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Research Article

Switchable Solvent Selective Extraction of Hydrophobic Antioxidants from *Synechococcus bigranulatus*

Luigi D'Elia, Paola Imbimbo, Davide Liberti, Francesco Bolinesi, Antonino Pollio, Olga Mangoni, Wim Brilman, Giuseppe Olivieri, and Daria Maria Monti*

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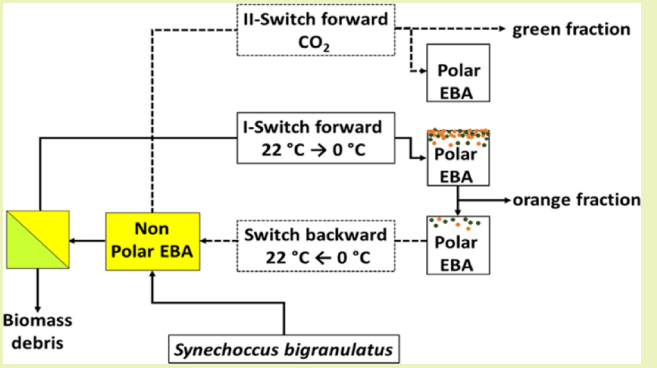
ABSTRACT: Hydrophobic molecules, in particular, carotenoids, have been directly extracted from *Synechococcus bigranulatus* ACUF680 by means of secondary amine switchable solvent *N*-ethylbutylamine (EBA) without any other pretreatment. EBA was able to extract hydrophobic molecules from both fresh and frozen biomass at the same extent of the conventional procedure (about 20% of dry biomass). In particular, selective extraction of a zeaxanthin-enriched fraction (green fraction, GF) and a β-carotene-enriched fraction (orange fraction, OF) was obtained. The ratio between zeaxanthin and β-carotene was 4.4 ± 1.5 for GF, 0.07 ± 0.06 for OF, and about 1 for conventional extraction. These fractions showed *in vitro* antioxidant activity (IC_{50} values of 0.056 ± 0.013 and 0.024 ± 0.008 mg mL^{-1} for GF and OF, respectively) and biocompatibility on immortalized cells. Moreover, OF and GF were able to protect cells from oxidative stress, both before and after thermal treatment. Results clearly indicate that EBA is a good candidate to specifically extract β-carotene and zeaxanthin from the wet biomass of *S. bigranulatus* without affecting their biological activity. Skipping energy-intensive operations to break the cells and using either fresh or frozen biomass may be the driving factors to use EBA switchable solvent on an industrial scale.



KEYWORDS: *antioxidants, switchable solvents, microalgae, N-ethylbutylamine, green chemistry*

INTRODUCTION

In the last decades, the search for energy from renewable sources and the increasing demand of consumers for healthy foods have driven the attention toward microalgae and cyanobacteria.[1−4] These phototrophic microorganisms can be rich in high-value biological compounds, such as proteins, polysaccharides, polyunsaturated fatty acids, vitamins, and pigments with special biological activities, and thus they can potentially be exploited in several industrial sectors and meet many new consumer wishes.[4−6]

Today, algae and cyanobacteria are used only in the food industry as additives for functional food and as food supplements. In this context, the increasing demand for natural antioxidants, as a healthy alternative to synthetic additives in the food industry, has strengthened the interest for microalgae and cyanobacteria as a valid source of natural antioxidants, such as polyphenols and pigments.[7−14] Among pigments, carotenoids show the highest antioxidant activity. They are divided into two groups: carotenes and xanthophylls. So far, in the global market, the most commercialized carotenoids are astaxanthin, β-carotene, lutein, canthaxanthin, lycopene, and zeaxanthin.[15]

Till date, antioxidant extraction is usually performed by using organic solvents.[10,16,17] However, these benchmark technologies suffer from several drawbacks, making the whole process unsustainable: (i) the use of high amounts of solvent; (ii) the need for a large amount of energy to recover the solvent by evaporation; (iii) the need for more than one extraction step; (iv) the recovery of a mixture of molecules with similar polarity.[18−20]

These drawbacks are even more consistent in the case of antioxidants from microalgae as they already suffer from upstream process costs.[21] Thus, the optimization of a green extraction technique able to replace conventional procedures seems to be a good starting point for lowering the costs. So far, it has been reported that the new green extraction techniques performed at high pressure, such as supercritical fluid extraction and pressurized liquid extraction, are more sustainable and can be competitive in the effectiveness of the extraction of hydrophobic molecules.[22−24] However, these innovative tech-

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nologies show some disadvantages: (i) need of biomass pretreatment often, (ii) difficulty to extract hydrophilic molecules ; (iii) high investment costs, and (iv) difficulty to scale up the process at an industrial scale.[20,25]

In this context, a new class of solvents is emerging: switchable solvents. Switchable solvents (SSs), first reported by Jessop and Liotta,[26,27] are liquids that can be converted from a nonpolar form to a polar form and vice versa. The switching of the solvent in the hydrophilic form (switch forward) is done by bubbling CO_2 through the solvent. The reverse reaction is induced by completely removing CO_2 from the system with an inert gas, such as N_2, and is enhanced by the heating temperature.[28] This unique feature allows the solvent, when in the nonpolar form, to extract hydrophobic components, and, when in the polar form, to extract hydrophilic components. Furthermore, when in the polar form or in the nonpolar form, the solvent should be easily separated by the hydrophobic or hydrophilic extracts, respectively. Noteworthy, the circularity of the extraction process on the wet biomass involves a significant reduction in energy consumption because the separation of the solvent from the extract is not performed by evaporation.[29,30]

Recently, a study carried out by Du et al. reported that the secondary amine *N*-ethylbutylamine (EBA) shows a lower critical solution temperature (LCST) behavior, which means that a change in EBA polarity is possible only by changing the temperature.[31] This alternative operation is expected to be a cost and energy efficient alternative to the CO_2 switching system.[31] However, the feasibility of the process to effectively and sequentially extract lipids and proteins from microalgae by using a SS characterized by LCST behavior has not been clearly defined yet.[32]

Here, an innovative procedure to selectively extract hydrophobic antioxidants from the cyanobacterium *Synechococcus bigranulatus* was set up. *S. bigranulatus* is a good candidate for the production of thermo-resistant antioxidants as it is able to grow at different temperatures.[33] Experiments were performed by using EBA as the extracting solvent, and three different strategies were tested (Figures S1−S3). Hydrophobic fractions were characterized from a chemical and biological point of view, and the antioxidant activity was tested before and after high-temperature short-time (HTST) pasteurization.

MATERIALS AND METHODS

Reagents. All solvents, reagents, chemicals, and culture media, unless differently specified, were from Sigma-Aldrich (St Louis, MO, USA).

