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BANCO ESPAÑOL DE ALGAS

Working on a standard protocol for the quantification of phycocyanin from *Arthrospira platensis*: first attempts for validation

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Abstract

It is a fact that the use of microalgae for various commercial uses is becoming more and more common, such as cosmetics, nutraceuticals or pharmacology. *Arthrospira (Spirulina) platensis* is one of the main sources of these components, such as proteins, fatty acids, carotenoids and phycobiliproteins. Spirulina has a wide range of pharmacological uses due to its phycobiliprotein content, especially C-phycocyanin, which is also used as a natural dye. Its potential use for anticancer treatments is still under study. Over the years, many methods have been developed, such as freezing and thawing, sonication and mechanical disruption for cell disruption, and high-speed cent rifugation, ammonium sulfate precipitation and ion exchange chromatography for extract purification. This has resulted in many ways to extract this pigment, with different techniques, yields and degrees of purity, but without a common methodology. Therefore, the development of standards for algal protocols and algae products is proposed. The objective of this study is to evaluate a common methodology, so that the extraction follows a single method with the best possible yield and purity. In this way, we will be able to compare the results obtained in different laboratories.

KEYWORDS: Aquaculture, microalgae, standard, cyanobacteria, Spirulina, extract, phycobiliproteins, C-phycocyanin, carotenoids, food grade, analytical grade, absorbance, natural pigments.

Resumen

Es un hecho que la utilización de microalgas para diversos usos comerciales es cada vez más común, como los cosméticos, los nutracéuticos o la farmacología. *Arthrospira (Spirulina) platensis* es una de las principales fuentes de estos componentes, como proteínas, ácidos grasos, carotenoides y ficobiliproteínas. La Espirulina tiene una amplia gama de usos farmacológicos debido a su contenido en ficobiliproteínas, especialmente la C-ficocianina, que se utiliza también como colorante natural. Su uso potencial para los tratamientos anticancerígenos sigue siendo objeto de estudio. A lo largo de los años, se han desarrollado muchos métodos, como la congelación y descongelación, la sonicación y la disrupción mecánica para la disrupción celular, y la centrifugación de alta velocidad, la precipitación con sulfato de amonio y la cromatografía de intercambio iónico para la purificación del extracto. Esto ha dado lugar a muchas formas de extraer este pigmento, con diferentes técnicas, rendimientos y grados de pureza, pero sin una metodología común. Por lo tanto, se propone el desarrollo de estándares para protocolos de algas y productos de algas. El objetivo de este estudio es evaluar una metodología común, para que la extracción siga un único método con el mejor rendimiento y pureza posibles. De este modo, podremos comparar los resultados obtenidos en diferentes laboratorios.

KEYWORDS: Acuicultura, microalgas, estándar, cianobacterias, Spirulina, extracto, ficobiliproteínas, C-ficocianina, carotenoides, grado alimentario, grado analítico, absorbancia, pigmentos naturales.

1. Introduction

There is currently a need to look for healthy and environmentally friendly food sources. The evolution of biorefineries is considered a key point to generate a sustainable economy for nutraceuticals, pharmaceuticals, cosmetics, food additives and biofuels (Jiang, 2021). Photosynthetic organisms, among which we find cyanobacteria and microalgae, are a potential source of healthy foods due to their high content of bioactive compounds, such as essential amino acids, proteins, lipids and carbohydrates (Nisticò, 2022). These organisms present certain advantages, such as low water use and therefore the possibility of growing in environments with unfavorable conditions, great biodiversity, and manageability of their metabolism, where the production of molecules of interest can be induced (Nisticò, D.M, 2022). There are different markets in which microalgae are used, such as cosmetics, textile industry, and even as high nutritional food. Despite the evolution in studies, and the fact that the first Spirulina production plant was built 50 years ago, there is still a lack of food of the future. There have been numerous developments, such as drinks with chlorophyll, oil mermelades with spirulina, or its use as a blue dye.

Among the molecules of interest are phycobiliproteins, which are pigments that aid in light uptake by photosynthetic organisms (Sobiechowska-Sasim et al., 2014). According to their characteristics, they are divided into phycoerythrins (PEs), phycocyanins (PCs) and allophycocyanins (APCs) (Bermejo & ramos, 2012; Samsonoff & MacColl 2001). Of these, C-phycocyanin (C-PC) is the most interesting, due to the fact that it contains a wide variety of essential amino acids (Yu, 2017), to its anticancer, antioxidant, antiviral and anti-inflammatory activity (Jiang, 2021).

1.1. Importance and properties of Spirulina

Arthrospira platensis (Spirulina, Figure 1) is a spiral-shaped cyanobacteria (photoautrophic, mixotrhophic or heterotrophic prokaryotic organisms, able to obtain energy by using chromophores (EN 17399: 2020 E), which has attracted attention due to its high content of proteins, vitamins, minerals, many essential amino acids and essential fatty acids (Grosshagauer et al., 2020). Spirulina is a unicellular alga, with multicellular cylindrical trichomes. It is rich in bioactive components, such as C-PC, chlorophyll, carotenoids and fatty acids (Ismaiel et al., 2016).

Figure 1: photos of the spirulina cells used in the experiment

Spirulina shows high protein content (55-65% of dry weight), that is why it is considered a high nutritional food (Mishra et al., 2008). 20% of the protein dry weight is C-PC (Patil & Raghavarao, 2007).

1.2. Importance and properties of C-PC

Depending on the taxonomical characteristics and groups there are 3 main classes of phycobiliproteins, but Spirulina mainly synthetize C-phycocyanin (C-PC). The following figure (Figure 2) shows the absorption spectra of the different phycobiliproteins:

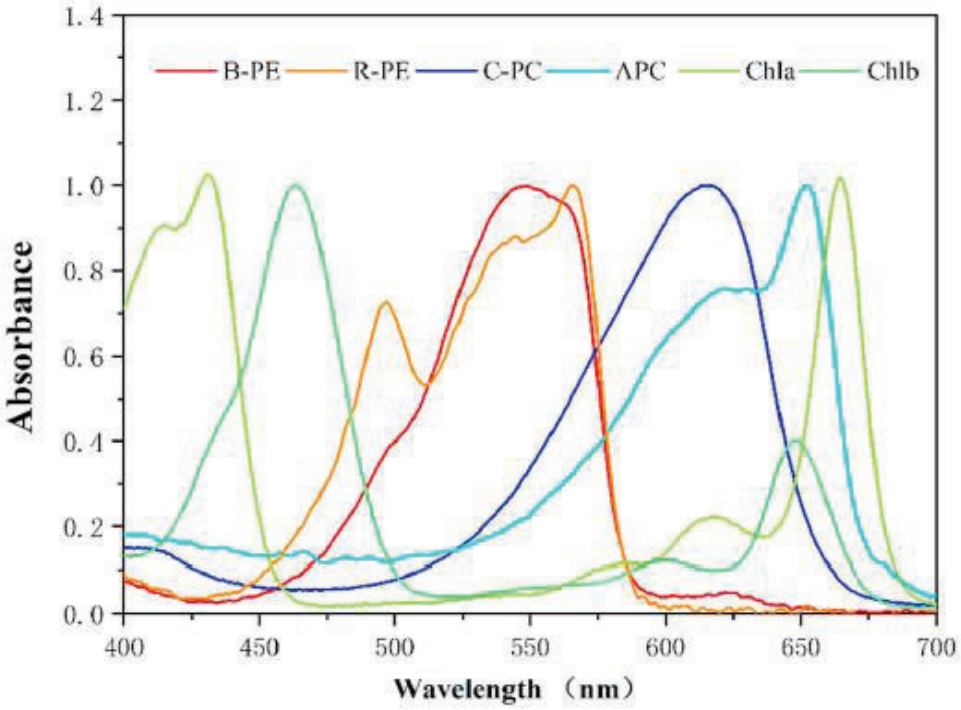


Figure 2. Absorbance spectra of B-PE, R-PE, C-PC, APC, Chla, and Chlb. Source: S.Qin, 2019

