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Carotenoids, Chlorophylls and Phycocyanin from Spirulina. Supercritical CO_2 and Water Extraction Methods for Added Value Products Cascade

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20th November, 2019

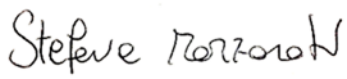
Sub: Re-Submission of a research paper to Green Chemistry
Manuscript ID: GC-ART-09-2019-003292.R1

Dear Editor,

Kindly find enclosed the research paper titled "Carotenoids, Chlorophylls and Phycocyanin from Spirulina. Supercritical CO_2 and Water Extraction Methods for Added Value Products Cascade" for publication in your esteemed journal "Green Chemistry".
Authors: Stefania Marzorati, Andrea Schievano, Antonio Idà, Luisella Verotta.
We have made the revision that the reviewer pointed out in his comments.
In the manuscript, the revised text is highlighted in RED color so that it will be easier for you to notice the changes.
We are grateful for the time spent to guarantee the improvement of our manuscript in the present form and we thank you for accepting our paper.

Best regards,

(Stefania Marzorati)



Referee: 3

Comments to the Author

The authors have revised and greatly improved the manuscript addressing the different observed weaknesses. Questions on extraction process steps, time, optimization, concentration, etc., have been clarified, and a new section on “Overview on Extractions” added in the article. Manuscript is now ready for publication in Green Chemistry and I recommend to accept it without further changes.

We thank the reviewer for his/her time and for the further time spent on our manuscript

A simple minor correction would be to change the symbol “-“ in “Table S1 – Optimization trials in supercritical CO2 for extraction of carotenoids and chlorophylls from Spirulina”, in the column for “% etanol” for a “0”, when 0% of ethanol was used, as this symbol “-“ means that no extract was obtained” in the next two columns of this Table.

Done

ARTICLE

Carotenoids, Chlorophylls and Phycocyanin from Spirulina. Supercritical CO_2 and Water Extraction Methods for Added Value Products Cascade

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In the last decade, the cyanobacterium Spirulina has gained high commercial interest as a food supplement, mainly due to its high content in proteins, but also in pigments, such as carotenoids, chlorophylls and phycocyanins. In particular, phycocyanin has been widely considered as a precious food-dye, because of its protein-based structure and rare intense-blue color. Different strategies were developed for the isolation and purification of phycocyanin. The main drawback of such processes is that carotenoids and chlorophylls are generally wasted, together with the residual biomass. In this work, a different approach is proposed, suggesting an integrated pigments extraction chain. The body of the strategy involves two consecutive steps of supercritical-CO_2 extraction of carotenoids and chlorophylls, before phycocianin extraction. The total carotenoids, chlorophyll a and chlorophyll b content in the extracts were equal to 3.5 ± 0.2 mg g^{-1} (by dry Spirulina weight), 5.7 ± 0.2 mg g^{-1} and 3.4 ± 0.3 mg g^{-1}, respectively. The biomass residue, exhausted in terms of carotenoids and chlorophylls, was then extracted in water to yield phycocyanin. Consecutive steps were developed in order to ehance phycocyanin purity, including electrocoagulation, dialysis and protein salting-out. These processes yielded 250 mg g^{-1} of phycocyanin (by dry Spirulina weight). A potentially scalable strategy to obtain the blue pigment with high purity (A_{620}/A_{280}=2.2) was developed. The practical application of the extracted blue phycocyanin pigment as cotton-based tissues colorant was also experimented.

Introduction

Already in the ninth century, Kanem's empire in Chad discovered the benefits of *Arthrospira platensis* [1,2]. Nowadays, blue-green algae of the genus *Arthrospira*, commonly known as Spirulina, are commercially grown all around the world for their nutritional properties [3]. The popularity of Spirulina as a food supplement is mainly due to its high protein content (up to about 70% by dry weight) and its richness in minerals, vitamins and provitamins, phytochemicals, essential amino acids, fibers and pigments [4]. About this latter point, Spirulina contains distinctive natural orange, green and blue pigments, namely carotenoids, chlorophylls and phycocyanins, respectively. Spirulina commercialization has aligned year by year with consumer awareness regarding the importance of natural agents [5]. Obtaining pigments from natural sources is in fact a well-known strategy, due to legislation restrictions in terms of synthetic dyes [6].

Carotenoids are natural lipophilic pigments responsible for the red, yellow, and orange colors found in many living organisms. They are popular as food and feed dyes and flavorings, and in nutritional supplements [5]. Moreover, Spirulina may contain about 2 wt % of chlorophylls, ten times more than in ordinary terrestrial plants and hence accounting for an enhanced photosynthetic conversion efficiency, equal to 8-10%, if compared to land plants, characterized just by 3% conversion efficiency [7]. Chlorophylls derivatives, being heat-, light-, acids- and bases- stable, are potentially applicable in food, cosmetics, and pharmaceutical fields as additives or colorants [8]. According to many studies, the free radical-scavenging and strong antioxidant activity of Spirulina, are mostly attributable to the blue-colored phycocyanin. The extraction, isolation and purification of phycocyanin have been the focus for many years, resulting in diverse strategies leading to different results [4,9–14].

Many works in the literature have been focusing specifically on phycocyanin extraction from Spirulina biomass [4,9–12,14–18] due to phycocyanin high commercial value [19,20]. The main drawback of these processes is the discard of the remaining added-value pigment content, as carotenoids and chlorophylls, wasted in the biomass residues.

In this work, a different approach is proposed, suggesting an extraction chain that only finally leads to phycocyanin. The body of the strategy involves a double-step extraction of carotenoids and chlorophylls using supercritical-CO_2 ($scCO_2$), with minimized modifications on the biomass. The biomass residue, exhausted in terms of lipophilic compounds and chlorophylls, is ready to undergo extraction of phycocyanin.

The absence of any heating or destructive step allowed the reuse of the $scCO_2$-treated Spirulina biomass for a further

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extraction step in aqueous media, aiming at obtaining phycocyanin. On the basis of the recent and past literature on the topic, a strategy to yield the blue pigment at high purity was finally developed, keeping an eye on the scalability of the overall process in terms of costs and time savings [9,21,22]. The practical application of the extracted blue phycocyanin pigment as cotton-based tissues colorant was also experimented.

Materials and Methods

Chemicals and starting materials

Milli-Q water was obtained from an Elix Essential Millipore SAS water system. NH_4Cl (BioUltra, for molecular biology, ≥99.5%), $(NH_4)_2SO_4$, $KAl(SO_4)\cdot 12H_2O$, Na_2CO_3 and ethanol (HPLC grade) were purchased from Sigma-Aldrich. Carbon dioxide tube (CO_2 purity 5.0) was from Sapio, Italy. Dialysis tubing cellulose membrane were purchased from Sigma-Aldrich. All the extracts have been analyses as soon as they were obtained and then kept at 4°C in the dark.

Spirulina platensis dried flakes (Spireat®) were supplied from Algaria slr (Italy) and stored at 4°C in the dark. Phycocyanin from Sigma-Aldrich (12.3 mg ml^{-1}, suspended in 150 mM sodium phosphate, 60% ammonium sulfate, 1 mM EDTA, 1 mM sodium azide, pH 7.0), labelled as PC_{std}, was used as the standard to build a calibration line in the PC_{std} concentration range: 20-150 µg ml^{-1} (water solutions).

Supercritical CO_2 extractions of carotenoids and chlorophylls

Supercritical fluid extractions were performed using the pilot unit SFT110XW System supplied by Supercritical Fluid Technologies, Inc. (USA). It consists in a 100 cm^3 stainless steel extractor inserted in an oven, SFT10 CO_2 pump with a Peltier Cooler, a Waters 515 HPLC pump for the co-solvent and a collection vessel.

Spirulina platensis dried flakes were grounded into powder (about 50 µm particle size) by a kitchen blender for 1 min at the maximum velocity. To avoid powder heating during blending and a consequent degradation of thermolabile species, liquid nitrogen was added.
The extraction vessel was filled with 31.4 g of spirulina powder. The system was sealed, the oven and restrictor block temperatures were set at 45°C and 70°C, respectively. Fig. 1 displays the picture of the $scCO_2$ extraction instrument. Some experimental trials, reported in Table S1, based on literature works [23,24] were performed in order to assess the best procedure. This was performed in consecutive steps, keeping the biomass inside the vessel. A larger scale extraction would need the support of a mathematical model, in order to really optimize the parameters [25].

Carotenoids extraction by $scCO_2$: The pump pressurized the sample vessel at 300 bar at a flow rate of 15 ml min^{-1}. Once the set pressure was reached, after a static period of 30 min, the valves were opened to collect the sample for 1 h in dynamic condition (CO_2 flow rate of 15 ml min^{-1}). The vessel was then depressurized. The biomass was kept inside the vessel for the next extraction of chlorophylls.