Microalgal Strain and Culture Conditions. *S. bigranulatus* was provided by the Algal Collection of the University Federico II (ACUF number 680).[34] Cells were grown in autotrophic conditions in 800 mL working volume bubble column photobioreactors in BG11 medium. The photobioreactors were housed in a climate chamber at 37 ± 1 °C equipped with fluorescent lamps with a constant light intensity of 300 PAR $\mu mol_{photons}\ m^{-2}\ s^{-1}$. The aeration of cultures was provided by supplying air at the bottom of the photobioreactors. Cell density was inferred from the absorbance measured at a wavelength of 730 nm. The culture was harvested at the end of the exponential phase, and the biomass concentration was about 0.8 g L^{-1}.

Water Content of *S. bigranulatus*. The water content of the harvested wet microalgae paste after centrifugation was determined by weighing the sample before and after drying at 60 °C for 24 h. The water content did not change significantly among samples and was 73.1 ± 3.3%.

Protein Extraction and Quantification. The biomass was harvested by centrifugation at 1200*g* for 30 min at room temperature. Then, 1.5 g of wet biomass, which corresponds to about 400 mg d.w., was resuspended in 50 mM sodium acetate buffer pH 5.5.[35] Cells were disrupted by ultrasonication (30 s on, 30 s off, 40% instrument amplitude) for 45 min on ice. After centrifugation at 5000*g* at 4 °C for 30 min, the proteins were recovered in the supernatant and their concentration was measured by the BCA Assay Kit (Thermo Fisher Scientific, Waltham, MA USA).

Conventional Extraction of Total Hydrophobic Molecules. Conventional extraction of hydrophobic molecules from the dry biomass (~0.3 g) was performed according to the original Bligh & Dyer (B&D) method.[36]

Hydrophobic Molecule Extraction by EBA. To extract hydrophobic molecules from *S. bigranulatus* by EBA, the wet biomass (~1.5 g) was mixed with EBA in a ratio of 1:2 (wt/wt). The mixture was magnetically stirred at room temperature for 18 h to ensure extraction of hydrophobic molecules. Then, the mixture was centrifuged at 9000*g* at room temperature, and the supernatant (which contains EBA and hydrophobic molecules) was mixed with water in a ratio of 1:2 (wt/wt), forming a biphasic system. Then, three experimental approaches were followed and are schematically represented in Figures S1−S3.

Strategy 1: CO_2−N_2 Switching Method. CO_2 was insufflated into the biphasic system for 30 min at 2 vvm (volume volume per minute) to allow EBA to switch in its polar form (switching forward). The sample was centrifuged at 9000*g* at room temperature. At the end of centrifugation, EBA and water formed a homogeneous phase (water phase) with a small lipid layer on the top. The hydrophobic molecules were recovered by chloroform. The total amount of the extracted product was measured gravimetrically (after evaporating the solvent) and reported as a percentage of the algae dry weight.

To extract hydrophilic molecules, the water phase was incubated in the presence of the residual biomass for 2 h at room temperature. The sample was centrifuged at 9000*g* at 0 °C for 10 min. Then, to recover the extracted hydrophilic molecules, N_2 was bubbled through the supernatant to remove CO_2 (switch back) and to allow EBA returning in the hydrophobic form. The sample was centrifuged at 9000*g* at room temperature. However, no formation of the two phase system was observed.

Strategy 2: Temperature switching method (LCST). The supernatant was cooled to 0 °C for 2 h to allow the switch of EBA in its polar form. The sample was centrifuged at 9000*g* at 0 °C. The result was a water phase with a hydrophobic layer on the top of the tube. This layer consisted of two parts, a green layer on the top and an orange layer on the wall of the tube. To recover the hydrophobic phase, chloroform was added, mixed, and centrifuged at 9000*g* at 0 °C for 10 min. A two-phase system was observed with the hydrophobic molecules on the bottom. The total amount of the extracted products was measured gravimetrically (after evaporating the solvent) and reported as a percentage of the algae dry weight. To extract hydrophilic molecules, the water phase was incubated in the presence of the residual biomass for 2 h at 0 °C. The sample was centrifuged at 9000*g* at 0 °C for 10 min. Then, to collect hydrophilic molecules, the supernatant was heated to 22 °C to allow EBA switching back to the nonpolar form. The sample was centrifuged at 9000*g* at 0 °C for 10 min to allow the formation of two phases. The aqueous fraction containing hydrophilic molecules was collected, the total proteins were determined by the BCA assay, and then an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed.

Strategy 3: Switch Forward-Switch Back-Switch Forward. To avoid the use of chloroform during the recovery of the hydrophobic molecules, heptane was used. By this procedure, the orange layer was recovered after centrifugation at 9000*g* at 0 °C. The green layer was found to be mixed again with EBA. To recover them, a double switch was needed. Thus, the system was heated to 22 °C to allow EBA switching back to the nonpolar form. The sample was centrifuged at 9000*g* at room temperature to allow the formation of two phases. The upper phase contained EBA and the hydrophobic molecules. The upper phase was transferred to a new tube and mixed with water in a ratio of 1:2 (wt/wt) forming a biphasic system. Then, CO_2 was insufflated into the system for 30 min at 2 vvm, and the sample was centrifuged at 9000*g* at room temperature to recover the green fraction (GF) on the top by chloroform.

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The total amount of the extracted products was measured gravimetrically (after evaporating the solvent) and reported as a percentage of the algae dry weight.

In a parallel experiment, heptane was replaced by methanol.

ABTS Assay. The *in vitro* antioxidant activity of the orange fraction (OF) and GF was evaluated by the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS assay according to Rigano et al. with some modifications.[37] Briefly, 7.4 mM $ABTS^{\bullet+}$ was mixed with 140 mM $K_2S_2O_8$, and the solution was stabilized for 16 h at room temperature in the dark. The mixture was then diluted with deionized water to obtain an absorbance of 0.70 ± 0.02 unit at 734 nm using a spectrophotometer. Each extract (50 μL) was allowed to react with 250 μL of diluted $ABTS^{\bullet+}$ solution for 7 min, and then the absorbance was read at 734 nm. The standard curve was linear between 0 and 20 μM Trolox. Results are expressed as IC_{50} (mg mL^{-1}), that is, the concentration required to scavenge 50% of free radical ABTS.

Cell Culture and Biocompatibility Assay. Human immortalized keratinocytes (HaCaT, Innoprot, Spain) and immortalized murine fibroblasts (BALB/c 3T3, ATCC, Manassas, VI, USA) were cultured in 10% fetal bovine serum in Dulbecco's modified Eagle's medium in the presence of 1% antibiotics and 2 mM L-glutamine in a 5% CO_2 humidified atmosphere at 37 °C. HaCaT cells were seeded in 96-well plates at a density of 2×10^3 cells $well^{-1}$, and BALB/c 3T3 were seeded at a density of 3×10^3 cells $well^{-1}$. 24 h after seeding, increasing concentrations of OF and GF (from 10 to 200 μg mL^{-1}) were added to the cells for 48 h. At the end of the incubation, cell viability was measured by the tetrazolium salt colorimetric assay (MTT assay), as previously described.[38] Cell survival was expressed as the percentage of viable cells in the presence of the extracts compared to control cells (represented by the average obtained between untreated cells and cells supplemented with the highest concentration of the buffer). Each sample was tested in three independent analysis, each carried out in triplicate.

