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.

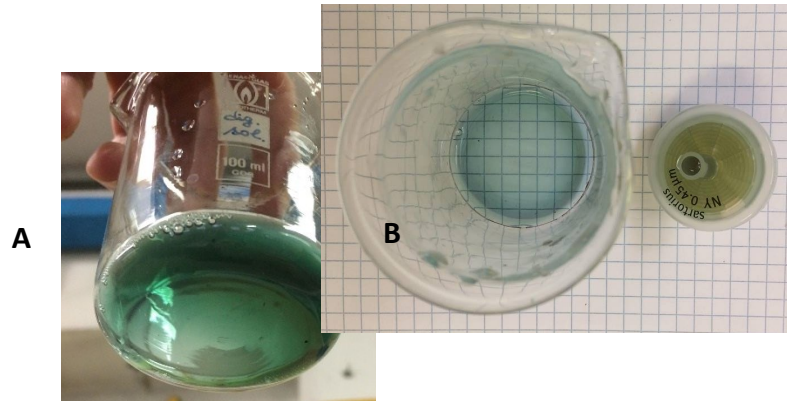

Figure S1 - A) blue/green suspension after centrifugation; B) light blue solution after filtration and the filter retaining the green particulate.

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.

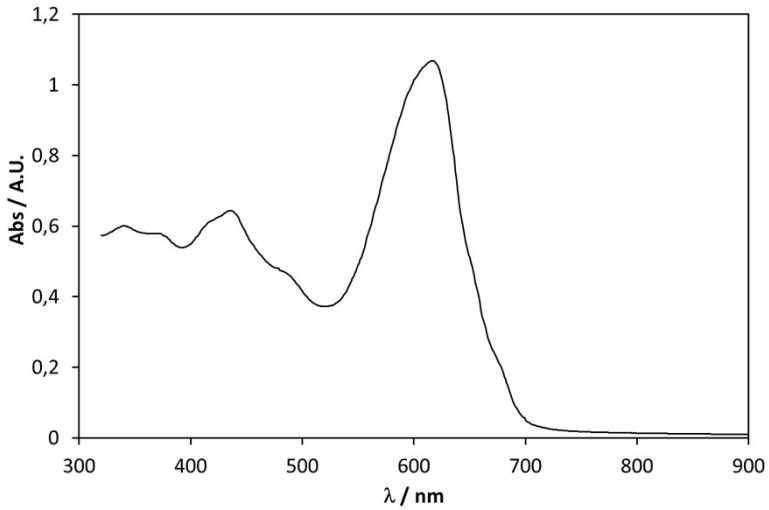

Figure S2 - Spectrum acquired for the final solution in the visible region.

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).

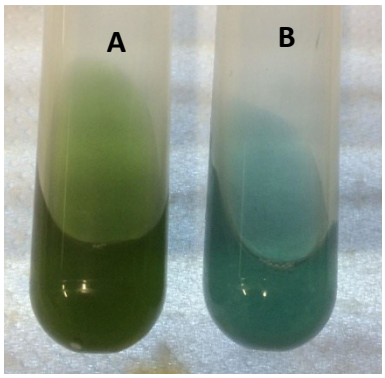

Figure S3 - A) Final solution obtained by sonication method; B) final solution obtained by the previous method (stirring).

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.

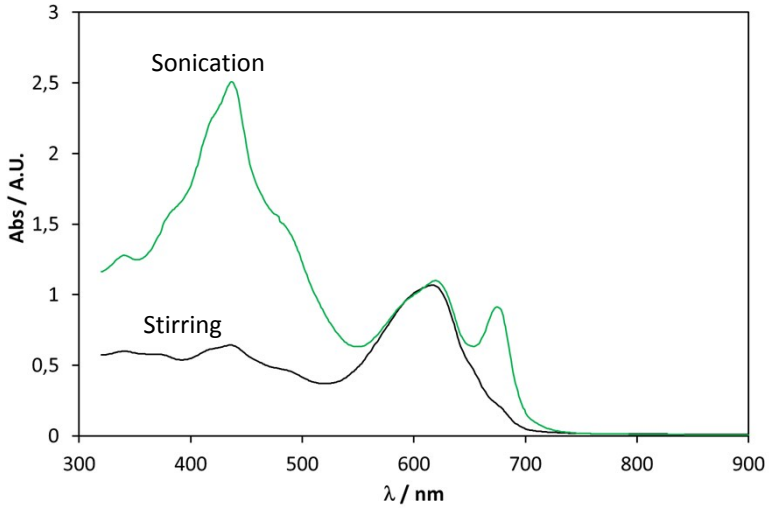

Figure S4 - Spectra acquired for the final solutions in the visible region; stirring and sonication methods are compared.