Despite being water-soluble molecules with intense color, their application in food is still under investigation due to their low stability (Falkeborg et al., 2018) to light (a high tendency to lose chromophores has been demonstrated, (Wu et al., 2016)), pH values, high temperatures (above the optimum temperature, denaturation occurs, (Kannaujiya & Sinha, 2016), presence of alcohols and strong ionic strength (Chaiklahan et al., 2012) , (Rahman et al., 2017). The extraction of C-PC is still under study, due to the resistance of the cell walls of the algae (*A. pltensis*), which implies lower efficiency (Jiang, 2021).

The ratio A620/A280 determines the specific use of the C-PC extract. The following table (Table1) shows the different purity grades (Eriksen, 2008; Patil et al., 2008; Roy & Pabbi, 2022; Sonani, 2016; Rito-Palomares et al. 2001).

Table1: C-PC applications according to the A620/A280 ratio

A620/A280 value	Purity grade
>0.7	Food and cosmetic grade
>3.9	Reagent grade macromolecule
>4.0	Analytical grade (pharmaceutical products)

Figure 3 shows the structure of the phycocyanin found in cyanobacteria.

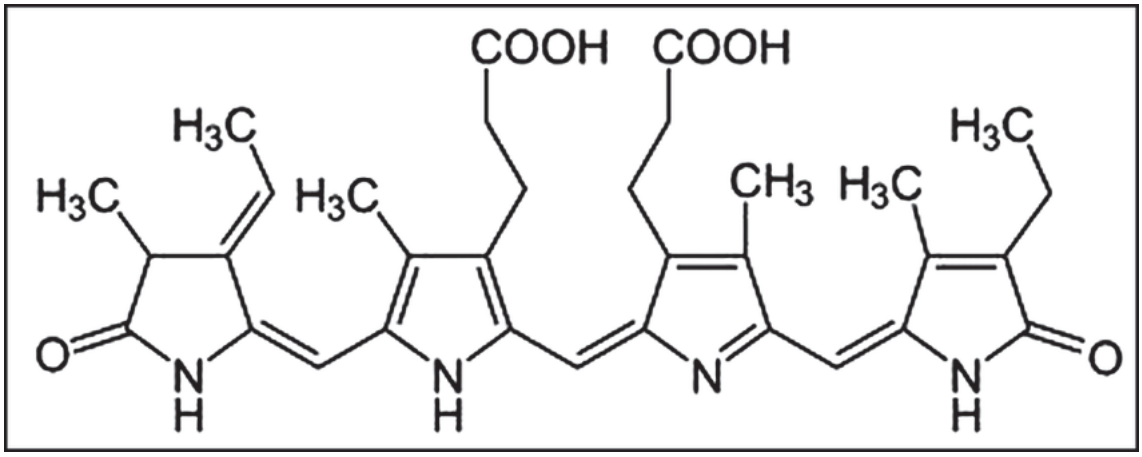


Figure3: Structure of phycobilins found in cyanobacteria. Source:(Hoseini et al, 2013)

Phycocyanin is also found in Cryptophyta, Rhodophyta, Glaucophyta and Dinophyta (Yepremian et al, 2017). It has been determined spectrophotometrically in cyanobacteria such as Synechocystis (Zavřel et al., 2018).

1.3. Extraction methods of phycocyanin in Spirulina

Upon doing a literature review you discover that there is no common method to proceed with the extraction of phycocyanin in this cyanobacterias. Initial techniques such as the study of precipitates from ammonium sulfate for chromatography, or crystallization, were not specific, because it was not possible to obtain each pigment separately (Chamorro-Cevallos et al., 2016). One tentative has already been made in 2018 for the FAO/WHO Expert Committee on Food Additives (JECFA) for the extraction of phycobilins (phycocyanin C-PC and allophycocyanin a-PC). There are more protocols for the determination of phycobiliproteins, as the Steward and Farmer (1984) method and the Yepremian et al (2017) protocol for extraction and determination of phycocyanin.

There are a variety of methods currently available, but the problem remains the low yield and purity of the extract. (Hsieh-Lo et al., 2019). It is necessary to find a way to obtain phycocyanin extracts from blue-green microalgae with a low economic and temporal cost, and with a high yield and purity (Şahin Oya Irmak, 2020).

1.4. Importance of standardizing processes

Given the growing interest in algae and algae products in Europe as a source of protein, lipids and carbohydrates, the European Committee for Standardization (CEN) has been asked to carry out standards for algae and algae products, thus creating CEN/TC 454 "Algae and algae products". Standardization is done to promote the use of algae and derived products, in order to compare results after performing the same methodology.

The aim is to enable laboratories to conduct a standardized procedure for sample preparation to be used for analysing samples of algae and algae products.

1.5. Objectives

The main objective of the study is to validate the first protocol developed as a standard for the determination of phycocyanin from *Arthrospira platensis,* and then, compare the results of the extraction by the different methods established in the CEN protocol, to assess the standard method.

As specific objectives, we want to see the differences between the use of fresh biomass and the use of dry biomass; to evaluate the protocol established by CEN and to see if modifying some of the applied methodologies improves yields.

The study is organized into the following sections: section 2 details the materials and the method followed in the study, as well as the improvements added. Section 3 shows the results obtained after performing the experiment, and the discussion of them. Finally, section 4 presents the conclusions and the validity of the method.

2. Material and methods

The study was carried out at the BEA (Banco Español de Algas). Is an infrastructure belonging to the University of Las Palmas de Gran Canaria, which in turn is managed by the Fundación Canaria Parque Científico Tecnológico (FCPCT). Here, certain species of microalgae and cyanobacteria are isolated, conserved and characterized. In addition to developing new cultivation techniques and applications for macro and microalgae. It belongs to the European Culture Collections Organization (ECCO) and is included in the World Data Center for Microorganisms (WFCC-MIRCEN). Finally, it is an international authority for the deposit of microorganisms (according to the Budapest Treaty), being one of the 3 entities in Europe that fulfills this characteristic.

2.1. Protocol attempt

In the established protocol is specified the extraction procedures for the phycobiliprotein C-phycocyanin (C-PC) from *Arthrospira*. It also describes how to do the determination of C-PC.

The necessary equipment for the method is: Analytical balance with an accuracy of 0.01 mg, Vortex, Magnetic stirrer, Centrifuge, UV-VIS Spectrophotometer for 1 cm cells. 1mm glass beads and phosphate buffer are also needed as reagents and materials.

Preparation of samples:

We must consider whether the samples are frozen, fresh or dried. The extraction process will be done in triplicate for statistical purposes. It also will be done with dim light and temperature control (below 40 degrees) to avoid photochemical and temperature degradation of proteins (C-PC) (Woo Sung Park et al, 2018).

