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Abstract

C-phycocyanin is a highly valuable phycobiliprotein from *Arthrospira platensis*. However, its extraction, purification and conservation currently limit its commercial use. We review here the most common techniques and less conventional methods. Simple incubation in phosphate buffer at neutral pH can give high yields (>100 mg/g) and even high protein purity (> 0.7) if the parameters (buffer concentration, temperature, incubation time, *Arthrospira platensis.* state etc.) are chosen correctly. This method is preferable to incubation in distilled water or acid solutions. Some mechanical, physical or thermal treatments can improve the extraction yield and accelerate the release of C-phycocyanin. Ultrasound-assisted extraction has been widely studied and probes

generally give higher extraction yields than baths (> 100 mg/g) in less than 30 minutes. This technique can be coupled with others, such as freeze-thaw methods, to improve protein release. Indeed, freeze-thaw cycles are an efficient destructuring technique that can be used alone or as a pretreatment. Bead mills or pulsed electric fields can also be used to extract C-phycocyanin, but there is room for improvement in the choice of operating parameters. For purification of the extracted pigments, salting out or aqueous two-phase extraction can be used to increase phycocyanin grade. More advanced purification methods, mostly based on chromatography, can provide addition improvement. C-phycocyanin stability is dependent principally on pH and temperature, and is higher between pH 5 and pH 7 and at temperatures below 40°C, but the use of various preservatives or conditioning can increases its lifetime.

Keywords

Spirulina, Ultrasound, Salting out, Aqueous two-phase extraction, Ion-exchange chromatography, Freeze-thaw,

Introduction

Microalgae grow faster than other plants and can accumulate valuable compounds over short time periods [1]. They are used in many different applications, as health foods, colorants or therapeutic agents [2]. *Arthrospira platensis* (simplified to “spirulina”, due to its spiral-shaped structure), is the most widely consumed microalgae worldwide. Its benefits were discovered more than a thousand years ago, initially in Chad, and then in Mexico [3, 4]. It is rich in proteins (50 to 70% of its dry weight) and also contains minerals, vitamin B12, fibers and pigments. It has been reported to have positive effects on diabetes and obesity [5] and is used mostly for its beneficial nutritional properties. C-phycocyanin (C-PC) is one of the major high-added value molecules present in *Arthrospira platensis.* It is a blue, water-soluble protein that harvests light energy at wavelengths

(615-620 nm) poorly absorbed by chlorophyll. It is also present in several other microorganisms, such as *Spirulina fusiform* [6] and *Spirulina maxima* [7]. *Arthrospira platensis* also contains high concentrations of other phycobiliproteins, such as allophycocyanin (absorbance at 650 nm) and, to a lesser extent, pycoerythrin (absorbance at 565 nm). C-phycocyanin has been the subject of many studies in recent decades [8] and is now used in many applications [9]. It has been reported to have positive effects against ischemia-induced myocardial injury [10], inflammation [11, 12], liver injury [13, 14], and diabetes [15], and may also protect against cancer [16–18]. In addition to its pharmaceutical applications, C-PC can be used in nutrition [19] and as a natural coloring for food or cosmetics [20, 21]. Its fluorescence is also used in scientific research and in diagnostic applications for medicine [22, 23]. Its effective extraction from microalgae is, therefore, viral, but its purification and storage are also important for its scientific and commercial value. The quantitative characterization of C-PC is generally performed by spectrophotometric methods [24].

The cell wall of *Arthrospira platensis* is more fragile than those of several other microalgae, such as *Chlorella vulgaris* and *Haematococcus pluvialis* [25]. It may therefore be possible to release C-PC at higher rates through its membrane, provided that the conditions for its diffusion are optimized. The membrane of this microalga consists of four layers, the outermost of which is analogous to the cell wall of gram-negative bacteria; this layer is covered by protein fibrils, followed by a peptidoglycan layer and an innermost fibril layer [3]. The peptidoglycan layer is the principal contributor to cell rigidity [26].

Extraction at a high yield, purification to a high grade, and the storage of C-PC for long periods, together with waste minimization, are the limiting steps in the commercial use of this compound. We review here the most common techniques used. Even during simple incubation in water, increasing the temperature can increase the diffusion rate, and modifications to pH can increase the solubility of C-PC in water and may even partly solubilize the membrane. Enzymes can be

used to lyse the cell walls, and surfactants can help to destructure the membrane and partition the dye. Mechanical or physical chemistry methods can also be used to disrupt the cell wall and/or extract C-PC at a higher rate. Ultrasound, freeze-thaw, bead mills, and pulsed electric fields can be used. The purity of the pigment depends on the methods used, as these methods can extract a broad range of compounds. Extracts generally contain large amounts of chlorophyll. However, the large difference in the solubilities of C-PC and chlorophyll in water make it far easier to separate these two compounds than to separate C-PC from other soluble chromophores or proteins. The purity indicated in the literature therefore generate relates solely to C-PC as a proportion of the total amount of protein extracted, as estimated spectrophotometrically. This purity can subsequently be improved with the various techniques described here. Once C-PC is extracted, judicious conditioning, with careful control of pH and temperature, is required to ensure that it remains stable.