HTST Pasteurization. Thermal pasteurization was performed accordingly to the protocol reported by Ferraro et al.[39] Briefly, OF and GF were heated at 75 °C in a water bath. After 10 min incubation, the samples were transferred to a second water bath at 20 °C and then stored at 4 °C until analysis.

Cellular Reactive Oxygen Species Assay. To evaluate the protective effect of OF and GF against oxidative stress, DCFDA assay was carried out according to the protocol reported by Petruk et al.[40] with some modifications. Briefly, 24 h after seeding, the cells were incubated in the presence of 25 μg mL^{-1} of either raw or pasteurized OF and GF for 2 h and irradiated by UVA light for 10 min (100 J cm^{-2}). Fluorescence intensity of the probe (2′, 7′-dichlorofluorescein, DCF) was measured at an emission wavelength of 525 nm and an excitation wavelength of 488 nm using a PerkinElmer LS50 spectrofluorometer. Emission spectra were acquired at a scanning speed of 300 nm min^{-1} with a 5-slit width for both excitation and emission. Reactive oxygen species (ROS) production was expressed as the percentage of DCF fluorescence intensity of the samples under test compared to untreated samples. Three independent experiments were carried out, each one with three determinations.

Pigment Identification by High-Performance Liquid Chromatography. For the determination of the pigment content, the extracts were resuspended in 100% methanol and analyzed by isocratic high-performance liquid chromatography (HPLC) (Hewlett Packard, 1100 Series) in a reverse phase (C8 column 3 μm Hyperloop MOS, 10 cm, 4.6 mm internal diameter, Shandon) as described by Vidussi et al.[41] The mobile phase consisted of MeOH: 0.5 N aqueous ammonium acetate, 70:30% v/v (solvent A) and MeOH (solvent B), with a gradient (time expressed in minutes; percent of solvent A-percent of solvent B): 0:75−25; 1:50−50; 15:0−100; and 19:75−25. For the determination of chlorophylls and carotenoids, a spectrophotometer with a diode array detector was set at 440 nm, making it possible to determine the absorption spectrum of the 350−750 nm interval for each peak in order to check the purity of single pigments. The calibration of the instrument was carried out using external standard pigments provided by the International Agency for 14C determination-VKI Water Quality Institute. The identification of pigments was based considering the retention time, spectral characteristics, and chromatography with certified commercial standards (International Agency for 14C determinations, Denmark). Quantification was based on the absorbance at 440 nm and the factor response (peak area/pigment concentration) value for each pigment, as described in the study by Mantoura and Repeta.[42]

Statistical Analysis. Samples were analyzed in triplicate. Results are presented as mean of results (means ± SD) and compared by one-way ANOVA according to the Bonferroni's method (post hoc) or *t*-test using Graphpad Prism for Windows, version 6.01.

RESULTS AND DISCUSSION

Extraction and Recovery of Hydrophobic Molecules from Synechococcus bigranulatus through SSs. To verify the ability of EBA SS to extract hydrophobic molecules from the frozen wet biomass of *S. bigranulatus*, a direct comparison with a reference extraction method (B&D) on dry biomass was performed. Thus, frozen wet biomass was mixed with the solvent. Then, to separate hydrophobic molecules from EBA, the solvent was switched to the hydrophilic form (switch forward) by two procedures: (i) by bubbling CO_2 through the system (SS−CO_2 switch-forward, reported in Figure S1) and (ii) by decreasing the temperature (SS-LCST switch-forward, LCST, reported in Figure S2).

The extracted hydrophobic molecules were measured gravimetrically and reported as percentage with respect to the dry weight biomass (Figure 1). Results clearly show that EBA was

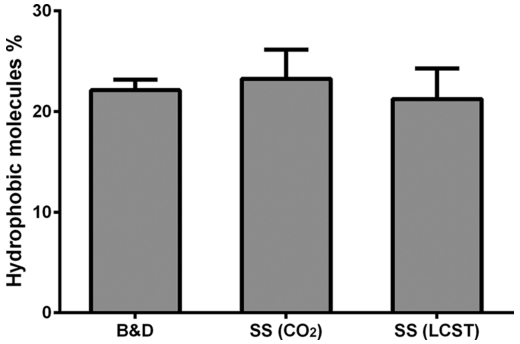

Figure 1. Hydrophobic molecules from *S. bigranulatus* biomass. Yields are reported as % with respect to the dry weight biomass. SS (CO_2) refers to extraction by SS and CO_2; SS (LCST) refers to extraction by SS and LCST. Results are reported as means ± SD of at least three independent experiments.

able to extract hydrophobic molecules from the algae at the same extent of the B&D procedure. This result indicates that it is possible to obtain hydrophobic molecules starting from a wet biomass, thus potentially reducing the costs of the whole process, which are affected by drying, milling, and temperature.

Protein Extraction. After the extraction of hydrophobic molecules, EBA solvent, in its polar form, was used to extract proteins on the residual biomass, as described in the Materials and Method section. In this case, ultrasounds were used as the reference procedure. When the SS-CO_2 switch-forward strategy was performed, no formation of the two-phase system was observed after N_2 insufflation and temperature increase, probably due to stripping of switched (hydrophobic) EBA from the solution by the warm N_2 flow. In the case of SS-LCST switch-forward strategy, in which only the temperature was changed, a hydrophilic phase was recovered and characterized. According to the BCA assay, the SS (LCST-CO_2) method allowed to recover about 10% proteins, a value 3-fold lower than

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in the case of ultrasonication. However, when the samples were analyzed by SDS-PAGE, no proteins were observed in the sample SS (LCST-CO_2) (Figure 2), thus suggesting that, during

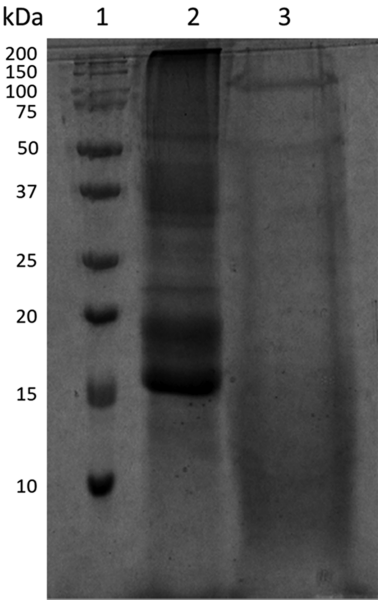

Figure 2. Proteins extracted from *S. bigranulatus*. SDS-PAGE analysis of proteins extracted by ultrasounds and SS (LCST-CO_2) extraction. Lane 1: molecular weight markers; lane 2: soluble proteins extracted by ultrasounds (30 μg); lane 3: soluble proteins extracted by EBA (30 μg). SDS-PAGE was stained by Blue Coomassie.

the extraction with nonpolar EBA, proteins may be degraded and/or unfolded and precipitate in the centrifugation step. The results indicate that the yield calculated by the BCA procedure is affected by the presence of EBA itself. In a parallel experiment, the polar form of EBA was used on the raw biomass to verify its ability to extract hydrophilic molecules. However, no hydrophilic molecules were obtained, maybe because polar EBA was not able to destroy the biomass in the absence of any physical and/or mechanical treatment (data not shown). On the other hand, nonpolar EBA better penetrates the biomass of microalgae with a fragile cell wall, such as the one of *S. bigranulatus*. The cyanobacterium has a cell wall similar to that of Gram-negative bacteria but equipped with a thick peptidoglycan layer.[43,44] Moreover, the absence of cellulose renders the process feasible under mild conditions.