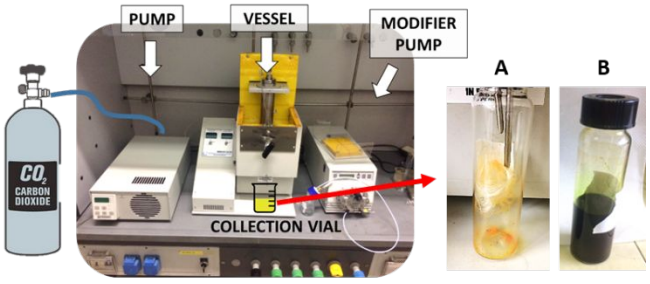

Figure 1 - Pictures of the instrumental schematic (left) and of collection flasks during (A) $scCO_2$ extraction and (B) during $scCO_2$ + 10% ethanol extraction.

Chlorophylls extraction by $scCO_2$ + 10% EtOH: 10 ml of ethanol were loaded in the vessel before pressurization at 300 bar at a flow rate of 15 ml min^{-1}. Once the set pressure was reached, after a static period of 30 min, valves were opened to collect the sample for 1 h in dynamic condition. During the dynamic extraction, an ethanol flow rate of 1.5 ml min^{-1} was maintained (CO_2 flow rate of 13.5 ml min^{-1}). The vessel was then depressurized and the residual biomass was collected.

The following equations (1), (2) and (3), proposed by Wellburn [26], were used to determine the concentration of total carotenoids, chlorophyll a and chlorophyll b in the samples from visible spectra acquired through a Varian Cary 50 Bio UV-Vis:

$$C_{total\ carotenoids}\ (\mu g\ ml^{-1}) = (1000\ A_{470} - 2.86\ C_{chlorophyll\ a} - 129.2\ C_{chlorophyll\ b}) / 221 \quad (1)$$

where A_{470} is the absorbance at 470 nm, and $C_{chlorophyll\ a}$ and $C_{chlorophyll\ b}$ are the concentrations of chlorophyll a and b, respectively, calculated by:

$$C_{chlorophyll\ a}\ (\mu g\ ml^{-1}) = 15.65\ A_{666} - 7.34\ A_{653} \quad (2)$$

$$C_{chlorophyll\ b}\ (\mu g\ ml^{-1}) = 27.05\ A_{653} - 11.21\ A_{666} \quad (3)$$

where A_{666} and A_{653} are the absorbance values at 666 nm and 653 nm, respectively. These equations are valid when the solvent is methanol and the spectrophotometer has a resolution of 1-4 nm.

UPLC-MS methods for carotenoids identification

The $scCO_2$ extract composition was analyzed by UPLC using a Dionex Ultimate 300 equipped with a UV detector. The separation was carried out through a ACQUITY UPLC® BEH C18 column (1.7 µm, 2.1x50mm). The mobile phase was a mixture of acetonitrile:methanol=70:30, at a flow rate of 0.2 ml min^{-1}, in isocratic mode for 10 min. 10 µl of a methanolic solution of the extract were filtered (0.2 µm nylon filters), injected and monitored at 445 nm. Chromatographic separation was followed by mass spectrometry (LCQ Fleet Thermofisher). Positive electrospray mode was used for ionization of molecules with capillary voltage at 80 V, at a capillary temperature of 275°C. The heater temperature was set at 80°C, the gas flow rate was 35 (arb) and the spray voltage was 5.50 kV. The monitored mass range was from m/z 50 to 1000. Before sample injection, mass spectrometry parameters were optimized using a standard of beta-carotene (purity > 97%) from Sigma.

Phycocyanin extraction

30.0 g of the biomass residue in the vessel, post $scCO_2$ extraction, underwent phycocyanin extraction optimization trials in water (see Supporting information).
Then, in order to eliminate the suspended biomass residues, an electrocoagulation method was performed on 50 ml of the prepared suspension by immersing two Al electrodes (3 cm x 3 cm) at a distance of 3 cm inside the liquid. A potential difference of 14 V (0.8 A) was set between electrodes for 2 min by a power supply (Tech Star®, TPR3005-2D). An ice bath was used to avoid temperature increase. A cotton tissue was enough to filter away the vegetal residue out of the blue solution.
The blue filtrate was forced to pass through 12 mesh paper by a peristaltic pump in order to get rid of the remaining cells debris suspended in the solution.

Phycocyanin purification methods

Dialysis: A lab-scale dialysis tubing cellulose membrane with a cutoff at 15 kDa was employed. The membrane was pre-treated as follows: tubes were washed in running water for 3-4 hours, then with hot water (60°C) for 2 minutes, followed by acidification with a 0.2% (v/v) solution of sulfuric acid, then rinsed with hot water to remove the acid.
1 ml of sample solution was load inside the membrane. 50 ml of milli-Q water were place in external contact with the dialysis membrane and replaced three times every 24 hours. External solutions and the solution inside the dialysis membrane were lyophilized (Edwards, Pirani 1001).

Ammonium sulfate precipitation: the previously lyophilized powder was dissolved in the minimum amount of milli-Q water (45 mg ml^{-1}). Ammonium sulfate powder was slowly added to the solution, until it reached 24%, 39% or 50% of its saturation, with continuous stirring. After 2 h standing in the fridge, the solution got turbid and it was centrifuged for 30 min at 6000 rpm (Hettich Rotofix 32A). A blue pellet was collected while the light blue supernatant was discarded.

Phycocyanin purity determination

The purity of phycocyanin was evaluated spectrophotometrically, on the basis of the ratio between phycocyanin absorbance at 620 nm (A_{620}) and absorbance from aromatic amino acids in all proteins at 280 nm (A_{280}). As described by Rito-Palomares *et al.* [19], phycocyanin preparations with A_{620}/A_{280} lower than 0.7 are considered food grade, while A_{620}/A_{280} between 0.7 and 3.9 are reagent grade and A_{620}/A_{280} greater than 4.0 are considered of analytical grade.

Application of Phycocyanin extract as blue dye for tissues

Two different cotton fibers have been experimented: commercial sterile gauzes and a cotton bed linen. In separate beckers, a weighted amount of each tissue was immersed in milli-Q water containing 20 % w/w of $KAl(SO_4)\cdot 12H_2O$ (calculated on the basis of the weight of the tissue) as a mordant and 6% w/w of Na_2CO_3. The temperature was increased up to 100°C and kept for 1 h. Then, tissues were rapidly rinsed with milli-Q water and dried at room temperature.

Six weighted pieces of each type of tissue were put in contact for 1 h with different amounts of phycocyanin in water solution to build adsorption isotherms (from 24 to 1 $\mu g_{phycocyanin}\ g^{-1}_{tissue}$). After this time, tissues were extracted from the solution, rinsed and dried at room temperature. The absorbance at t=0 and t=1 h was measured at 620 nm for each solution and absorption isotherms have been built. TIMCAL ENSACO activated carbon was employed as a reference adsorbing material.
Colored tissues have been rinsed with tap water after 1, 2 and 3 weeks after the dying procedure.

Results and Discussion

sc-CO_2 extraction of carotenoids

High contents of carotenoids (β-carotene, cryptoxanthin, zeaxanthin, among others) were reported for Spirulina, as compared to other natural sources [27]. Their relative percentages may vary with Spirulina growth and environmental conditions, processing, etc.. El-Baky *et al.* grew Spirulina in the presence of different concentration of nitrogen and varying the ionic strength of the growth medium. These factors were responsible for variations in the relative percentages of carotenoids [28]. $scCO_2$ has been successfully used for isolation of carotenoids from various fruit and vegetables matrices in the past and more recent literature [29–32]. This technique is able to provide extracts totally free of organic solvents (avoiding oxygen contact) at relatively low critical temperature and pressure. Since various parameters potentially affect the $scCO_2$ extraction process, the optimization of the experimental conditions represents a critical step in the development of the method. Based on preliminary experiments (reported in Table S1) and literature data [23,24], some operational parameters were optimized. 300 bar and 45°C were set as optimized process parameters. Pressures lower than 300 bar were not high enough to yield carotenoids-enriched extracts. Temperature increase from 45°C to 55°C did not allow any significant yield improvement. No organic modifier was added since $scCO_2$ alone displays a solvating power similar to n-hexane, hence able to dissolve lipophilic compounds such as carotenoids [33].
After the static period, the deep orange extract was collected in a weighted flask in dynamic conditions. The extraction has been performed under supercritical CO_2 and extracts have been analysed as soon as they were obtained, implying that oxygen has never influenced the biomass or the extracts. This is also valid for light and temperature, since extracts have been stored at 4°C in the dark.
Fig. 1A shows the picture of the obtained extract: a deep orange viscous extract (in the presence of red, darker spots) was collected after 30 min, corresponding to an extraction yield of around 0.5%. The visible spectrum of carotenoids-enriched extract is displayed in Fig. 2A. By applying the previously mentioned equations (1), (2) and (3), C_a and C_b were approximated to zero, in agreement with the hypothesis that no chlorophylls were extracted under these conditions. The concentration of carotenoids was equal to 3.5 ± 0.2 mg g^{-1} of dry spirulina biomass. These results are in agreement, in terms