C-PC extraction

Simple extraction in aqueous solutions

Incubation in water

The extraction yield of C-PC is calculated almost exclusively by measuring absorbance at specific wavelengths. In water, the concentration of C-PC can be calculated as follows [27]:

$C_{C\text{-}PC}$ (mg/mL) = $\frac{A_{617}-0.474\times A_{652}}{5.34}$

With:

$C_{C\text{-}PC}$: Concentration of C-PC (mg/mL)

A_{617}: Absorbance at 617 nm

A_{652}: Absorbance at 652 nm

This equation is used in almost all articles on phycocyanin extraction, but it dates back to 1973 and was initially developed for C-PC extracted from *Fremyella*, and extinction coefficients may change differ between substrates and extraction conditions [28]. Furthermore, it does not take into account the influence of chlorophyll pigments on the absorbance values obtained. A correction has, therefore, recently been applied to this equation [29, 30], taking into account the specific absorption values of chlorophyll at these wavelengths. The equation thus becomes:

$$C_{C\text{-}PC}\ (mg/mL) = \frac{A_{617} - A_{617}(chla) - 0.474 \times (A_{652} - A_{652}(chla)}{5.34}$$

With:

$A_{617}(chla)$: contribution of chlorophyll *a* to the absorbance measured at 617 nm

$A_{652}\ (chla)$: contribution of chlorophyll *a* to the absorbance measured at 652 nm

The extraction yield is then calculated as follows:

$$Y_{C\text{-}PC}\ (mg/g) = \frac{C \times V}{m}$$

With Yc-pc: C-PC extraction yield (mg/g), c: concentration of C-PC (mg/mL), V: volume of the extract (mL) and m: mass of spirulina (g). Protein purity is evaluated by determining the ratio of C-PC absorbance at 620 nm and the absorbance of the aromatic amino acids of all proteins at 280 nm.

Several authors have performed extraction involving only incubation or gentle stirring in different solutions. The influences of time, microalga conditioning and concentration have also been investigated, sometimes through experimental design. The most interesting results of these studies are reported here, but we have deliberately excluded studies for which it was not possible to calculate an extraction yield (mg C-PC/g of biomass).

In distilled water

Table 1. Yield (Y) and purity (P) of C-PC after incubation of spirulina in distilled water, as a function of initial state of spirulina, concentration (C), incubation, temperature and time (N.I : not indicated).

#	Spirulina state	C (mg/mL)	Conditions	Incubation time (H)	Y (mg/g)	P	Ref.
1	Dried	1 - 170	Stirring 30 min	24	17-18	N.I.	[31]
2	Dried	33	N.I.	4x12	24	0.7	[32]
3	Wet	N.I.	37°C	14-16	11	0.2	[33]
4	Wet	N.I.	4°C	24	13	0.5	[34]
5	Dried (40°C, 48 h) then frozen (-18°C)	70	24°C	24	47	0.4	[35]
6		80	32.5°C	24	40	0.5	
7		40	30°C, rotary shaker	24	93	N.I.	

Spirulina is cultured in solutions with high concentrations of minerals [36, 37]. Its subsequent incubation in distilled water could, therefore, promote osmotic shock and enhance the diffusion of its pigments. However, the use of distilled water in the absence of other treatments (Table 1), with dried or wet spirulina, results in low yields (<25 mg/g) (entries 1-4) and low purities (< 0.8) [31–34], even with a long incubation time (24 hours) (entries 1 and 4). Thus, wet or dried spirulina is not significantly broken down by incubation in a hypotonic medium. With a single freeze-thaw cycle (entries 5-7), much higher yields can be obtained [35], but purity remains low. In this last study, a higher yield (93 mg/g) was obtained with the lowest microalga concentration (40 mg/mL). This indicates that distilled water does not provide sufficient osmotic pressure to enhance the diffusion of C-PC unless the membrane has already been degraded.

In acid aqueous solutions

An acid solution can be used to protonate C-PC and increase its solubility. It may also help to hydrolyze the cell wall. However, strong acids may also hydrolyze C-PC. According to published results (Table 2), the yield may increase with acid concentration (entries 1-4 and 6-7), but it remains low (< 50 mg/g) (entries 1-6). The cell membrane remains resistant to even high-concentration solutions of hydrochloric acid [38, 39], and the pigment is not stable at the resulting pH and cannot be extracted with a high yield. The yield obtained in sodium acetate, at a pH of 5, is lower than that obtained with distilled water (entry 9) [35]. It remains low (13 mg/g) (entry 8) even after successive extraction cycles [32]. Freeze-thaw cycles increase the extraction yield (46 mg/g) (last entry).