Switch Forward-Switch Back-Switch Forward Strategy. In order to replace chloroform with a green solvent, a third strategy was set up (Figure S3), and the detailed procedure is described in the Materials and Method section. The replacement of chloroform with heptane allowed to recover an OF, whereas the green layer was mixed again with EBA. Thus, the GF was recovered after a second cycle of switch-forward by using chloroform. Fresh and frozen (stored at −20 °C) biomass were analyzed in parallel experiments. As reported in Figure 3, the yields of OF and GF do not show any significant difference between the two starting materials. This result suggests that the extraction can be performed according to the experimental purpose, that is, on either just harvested or stored biomass. Thus, from an industrial point of view, even if storing the biomass can be a cost, it could allow a more flexible scheduling of the downstream process.

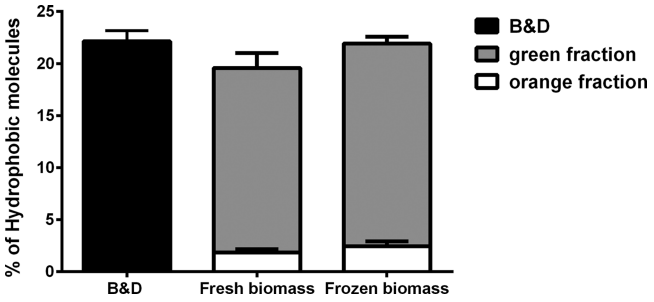

Figure 3. Yields of hydrophobic molecules in OF and GF. Yields are referred to extracts from fresh or frozen biomass and reported as % with respect to the dry weight biomass. Black bar refers to B&D; gray bars refer to the GF; white bars refer to the OF. Results are reported as means ± SD of at least three independent experiments.

HPLC Analysis. HPLC analysis was performed to identify the molecules present in OF and GF. Results are reported in Figure 4 and they clearly show that OF is enriched in β-carotene, whereas GF is enriched in zeaxanthin. Indeed, when the ratio zeaxanthin/β-carotene was measured in both fractions, a ratio of 0.07 ± 0.06 was found in the OF and 4.4 ± 1.5 in the GF, whereas the ratio in the raw extract obtained by conventional extraction was about 1. In particular, zeaxanthin values ranged between 1.3 and 20.7 mg g_{dw}^{-1} in the GF, whereas β-carotene ranged between 0.3 and 0.6 mg g_{dw}^{-1} in the OF. A mean value of 0.37 mg g_{dw}^{-1} was observed instead for both molecules in the extract obtained by a conventional method, suggesting a better extraction yield of at least zeaxanthin with EBA with respect to conventional methods.

ABTS Assay. To verify if the extraction technique affected the biological activity of OF and GF, the *in vitro* antioxidant activity was tested by the ABTS assay. Results are shown in Figure 5 and clearly indicate that both fractions had antioxidant activity. The IC_{50} values, which correspond to the concentration of the extract that can inhibit 50% of the radical, were 0.024 ± 0.008 and 0.056 ± 0.013 mg mL^{-1} for the OF and GF, respectively.

Evaluation of the Biocompatibility of OF and GF on Eukaryotic Cells. The biocompatibility of OF and GF was evaluated by the MTT assay on two immortalized eukaryotic cell lines: HaCaT (human keratinocytes) and BALB/c 3T3 (murine fibroblasts). Cells were incubated in the presence of each extract for 48 h, and cell survival was determined as described in the Materials and Methods section. As shown in Figure 6, the GF did not show cell mortality on both cell lines tested up to 200 μg mL^{-1} (A,B), whereas the OF (C,D) showed a low level of toxicity only on HaCaT cells (IC_{50} value of 152 ± 7 μg mL^{-1}). Noteworthy, the solvent used to recover the OF is important as when methanol was used, cell mortality was achieved at a very low concentration of the extract, with an IC_{50} value of 31 ± 8 μg mL^{-1} and 43 ± 4 μg mL^{-1} on HaCaT cells and BALB/c 3T3 cells, respectively (E,F).

Evaluation of Antioxidant Activity of OF and GF on Eukaryotic Cells. To verify if the two extracts could be used in the food industry and if they are resistant to high-temperature treatments, a comparison between the antioxidant activity of raw and pasteurized extracts (i.e., HTST pasteurization) was performed on a cell-based system. HaCaT cells were stressed by UVA, chosen as a source of oxidative stress. Cells were incubated for 2 h with 25 μg mL^{-1} of each extract prior to UVA exposure. Then, the DCFDA probe was used to measure the

