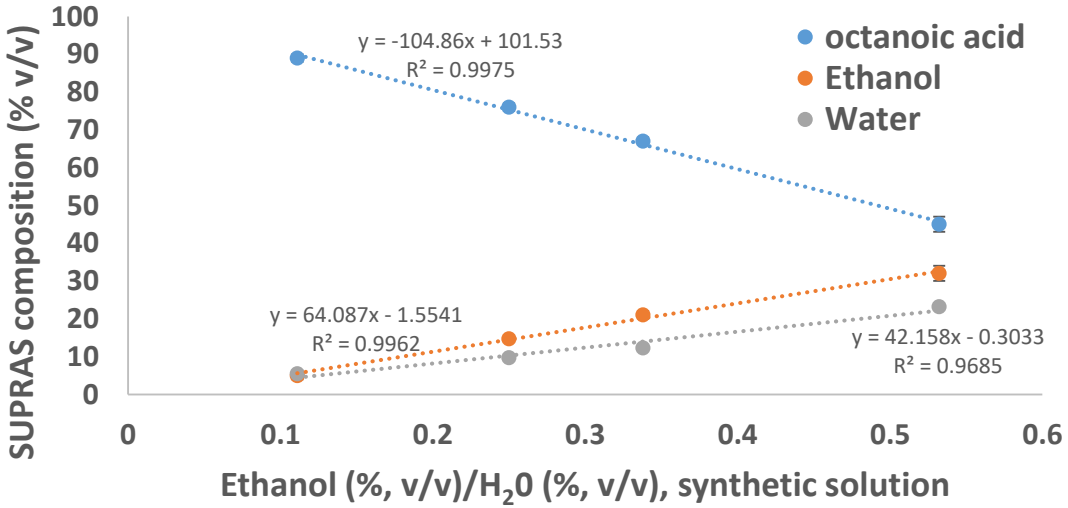


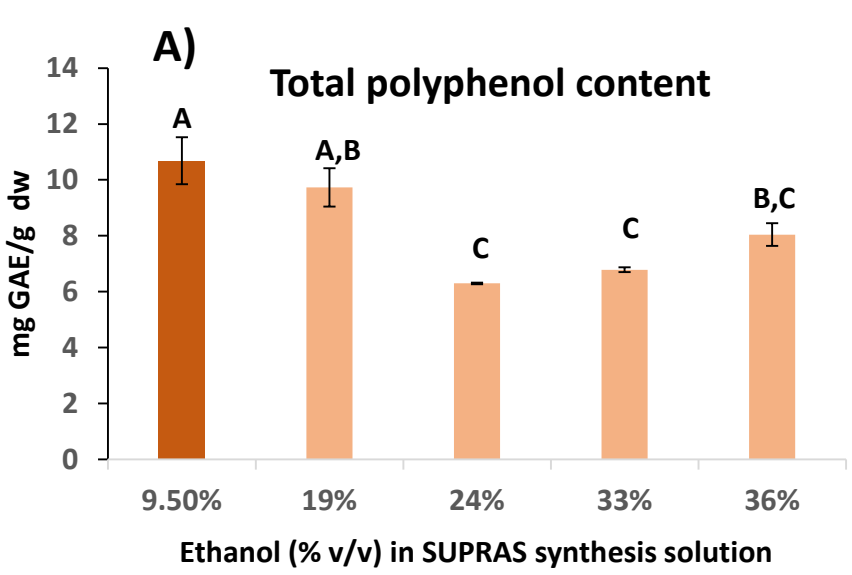
Vortex-shaking
(5 min)
+
Centrifugation
(10,000 rpm)