Table 2. Yield (Y) and purity (P) of C-PC after spirulina incubation in acid solutions, as a function of initial spirulina state, concentration (C) ,extraction medium, incubation temperature and time (N.I. : not indicated)

#	Spirulina state	C (mg/mL)	Extraction medium	Conditions	Incubation time (H)	Y (mg/g)	P	Ref.
1	Wet	5000	HCl 4 M	25°C	24	<2	N.I.	[38]
2			HCl 6 M			17		
3			HCl 8 M			12		
4			HCl 12 M			40		
5			Acetic acid, 1 M			<2		
6	Wet	N.I.	HCl 2-8 N	25°C	24	< 0.5	N.I.	[39]
7			HCl 10 N			2.1		
8	Dried	33	Sodium acetate (pH 5.0)	4°C	12 cycles	13	0.6	[32]
9	Dried (40°C, 48 h) then frozen	40	Sodium acetate (10 mM, pH 5.0)	30°C, rotary shaker	24	46	N.I.	[35]

	(-18°C)							

At neutral pH, with phosphate buffer

Table 3. Yield (Y) and purity (P) after the incubation of spirulina in phosphate buffer, as a function of initial spirulina state, concentration (C) , buffer molarity, incubation temperature and time (N.I. : not indicated)

#	Spirulina state	C (mg/mL)	Buffer molarity	Conditions	Incubation time (H)	Y (mg/g)	P	Ref.
1	Dried (Water bath, 50°C)					17	1.0	
2	Sun-dried (35°C, 1 h)	N.I.	0.1 M	4°C	24	65	0.9	[34]
3	Air-dried (25°C, 1 h)					80	1.8	
4	Wet	5000	10^{-4} M + EDTA + lysozyme	25°C	24	<2	N.I.	[38]
5	Oven-dried	60	0.01 M	25°C	24	96	3.5	[40]
6		40				104	3.2	
7		20				105	3.3	
8		60	0.1 M		48	121	0.8	
9		40			24	111	0.7	
10		20			12	89	0.7	
11	Freeze-dried	60	0.01 M	25°C	24	193	0.9	
12		40				197	1.0	
13		20				187	1.0	
14		60	0.1 M	4°C	24	151	0.8	
15		40				128	0.8	

16		20		25°C	12	313	0.6	
17	Dried (40°C, 48 h) then frozen (-18°C)	40	0.01 M	30°C, rotary shaker	24	105	N.I.	[35]
18	Dried	66	0.1 M	Stirring	2	17	0.9	[41]
19	Wet	10	0.1 M	Stirring	4	109	1.0	[42]
20	Freeze-dried (-80°C, 25°C, 8 h)					133	0.8	
21	Sun-dried (30-35°C, 7 h)					125	0.8	
22	Oven-dried (70°C, 7H)					24	0.2	
23	Freeze-dried					83	0.6	[43]

With the use of a phosphate buffer (PB) (Table 3), at neutral pH, it is possible, in some cases, to obtain very high extraction yields (more than 100 mg/g), even at room temperature. A high concentration gradient is required, together with a very low concentration of spirulina in water, to achieve a high diffusivity of C-PC. Very low yields are therefore obtained (entry 4) for a surprisingly high spirulina concentration (5000 mg/mL) [38]. Diffusion across intact cell membranes may also require a long incubation time. With dried spirulina at a concentration of 66 mg/mL and a short incubation time (2 hours) the yield obtained is lower (17 mg/g) (entry 18) [41] than that obtained at a concentration of 10 mg/mL and over a longer incubation time (from 109 to 133 mg/g) (entries 19 to 21) [42]. However, this difference may also reflect the pretreatment of the spirulina. Dried spirulina can give results similar to those obtained with wet spirulina, but the mode of drying must be chosen with care. For example, the exposure of spirulina to the sun, at a temperature of 30-35°C, for seven hours does not result in significant C-PC denaturation, as a yield of 125 mg/g can be obtained (entry 21) [42]. However, lower temperatures may be

advantageous, as a yield of 80 mg/g (entry 3) is obtained with air-dried spirulina at 25°C, versus 65 mg/g with sun-dried spirulina at 35°C (entry 2) [34]. Yield is strongly decreased by drying temperatures above 40°C, with a yield of 17 mg/g obtained at 50°C (entry 1), versus 80 mg/g at 25°C (entry 3), or 24 mg/g at 70°C (entry 22) versus 125 mg/g at 35°C (entry 21).

One research team [40] has performed a large number of extraction trials (108), varying the state of the spirulina (oven-dried or freeze-dried), the concentration of the phosphate buffer (0.1 M and 0.01 M), biomass concentration (20, 40 and 60 mg/mL), incubation time (12, 24 and 48 h) and temperature (-20°C, 4°C, 25°C). The best results for each biomass concentration are indicated here (entries 5-16). The freeze-drying of spirulina weakens the cell membrane, potentially accounting for the better yields obtained with this spirulina pretreatment (entries 11-16, with yields between 128 and 313 mg/g, whereas, for entries 5-10, yield is between 89 and 121 mg/g, although repeatability tests are required to confirm the maximum yield of 313 mg/g). These yields are associated to high purity values (>0.7). Diffusion should increase with temperature, and the highest temperature tested (25°C) does not seem to degrade the dye, even after incubation for 24 h (entries 11-13). This duration seems to be largely sufficient to obtain a high extraction yield and prolonging the incubation time to 48 h may jeopardize C-PC integrity. The concentration of spirulina (from 20 mg/mL to 60 mg/mL) does not seem to be a significant factor (entries 5 to 16).