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.

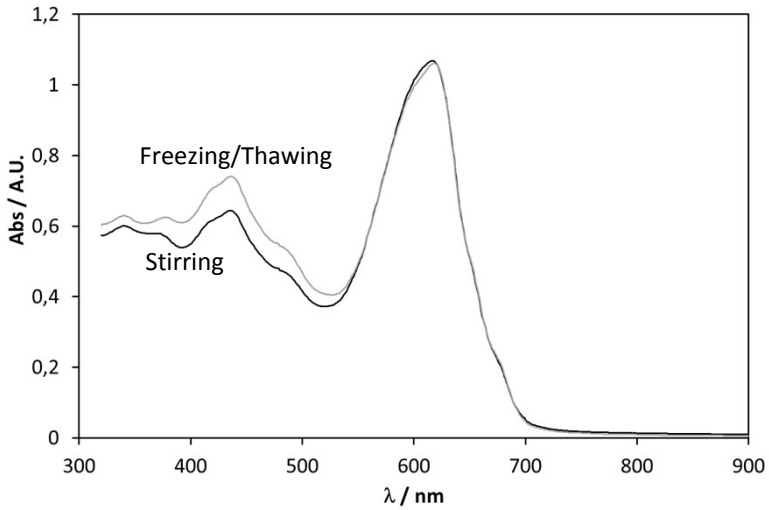

Figure S5 - Spectra acquired for the final solutions in the visible region; stirring and freezing/thawing methods are compared.

- 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.

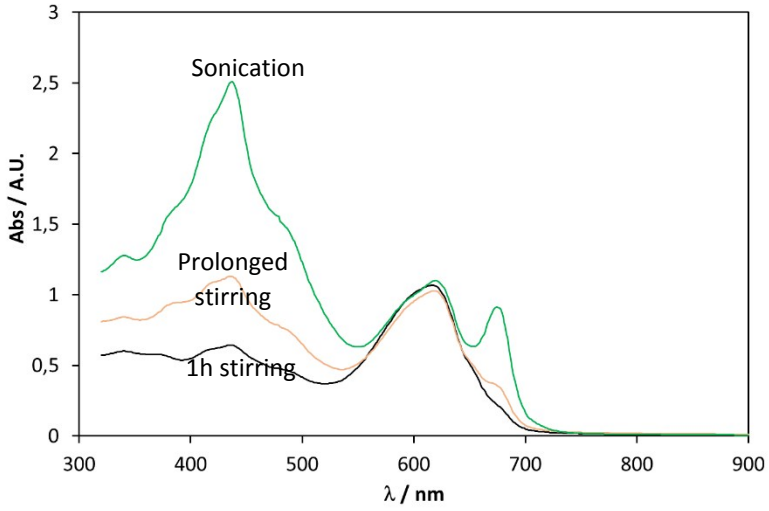

Figure S6 - Spectra acquired for the final solution in the visible region; stirring and sonication methods are compared.

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.

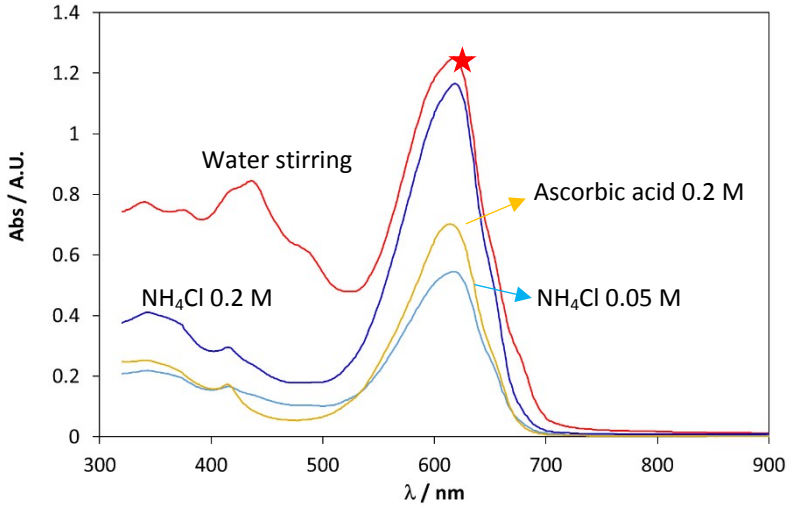

Figure S7 - Spectra acquired for the final solutions in the visible region; the water stirring method, highlighted by a red star, is displayed for comparison.