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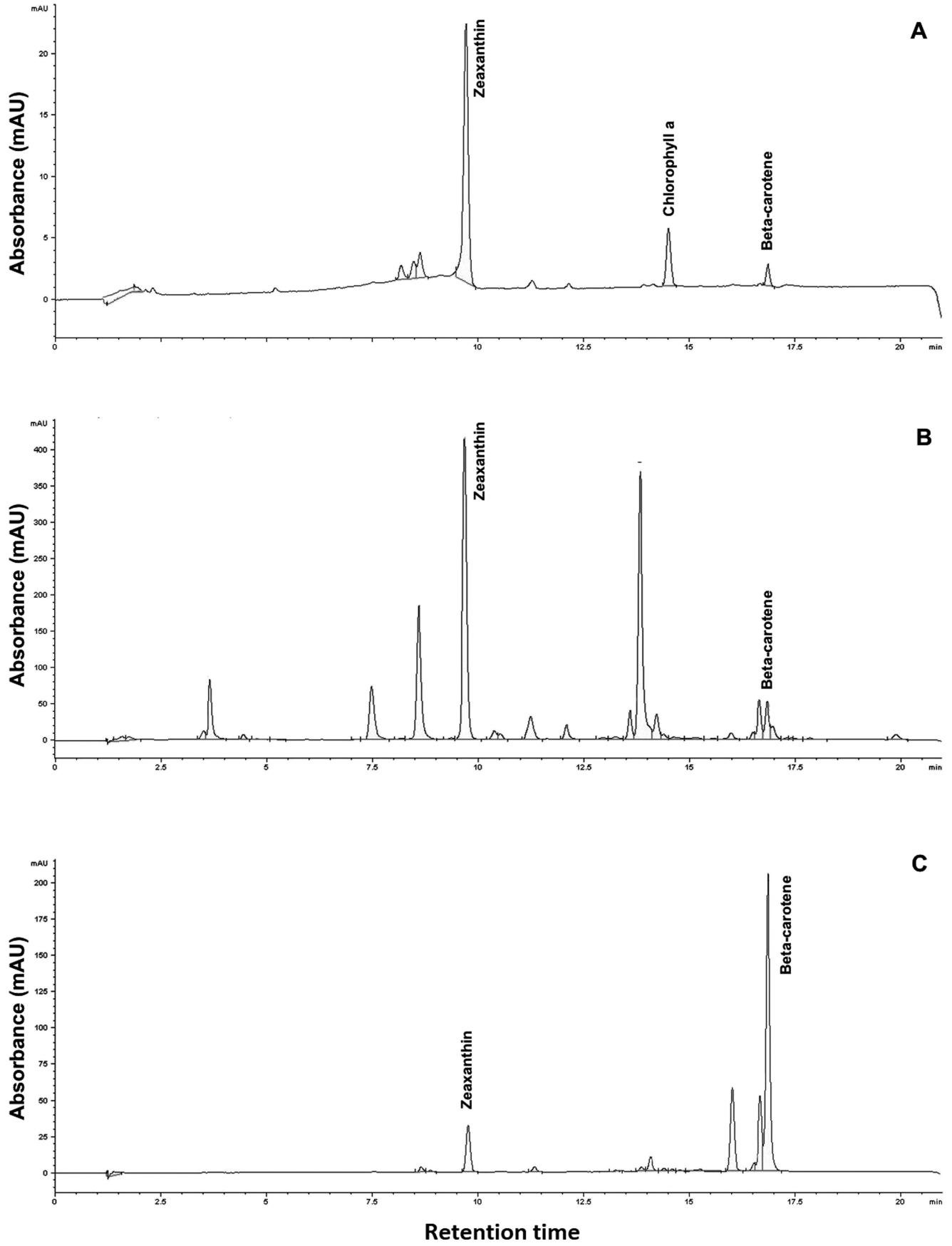

Figure 4. Representative HPLC chromatograms of antioxidants extracted from *S. bigranulatus*. (A) Raw extract obtained by conventional extraction, (B) GF, and (C) OF.

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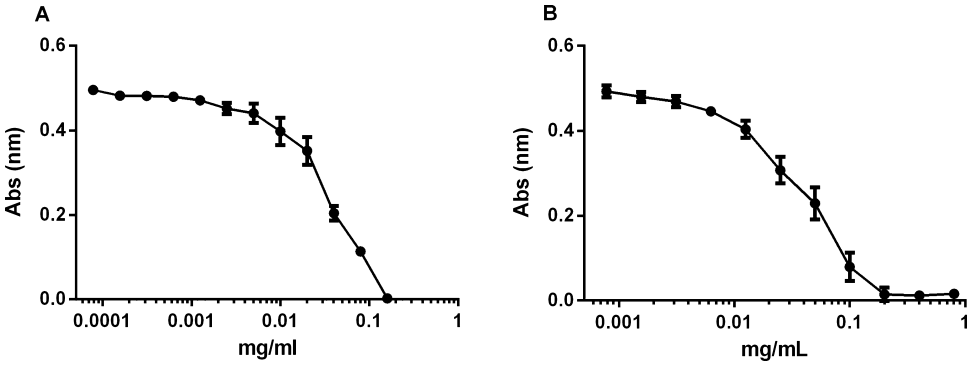

Figure 5. ABTS assay on OF and GF. The ABTS scavenging activity of OF (A) and GF (B) (mg mL^{-1}) from *S. bigranulatus*. Data shown are means ± SD of three independent experiments.

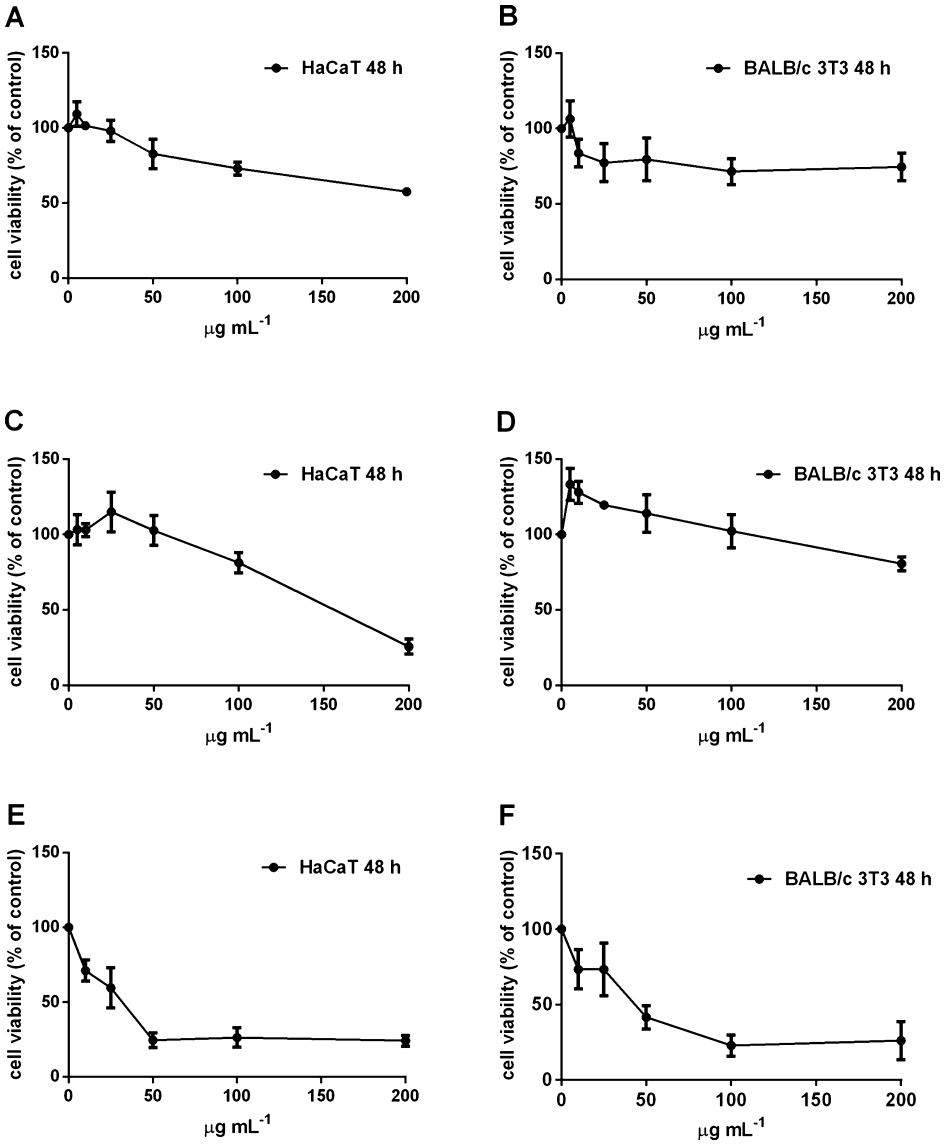

Figure 6. Cell viability of OF and GF on eukaryotic cells. HaCaT and BALB/c 3T3 were incubated for 48 h with increasing concentrations (10−200 μg mL^{-1}) of GF (A,B), OF collected with heptane (C,D), and OF collected with methanol (E,F). Cell viability was assessed by the MTT assay, and cell survival expressed as a percentage of viable cells in the presence of the extracts under test with respect to control cells grown in the absence of the extracts. Data shown are means ± SD of three independent experiments.