In summary, better results are generally obtained with freeze-dried spirulina, extraction at ambient temperature (25°C) and in 0.01 M phosphate buffer at pH 7 (entries 11-13) [40].

With enzyme or surfactant

Table 4. Yield (Y) and purity (P) of C-phycocyanin after the incubation of spirulina in different enzymatic or surfactant solutions as a function of initial spirulina concentration, incubation time and temperature

#	Concentration (mg/mL)	Medium	Conditions	Y	P	Duration (H)	Ref.
1	167	Tween 80 (0.6%)	27°C	84	0.7	0.8	[44]
2	167	Tween 20 (0.6% pH 7)	27°C	77	0.8	1	
3	167	Triton X-100 (0.6%)	27°C	50	0.6	0.8	
4	5	Lysozyme (0.6%, pH 7)	37°C	70	1.2	0.8	
5	167	Lysozyme (1%, pH 7)	37°C	80	1.2	16	

Detergents and enzymes are often used for cell lysis [45]. Detergents are composed of amphiphilic molecules that can be incorporated into membrane bilayers, disrupting lipid-lipid and lipid-protein interactions. Above a certain concentration, they can totally disorganize the structure of the membrane, and even solubilize membrane proteins [46]. Enzymes, such as lysozyme, can digest the polysaccharide components of some cell walls at certain temperatures, pH values and activities [47]. In *Arthrospira platensis*, these enzymes can be used to digest the peptidoglycan layer. The various articles dealing with the enzyme-assisted extraction of C-PC do not precisely indicate yield or purity. In one study [44], the results obtained with lysozyme or surfactant were not superior to obtained with phosphate buffer at pH 7 (Table 4). However, lysozyme treatment may increase the purity of the C-PC obtained (entries 4 and 5). This enzyme could certainly be used to break down the peptidoglycan layer of the membrane further in optimized conditions. In the conditions (pH, concentration) used here, the surfactants tested may not have been able to solubilize the membrane proteins and seem to have had little effect on the peptidoglycan layer.

Extraction assisted by thermal and/or technological methods

C-PC extraction with ultrasound

Ultrasound without other treatment

Ultrasound can create high shearing forces, with the implosion of cavitation bubbles creating hotspots of high temperature and pressure capable of generating jets of liquid. When using ultrasound technology, it is important to differentiate between ultrasound baths with poorly localized waves and a low volumetric power (generally < 100 W/L) and ultrasound probes, which are generally used to treat smaller volumes, but at much higher power (>1000 W/L). Various parameters are crucial to obtaining a better yield. These parameters include probe diameter and amplitude, immersion depth, container shape, volume of solution, organelle concentrations, temperature, treatment time and pulse mode. However, the values of these parameters are not generally reported in studies dealing with the ultrasound-assisted extraction of C-PC. The concentration of the extract is generally indicated (in mg/mL), but the total volume is not always mentioned, precluding yield comparisons. Here, we separate the results obtained with ultrasound probes (Table 5) from those obtained with ultrasound baths (Table 6), as the energies involved are more than one order of magnitude differed. We indicate only the best yields obtained in each study.

Table 5. Yield (Y) of C-phycocyanin after extraction assisted by ultrasound probe,s as a function of the initial spirulina concentration (C), probe diameters (probe diam.), volume (V), treatment temperature (T) and time, extraction medium, pulsation (Puls.), power and/or amplitude of the ultrasound wave (N.I. : not indicated)

#	Spirulina state	C (mg/mL)	Probe diam. (mm)	V (mL)	T (°C)	Extraction medium	Power (W) Amplitude (%)	Puls.	Duration (min)	Yield (mg/g)	Ref.
1	Wet	0.5 (dry)	4	8	4	TRIS-SO4 (pH 7.5, 50 mM)	60 W ; N.I.	N.I	12	174	[48]
2	Freeze-dried	200	30	5	25	Britton-Robinson	130 W N.I.	75%	20	131	[49]

						(pH 7, 0.05 M)					
3	Wet	5	4	~ 10	4	Tris-HCl (pH 8.3, 10 mM)	60 W N.I.	N.I.	10	90	[50]
4	Dried (40°C, 48 h)	2.2	3 or 7	4	25	$CaCl_2$ 1%	50 W 80%	50%	9.3	91	[51]
5						Distilled water	50 W 74%			92	
6	Dried	10	13	100	4	Distilled water	600 W 20-40%	50% (60 s/60 s)	20	68-73	[52]
7	Freeze-dried	N.I. 15 mg	2	N.I.	4	PB (pH 6.8, 50 mM)	130 W 80% (best 20-100%)	67% (10 s/5 s)	4 (best 2-12)	67	[53]
8	Rehydrated (120 min)	167	N.I.	N.I.	25	PB (pH 6.8, 0.1 M)	200 W N.I.	50% (1 s/1 s)	2.5	52	[54]
9	Freeze-dried	67	25	N.I.	N.I.	PB (pH 7, 10 mM)	750 W 50%	(60 s/30 s)	5	60	[55]

Treatment duration was necessarily short (less than 20 min) in all trials with an ultrasound probe. Extraction yields could reach values exceeding 100 mg/g (entries 1 and 2).