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.

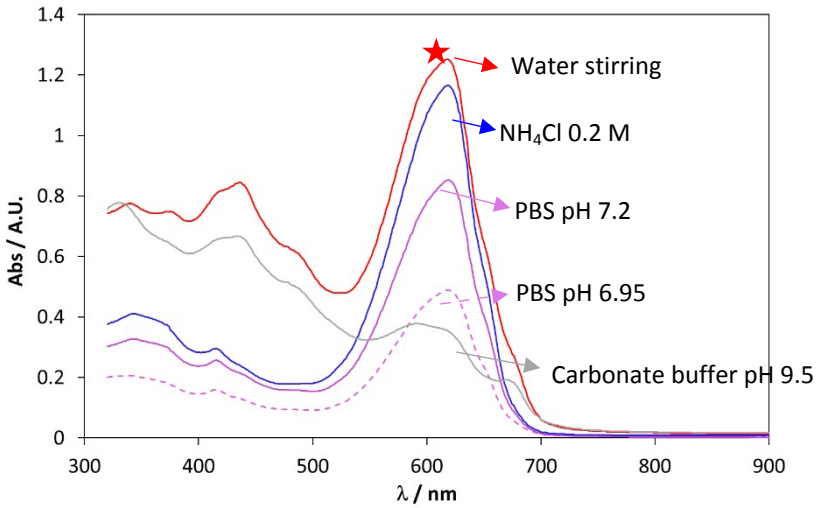

Figure S8 - Spectra acquired for the final solutions in the visible region; the optimized water stirring method, highlighted by a red star, is displayed for comparison.

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.

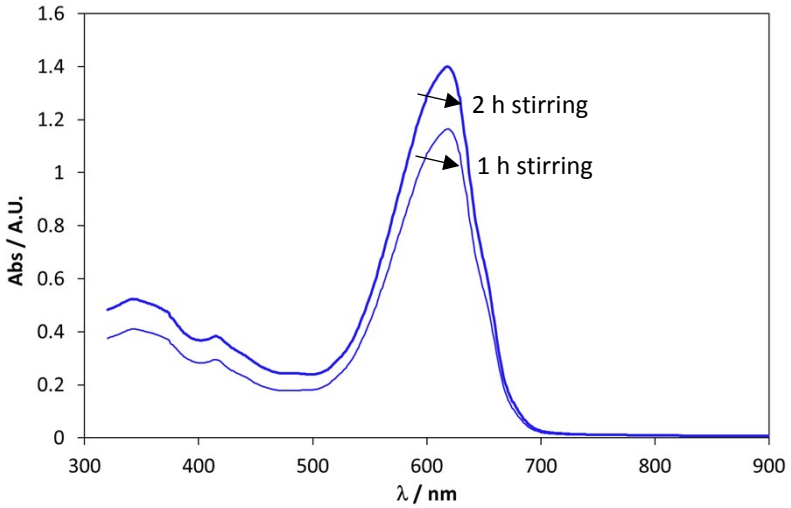

Figure S9 - Spectra acquired for the final solutions in the visible region.

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.

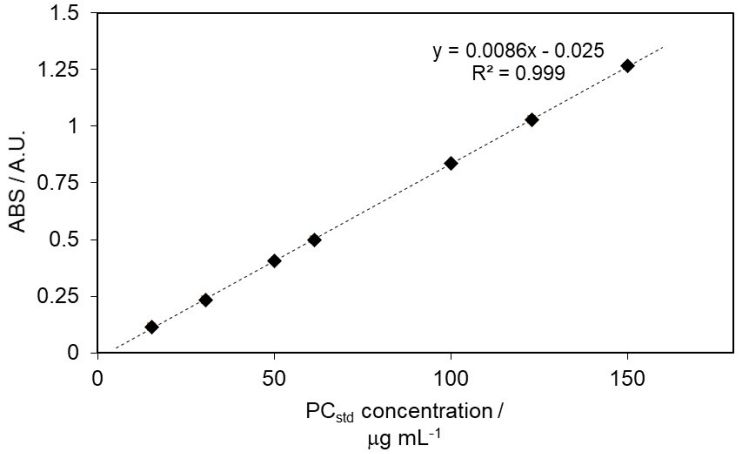

Figure S10 - Experimental data with error bars and calibration line of PC_{std} by absorbance measurement at 620 nm.

References

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