Note: if samples are frozen, note that the first cellular break has been made.

For fresh samples, start with 10-20 minutes of centrifugation (3000-15000 x g at 4ºC). Repeat it until getting a compact pellet. When the supernatant is clear and homogeneous, discard it. Once biomass has been obtained, rinsed it twice with distilled water and centrifugate it again. Next step is to freeze the samples, at -20ºC or -80ºC (recommended)

to use it again in the future. This process can last 1 day. After that, when we have the final pellet/filter, resuspend it in the extraction solvent (phosphate buffer 0.1M, pH=7.0). Use it in a proportion 50:1 (biomass:solvent). Then is needed to do cell disruption. We obtain it by repeating 2 freeze-thow cycles at -20ºC to 4ºC in darkness (incubated until solid). Vortex the mixture for 1 minute between the cycles. This is followed by centrifugation of the samples at 15000 x g for 20 minutes at 4ºC, and the pellet and supernatant are separated. C-PC will be given by the supernatant.

For dried samples, weight 50 mg of sample and resuspend it in 10 mL phosphate buffer (0.1M, pH=7.0) as the extraction solvent (proportion= 5:1). Vortex the mixture for 1 minute maximum and repeat it twice (avoiding overheating). In this case, cell disruption is obtained by just 1 freeze-thow cycle at -20ºC to 4ºC in darkenss (incubated until solid). Vortex the mixture like fresh samples between each cycle. After that, the slurry is agitated for 2-12 h (overnight) in a magnetic stirrer at constant speed and at 4-8ºC in darkness. A glass beads (1 mm) in the solution could increase cell lysis. Then, centrifugate the samples at 15000 x g for 20 minutes at 4ºC, and the pellet and supernatant are separated. C-PC will be determined in the supernatant.

Finally, there are two optional methodologies for extraction of C-PC: ultrasound and bead milling. For ultrasound, submit samples to 3 ultrasonic cycles, at 20KHz of 1 minute each. Do it in an ice bath to avoid overheating. For bead milling breaking procedure subject the samples to a 5 minutes cycle at 30Hz.

Once the extract is obtained, the C-PC is quantified.

1. C-PC determination:

   Supernatant is collected and spectrophotometric measured at 280nm, 615nm and 652nm. The blank is made with the extraction buffer (phosphate buffer 0.1M, pH=7.0). The quantification of phycocyanin is made using the following equation by Bennett and Bogorad (1973):

   $$\text{C-PC (mg mL}^{-1}\text{)} = (A615 - 0.474 \times A652) / 5.34$$

2. Purity criteria:

   By the value of A_{max}/A_{280} we can determinate the purity of the phycobiliproteins, and in this case, of the phycocyanin in the samples. We measure:

   A_{615}/A_{280}nm for phycocyanin respect to other proteins in the solution.

   A_{650}/A_{615}nm for allophycocyanin respect to phycocyanin in the solution.

   A_{680}nm for the detection of chlorophylls contamination.

   The purity criteria scale is shown in Table 1.

3. Calculations:

For the wet/fresh samples, we calculate the dry weight-fresh weight ratio.

$$d.w. = Dry\ weight\ (kg) = \frac{Weight_{dried\ sample} \times Dry\ matter\ content}{100}$$

After the extraction, we calculate the amount of C-PC in mg mL^{-1} (using Bennett and Bogorad equation). We can also calculate it in percentage:

%= (g C-PC/g dry weight biomass) x 100

Yield can also be estimated in terms of mg/g as

$$Yield\ \left(\frac{mg}{g}\right) = \frac{C - PC(\text{mg mL} - 1\ ) \times Volumen\ extract\ (ml)}{dry\ weight\ (g)}$$

Moisture content is used to determine the water content of the sample. It can be calculated as:

$$Dry\ matter\ content\ (\%) = \frac{Weight_{crucible+dry\ sample} - Weight_{crucible}}{Weight_{sample\ as\ received}}\ \text{x } 100$$

$$Moisture\ content\ (\%) = 100 - Dry\ matter\ content$$

$$= 100 - \left(\frac{Weight_{crucible+dry\ sample} - Weight_{crucible}}{Weight_{sample\ as\ received}}\ \text{x } 100\right)$$

As the experiment was progressing, new steps were added to the methodology, to improve the efficiency of the method. It was observed that not all of the phycocyanin was extracted from the sample. For fresh samples, it is necessary to perform 4 freeze-thaw cycles to obtain a total cell rupture, centrifuging in medium to separate the supernatant from the biomass. The total phycocyanin content of the sample will be the sum of the phycocyanin extracted in each of the 4 centrifugations.

For dried samples, it is necessary to perform 2 more freeze-thaw cycles for a total of 3 centrifugations. The total phycocyanin content of the sample will be the sum of the phycocyanin extracted in each of the 3 centrifugations.

The next flow chart (Figure 4) shows the steps to be followed for phycocyanin extraction form the Spirulina.