Many parameters differed between the various experiments, so it is difficult to determine precisely which have the greatest influence on extraction yield. Spirulina concentration may affect yield as, with a very low spirulina concentration, 0.5 mg/mL (entry 1), it is possible to obtain a yield exceeding 150 mg/g, whereas yield decreases to 90 mg/g at a concentration of 5 mg/mL (entry 3).

However, differences in the buffers used in the two studies may also have contributed to this result. The addition of $CaCl_2$ (1%) to distilled water seems to have no effect on extraction yield (entries 4 and 5). Spirulina concentration does not seem to be crucial, as even high concentrations of freeze-dried spirulina (entry 2) can give a high extraction yield (131 mg/g), as indicated in a detailed study [49]. The use of high power with low temperature resulted in a low yield (~ 70 mg/g) (entry 6) [52], raising questions about the possible degradation of C-PC or decreases in cavitational activity at high ultrasound energy [53]. The low yields (67 mg/g, 52 mg/g and 60 mg/g) obtained in three studies [53] (entry 7) [54] (entry 8) [55] (entry 9) may reflect a short treatment time, but data for some parameters are missing. In the last of these studies, a relatively low yield (60 mg/g) was obtained after a short extraction time (5 min) despite the use of high power (750 W). Better yields were obtained with a low biomass-to-solvent ratio (1:15 was found to be better than 1:25 and 1:50). After the extraction of C-PC, some authors also treated the residual biomass with ultrasound to extract β-carotene [53].

Table 6. Yield (Y) and purity (P) of C-phycocyanin after ultrasound bath-assisted extraction, as a function of spirulina initial state, concentration (C), treatment time and temperature, extraction medium, ultrasound frequency (f) and power (N.I.: not indicated)

#	Spirulina state	C (mg/mL)	T. (°C)	Extraction medium	f (kHz)	Power (W/L)	Duration (min)	Yield (mg/g)	P	Ref.
1	Oven-dried	2	25	PB (pH 7, 0.01 M)	35	160	30	105	0.67	[56]
2	Freeze-dried							83	0.54	
3	Dried	48	<10	PB (pH 7, 0.1 M)	28	100	6 (plateau)	67	High*	[57]
4	Oven-dried (60°C , 3 h)	50 (dry)	30	Distilled water	N.I.	225	5	32	N.I.	[58]
5	Wet	60	45	Ethanol 96%	42	400	35	16	N.I.	[59]
6	Air-dried	N.I.	25	PB	37	112	25	8	0.6	[60]

	(2-3 days)			(pH 7, 0.1 M)						
7	Dried	40	35	Distilled water	35	300	180	81	0.86	[61]

*In this study, purity was defined as the ratio between C-PC concentration before and after ultracentrifugation

With an ultrasound bath, frequency may have an important effect on the purity of the extracts, or at least on the C-PC/chlorophyll ratio, as indicated by the reports that a frequency of 28 kHz is better than 20 kHz or 40 kHz (entry 3) [57]. Yields can be high (>100 mg/g) at high dilution (entries 1 and 2) [56], but many parameters, including spirulina state, concentration and the extraction medium used, may explain the lower yields obtained by other authors (entries 4-6) [58–60]. The positioning of the sample relative to the transducers should also be taken into account. With a long duration of ultrasound treatment (3 hours) at a high temperature (35°C), a good yield (81 mg/g) and purity (0.86) can be obtained from dried spirulina (entry 7). Better results were obtained with dried spirulina than with a freeze-dried or frozen sample, but the sources of spirulina were also different. The yields and purities obtained after three hours of sonication were similar to those after 24 or 48 h of classical extraction (incubation in distilled water with constant stirring, at the same temperature and spirulina concentration) [61].