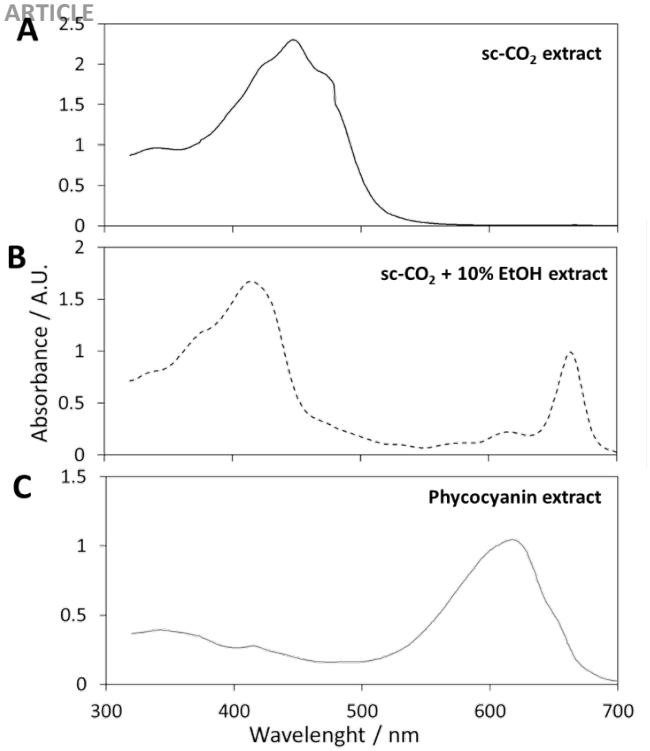

Figure 2 - *A: visible spectrum of the carotenoids extract; B: visible spectrum of the chlorophylls extract; C: phycocyanin extract.*

of orders of magnitude, with Careri *et al.* They employed supercritical fluid extraction of carotenoids in *Spirulina pacifica* algae [34]. 1.7 mg g^{-1} of carotenoids (zeaxanthin, β-cryptoxanthin and β-carotene) could be extracted from the biomass through a chemiometric approach. The same authors, via comparison with a standard method based on conventional solvent extraction, obtained similar values, confirming the efficacy of the selected supercritical conditions. Similar results, assessing the suitability of $scCO_2$ to extract carotenoids, were obtained by cías-Sánchez *et al*.[35]. They performed a conventional methanol extraction and their results were in agreement with what obtained though $scCO_2$ optimization trails.

The composition of the carotenoids-enriched extract has been further investigated by UPLC-MS analysis. The UV-Vis chromatogram is displayed in Fig. 3. The identified peaks, highlighted in the figure, corresponded to zeaxanthin (retention time of 3.3 min), β-cryptoxanthin (retention time of 4.6 min) and β-carotene (retention time of 7.4 min). The assignments were confirmed by mass spectrometry results. Peak 1 is characterized by two signals at m/z= 551.5 and m/z= 583.6 corresponding to zeaxanthin $[M\text{-}H_2O+H]^+$ and $[M\text{-}H_2O+MeOH+H]^+$ species. Regarding peak 2, the main signal at m/z= 552.4, corresponds to the β-cryptoxanthin $[M]^{+\bullet}$ molecular species; about peak 3, the main signal at m/z=536.4 corresponds to β-carotene $[M]^{+\bullet}$, the secondary signal at m/z=569.4 might correspond to the coordination of methanol molecule. Results are in agreement with literature data [36,37].

$sc\text{-}CO_2$ extraction of chlorophylls

Usually, chlorophyll a is extracted from raw materials based on solvent extraction [38]. Although the conventional process has been well investigated, drawbacks like the high extraction temperature and long extraction time, large amount of solvent, low extraction yield, claim the need for alternative techniques. Nevertheless, only few investigations are available on the extraction of chlorophyll a using $scCO_2$. Some works in the literature employed a co-solvent to enhance the supercritical fluid solvating power towards the molecule [39,40].

Carotenoids-free spirulina biomass underwent $scCO_2$ extraction with the addition of ethanol as co-solvent, to enhance the extraction yield towards more polar compounds.

The experimental parameters such as temperature and pressure, as well as the static and dynamic periods in supercritical conditions, have been kept equal to the $scCO_2$ carotenoids extraction process. This approach was adopted to shift the target compounds to be extracted from carotenoids to chlorophylls. 10% of ethanol was selected as co-solvent amount. A further increase of the ethanol percentage did not improve the extraction yield, meaning that chlorophylls have been completely extracted by using 10% of co-solvent (Table S1). The collected extract solution (in ethanol) was dark green in color, as displayed in Fig. 1B. A visible spectrum was

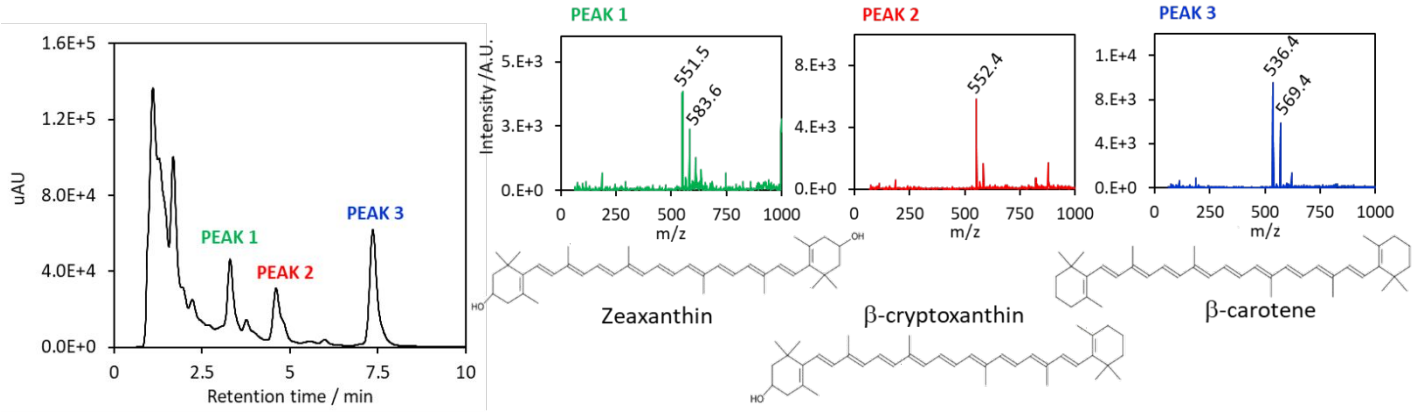

Figure 3 – *Left: UV-Vis chromatogram of the carotenoids-containing extract by $scCO_2$. Left: mass spectroscopy analyses and molecular structures of assigned carotenoids.*

Table 1 - Carotenoids and chlorophylls content calculated by equations (1), (2) and (3). The purity was calculated as the ratio between the mass of specific compound and the total mass of the extract.

$scCO_2$ operative conditions	**$C_{total\ carotenoids}$ / mg g^{-1}** (by dry Spirulina weight)	**$C_{chlorophyll\ a}$ / mg g^{-1}**	**$C_{chlorophyll\ b}$ / mg g^{-1}**
T_{vessel} = 45°C p = 300 bar	3.5 ± 0.2 (86% purity)	-	-
T_{vessel} = 45°C p = 300 bar + 10% ethanol	-	5.7 ± 0.2 (63% purity)	3.4 ± 0.3 (37% purity)

recorded and it is displayed in Fig. 2B. Chlorophyll a displays maxima at wavelengths of 400-450 nm and 650-700 nm, while the absorption spectra of chlorophyll b are at 450-500 nm and 600-650 nm [41].

The total concentration of carotenoids and chlorophylls was determined by measuring the absorbance of a weighted amount of sample in the range 320-700 nm and applying the Wellburn equation. As expected, this extract did not contain any carotenoid, and $C_{total\ carotenoids}$ was approximated to zero. This interesting result confirmed the high selectivity of the method.

Chlorophyll a content was calculated to be equal to 5.7 ± 0.2 mg g^{-1} (by dry Spirulina weight). Chlorophyll b content was calculated to be equal to 3.4 ± 0.3 mg g^{-1}.

Tong *et al.* run single-factor and response surface analysis experiments for the supercritical fluid extraction of chlorophyll from *Spirulina platensis*. Our work is in agreement with their results: they found out the necessity of adding a modifier in order to obtain the desired product. Their comparison experiments demonstrated that $scCO_2$ extraction with modifier was more efficient than conventional solvent methods. Their results are also in numerical agreement with the present work (their extraction yield was 6.84 mg g^{-1}) [39]. Macías-Sánchez *et al.*, recovered only low amount of chlorophylls, if compared to results obtained in this work, due to the absence of any modifier in the extraction process [40].

All the mentioned works already published in the literature were specifically focused on a class of target compounds, such as chlorophylls or carotenoids. Among them, methanol was also suggested as a modifier. However, solvents like methanol, prohibited in industrial processes, have been discarded in this work in the view of potential scale-up applications.