ROS levels. As shown in Figure 7, no effect on ROS levels was observed when the cells were incubated with either the raw OF or GF extracts, whereas when the cells were incubated with pasteurized orange and green extracts, an increase in intracellular ROS levels is observed in the absence of any treatment. UVA induced a significant increase in intracellular ROS levels

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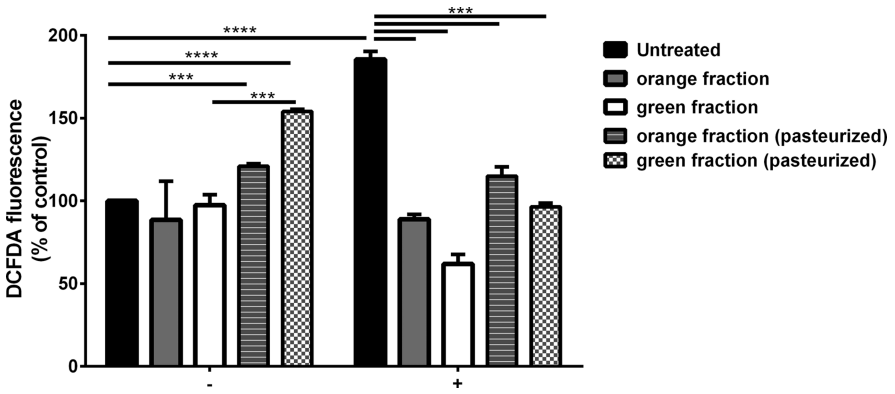

Figure 7. Antioxidant effect of the orange and green extracts from *S. bigranulatus* on UVA-stressed HaCaT cells. Cells were preincubated in the presence of 25 μg mL^{-1} of raw and pasteurized extracts for 2 h prior to be irradiated by UVA (100 J cm^{-2}). For each experimental condition, ROS production was measured, and the results are reported as percentage with respect to untreated cells. Black bars: untreated cells; gray bars: cells incubated with OF; white bars: cells incubated with GF; gray striped bars: cells incubated with pasteurized OF; white squared bars: cells incubated with pasteurized GF. Data shown are means ± SD of three independent experiments. *** indicates $p < 0.005$; **** indicates $p < 0.0005$.

(180%) with respect to untreated cells (black bars). When cells were preincubated with any of the extract, no alteration in ROS levels was observed, thus suggesting that the extracts were able to protect cells from stress injury. These results indicate that thermal procedures do not affect the antioxidant activity of both fractions.

CONCLUSIONS

SSs are widely used to extract hydrophobic molecules from algae raw biomass.[19,28,45] In this work, the secondary amine EBA was used on the cyanobacterium *S. bigranulatus* to test the ability to sequentially extract hydrophobic and hydrophilic components. Yields of hydrophobic molecules clearly show that EBA has the same extraction power as conventional methods. However, EBA was not able to extract proteins. This result seems to be in contrast with the data reported in the literature as about 40% of proteins have been reported to be extracted from different microalgae by using the tertiary amine *N,N*-dimethyl-cyclohexylamine.[30] Noteworthy, in the present paper, by using the same colorimetric assay, about 10% of proteins seemed to be present in the hydrophilic fraction, but no proteins were observed by SDS-PAGE analysis, thus suggesting an interference of EBA with the colorimetric assay.

Surprisingly, EBA allowed the selective extraction of β-carotene and zeaxanthin. The selectivity found was in agreement with data recently reported on astaxanthin extraction from *Haematococcus pluvialis*.[22] This selectivity allowed obtaining a β-carotene-enriched fraction and a zeaxanthin-enriched fraction, as clearly demonstrated by the HPLC analysis. Furthermore, the yields of both hydrophobic molecules were found to be much higher than those obtained by conventional methods.[19,46] In addition, EBA had the same extraction power on both frozen and fresh biomass. This result is very important from an industrial point of view as it is possible to process the biomass anytime with the same yields and, in case of fresh biomass, to lower the downstream costs.

From a biological point of view, the isolated molecules were fully biocompatible, active as antioxidants, and thermo-stable. In particular, the *in vitro* results showed lower IC_{50} values than those reported in the literature with different microalgae.[47,48] Both fractions were able to protect immortalized human keratinocytes from oxidative stress induced by UVA. This ability was fully maintained also after thermal treatment, that is, pasteurization.

In conclusion, EBA does not affect the biological activity of the extracted molecules, has a higher yield of extraction with respect to conventional methods, and does not require any pretreatment step or energy-intensive equipment to break the cells. Further investigation is instead needed to verify if the extraction of hydrophilic components can be improved by means of any chemical or mechanical pretreatment to make proteins more accessible for the extraction.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.1c04400.

Schematic representation of the three strategies adopted (PDF)

AUTHOR INFORMATION

Corresponding Author

Daria Maria Monti − *Department of Chemical Science, University of Naples Federico II, Naples 80126, Italy; Istituto Nazionale di Biostrutture e Biosistemi (INBB), Rome 00136, Italy;* orcid.org/0000-0002-1136-0576; Email: mdmonti@unina.it

Authors

Luigi D'Elia − *Department of Chemical Science, University of Naples Federico II, Naples 80126, Italy;* orcid.org/0000-0002-6557-1715

Paola Imbimbo − *Department of Chemical Science, University of Naples Federico II, Naples 80126, Italy*

Davide Liberti − *Department of Chemical Science, University of Naples Federico II, Naples 80126, Italy*

Francesco Bolinesi − *Department of Biology, University of Naples Federico II, Naples 80126, Italy*

Antonino Pollio − *Department of Biology, University of Naples Federico II, Naples 80126, Italy*

Olga Mangoni − *Department of Biology, University of Naples Federico II, Naples 80126, Italy*

Wim Brilman − *Sustainable Process Technology Group (SPT), Green Energy Initiative, Faculty of Science and Technology, University of Twente, AE Enschede 7500, The Netherlands*

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Giuseppe Olivieri − *Bioprocess Engineering Group, Wageningen University and Research, Wageningen 6700AA, the Netherlands*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssuschemeng.1c04400

Notes

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REFERENCES

(1) Spolaore, P.; Joannis-Cassan, C.; Duran, E.; Isambert, A. Commercial Applications of Microalgae. *J. Biosci. Bioeng.* **2006**, *101*, 87−96.