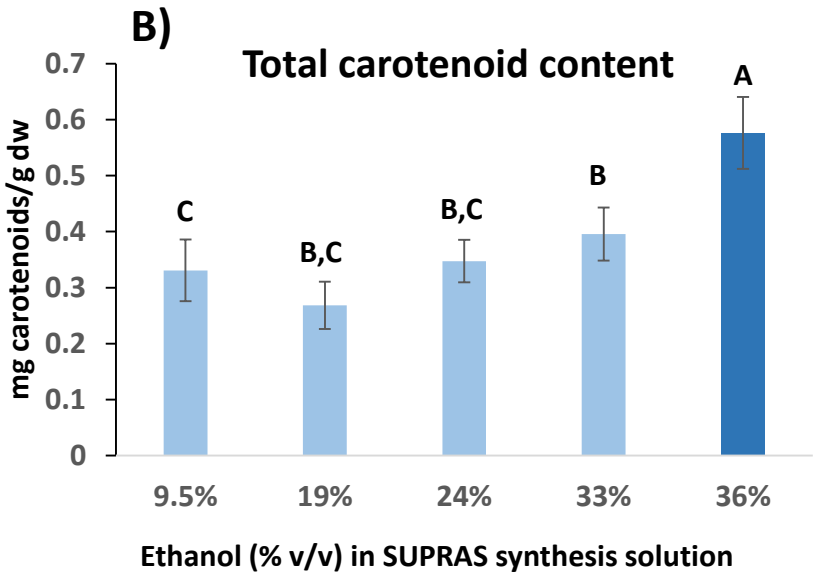
Bioactive-rich SUPRAS extract

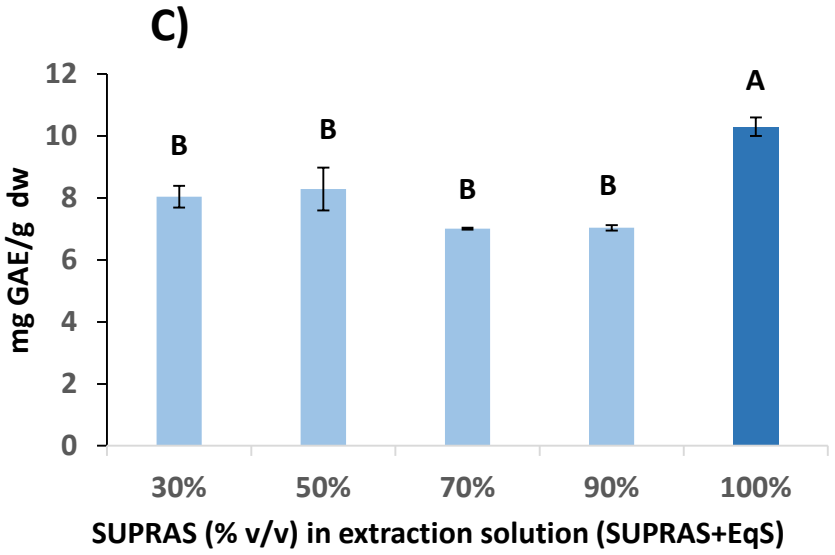
B






A)
Total polyphenol content
mg GAE/g dw
14
12
10
8
6
4
2
0
A
A,B
C
C
B,C
9.50%
19%
24%
33%
36%
Ethanol (% v/v) in SUPRAS synthesis solution


B)
Total carotenoid content
mg carotenoids/g dw
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
C
B,C
B,C
B
A
9.5%
19%
24%
33%
36%
Ethanol (% v/v) in SUPRAS synthesis solution


C)
mg GAE/g dw
12
10
8
6
4
2
0
B
B
B
B
A
30%
50%
70%
90%
100%
SUPRAS (% v/v) in extraction solution (SUPRAS+EqS)

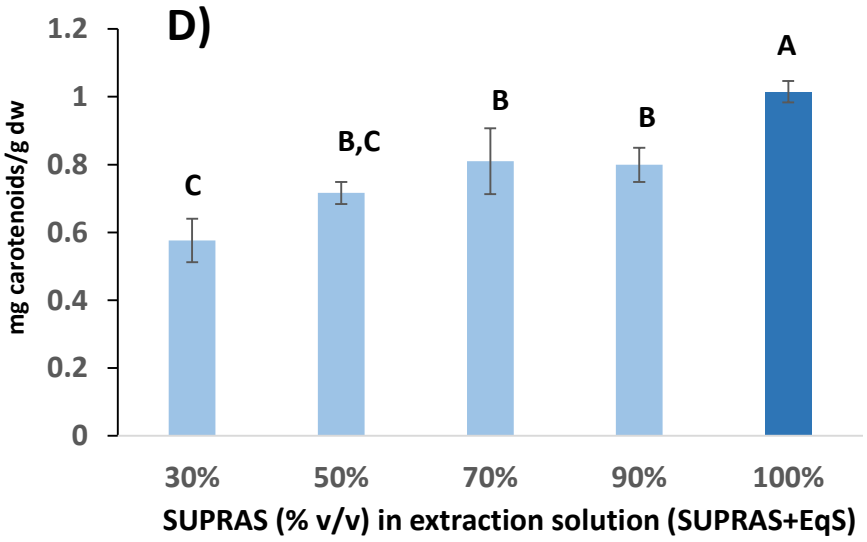

D)
mg carotenoids/g dw
1.2
1
0.8
0.6
0.4
0.2
0
C
B,C
B
B
A
30%
50%
70%
90%
100%
SUPRAS (% v/v) in extraction solution (SUPRAS+EqS)

Table 1

Tentative identification of carotenoids and polyphenols analysed by LC-MS/MS in SUPRAS extracts of *Scenedesmus sp.*

Chemical Class	Compound	RT (min)	Observed parent ion	Most abundant fragment	Peak Area	Abundance[a]
Carotenoids	Astaxanthin	16.0	597.4 $[M+H]^+$	147.1	595	0.068
	Lutein	17.8	551.4 $[M+H-H_2O]^+$	135.1	6052	**0.696**
	Unknown carotenoid[b]	19.8	565.4	109.1	2042	0.235
Polyphenols	Carnosic acid	53.4	331 $[M+H]^-$	287.1	1084	0.05
	Sinapic acid	38.4	223 $[M+H]^-$	179	2846	0.12
	Caffeic acid	1.3	179 $[M+H]^-$	135	8110	**0.35**
	Unknown polyphenol[c]	43.8	169 $[M+H]^-$	125	4289	0.19
	Vanillin	18.7	151 $[M+H]^-$	136	2968	0.13
	p-hydroxybenzoic acid or salicylic acid	11.9	137 $[M+H]^-$	93	2570	0.11
	Phloroglucinol	1.9	125 $[M+H]^-$	97	464	0.02
	Formononetin	52.8	267 $[M+H]^-$	252	757	0.03

[a]Calculated as the ratio: Compound area/total area of both compound classes; [b]The transition measured for the unknown carotenoid corresponded to a secondary transition of canthaxanthin but neither the retention time nor the relative abundance of the monitored transitions matched canthaxanthin; [c]The transition measured for the unknown polyphenol corresponded to gallic acid but the retention time did not matched the authentic standard

Sample CRediT author statement

M. N. Keddar: Investigation, Writing-Original Draft, Formal analysis; **Ana Ballesteros-Gómez**: Formal analysis, Methodology, Conceptualization, Supervision, Writing-Review & Editing, Funding acquisition; **M. Amiali**: Supervision, Writing- Review & Editing; **J.A. Siles**: Resources, Supervision, Writing- Review & Editing; **D. Zerrouki:** Supervision, Writing- Review & Editing; **M.A. Martín**: Resources, Supervision, Writing- Review & Editing; **Soledad Rubio**: Methodology, Conceptualization, Supervision, Writing-Review & Editing, Funding acquisition