Synergic use of ultrasound

Table 7. Yield (Y) and purity (P) of C-PC after ultrasound-assisted extraction coupled with another method (N.I.: not indicated)

#	Extraction method	Yield (mg/g)	Purity	Ref.
1	US alone (Probe, 24 kHz – 4000 W/L – 1/1-4°C 2.5 min)	52	0.6	[62]
2	Freeze/thaw alone (4 cycles F/T)	74	0.66	
3	US + Freeze/Thaw	109	0.4	
4	Maceration alone (8 min)	56	0.63	

5	US + maceration	99	0.63	
6	Homogenization alone (8 min , 25200 x *g*)	52	0.6	
7	US + Homogenization	91	0.65	
8	Tween 80 (0.6%, 50 min) alone	84	0.7	[44]
9	US + Tween 80	93	0.8	
10	Lysozyme (0.6%) alone	70	1.2	
11	US + Lysozyme	98	1.1	

Several authors have tried to associate the use of ultrasound with other extraction methods, such as freeze-thaw, maceration and homogenization, to increase extraction yield further, but only a few have studied the synergic effects of ultrasound and other treatments, as presented in Table 7 [44, 62]. In these studies, phosphate buffer (pH 6.8, 0.1 M) was used with spirulina rehydrated for 120 minutes and used at a concentration of 167 mg/mL.

Combinations of methods systematically provided a better extraction yield, particularly for freeze-thaw extraction (entries 2 and 3). However, purity decreased with increasing number of freeze/thaw cycles, as additional molecules are released with each cycle. Many studies have investigated freeze-thaw methods for C-PC extraction.

Freeze/thaw method

In freeze/thaw extraction, the freezing of water at temperature T_1 leads to an expansion of cell volume, whereas thawing induces a contraction. With repeated freeze/thaw cycles, the membranes are gradually weakened mechanically and may eventually rupture. The number of cycles is the parameter with the greatest influence, but other variables may also play a substantial role. The lowest temperature reached (T_1) must be lower than the freezing temperature of the main compounds present in the microalgae and is clearly of greater importance than the highest

temperature (T_2) but the rate of temperature change or the duration of the freezing and cooling steps (time at T_1 and time at T_2) may also be of the utmost importance. It is therefore essential to specify the freezing method used. A few articles have provided detailed information that can be exploited (Table 8).

Table 8. Yield (Y) and purity (P) of C-phycocyanin after freeze/thaw extraction as a function of initial spirulina state, concentration (C), extraction medium, temperature (T_1) and freezing time, temperature (T_2) and time of thawing, number of freeze/thaw cycles (N.I. : not indicated)

#	Spirulina state	C (mg/mL)	Extraction medium	T_1 (°C)	Time at T_1 (H)	T_2 (°C)	Time at T_2 (H)	Cycles	Y (mg/g)	P	Ref.
1	Wet	10	NH_4Cl (pH 4.4, 0.05 M)	-20	2	4	4	4	18	0.7	[63]
2		35	PB (pH 7, 0.125 M)						20	1.5	
3	Dried	50	PB (pH 6.5)	-20	4	25	0.33	4	83	N.I.	[64]
4	Wet	5	Tris-HCl (pH 8.3, 5 g/L)	-18	< 24	25	< 24	2	59	N.I.	[50]
5								4	101	N.I.	
6	Oven-dried	167	Distilled water	-18	2	25	0.5	N.I	17	N.I.	[31]
7		20							18		
8		10							18		
9	Dried	167	PB (pH 6.8, 0.1 M)	-40	4	25	1	4	74	0.7	[62]
10		100							75	0.6	

For short extraction times (≤ 2 h) at low temperature T_1, yields are low (about 20 mg/g), for both wet (entries 1 and 2) [63] and dried (entries 6-8) [31] spirulina, as some of the water present may not crystallize in these conditions. However, purity seems to depend on the extraction medium (entries 1 and 2) [63]. After 4 hours at low temperature, yields are significantly higher (entries 3, 9-11) [62, 64], whatever the concentration tested. The number of freeze-thaw cycles is also of the utmost importance, and these cycles can be repeated for as long as yield continues to increase (from 59 mg/g after 2 cycles to 101 mg/g after 4 cycles) (entries 4 and 5) [50], until a quantitative extraction is achieved.

Bead mills

Table 9. Yield (Y) and purity (P) of C-phycocyanin after bead milling, as a function of spirulina initial state, concentration (C), extraction medium, bead diameter, the percentage of the volume occupied by the beads, and operating conditions (N.I.: not indicated)

#	Spirulina state	C (mg/mL)	Extraction medium	Bead diameter	Bead volume percentage	Conditions	Y (mg/g)	P	Ref.
1	Wet	N.I.	PB (pH 7.2)	0.25 – 0.5 mm	N.I.	4 cycles, 25 s, 30 Hz	95	N.I.	[65]
2	Frozen	N.I.	None	0.106 - 0.125 mm	N.I.	Post milling: 100 rpm, 4 h, 25°C and S:L = 0.08:1 (distilled water)	64	0.8	[66]
3	Wet	12	$CaCl_2$ 10	0.8-5 mm	38%	1500 rpm	7	1.3	[7]

4	2	g/L			14	1.3	

In bead milling, the membranes are destroyed by mechanical impact with the beads, which are agitated to acquire a high kinetic energy. Smaller bead diameters results in smaller ground particles. For bead milling, the diameter of the beads must be specified, at the very least, but their number and total volume should also be indicated. Good yields (64-95 mg/g) (Table 9, entries 1 and 2) are obtained with beads that have a small diameter [65], even if no solvent is added during the crushing procedure [66]. Higher diameter beads give low yields (≤ 14 mg/g) (entries 3 and 4) [7].