(2) Zhu, L. Biorefinery as a Promising Approach to Promote Microalgae Industry: An Innovative Framework. *Renewable Sustainable Energy Rev.* **2015**, *41*, 1376−1384.

(3) Ruiz, J.; Olivieri, G.; de Vree, J.; Bosma, R.; Willems, P.; Reith, J. H.; Eppink, M. H. M.; Kleinegris, D. M. M.; Wijffels, R. H.; Barbosa, M. J. Towards Industrial Products from Microalgae. *Energy Environ. Sci.* **2016**, *9*, 3036−3043.

(4) Draaisma, R. B.; Wijffels, R. H.; Slegers, P.; Brentner, L. B.; Roy, A.; Barbosa, M. J.; Elsevier Current Trends April, 2013, *24*, pp 169−177. DOI: 10.1016/j.copbio.2012.09.012.Food Commodities from Microalgae. *Curr. Opin. Biotechnol.*

(5) Dolganyuk, V.; Belova, D.; Babich, O.; Prosekov, A.; Ivanova, S.; Katserov, D.; Patyukov, N.; Sukhikh, S. Microalgae: A Promising Source of Valuable Bioproducts. *Biomolecules* **2020**, *10*, 1153.

(6) Pulz, O.; Gross, W. Valuable Products from Biotechnology of Microalgae. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 635−648.

(7) Shebis, Y.; Iluz, D.; Kinel-Tahan, Y.; Dubinsky, Z.; Yehoshua, Y. Natural Antioxidants: Function and Sources. *Food Nutr. Sci.* **2013**, *04*, 643−649.

(8) Ribeiro, J. S.; Santos, M. J. M. C.; Silva, L. K. R.; Pereira, L. C. L.; Santos, I. A.; da Silva Lannes, S. C.; da Silva, M. V. Natural Antioxidants Used in Meat Products: A Brief Review. *Meat Science*; Elsevier Ltd. February, 2019, *148*, pp 181−188.

(9) Goiris, K.; Muylaert, K.; Fraeye, I.; Foubert, I.; De Brabanter, J.; De Cooman, L. Antioxidant Potential of Microalgae in Relation to Their Phenolic and Carotenoid Content. *J. Appl. Phycol.* **2012**, *24*, 1477−1486.

(10) Lourenço, S. C.; Moldão-Martins, M.; Alves, V. D. Antioxidants of Natural Plant Origins: From Sources to Food Industry Applications. *Molecules* **2019**, *24*, 4132.

(11) Zhao, Z.; Rasool, M. A.; Chen, C.; Ma, S.; Wang, L.; Huang, G. Identification and Screening of Multiple Tropical Microalgal Strains for Antioxidant Activity in Vitro. *Food Biosci.* **2020**, *36*, 100649.

(12) Villalobos-Delgado, L. H.; Mateo, J.; Caro, I.; Leal Ramos, M.-Y.; Mendez, N. G.; Cansino, R. G.; González Mondragón, E. G. *Sustainable Meat Production and Processing*; Elsevier, 2019, pp 207−236. DOI: 10.1016/b978-0-12-814874-7.00011-0.Natural Antioxidants in Fresh and Processed Meat

(13) Liu, X.; Luo, G.; Wang, L.; Yuan, W. Optimization of Antioxidant Extraction from Edible Brown Algae Ascophyllum nodosum Using Response Surface Methodology. *Food Bioprod. Process.* **2019**, *114*, 205−215.

(14) Sansone, C.; Brunet, C. Promises and Challenges of Microalgal Antioxidant Production. *Antioxidants* **2019**, *8*, 199.

(15) Jacob-Lopes, E.; Maroneze, M. M.; Deprá, M. C.; Sartori, R. B.; Dias, R. R.; Zepka, L. Q. *Current Opinion in Food Science*; Elsevier Ltd February, 2019, *25*, pp 1−7. DOI: 10.1016/j.cofs.2018.12.003.Bioactive Food Compounds from Microalgae: An Innovative Framework on Industrial Biorefineries

(16) Hidalgo, G.-I.; Almajano, M. Red Fruits: Extraction of Antioxidants, Phenolic Content, and Radical Scavenging Determination: A Review. *Antioxidants* **2017**, *6*, 7.

(17) Selvamuthukumaran, M.; Shi, J. Recent Advances in Extraction of Antioxidants from Plant By-Products Processing Industries. *Food Quality and Safety*; Oxford University Press March, 2017, *1*, pp 61−81.

(18) Mäki-Arvela, P.; Hachemi, I.; Murzin, D. Y. Comparative Study of the Extraction Methods for Recovery of Carotenoids from Algae: Extraction Kinetics and Effect of Different Extraction Parameters. *J. Chem. Technol. Biotechnol.* **2014**, *89*, 1607−1626.

(19) Imbimbo, P.; D'Elia, L.; Liberti, D.; Olivieri, G.; Monti, D. M. Towards Green Extraction Methods from Microalgae Learning from the Classics. *Applied Microbiology and Biotechnology*; Springer Science and Business Media Deutschland GmbH November, 2020, Vol.*104*; pp 9067−9077.

(20) Du, Y.; Schuur, B.; Kersten, S. R. A.; Brilman, D. W. F. Opportunities for Switchable Solvents for Lipid Extraction from Wet Algal Biomass: An Energy Evaluation. *Algal Res.* **2015**, *11*, 271−283.

(21) Peng, L.; Fu, D.; Chu, H.; Wang, Z.; Qi, H. Biofuel Production from Microalgae: A Review. *Environ. Chem. Lett.* **2020**, *18*, 285−297.

(22) Liu, H.; Huang, W.-C.; Guo, N.; Mao, X. Application of Secondary Amine Switchable Hydrophilicity Solvents for Astaxanthin Extraction from Wet Haematococcus pluvialis. *Algal Res.* **2020**, *48*, 101892.

(23) Sanzo, G.; Mehariya, S.; Martino, M.; Larocca, V.; Casella, P.; Chianese, S.; Musmarra, D.; Balducchi, R.; Molino, A. Supercritical Carbon Dioxide Extraction of Astaxanthin, Lutein, and Fatty Acids from Haematococcus pluvialis Microalgae. *Mar. Drugs* **2018**, *16*, 334.

(24) Imbimbo, P.; Bueno, M.; D'Elia, L.; Pollio, A.; Ibañez, E.; Olivieri, G.; Monti, D. M. Green Compressed Fluid Technologies To Extract Antioxidants and Lipids from Galdieria phlegrea in a Biorefinery Approach. *ACS Sustainable Chem. Eng.* **2020**, *8*, 2939−2947.