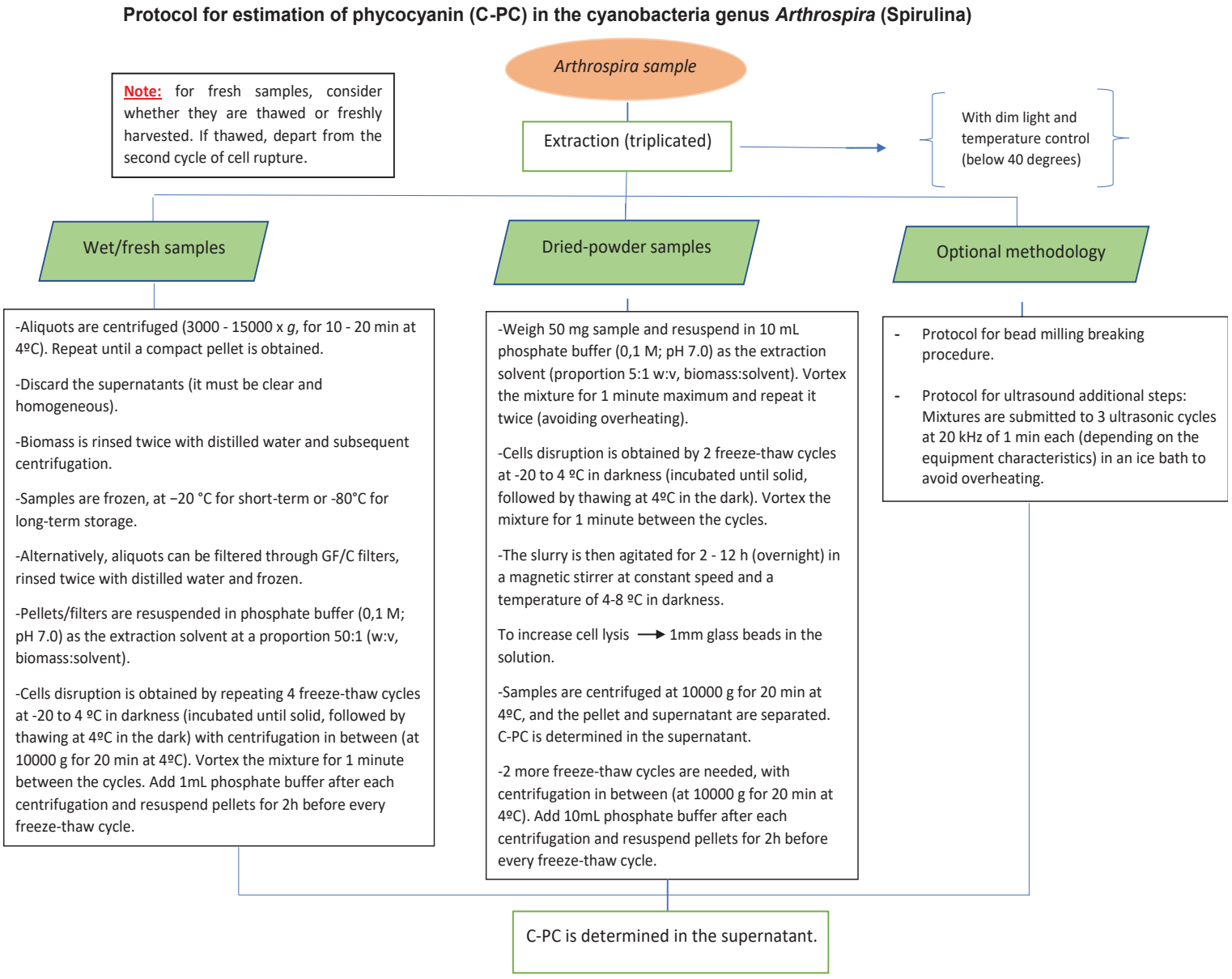


Figure 4: Flow chart Protocol for extraction of phycocyanin (C-PC) in the cyanobacteria genus *Arthrospira* (Spirulina). Self-created

A diagram of the steps to be followed for the spectrophotometric determination of the phycocyanin extract obtained is shown below (Figure 5).

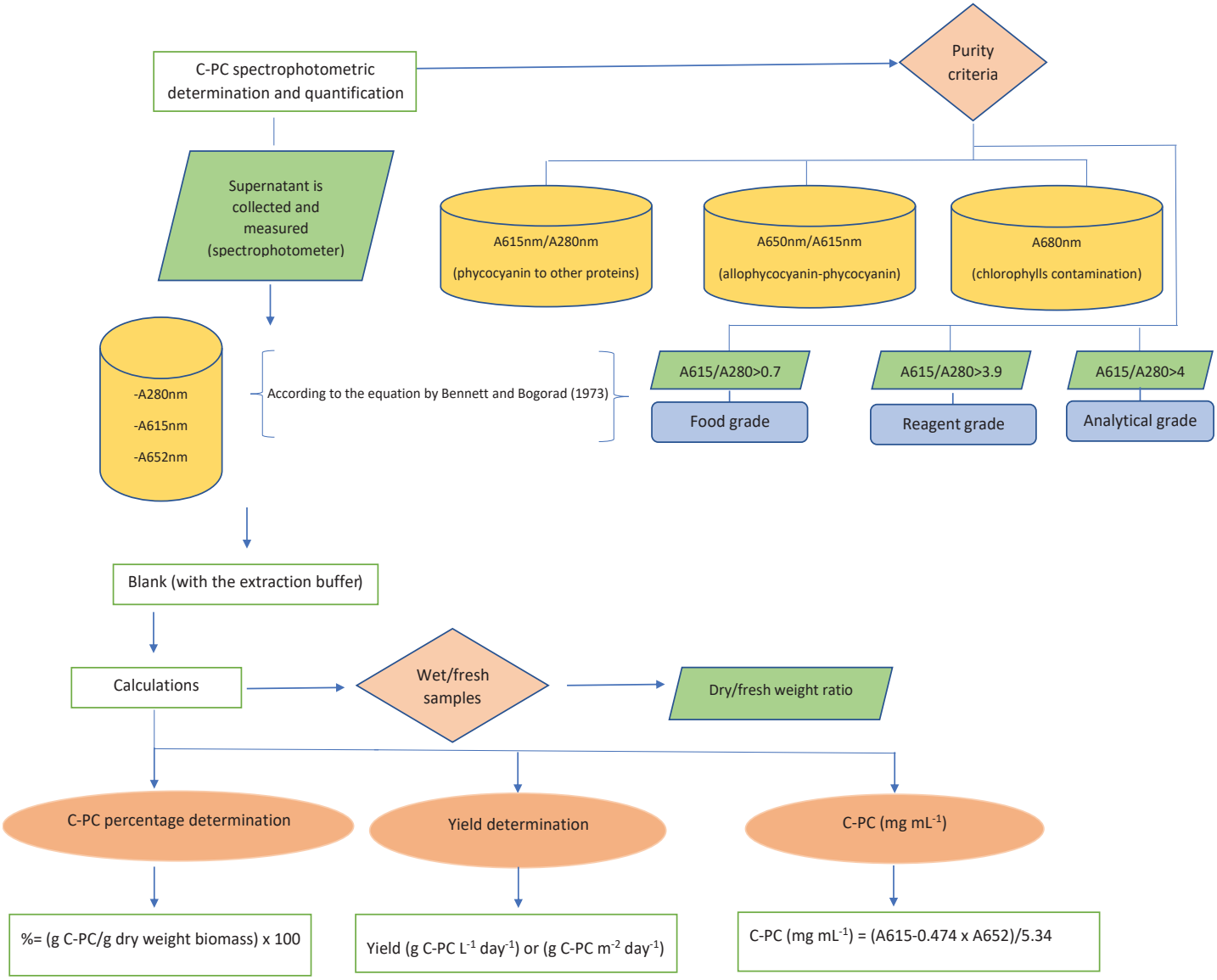


Figure 5: Flow chart Protocol for determination of phycocyanin (C-PC) in the cyanobacteria genus *Arthrospira* (Spirulina). Self-created

2.2. Experiments

Several experiments were conducted in order to improve the extraction performance. Biomass was obtained from a culture of the BEA culture room. It is culture BEA_0007 of the species *Arthrospira platensis*. It is in the activation chamber at a temperature of 25°C, 120 µmol photons/m^2s, with constant aeration and a photoperiod of 16:8 (light:dark).

A volume of culture was taken to obtain two grams of biomass. First of all, a sample of the culture is taken to look at the cells microscopically. During the whole experiment, after each process, a microscopic picture is taken to evaluate the level of cellular rupture.

The volume is filtered to remove the medium and the biomass is washed with distilled water. This is done by centrifuging (used model: Thermo Scientific™ 75004521) the biomass with a previous washing, to remove as much of the culture medium as possible and any salts that may be present.

The initial sample is divided into twelve samples. Three of them will be freeze-dried. The remaining nine will be fresh samples.

Fresh samples

Fresh samples are divided according to the three procedures to be followed: protocol for wet samples, protocol for bead milling breaking procedure and protocol for ultrasound.

-Protocol for wet samples

First, start with the biomass filtering and washing. Then biomass is frozen (-20ºC). After this first frozen-thawed cycle, the level of cellular rupture is checked under the microscope. Then pellets are resuspended in phosphate buffer for two hours as the extraction solvent at a proportion 50:1 (biomass:solvent). Freeze again pellets to do the second freeze-thaw cycle. Check again the cellular rupture after thawing. After this cycle, do the first centrifugation (20 min, 4ºC, 10000g). First supernatant is obtained. Measure it.

When performing the experiment, it was discovered that two more cycles of phosphate buffer (2h), freeze-thaw and centrifugation were necessary, obtaining a total of 4 supernatants. The total amount of phycocyanin in the sample is the sum of the phycocyanin obtained in each of the four extractions.