Table 1 summarizes the results in terms of carotenoids and chlorophyll a and b content from Sprirulina biomass by supercritical carbon dioxide (first) with and (then) without ethanol as modifier. Results confirm the selectivity of the technique: supercritical CO_2 alone was able to selectively extract carotenoids, while the addition of a co-solvent under the same operational conditions provided exclusively chlorophylls (a and b). By adjusting successive operational parameters, the technique is able to extract one by one valuable compounds from the biomass, without the need for purification steps.

For carotenoids and chlorophyll extraction, supercritical CO_2 ($scCO_2$) has to be preferred for its immediate advantages over traditional solvent-based techniques [42].

It allows efficient and selective extractions of lipophilic compounds, with no need of concentration steps, providing high purity products and avoiding the use of potentially harmful organic solvents [42].

The addition of a co-solvent (for example ethanol) in relatively low mixing ratio with $scCO_2$ is able to modify its polarity, allowing the extraction of more polar molecules [43,44]. Moreover, operative temperatures are low enough to avoid the degradation of thermolable substances. Finally, CO_2 is easily removed from the solid at ambient conditions and/or, on industrial scale, can be recovered through specific apparatus for its fresh reuse. Literature results show a substantial advantage with respect to a conventional extraction in terms of easy recovery, selectivity, compounds stability, time, and overall energy saving [23].

Extraction of Phycocyanin

Starting from a commercial standard product (PC_{std}), a calibration line was built in order to calculate the phycocyanin content in further extraction solutions. The best fit of experimental data in the plot "A_{620} *vs* PC_{std} concentration" (A_{620} is the absorbance at 620 nm and PC_{std} concentration is in μg ml^{-1}) was, as expected, a straight-line, represented by the following mathematical equation: y = 0.0086x - 0.0246, resulting in a R^2 equal to 0.999. The slope *m* and intercept *q* of the regression line with their respective standard deviations were (0.0086 ± 0.0001) and (-0.025 ± 0.006), respectively. The calculated equation of the regression line was then employed to determine the phycocyanin concentration in sample solutions (see Fig. S10 for experimental data).

Fundamental units of phycocyanin protein are α and β subunits, arranged in an (αβ) protomers. These structures, in turn, can be associated in trimers $(\alpha\ \beta)_3$ and hexamers $(\alpha\ \beta)_6$. For this reason, phycocyanin molecular weight lies between 18kDa [45] (considering single units) and 210kDa [9] (considering the hexamers). By using an average of the most frequently encountered molar mass of phycocyanin equal to 120kDa, the molar extinction coefficient ε was calculated to be equal to $1.0*10^6$ $cm^{-1}M^{-1}$ at 620 nm, in agreement with the literature [46].

The two-steps $scCO_2$ process, yielding first carotenoids and then chlorophylls, was able to keep the phycocyanin content of Spirulina biomass unchanged. The grinded powder in fact was deprived from its orange and green pigments, appearing, after $scCO_2$, enriched in blue color, mainly due to the removal of chlorophylls, responsible of its typical green aspect. Fig. 4A shows Spirulina powder before and after the $scCO_2$ extraction step.

Spirulina biomass, recovered after $scCO_2$ was then re-used to extract phycocyanin by aqueous methods. Optimization trials are reported in the Supporting Information section. Briefly, water stirring and sonication have been first experimented and compared: ultrasounds were found to excessively disrupt cells, hence yielding less pure extracts (see Fig. S4). Freezing and thawing method did not provide any advantage compared to water stirring and hence abandoned, being less simple and

more time-consuming. pH variations seemed to be the key point: stirring the suspension in ammonium chloride solution not only enhanced the yield (if compared to alkaline pH solutions), but was also able to provide purer extracts, lacking in the species adsorbing between 400 and 500 nm (Fig. S7) that could badly affect the intense blue color of the phycocyanin solution.

For these reasons, turbine stirring for 2 hours in 0.2 M NH_4Cl of 20 g l^{-1} biomass dispersion was set as the best extraction strategy. Ammonium chloride was found to be an effective and suitable salt solution for enhancing the phycocyanin purity in crude extracts. Furthermore, it reduces the extraction cost because it is cheaper than other salts [11].

Separation of suspended solids

After extraction, in order to develop a scalable procedure that could help in removing the great part of suspended solid residue, one of the most cost-effective techniques in algae harvesting was tested: electrocoagulation. In electrocoagulation, flocculation is induced through electrolytic release of metal ions from a sacrificial anode and avoids contaminations of anions. Operating costs of such technique are typically below 0.04 € m^3 [47]. The key for this technology is the application of current between a sacrificial anode (in this case aluminum) and an inert cathode (made of aluminum as well as the anode). Aluminum ions are released from the anode, able to neutralize the negative surface charge of the alga. In addition, oxygen and hydrogen bubbles are also produced by the electric current, thus promoting turbulence and helping the flocculation process. Electrocoagulation was indeed effective and the majority of cells fragments could be easily removed simply by filtration on a gauze.

Electrocoagulation was not however exhaustive in removing the smallest fraction of particulate residues of the cells walls from the solution. Depending on the specific use of the pigment (for example if the pigment application is related to food printing, where a channeling inside a needle is necessary), a further filtration might be necessary. Notwithstanding the large number of publications in the field of phycocyanin extraction, filtration topic has never been comprehensively faced by the literature. Many works employed ultrafiltration [16,19,48] on lab-scale experiments. This method is however expensive and hence hardly applicable on large scale. Here, the balance between effectiveness, large scale applicability and costs was identified in 12-mesh papers filtration procedure under pressure. Paper filters are in fact the cheapest disposable material in commerce and can be easily replaced when clogged.

After filtration, the obtained solution showed a phycocyanin content and purity of, respectively 82 mg g^{-1} (by dry weight) and A_{620}/A_{280}=1.2. A visible spectrum of the phycocyanin solution is displayed in Fig. 2C.

Table 2 - Phycocyanin content and purity (A_{620}/A_{280}) as obtained by salting-out procedure.

% saturation $(NH_4)_2SO_4$	$PC_{content}$ / mg $_{PC}$ g^{-1} (by dry weight)	A_{620}/A_{280}
24	217	1.9
39	250	2.2
50	252	2.2

A non-negligible amount of blue fraction was lost during the filtration procedure, due to adsorption on membranes, about 8% of the phycocyanin content.

Phycocyanin purification

The consecutive steps, performed to achieve high purity phycocyanin, are effective but also time consuming. With this awareness, different levels of purification are proposed in a way that once the purity is enough to satisfy the needs of a specific application, the next steps can be omitted.

The next step involved the protein purification by dialysis membrane. Dialysis is not frequently adopted in the literature, however it displays many advantages compared to other purification methods in terms of cost savings [49]. It could be an interesting option to concentrate phycocyanin, while washing out ammonium chloride and other solutes with molecular dimensions below 15 kDa. The dialysis was performed against milli-Q water, to maximize the osmotic pressure and with no addition of other chemicals. From the UV-Vis spectrum, the phycocyanin content and purity resulted in respectively 200 mg $_{PC}$ g^{-1} (calculated on dry weight) and A_{620}/A_{280}=1.8. The phycocyanin content was enhanced of more than the double, while increasing by 50% in purity.

As further step to improve PC purification, ammonium sulfate precipitation was employed for protein salting-out. The mechanism of salting-out is based on preferential solvation due to exclusion of the salt ions from the layer of water closely associated with the hydration layer of the protein. When a salt is added to the solution, the surface tension of the water increases, enhancing hydrophobic interaction between protein and water. The protein reacts by decreasing its surface area in an attempt to minimize contact with the solvent, resulting in folding and self-association, leading to precipitation [50].

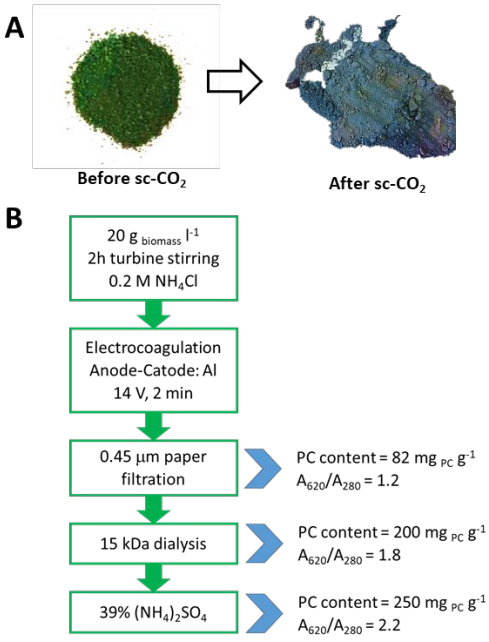

Figure 4 - *A: Grinded Spirulina powder before and after $scCO_2$ extraction processes; B: Scheme of the optimized steps in phycocyanin extraction and purification.*

Different ammonium sulfate concentrations were experimented: 24%, 39% or 50% of ammonium sulfate saturation. After centrifugation the blue pellet was collected and characterized. Table 2 summarizes the results. The optimized percentage of ammonium sulfate was identified in 39% of its saturation, since reaching 50% of its saturation did not improve significantly either phycocyanin content and its purity.