(25) Pawliszyn, J. Kinetic Model of Supercritical Fluid Extraction. *J. Chromatogr. Sci.* **1993**, *31*, 31−37.

(26) Jessop, P. G.; Heldebrant, D. J.; Li, X.; Eckert, C. A.; Liotta, C. L. Reversible nonpolar-to-polar solvent. *Nature* **2005**, *436*, 1102.

(27) Phan, L.; Andreatta, J. R.; Horvey, L. K.; Edie, C. F.; Luco, A.-L.; Mirchandani, A.; Darensbourg, D. J.; Jessop, P. G. *Switchable-Polarity Solvents Prepared with a Single Liquid Component*; ACS Publications, 2007.

(28) Du, Y.; Schuur, B.; Samorì, C.; Tagliavini, E.; Brilman, D. W. F. Secondary Amines as Switchable Solvents for Lipid Extraction from Non-Broken Microalgae. *Bioresour. Technol.* **2013**, *149*, 253−260.

(29) Du, Y.; Schuur, B.; Brilman, D. W. F. Maximizing Lipid Yield in Neochloris oleoabundans Algae Extraction by Stressing and Using Multiple Extraction Stages with N-Ethylbutylamine as Switchable Solvent. *Ind. Eng. Chem. Res.* **2017**, *56*, 8073−8080.

(30) Cicci, A.; Sed, G.; Jessop, P. G.; Bravi, M. Circular Extraction: An Innovative Use of Switchable Solvents for the Biomass Biorefinery. *Green Chem.* **2018**, *20*, 3908−3911.

(31) Du, Y.; Cyprichová, V.; Hoppe, K.; Schuur, B.; Brilman, W. Process Evaluation of Swing Strategies to Recover N-Ethylbutylamine after Wet Lipid Extraction from Microalgae. *Sep. Purif. Technol.* **2020**, *233*, 115819.

(32) Ismail, M.; Al-Zuhair, S. Thermo-Responsive Switchable Solvents for Simultaneous Microalgae Cell Disruption, Oil Extraction-Reaction, and Product Separation for Biodiesel Production. *Biocatal. Agric. Biotechnol.* **2020**, *26*, 101667.

(33) Davies, F. K.; Fricker, A. D.; Robins, M. M.; Dempster, T. A.; McGowen, J.; Charania, M.; Beliaev, A. S.; Lindemann, S. R.; Posewitz, M. C. Microbiota Associated with the Large-Scale Outdoor Cultivation of the Cyanobacterium Synechococcus sp. *Algal Res.* **2021**, *58*, 102382.

(34) D'Elia, L.; Del Mondo, A.; Santoro, M.; De Natale, A.; Pinto, G.; Pollio, A. Microorganisms from Harsh and Extreme Environments: A Collection of Living Strains at ACUF (Naples, Italy). *Ecol. Quest.* **2018**, *29*, 63−74.

(35) Wu, H.-L.; Wang, G.-H.; Xiang, W.-Z.; Li, T.; He, H. Stability and Antioxidant Activity of Food-Grade Phycocyanin Isolated from Spirulina platensis. *Int. J. Food Prop.* **2016**, *19*, 2349−2362.

(36) Bligh, E. G.; Dyer, W. J. A Rapid Method of Total Lipid Extraction and Purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911−917.

(37) Rigano, M. M.; Raiola, A.; Tenore, G. C.; Monti, D. M.; Del Giudice, R.; Frusciante, L.; Barone, A. Quantitative Trait Loci Pyramiding Can Improve the Nutritional Potential of Tomato

(Solanum lycopersicum) Fruits. *J. Agric. Food Chem.* **2014**, *62*, 11519−11527.
(38) Petruk, G.; Gifuni, I.; Illiano, A.; Roxo, M.; Pinto, G.; Amoresano, A.; Marzocchella, A.; Piccoli, R.; Wink, M.; Olivieri, G.; et al. Simultaneous Production of Antioxidants and Starch from the Microalga Chlorella sorokiniana. *Algal Res.* **2018**, *34*, 164−174.
(39) Ferraro, G.; Imbimbo, P.; Marseglia, A.; Illiano, A.; Fontanarosa, C.; Amoresano, A.; Olivieri, G.; Pollio, A.; Monti, D. M.; Merlino, A. A Thermophilic C-Phycocyanin with Unprecedented Biophysical and Biochemical Properties. *Int. J. Biol. Macromol.* **2020**, *150*, 38−51.
(40) Petruk, G.; Raiola, A.; Del Giudice, R.; Barone, A.; Frusciante, L.; Rigano, M. M.; Monti, D. M. An Ascorbic Acid-Enriched Tomato Genotype to Fight UVA-Induced Oxidative Stress in Normal Human Keratinocytes. *J. Photochem. Photobiol. B Biol.* **2016**, *163*, 284−289.
(41) Vidussi, F.; Claustre, H.; Bustillos-Guzmàn, J.; Cailliau, C.; Marty, J.-C. Determination of Chlorophylls and Carotenoids of Marine Phytoplankton: Separation of Chlorophyll a from Divinylchlorophyll a and Zeaxanthin from Lutein. *J. Plankton Res.* **1996**, *18*, 2377−2382.
(42) Mantoura, R. F. C.; Repeta, D. J. *Calibration Methods for HPLC*; Jeffrey, S. W.; Mantoura, R. F. C.; Wright, S. W., eds.; UNESCO Publ., Paris: 1997, 407−428.
(43) Callieri, C. Synechococcus Plasticity under Environmental Changes. *FEMS Microbiol. Lett.* **2017**, *364*, 1−8.
(44) Hoiczyk, E.; Hansel, A. Cyanobacterial Cell Walls: News from an Unusual Prokaryotic Envelope. *J. Bacteriol.* **2000**, *182*, 1191−1199.
(45) Samorì, C.; López Barreiro, D.; Vet, R.; Pezzolesi, L.; Brilman, D. W. F.; Galletti, P.; Tagliavini, E. Effective Lipid Extraction from Algae Cultures Using Switchable Solvents. *Green Chem.* **2013**, *15*, 353−356.
(46) Fabrowska, J.; Messyasz, B.; Szyling, J.; Walkowiak, J.; Łęska, B. Isolation of Chlorophylls and Carotenoids from Freshwater Algae Using Different Extraction Methods. *Phycol. Res.* **2018**, *66*, 52−57.
(47) Assunção, M. F. G.; Amaral, R.; Martins, C. B.; Ferreira, J. D.; Ressurreição, S.; Santos, S. D.; Varejão, J. M. T. B.; Santos, L. M. A. Screening Microalgae as Potential Sources of Antioxidants. *J. Appl. Phycol.* **2017**, *29*, 865−877.
(48) Guedes, A.; Gião, M.; Seabra, R.; Ferreira, A.; Tamagnini, P.; Moradas-Ferreira, P.; Malcata, F. Evaluation of the Antioxidant Activity of Cell Extracts from Microalgae. *Mar. Drugs* **2013**, *11*, 1256−1270.

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