-Protocol for bead milling breaking procedure

This procedure requires a ball mill (used model: Retsch MM 400) to perform the cellular breakage. In this case, after the first common freeze-thaw cycle following biomass washing, phosphate buffer is added to the sample in a 50:1 ratio and left to act for two hours. After this, the first centrifugation is performed (20 minutes, 4ºC, 10000g). A first supernatant is obtained. Then the cells are broken with the ball mill: a cycle of five minutes at 30Hz is performed. A picture is taken afterwards to observe the cell rupture. Buffer (50:1) is then added and left to act for two hours. This is followed by the second freeze-thaw cycle. A picture is taken at thawing and the second centrifugation is performed. The second supernatant is obtained and measured.

During the experiment it was found that a total of four centrifugations were needed, with the freeze-thaw cycles in between. The total amount of phycocyanin contained in the sample will be the sum of the phycocyanin obtained in the four supernatants.

-Protocol for ultrasound

This procedure is done with a sonicator (used model: Cole-Parmer 4710). After the first common freeze-thaw cycle following biomass washing, phosphate buffer (pH=7) is added to the sample in a 50:1 ratio. Then, samples are submitted to 3 ultrasonic cycles at 80KHz of one minute each (in an ice bath to avoid overheating). A picture is taken afterwards to observe the cell rupture. After this, the first centrifugation is performed (20 minutes, 4ºC, 10000g). A first supernatant is obtained and measured. Buffer (50:1) is then

added and left to act for two hours. This is followed by the second freeze-thaw cycle. A picture is taken at thawing and the second centrifugation is performed. The second supernatant is obtained and measured.

During the experiment it was found that a total of four centrifugations were needed, with the freeze-thaw cycles in between. The total amount of phycocyanin contained in the sample will be the sum of the phycocyanin obtained in the four supernatants.

Freeze-dried samples

The DW/FW ratio (dry weight/fresh weight) was obtained with the freeze-dried sample. Three samples with a fresh weight of 500 mg were prepared, frozen and freeze-dried. After obtaining the dry weight, the first freeze-thaw cycle is done. The freeze-thaw cycle is repeated, observing cell rupture between cycles. After this, the aqueous compound is stirred for two hours, in the dark with a magnetic stirrer (used model: Eppendorf Thermomixer Comfort), at a constant speed and a temperature between 4-8ºC. Cell rupture is checked again. The samples are centrifuged for 20 minutes, at 10000g and 4ºC. The supernatant is separated and measured. An additional 10mL of phosphate buffer (pH=7) are added and the pellet is suspended for two hours, after which it is frozen at -20ºC. The sample is thawed and centrifuged again. The second supernatant is measured. A last cycle is performed, and a third supernatant is obtained. The total phycocyanin content of the sample will be the sum of the three extractions.

2.3. Statistical analysis

Once the experiments have been completed, a statistical analysis is carried out in order to analyze the data and study the differences between the different extraction procedures. According to the data obtained, an ANOVA analysis is performed. It tests the null hypothesis that the means of K populations (K >2) are equal, against the alternative hypothesis that at least one of the populations differs from the others in terms of its expected value. In this case a one-way ANOVA is used to then check for differences between the means (C-PC concentrations obtained) and the different groups (processes).

3. Results and discussion:

First step is to determinate dry weight (DW)/ fresh weight (FW) ratio (with it we can perform the conversion from FW to DW). Data were taken with two samples (in triplicate): fresh and freeze-dried samples. 3 fresh samples were placed in the oven for 24h, at 60ºC. Their weight was measured before (FW) and after drying (DW). The data are shown in Table 2a. 3 fresh samples were taken; their weight was measured and freeze-dried. Then their dry weight was measured. The data are shown in table 2b.

Table 2a. DW/FW ratio with stove

DW/FW ratio with stove

Sample	FW(mg)	DW(mg)	DW/FW	DW/FW(%)
1	280.4	252.2	0.8994	8.9943
2	236.2	212.1	0.8980	8.9797
3	192.2	173.6	0.9032	9.0323
			Mean	9.0021
			Deviation	0.0271

Table 2b. DW/FW ratio with freeze-dried samples

DW/FW ratio with freeze-dried samples				
Sample	FW(mg)	DW(mg)	DW/FW	DW/FW(%)
1	532.7	452	0.8485	8.4851
2	471.4	452	0.9588	9.5885
3	513	452	0.8811	8.8109
			Media	8.9615
			Deviation	0.5668

Observing the data, we can conclude a good result for the DW/FW ratio. Practically the same values are obtained, with a low standard deviation.

As established in the protocol, a photo was taken after each process to observe under the microscope the level of cellular rupture. First check that the crop is in perfect condition (Figure 6a, 6b).

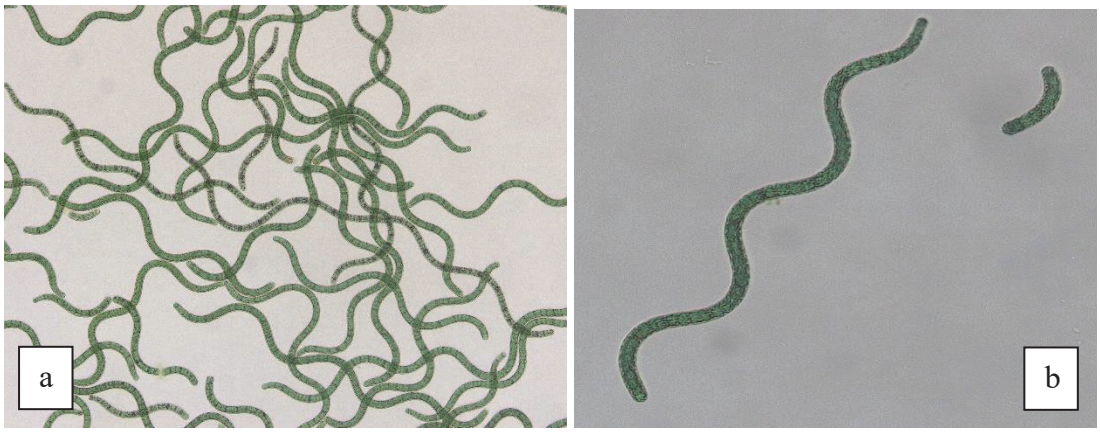
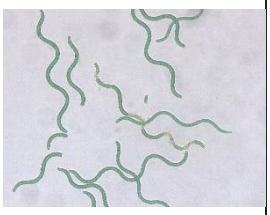
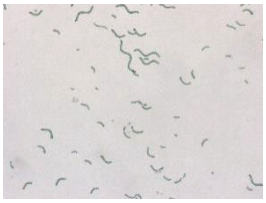
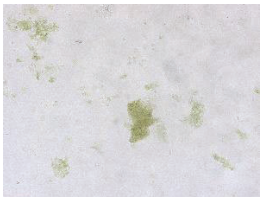
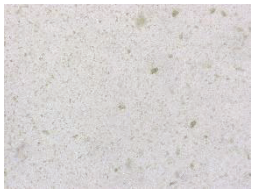
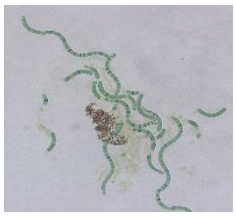
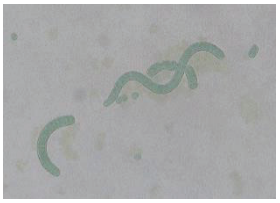
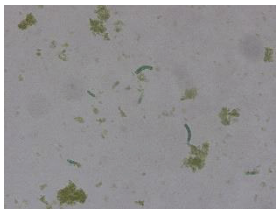
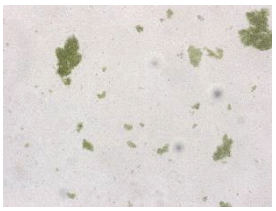
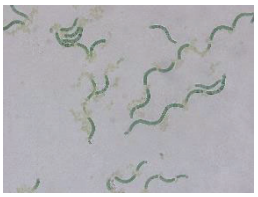
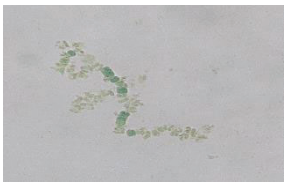
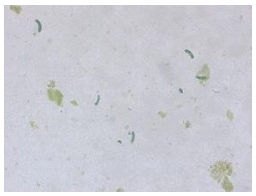
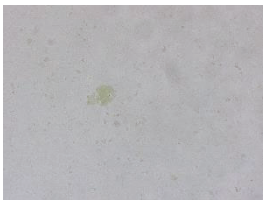
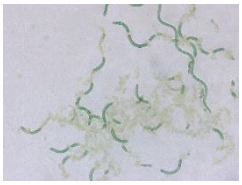
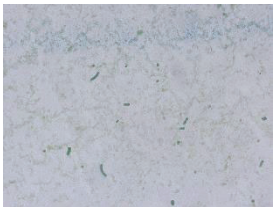
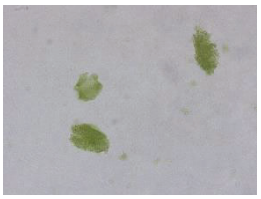
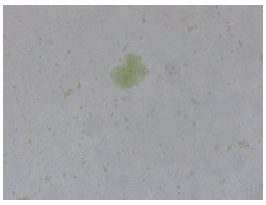