Overview on Extractions

In the first two steps of the process, carotenoids and chlorophylls were extracted in supercritical conditions in 180 min (90 min for carotenoids and 90 min for chlorophylls). The use of supercritical CO_2 is intrinsically a time-saving procedure, since it is more selective compared to traditional extraction methods with organic solvents.

Fig. 4B shows the operational scheme summarizing the procedure steps for phycocyanin extraction. The overall diagram leads to a product characterized by phycocyanin content and purity of middle-high grade, as described by Rito-Palomares *et al.* [19]. In fact, phycocyanin preparations with A_{620}/A_{280} between 0.7 and 3.9 are considered of reagent grade. The proposed extraction strategy can be hence suitable for larger scale applications. When the purity requirement is specified, the goal of the process design is to obtain maximum recovery at the lowest cost [51].

Depending on the need for purity level of the phycocyanin extract for a specific application, some of the proposed steps might be omitted, with time and energy costs savings. The 12-mesh papers filtration is enough to provide a purity of about 1.2, which lies in the reagent grade range as well as the final product that undergoes dialysis and protein salting-out as additional steps. If the procedure stops after electrocoagulation, a blue water solution of phycocyanin is obtained in 120 min. If the purity needs to be further enhanced by dyalisis, this needs three more days; if the ammonium sulfate precipitation is performed, this added 150 minutes more. The time needed to achieve a satisfying purity extract, strongly depends on the specific application.

Talking with numbers, the procedure presented in this work started with 31.4 g of Spirulina powder and provided a blue powder with a phycocyanin content of 7.85 g.

The added value of the process presented in this work is first related to the preliminary extraction of orange and green pigments, usually wasted in the discarded biomass. Second, the chosen processes in the phycocyanin extraction were selected setting apart, or at least minimizing, costly steps. Moreover, the overall process, yielding middle grade phycocyanin, lasts only few hours. The cost of phycocyanin is frequently determined by the quality (measured in terms of purity and concentration) which is strongly dependent on the steps used in the process. A minimization of production steps contributes to a reduction in of process costs and an increase in product yield. For instance, electrocoagulation and dialysis are already widely applied in industry and known to be easy-to-perform steps [47,52,53]. The paper filtration is preferable to ultrafiltration and other more echnological methods, in terms of time and costs savings. The commercial price of phycocyanin varies with increasing purity index; phycocyanin with reagent grade, as the one prepared in this work, was reported to cost from US 1 - 5$ per mg [11]. Through the strategy proposed here, phycocyanin is obtained without considerable product loss, maintaining a high protein recovery while potentially being able to reduce both processing cost and time. The finally discarded biomass, containing only vegetable residues and salts, has no more any added-value content.

Few works in the literature have claimed the development of large-scale methods for phycocyanin extraction from algal biomass. Some authors reported the extraction by osmotic shock, followed by purification by chromatographic techniques [9,21,22]. Their final products displayed purities around 5. The use of chromatography was discarded in the present work for the sake of robustness, cost-effectiveness and to keep the procedure as easy appliable as possible.

As a last mention, Gupta *et al.* employed chitosan or charcoal for further phycocyanin purification steps [54]. This method was also experimented in the present work. Chitosan and charcoal, although effective for purification procedure, are responsible for the loss of large amounts of phycocyanin, due to adsorption on the material itself. For this reason, chitosan and charcoal have not been further considered in the present work.

Application of Phycocyanin extract as blue dye for tissues

The first regulation in the field for dye additives was introduced in 1904 by the United States. Since then, a strict evaluation process of dyes was adopted by the Food and Drug Administration (FDA) [8]. The European Union followed a similar approach through the European Food Safety Authority (EFSA). The law is very restrictive concerning color additives but it has been favoring the use of natural or “nature identical” colorants [55]. The resulting growing awareness of importance of natural colors, especially in food and cosmetics colorants, accounts for the tendency in food manufacturing to use natural additives.

Spirulina and microalgae in general, due to their renowned pigments content, are perfectly able to answer these commercial expectations, with the added value of displaying, in many cases, health-beneficial effects.

The need to overcome the potential toxicological effect of synthetic dyes is a key issue in the coloring processes. Rose *et al.* extracted anthocyanins from blackcurrant waste from the fruit pressing industry and studied their adsorption on hair [56]. In this work, a similar approach was adopted to investigate the sorption properties of the blue phycocyanin pigment as tissue dye. The dye absorption from the phycocyanin solution onto the tissue fiber was measured before and after the dyeing procedure by recording visible spectra, monitoring the absorbance at 620 nm, typical of phycocyanin chromophore.

Adsorption results were applied to Langmuir and Freundlich isotherm models.

Langmuir isotherm is graphically characterized by a plateau, an equilibrium saturation point where once a molecule occupies a site, no further adsorption can take place. It considers a

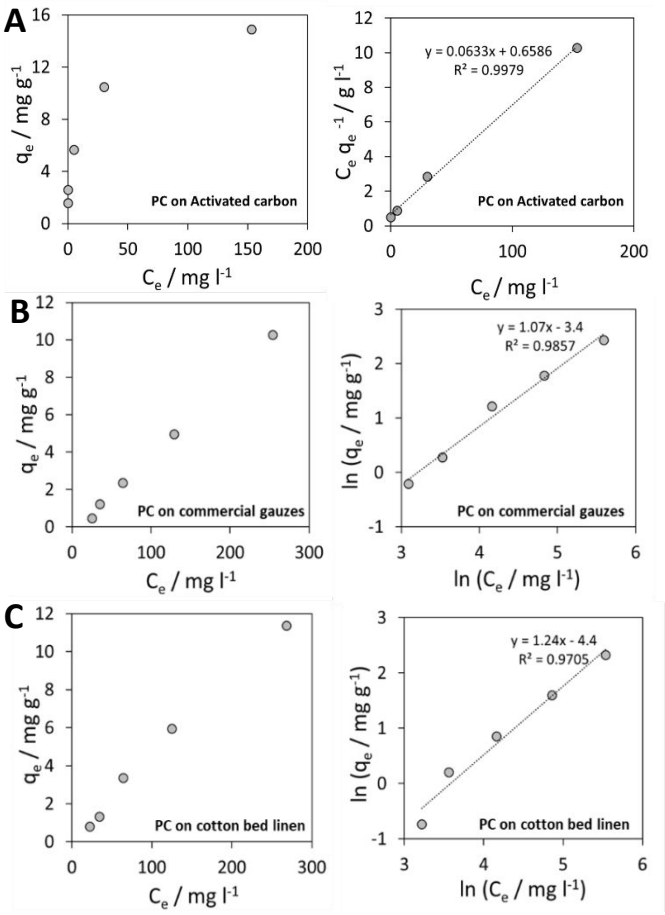

Figure 5 – Left: Adsorption isotherm of phycocyanin (PC) on different substrates; right: linearization of the Freundlich isotherm.

monolayer coverage, all adsorption sites equally probable and its linearized form is characterized by the following equation:

$$\frac{C_e}{q_e} = \frac{1}{KQ^0} + \frac{C_e}{Q^0}$$

where Q^0 gives the maximum adsorption capacity for forming single layer (in mg $g_{adsorbent}^{-1}$), C_e is the adsorbate concentration in solution at equilibrium (in mg l^{-1}) and q_e indicates the quantity adsorbed on the adsorbent at equilibrium (in mg $g_{adsorbent}^{-1}$). If the plot C_e/q_e vs C_e displays a linear trend, it means that the adsorption process can be described by the Langmuir model.

Results on activated carbon displayed a behavior that could be described by Langmuir isotherm. In Fig. 5A experimental results on activated carbon are shown.

The Langmuir isotherm linearization for the adsorption of phycocyanin on activated carbon provided a value of maximum adsorbing capacity of Q^0 = 15.8 mg $g_{adsorbent}^{-1}$. This could be also visually derived by the plateau reached by experimental data in Fig. 5A (right). Langmuir isotherm in fact considers a monolayer coverage: once a molecule occupies a site, no further adsorption is allowed [57].