Pulsed electric field (PEF)

This method has recently been used to increase the permeability of resistant cellular membranes. It has not been extensively developed for use with spirulina, which has a weaker cell wall than other microalgae and cyanobacteria. This method involves the application of an electric field in a treatment chamber. The field developed depends on the voltage applied between the electrodes and the distance between them. This electric field is applied in a pulsed mode; the pulses may have different widths and shapes and are applied for a particular treatment time. The specific energy is the electrical energy received by the sample [67]. Details are often provided as to the electric impulsions delivered to the system, but the concentration of spirulina is often not reported.

Table 10. Yield (Y) and purity (P) of C-phycocyanin after PEF extraction on wet spirulina as a function of the electric field applied, pulse mode, specific energy (Spec. energy) and post- treatment incubation time (Post-Incub time) (N.I.: not indicated)

#	Spirulina State	Extraction medium	Electric field	Puls./Time	Spec. energy	Post–Incub. time	Y (mg/g)	P	Ref.

			(kV/cm)		(J/mL)				
1	Wet	PB (pH 7.2)	40	Square, 1 µs	28	6 h (plateau at 30 min)	30	N.I.	[65]
2					56	6 h (plateau at 15 min)	85	N.I.	
3					112	6 h (plateau at 60 min)	72	N.I.	
4	Wet	Distilled water	25 (40°C)	Monopolar square: 3 µS Time: 150 µs	110	7 H (plateau at 6 h)	152	0.5	[68]
5			25 (10°C)		110	7 h	111	N.I.	

The electrical treatment is very short (less than 150 µs), but a longer post-stimulation incubation is then applied to give the desired compounds time to pass through the damaged membrane (Table 10). High yields (entry 4) were also obtained (152 mg/g) with this technique with the parameters tested [65, 68], but further optimization is undoubtedly possible.

High-pressure homogenization

This technique has rarely been used for C-PC extraction from spirulina. In one recent study [69], using spirulina dried in an oven at 40°C, moderate pressures, from 15 to 100 bars, were applied, for various time periods (up to 360 min) to a 100 mg/mL solution of spirulina in sodium phosphate buffer (pH 7, 0.1 M). Extraction yield increased with time and pressure, reaching 44 mg/g at 100 bars and 360 min, and the integrity of C-PC appeared to be preserved. A very high purity was achieved (3.6). Additional studies of this method are required to assess its effect on the selective extraction of C-PC more precisely.

Some of the various methods detailed above for the extraction of C-PC may be suitable for use alone or in synergy (particularly with freeze-thaw pretreatment) to obtain high extraction yields. It even appears possible to obtain both a good extraction yield and high purity (as observed in table 3, entries 11-19), provided that optimal spirulina pretreatment and extraction parameters are used, rendering C-PC extraction more economically viable. However, most studies have focused exclusively on extraction yield, the purity of C-PC either being totally ignored or considered a minor issue due to the large panel of purification techniques available.

C-PC purification

The purity of C-PC is evaluated by determining its concentration as a proportion of all the proteins extracted. This can be achieved by determining a simple absorbance ratio (A_{620nm}/A_{280nm}). Food-grade C-PC must have a purity greater than 0.7, whereas reagent-grade C-PC must have a purity of about 3.9. Analytical grade is attributed to any C-PC preparation with a purity higher than 4 [70, 71]. Depending on the purity expected, various methods can be used after extraction, and most of these methods consist of several successive steps [9, 72]. Extraction by simply incubating spirulina in phosphate buffer can result in good purity values, of 1 to 1.8 [34, 40, 42], but more disruptive methods rarely result in high purity. A simple salting-out technique is often sufficient to increase purity. Whenever a purification method is used, both final purity and the recovery percentage should be given. However, this second value is often missing from published reports. We will now considered the most relevant purification techniques described in previous studies, principally salting out, aqueous two-phase extraction and chromatographic methods.

Purification by salting out

Table 11. Purification of C-PC by salting out as a function of C-PC extraction mode, the salt used, initial purity, final purity and recovery percentage (Rec.)

#	C-PC extraction mode	Salt used	Initial Purity	Final purity	Rec. (%)	Ref.
1	FT	Sat. $(NH_4)_2PO_4$	0.9	2.4	N.I.	[63]
2	FT + 0.125 M sodium phosphate	Sat. $(NH_4)_2SO_4$	0.8	1.9	N.I.	
3	FT + 0.05 M NH_4Cl		1.5	2.8	N.I.	
4	Enzymatic extraction	50% $(NH_4)_2SO_4$	0.9	1.3	N.I.	[28]
5	FT + US	30% $(NH_4)_2SO_4$ then 50% and 65%	1.1	3.1	N.I.	[73]
6	Sc-CO_2 + dialysis	24% $(NH_4)_2SO_4$	1.8	1.9	N.I.	[74]
7	Sc-CO_2 + dialysis	39% $(NH_4)_2SO_4$	1.8	2.2	N.I.	
8	Sc-CO_2 + dialysis	50% $(NH_4)_2SO_4$	1.8	2.2	N.I.	
9	FT + US	25% $(NH_4)_2SO_4$ then 70%, 1 h, 4°C	1.4	1.8	N.I.	[21]
10	FT and first purification with chitosan	25% $(NH_4)_2SO_4$, 1 h	1.6	2.9	81%	[75]
11	Spray-dried	25% $(NH_4)_2SO_4$, 1 h + diafiltration	1.2	3.0	N.I.	[76]

Note. FT = freeze-thaw ; US = ultrasound ; Sc-CO_2 = supercritical CO_2.