Figure 6a,6b: Freshly harvested crop

After following the steps of the protocol and applying the different methodologies, a picture was taken after each procedure to observe under the microscope the level of cellular rupture. The following table (Table 3) summarizes in images the cellular rupture after each process. In them we can see how, starting from perfectly formed filamentous cells, we obtained a cellular rupture that left our pigment of interest free and only biological material is left.

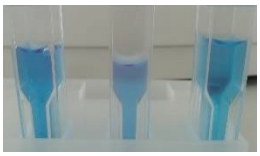
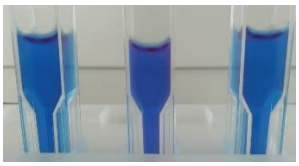
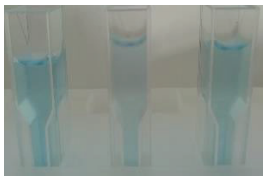
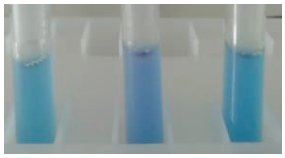
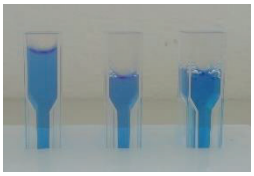
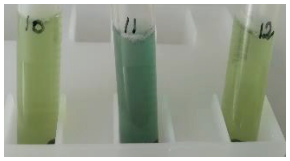
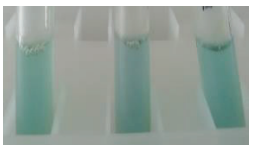
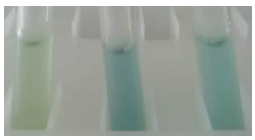
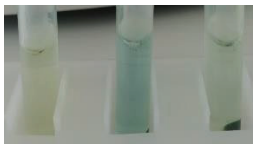
Table 3. Images of cellular rupture taken during experiment

Samples	Method	1º cycle	2º cycle	3º cycle	4º cycle
Dried-powder	Freeze-thawing				
Fresh	Freeze-thawing				
	Bead milling				
	Sonication				

It can be seen how, in the second cycle, the freeze-dried and ultrasound-treated samples seem to offer better cell rupture results.
It is also observed that there is no significant difference after performing the fourth freeze-thawing process.

Next Table (Table 4) collects the images taken of all the extracts obtained during the experiment, so that the purity of the extracts can be observed according to the cycles of the protocol.

Table 4: Images of the extracts obtained in each cycle

Samples	Method	1º cycle	2º cycle	3º cycle	4º cycle
Dried-powder	Freeze-thawing				
Fresh	Freeze-thawing				
	Bead milling				
	Sonication				

If we look at the photos of the freeze-dried sample, we can see that the extract obtained after the second cycle appears to be more concentrated than the first and third extracts. Likewise, it is observed that the fourth cycle is useless, since an almost transparent supernatant is obtained.

The same difference is observed in the extracts of the sample following the standard protocol: frozen-thawed. It is observed that the extract obtained after the second cycle has lower concentration than the third one.

As for the samples that follow the milling process, the images seem to indicate that this process could be too aggressive, contaminating the samples with chlorophyll and giving rise to this greenish color.

Samples following the ultrasound procedure show a super concentrated first extract. In this case, its absorbance could not be measured, since the pellet was not well formed. After the second cycle, yellow-greenish colors begin to appear, again indicating contamination by other pigments.

After analyzing the procedures qualitatively by means of images, we proceed to the quantification of the results. Table 5 shows the amount of C-PC (mg/ml) obtained in each cycle.

Table 5

Amount of C-PC (mg/ml) obtained in the experiment (n=3)

Processes	Cycle 1	Mean±deviation	Cycle 2	Mean±deviation	Cycle 3	Mean±deviation	Cycle 4	Mean ±deviation	C-PC (mg/ml)
Freeze-thaw	0.3029		0.1137		0.0568		0.0047		
	0.3205	0.3081±0.01	0.1627	0.1441±0.03	0.2726	0.2075±0.13	0.0708	0.0323±0.03	2.1092
	0.3010		0.1559		0.2932		0.0214		
Ultrasound	0		0.2780		0.0255		0.0054		1.1639
	0	0	0.3131	0.3026±0.02	0.0974	0.0597±0.04	0.0568	0.0256±0.03	
	0		0.3167		0.0562		0.0147		
Bead mill	0.1985		0.0574		0.0774	0.0817±0.007	0.0092		
	0.1471	0.1901±0.04	0.1669	0.0902±0.07	0.0898		0.1162	0.0479±0.06	1.2297
	0.2247		0.0464		0.0779		0.0183		
Freeze-dried	0.2098		0.2805		0.0413		0.0110		
	0.1314	0.1590±0.04	0.2901	0.2832±0.006	0.0258	0.0331±0.008	0.0095	0.0103±0.0	1.4567
	0.1357		0.2789		0.0323		0.0105		

Observing the results, a higher amount of C-PC was obtained after the first cycle of the standard protocol for fresh samples (frozen-thawed). For the second cycle, the most concentrated extracts were obtained in the freeze-dried samples and the fresh samples treated with the ultrasound protocol. In the third cycle the concentrations decreased, the maximum being again that of the standard process for fresh samples. In the fourth cycle, very low concentrations were obtained, being the maximum value theone of the milling protocol. Finally, the process by which the highest amount of C-PC (mg/ml) was obtained is freeze-thaw for fresh samples.