Among the experimented cotton-based materials, none of them could be fit by a Langmuir model isotherm for phycocyanin adsorption process. Many practical cases in fact cannot be

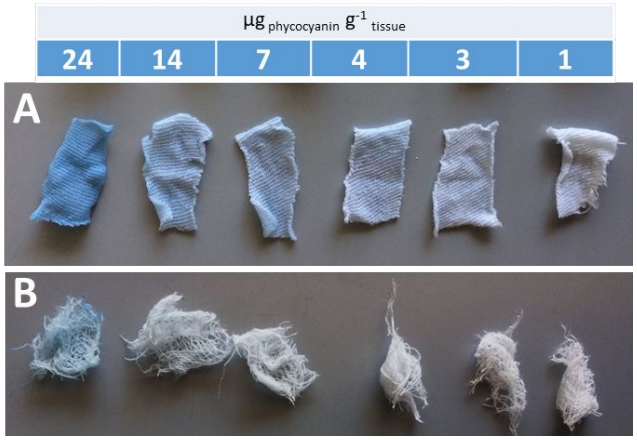

Figure 6 - *Pictures of the colored bed linen (B) and commercial gauzes (B) after phycocyanin adsorption experiments. Each colored tissue is the result of a different ratio phycocyanin:amount of tissue.*

described the Langmuir model. Freundlich isotherm is empirical and very widely used to describe the adsorption characteristics for the heterogeneous surface. Freundlich equation implies that the energy of adsorption on a homogeneous surface is independent of surface coverage. Its linearized form is described by the following equation:

$$ln\ q_e = ln\ K_F + \frac{1}{n} ln\ C_e$$

where K_F is an indicator of adsorption capacity: higher K_F, higher the maximum capacity. *1/n* is a measure of intensity of adsorption. Higher the *1/n* value, more favorable is the adsorption. Generally, $n < 1$. n and K_F are system specific constants. If the plot $ln\ q_e$ vs $ln\ C_e$ displays a linear trend, it means that the adsorption process can be described by the Freundlich model [57].

Fig. 4B-C display experimental results and Freundlich model linearization obtained for phycocyanin adsorption on commercial sterile gauzes and colorless cotton bed linen.

The linear regression lines in Fig. 5B-C (right) demonstrate that the linearization of Freundlich isotherm is able to fit experimental data.

Fig. 5 displays the coloring result varying the ratios phycocyanin:amount of tissue.

By calculating the *1/n* from the slopes of regression lines, 1.06 and 1.24 values were obtained for phycocyanin adsorption on commercial gauzes and cotton bed linen, respectively. This suggests that adsorption is more favorable on cotton bed linen, as also visible by the more pronounced coloring of bed linen tissues displayed in Fig. 6A if compared to Fig. 6B, relative to commercial gauzes. This might be explained by the presence of polymeric additives in commercial gauzes, hindering the phycocyanin adsorption process.

These experiments are meant exclusively as preliminary results to develop potential applications of phycocyanin as a stable dye after some rinsing steps. In fact, all colored tissues underwent three rinsing step in three successive weeks, and the color did not significantly dissolve in water.

For translation to a real tissues dye processing, the extracted blue pigment need to be incorporated into a base formulation.

Conclusions

Spirulina distinctive natural orange, green and blue pigments have been extracted from the dry biomass through an integrated chain. First, supercritical CO_2 alone was able to selectively extract carotenoids. Then, the addition of ethanol as co-solvent provided exclusively chlorophylls.

A strategy to finally extract high purity phycocyanin (A_{620}/A_{280}=2.2) from the biomass residue was developed and the potential application as tissue dye was studied.

The benefit of the suggested extractions cascade is the approach, where almost all the relevant content has been efficiently recovered, stage by stage, in a potentially scalable process with no loss of pigments or added-value compounds.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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SUPPORTING INFORMATION

Carotenoids, Chlorophylls and Phycocyanin from Spirulina.

Supercritical CO2 and Water Extraction Methods for Added Value Products Cascade

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OPTIMIZATION OF EXTRACTION PARAMETERS IN SUPERCRITICAL CO_2

Table S1 – Optimization trials in supercritical CO_2 for extraction of carotenoids and chlorophylls from Spirulina

Test	Pressure / bar	Vessel temperature / °C	% ethanol	$C_{carotenoids}$ / mg g^{-1} dry Spirulina	$C_{chlorophyll\ a+b}$ / mg g^{-1} dry Spirulina
1	100	45	0	-	-
2	200	45	0	-	-
3	300	45	0	3.5 ± 0.2	-
4	300	55	0	0.4 ± 0.1	-
5	300	45	10	-	9.1 ± 0.5
6	300	45	15	-	-

"-" means that no extract was obtained.

PHYCOCYANIN EXTRACTION PROCEDURES

Different procedures were adopted and compared aiming at optimizing the process of extracting phycocyanin (PC) from Spirulina powder. The experimental details and results are reported here below.

- Stirring and centrifugation: dehydrated spirulina powder was suspended in milli-Q water (20 mg mL^{-1}) and stirred for 1 hour. 0.5 mL of the suspension was diluted 1:9. After 15 min centrifugation at 5000 rpm, the blue/green supernatant suspension was filtered through 0.45 μm nylon syringe filters. Filters retained a green particulate. The resulting light blue solution was analysed by spectrophotometer acquiring the absorbance between 300 and 900 nm wavelengths. In Fig. S1 is displayed the suspension before filtration and the solution after filtration, together with the used filter.

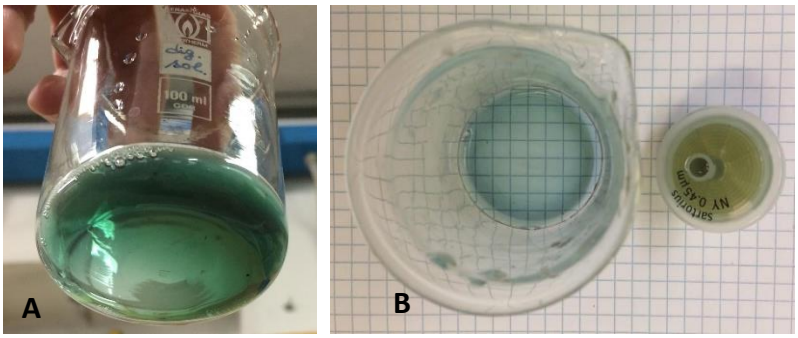


Figure S1 - A) blue/green suspension after centrifugation; B) light blue solution after filtration and the filter retaining the green particulate.

In Fig. S2 is displayed the acquired spectrum in the visible region. Spectrum shows the characteristics peak at 620 nm and other smaller peaks at lower wavelengths (lower than 500 nm), likely assignable to carotenoids or other compounds.

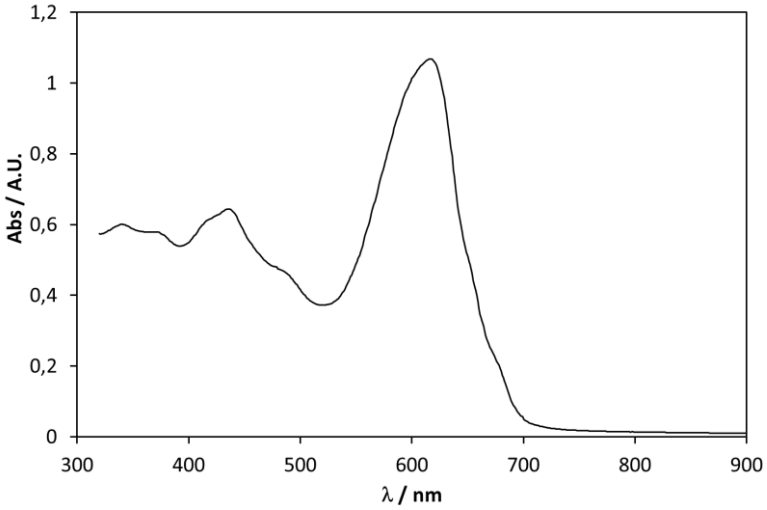


Figure S2 - Spectrum acquired for the final solution in the visible region.

The PC yield, by this extraction method was **5.4 ± 0.7 % w/w** (54 mg of PC every gram of Spirulina powder).

- Sonication: dehydrated spirulina powder was suspended in milli-Q water (20 mg mL^{-1}) and sonicated for 1 hour. 0.5 mL of the suspension was diluted 1:9. After 15 min centrifugation at 5000 rpm, the blue/green supernatant suspension was filtered through 0.45 μm nylon syringe filters. Filters retained a green particulate. The resulting green solution was analysed by spectrophotometer acquiring the absorbance between 300 and 900 nm wavelengths. In Fig. S3 is displayed the final solution and a comparison with that one resulting from the previous procedure (stirring).

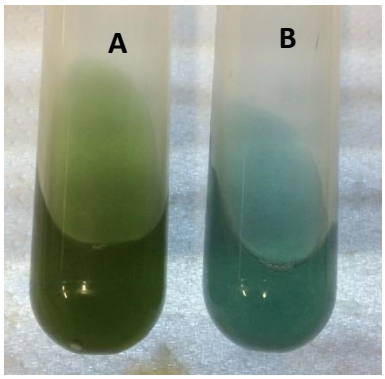


Figure S3 - A) Final solution obtained by sonication method; B) final solution obtained by the previous method (stirring).

In Fig. S4 is displayed the acquired spectrum in the visible region and compared to the previous one obtained by stirring procedure. Spectrum shows the same characteristics peak at 620 nm and the other peaks at lower wavelengths (lower than 500 nm), likely assignable to carotenoids or other compounds, are now much more intense. Another peak at 680 nm is present.

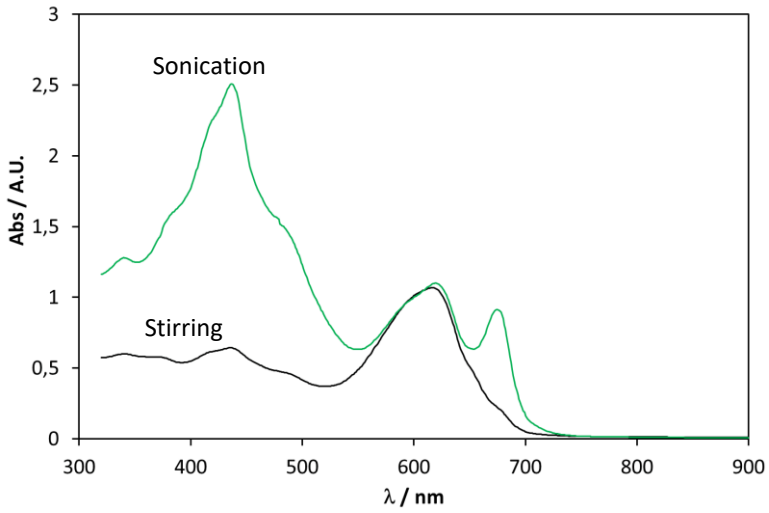


Figure S4 - Spectra acquired for the final solutions in the visible region; stirring and sonication methods are compared.

The PC yield, by this extraction method was **5.8 ± 0.4 % w/w** (58 mg of PC every gram of Spirulina powder). Even if the percentage of PC is similar to the previous stirring method (peaks at 620 nm are overlapping), this last extraction method should not be recommended, because of the strong effect of cavitation bubbles during sonication, which might have forced other compounds to be extracted. The final PC solution resulted less pure.

- Freezing and thawing: dehydrated spirulina powder was suspended in milli-Q water (20 mg mL^{-1}) and stirred for 1 hour. The suspension was frozen and unfrozen two times. 0.5 mL of the suspension was diluted 1:9. After 15 min centrifugation at 5000 rpm, the blue/green supernatant suspension was filtered through 0.45 μm nylon syringe filters. Filters retained a green particulate. The resulting light blue solution was analysed by spectrophotometer acquiring the absorbance between 300 and 900 nm wavelengths.
In Fig. S5 is displayed the acquired spectrum in the visible region and compared to the first one obtained by stirring procedure. Spectra are comparable.
The PC yield, by this extraction method was **5.5 ± 0.3 % w/w** (55 mg of PC every gram of Spirulina powder). Even if the percentage of PC is similar to the previous stirring method, this last extraction method should not be recommended, because of the non-useful addition of the freezing/thawing steps.

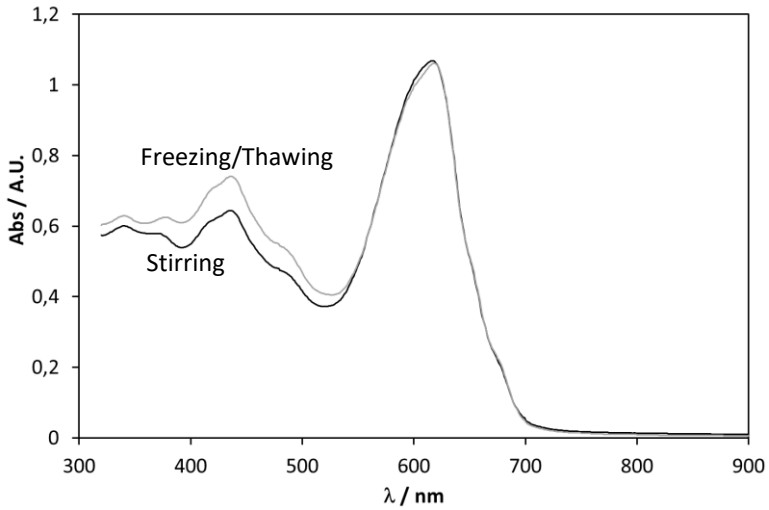


Figure S5 - Spectra acquired for the final solutions in the visible region; stirring and freezing/thawing methods are compared.

- Prolonged stirring: since the stirring was identified as a key step for PC extraction, dehydrated spirulina powders was suspended in milli-Q water (20 mg mL^{-1}) and stirred for a prolonged time: 20 hours. 0.5 mL of the suspension was diluted 1:9. After 15 min centrifugation at 5000 rpm, the blue/green supernatant suspension was filtered through 0.45 μm nylon syringe filters. Filters retained a green particulate. The resulting light blue solution was analysed by spectrophotometer acquiring the absorbance between 300 and 900 nm wavelengths.

In Fig. S6 is displayed the acquired spectrum in the visible region and compared to the ones obtained by stirring and sonication procedures. The resulting spectrum possesses the PC characteristic peak at 620 nm, with a comparable intensity. The other peaks (at wavelengths lower than 500 nm) are now less intense than those ones obtained by sonication, but more intense than those ones obtained just by 1 h stirring. The same peak observed after sonication procedure, at 680 nm, appears in the spectrum after prolonged stirring as a shoulder.

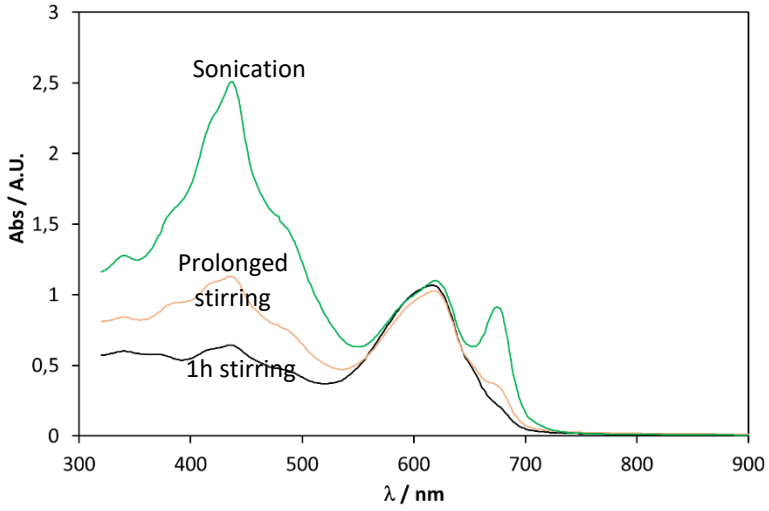


Figure S6 - Spectra acquired for the final solution in the visible region; stirring and sonication methods are compared.

The PC yield, by this extraction method was **5.5 % w/w** (55 mg of PC every gram of Spirulina powder). This last extraction method should not be recommended, because of the presence of other compounds dissolved in the solution, hence resulting in a less pure final phycocyanin solution.

- Acidic pH: following some literature methods [1,2], acidic pH were employed to verify if these could improve the extraction yield. At first, two organic acids were employed: citric acid and ascorbic acid, each one at 0.02 M concentration (pH were 2.38 and 2.86 for citric acid and ascorbic acid solutions, respectively). Then, another solution was employed: ammonium chloride solution at two different concentrations, 0.05 M and 0.2 M (pH were 5.61 and 4.94 for 0.05 M and 0.2 M NH_4Cl solutions, respectively). Dehydrated spirulina powder was suspended in the acidic solutions (20 mg mL^{-1}), stirred for 1 h, followed by centrifugation at 5000 rpm (before centrifugation 0.5 mL of the suspension was diluted 1:9). After centrifugation, when using citric acid, the supernatant was transparent and this solution was not further considered. In the case of ascorbic acid and ammonium chloride solutions, the light blue supernatant was filtered through 0.45 μm nylon syringe filters. Filters retained a weak green particulate. The resulting light blue solution was analysed by spectrophotometer acquiring the absorbance between 300 and 900 nm wavelengths. In Fig. S7 are displayed the acquired spectra.

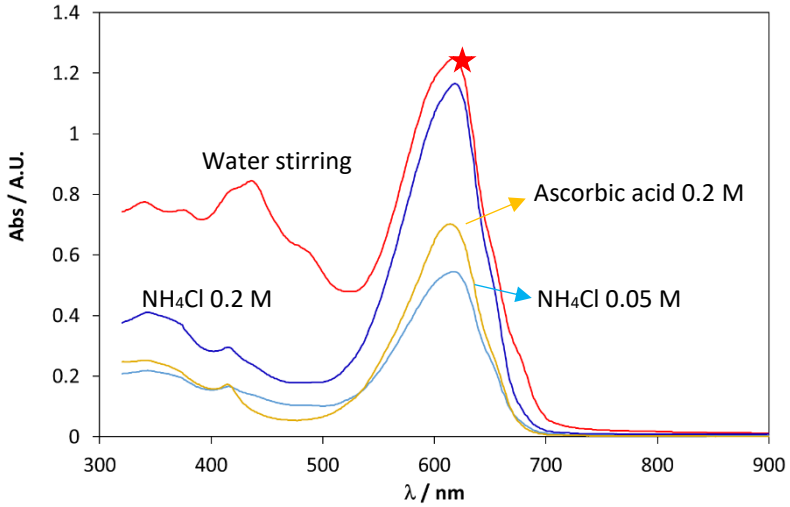


Figure S7 - Spectra acquired for the final solutions in the visible region; the water stirring method, highlighted by a red star, is displayed for comparison.