After this, the percentage of C-PC is calculated. Table 6 shows the C-PC (%) obtained by cycles.

Table 6

C-PC (%) obtained in the experiment

Processes	Cycle 1	Mean±deviation	Cycle 2	Mean±deviation	Cycle 3	Mean±deviation	Cycle 4	Mean±deviation	C-PC (%)
Freeze-thaw	5.4080		2.0304		1.0133		0.0836		
	3.0540	4.0388±1.22	1.5502	1.8274±0.25	2.5975	2.3904±1.89	0.6747	0.3393±0.30	25.7801
	3.6545		1.8936		3.5605		0.2598		
Ultrasound	0		6.2164		0.5712		0.1212		
	0	0	5.7656	6.3932±0.73	1.7940	1.2144±0.61	1.0467	0.5004±0.48	23.4049
	0		7.1976		1.2782		0.3332		
Bead mill	3.9843		1.1514		1.5544		0.1845		
	2.5248	3.7203±1.09	2.8655	1.6590±1.05	1.5412	1.5698±0.04	1.9950	0.8525±0.99	24.3239
	4.6518		0.9602		1.6138		0.3780		
Freeze-dried	4.3951		5.8767		0.8656		0.2296		
	3.1106	3.4859±0.79	6.8672	6.2702±0.53	0.6114	0.7265±0.13	0.2239		
	2.9519		6.0667		0.7025		0.2281	0.2272±0.00	32.1293

Seeing data (Table 6), the observations in the table above (Table 5) are checked. In the first cycle a higher percentage of C-PC is obtained in the fresh samples following the standard protocol. There is a small difference between this process and the others, showing a lower percentage. In the second cycle the percentages of C-PC obtained are maximum in the ultrasound and lyophilized processes. The first one shows the null extraction in the first cycle, increasing the concentration in the second one. As for the freeze-dried sample, the maximum yield is observed in the first two extraction cycles. As for the third cycle, the standard process for fresh samples again shows the maximum value. The fourth cycle shows the lowest concentrations, being the ball mill the one that obtains the best concentrations for this cycle. Looking at the total percentage obtained by each process, the process for freeze-dried samples presents the maximum, followed by the standard process for fresh samples. The process with the lowest %C-PC is the ultrasound process.

We can already calculate the yield (mg/g). The following table collects the yields (Table7)

Table 7

Yields(mg/g) obtained after each cycle

Processes	Cycle 1	Mean±deviation	Cycle 2	Mean±deviation	Cycle 3	Mean±deviation	Cycle 4	Mean±deviation	Total yield (mg/g)
Freeze-thaw	5.9488	4.4427±1.35	2.0304	1.8247±0.25	1.0133	2.3904±1.29	0.0836	0.3393±0.30	8.9972
	3.3594		1.5502		2.5975		0.6747		
	4.0200		1.8936		3.5605		0.2598		
Ultrasound	0	0±0	9.9462	10.2291±1.17	0.5712	1.2144±0.61	0.1212	0.5004±0.48	11.9439
	0		9.2250		1.7940		1.0467		
	0		11.516		1.2782		0.3332		
Bead mill	4.3827	4.0923±1.20	1.8422	2.6545±1.68	1.7098	1.7268±0.04	0.1845	0.9190±1.11	9.3926
	2.7773		4.5849		1.6954		2.1945		
	5.1170		1.5363		1.7752		0.3780		
Freeze-dried	4.3951	3.4859±0.79	5.8767	6.2702±0.53	0.8656	0.7265±1.13	0.2296	0.2272±0.00	10.7098
	3.1106		6.8672		0.6114		0.2239		
	2.9519		6.0667		0.7025		0.2281		

Attending to Table 7 and yields obtained, highlights the yield of the second cycle for the ultrasonic process. Since no extraction was performed in the first cycle, this value is not expressing a real maximum, but the sum of the yields of the two cycles.

Regardless of the above, the performance of the different processes is homogeneous per cycle and in terms of the total count, with the ultrasound process being the most efficient, followed by the protocol for dry samples.

Once the absorbances have been measured, the calculation for the purity of the extracts is done. Next table (Table 8) summaries the purity of the extracts obtained.

Table 8: Purity grades from extracts obtained during the experiment

Processes	cycle 1	cycle 2	cycle 3	cycle 4
Freeze-thawing	0.9	1.9	1.0	0.3
	0.9	1.1	1.0	1.1
	0.9	1.2	1.5	0.6
Ultrasound	0	0.8	0.5	0.2
	0	0.9	1.0	0.8
	0	1.1	0.9	0.4
Bead mill	1.3	0.5	1.0	0.3
	1.0	0.9	1.0	1.1
	1.2	0.5	1.1	0.5
Freeze-dried	1.0	1.2	0.9	0.5
	0.9	1.2	0.7	0.6
	0.9	1.1	0.8	0.5

>0.7= food grade
>3.9 reagent grade
>4=analytical grade

After working the results, it is obtained that the degree of purity of the extracts obtained during this experiment corresponds to food grade, being viable its use for food and cosmetics.

The phycocyanin obtained (Figure 7a, 7b, 7c) was stored in one of the BEA's refrigerators, protected from the action of light to avoid its degradation.

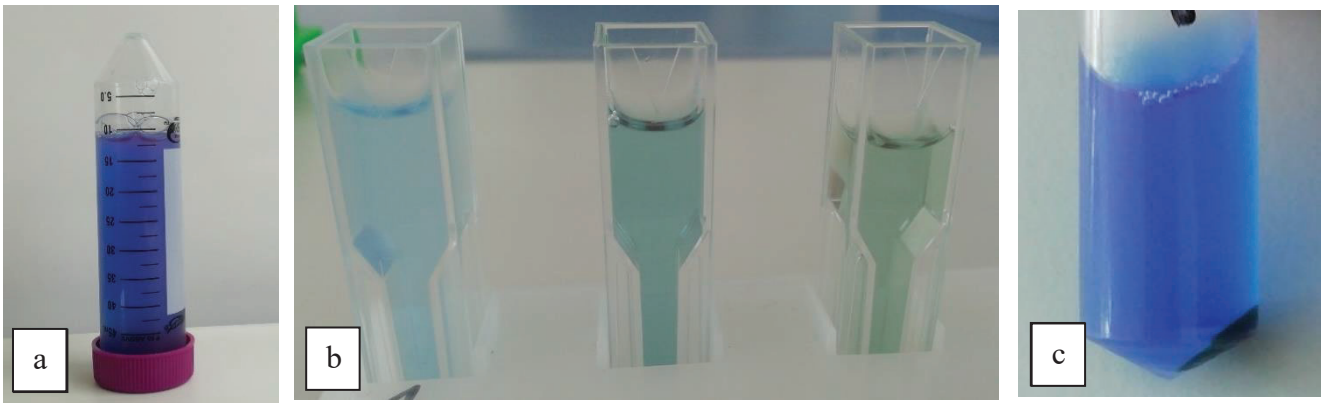


Figure 7a,7b,7c: some examples of the C-PC extracts obtained in the experiments

With a spectrophotometer, two absorption spectrum of the extracts obtained were performed (Figures 8a, 8b). Figure 2 shows us the absorption spectrum of the different phycobiliproteins. Two absorption spectra of the extracts are performed to verify that what we obtain corresponds to the absorption of the pigment of interest.

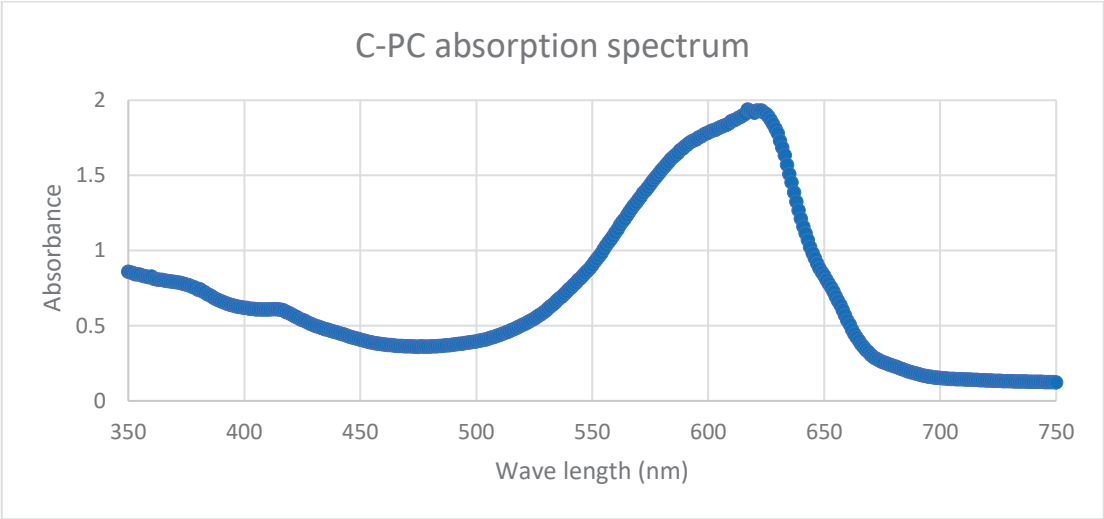


Figure 8a: absorption spectrum of the C-PC obtained during the experiments.

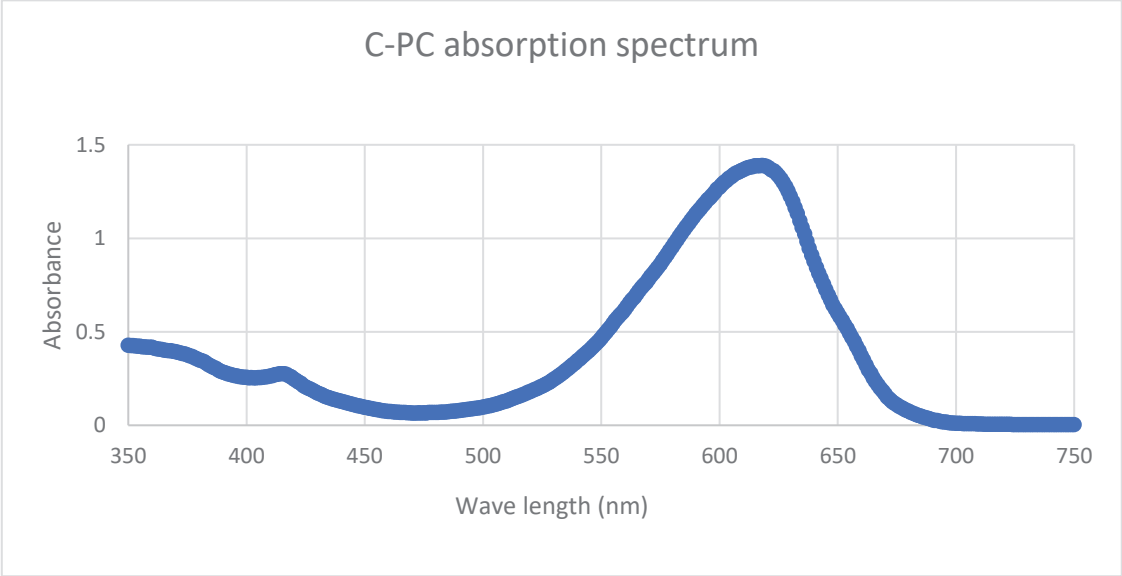


Figure 8b: absorption spectrum of the C-PC obtained during the experiments

The spectrum shows a phycocyanin extract, showing the maximum absorption for a wavelength of approximately 620nm.We can see that Figure 8a shows a pigment more concentrated than Figure 8b.

As mentioned in section 2, an ANOVA statistical analysis was performed to study the differences between the results obtained with the different processes. The summary table (Table 11) is shown below.

Table11: Summary table from ANOVA statistical analysis

Groups	*Count*	*Sum*	*Average*	*Variance*
Freeze-thaw	12	2.076026966	0.173002247	0.01441275
Ultrasound	12	1.163857678	0.09698814	0.01631655
Bead mill	12	1.229714232	0.102476186	0.0048046
Freeze-dried	12	1.456738951	0.121394913	0.0133831

We can see that the freeze-thawing process shows the maximum sum of C-PC (mg/ml) obtained.
The table for the analysis of variance (Table 12) is shown below.

Table12: Variance analysis table from ANOVA statistical analysis

Origin of variations	*Sum of squares*	*Degrees of freedom*	*Averga of the squares*	*F*	*Probability*	*Critical value for F*
Between groups	0.043197343	3	0.014399114	1.17743223	0.32920182	2.816465817
Within groups	0.53808704	44	0.012229251			
Total	0.581284383	47				

The statistical population (n=3) is enough for a reliable and valid data analysis. Considering the F value, it is observed that the value calculated with the data is lower than the critical F value, which indicates that the null hypothesis, which indicates that there is no significant difference between the means, that is, that there are no significant differences between the processes, is accepted.

4. Conclusions

After the work carried out and the subsequent data analysis it is concluded that the data obtained are good and comparable. Furthermore, the data of fresh and dry samples can be compared thanks to the DW/FW ratio, obtaining a better yield result for the fresh samples treated according to the ultrasound protocol, with a final yield of 11.9439 mg/g, followed by the fresh samples with a yield of 10.7898 mg/g. According to dry weight, the percentage of C-PC present in *A. platensis* is 20% of the protein dry weight (Patil & Raghavarao, 2007). The yield is very good although it is not the maximum that can be obtained

It is also concluded that the results are quite homogeneous. Thanks to the statistics and the analysis of variance (ANOVA), it is established that there are no significant differences between the processes, all being considered equally valid, in spite of the small differences in yields and %C-PC.

As for alternative protocols, ultrasound has the best performance, thus improving extraction. Mill protocol does not improve extraction performance.

Attending to Table 4, extracts, in general, are less pure as we perform more cycles, except for the exceptions in cycle 2 for dry samples and in cycle 2 for frozen-thawed samples. The fourth cycle was made to check that there were no significant quantities of C-PCs in the samples. After analysing the results, it is found that performing a fourth cycle does not increase the yield in the extraction by assessing the economic and temporal cost.

Therefore, the validity of the protocol is established, being this useful to perform extractions of C-PC of Spirulina through any of its protocols, presenting these homogeneous and comparable results.

5. Acknowledgments

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6. References

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EN17399 European Committee for standardization (CEN): Algae and algae products-Terms and definitions

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