Ammonium chloride was more effective in the extraction, compared to ascorbic acid. As shown in the figure, the main peak intensity is slightly lower than what obtained by the water stirring method using just milli-Q water. It is however also noticeable that the peaks at lower wavelengths decreased considerably their intensities in all cases. The final extract can be considered less contaminated by other compounds. The PC yields, by these extraction methods were:

- Ascorbic acid 0.02 M: **3.8 % w/w**
- NH_4Cl 0.05 M: **3.2 % w/w**
- NH_4Cl 0.2 M: **5.8 ± 0.4 % w/w**

- Neutral/alkaline pH: following literature methods [1], pH was adjusted to neutral and alkaline values. Two solutions of phosphate buffer (PBS) at pH 6.95 and 7.2 were prepared. A solution of carbonate buffer at pH 9.5 was also prepared. Dehydrated spirulina was suspended in the solutions (20 mg mL^{-1}), stirred for 1 h, followed by centrifugation at 5000 rpm (before centrifugation 0.5 mL of the suspension was diluted 1:9). After less than 1 h stirring, the suspension in carbonate buffer turned to a brown/green color and the method was discarded. In the case of PBS solutions, the light blue supernatant was filtered through 0.45 μm nylon syringe filters. Filters retained a weak green particulate. The resulting light blue solution was analysed by spectrophotometer acquiring the absorbance between 300 and 900 nm wavelengths. In Fig. S8 are displayed the acquired spectra.

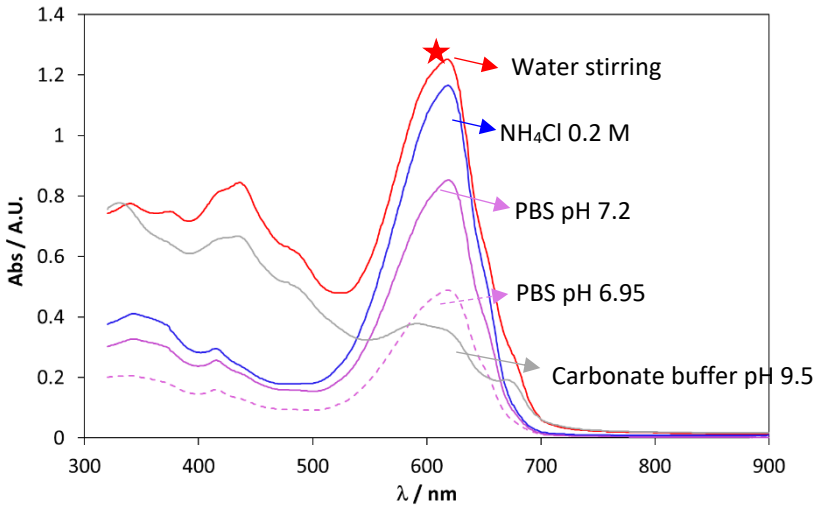


Figure S8 - Spectra acquired for the final solutions in the visible region; the optimized water stirring method, highlighted by a red star, is displayed for comparison.

The PC yields, by these extraction methods were:

- PBS pH 6.95: **2.7 % w/w**
- PBS pH 7.2: **4.6 % w/w**

- Ammonium chloride, effect of the stirring time: since the best result previously exposed was achieved by extraction in 0.2M NH_4Cl solution, the effect of the stirring time was studied. Dehydrated spirulina powder was suspended in the acidic solutions (20 mg mL^{-1}), stirred for 1 h or 2h, followed by centrifugation (before centrifugation 0.5 mL of the suspension was diluted 1:9). The light blue supernatant was filtered through 0.45 μm nylon syringe filters. Filters retained a weak green particulate. The resulting light blue solution was analysed by spectrophotometer acquiring the absorbance between 300 and 900 nm wavelengths. In Fig. S9 are displayed the acquired spectra.

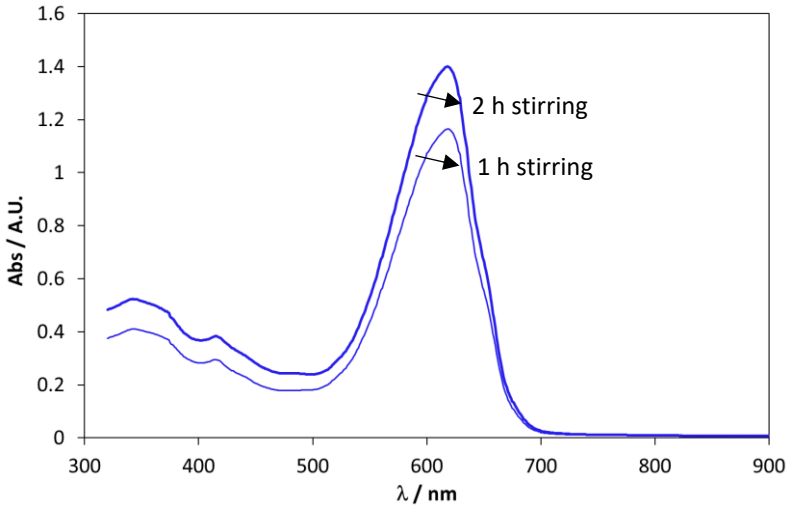


Figure S9 - Spectra acquired for the final solutions in the visible region.

As shown in the figure, the intensity of the main peak increased by increasing the stirring time. Consequently, by duplicating the stirring time, the PC yield, increased to **7.5 ± 0.8 % w/w**. The yield

underwent 20% increased. Other attempts to further increase the yield by increasing the stirring time were ineffective.

STANDARD PHYCOCYANIN CALIBRATION LINE

Starting from a commercial standard product (PC_{std}), a calibration line was built in order to calculate the phycocyanin content. The best fit of experimental data in the plot "A_{620} *vs* PC_{std} concentration" (A_{620} is the absorbance at 620 nm and PC_{std} concentration is in μg ml^{-1}) was a straight-line, represented by the following mathematical equation: y = 0.0086x - 0.0246, resulting in a R^2 equal to 0.999. The slope *m* and intercept *q* of the regression line with their respective standard deviations were (0.0086 ± 0.0001) and (-0.025 ± 0.006), respectively. Fig. S10 displays the experimental results and the best fit obtained by linear regression line.

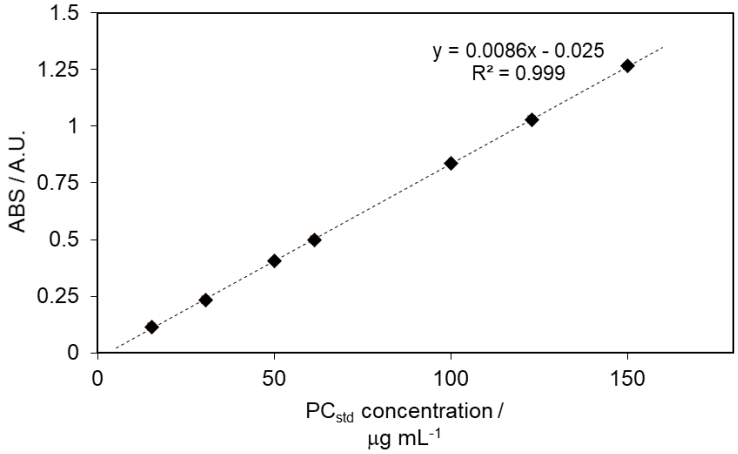
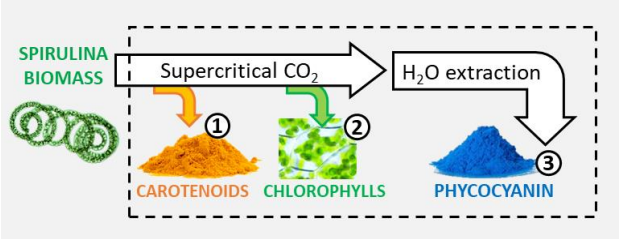


Figure S10 - Experimental data with error bars and calibration line of PC_{std} by absorbance measurement at 620 nm.

References

1 E. Manirafasha, T. Murwanashyaka, T. Ndikubwimana, Q. Yue, X. Zeng, Y. Lu and K. Jing, *J. Appl. Phycol.*, 2017, **29**, 1261–1270.

2 W. Pan-utai, W. Kahapana and S. Iamtham, *J. Appl. Phycol.*, 2018, **30**, 231–242.



This work investigates a green approach for an integrated extraction-chain of added-value compounds from Spirulina