In salting-out purification (Table 11), the aim is to decrease the solubility of the protein in water by adding a more soluble substance, thereby rendering protein hydration more difficult. Ammonium sulfate is the salt most frequently used for this purpose. Concentrations below 30% seemed to be insufficient in some studies (entry 6) [74], even if salting out was associated with other purification techniques (entries 10 and 11) [75, 76] with the aim of obtaining a high final purity. With a concentration of 39% to 50%, purity can be increased from 0.9 to 1.3 (entry 4) or

from 1.8 to 2.2 (entries 6-8) [28, 74]. The use of concentration gradients seems to give results that are at least as good, with a purity of 1.4 to 1.8 (entry 9) [21], or 1.1 to 3.1 (entry 5) [73] . These results are better than those obtained with a saturated solution, which gave purities from 0.9 to 2.4, 0.8 to 1.9, or 1.5 to 2.8 (entries 1-3) [63]. The purity indicated relates exclusively to the proteins present in the product. Dialysis may be performed to decrease the salt concentration in the extracted and/or purified C-PC.

Purification by aqueous two-phase extraction (ATPE)

Another simple separation technique widely used for C-PC purification is aqueous two-phase extraction (ATPE) with a polar polymer and a salt. Depending on the parameters used (type and concentration of salt, molecular weight of the polymer, pH, phase volume ratio, etc.), the desired proteins can be concentrated into a single aqueous phase (Table 12). With phase diagrams and a systematic approach, testing different molecular weights of polyethylene glycol "PEG" (4000, 1500, 6000, 20000 Da), different salts, pH, and volume ratios, the purity of C-PC was increased from 1.18 to 3.52 (entry 2) [77], with a high recovery percentage. With similar parameters, but starting from a lower initial purity and with a lower concentration of PEG 4000, another study failed to achieve such high purity (entry 3) [78]; this was also the case for the use of a polymer of lower molecular weight (entry 1) [7]. Purification *in situ* with PEG4000 and KH_2PO_4 at pH 7 was also found to be highly efficient (entry 5) [79]. With the use of an ionic liquid instead of PEG, some researchers reported obtaining both a high final purity and a high recovery percentage (entry 4) [80].

With lower-molecular weight PEGs and sodium phosphate, at a pH of 5.8, it was possible to increase purity from 0.42 to 1.31, and a third ATPE made it possible to increase purity to 2.11 [81] (entry 6). The use of trimethylamine (TMA) with PEG 1000 and Na_3PO_4 at pH 6.25 and 25°C

gave a better purification. Final purity was 4.36 with non-optimized parameters, and reached 5.21 after a response surface methodology, and even 6.71 for two-stage ATPE (entry 7) [82].

Table 12. Purification of C-PC by ATPE methods, according to initial and final purity and recovery percentage (Rec.) , by C-PC extraction mode, polymer nature, molecular weight, the nature and concentration of the salt used, and operating conditions.

#	C-PC extraction mode	Polymer & MW	Salt used	Conditions	Initial purity	Final purity	Rec. (%)	Ref.
1	Extraction with glass beads *in situ* with ATPE	PEG 1450 7%	KH_2PO_4/K_2HPO_4 20%	12 g/L of spirulina, pH 6.5	N.I.	1.3	N.I.	[7]
2	HPH	PEG 4000, 13%	KH_2PO_4/K_2HPO_4 14%	Volume ratio 0.8, pH 6	1.18	3.52	90.6%	[77]
3	Conventional	PEG 4000 5%	KH_2PO_4/K_2HPO_4 18%	pH 6	0.59	0.79	N.I.	[78]
4	FT	23% [Bmim]Cl	29% K_2HPO_4	3 mg/mL	N.I.	3.98	90.2%	[80]
5	*In situ*, air flotation	PEG 4000 (250 g/L)	K_2HPO_4 25%, pH 7	0.625% of crude extract	N.I.	Purif. 3.16-fold	87%	[79]
6	FT	PEG 1000	Na_3PO_4	pH 5.8	0.42	1.31	89.52	[81]
7	FT	PEG1000/ TMA-PEG1000	Na_3PO_4	pH 6-7, T<35°C	2.08	5.21	>97%	[82]

Note: HPH = high-pressure homogenization

Purification by chromatography or other techniques

Other methods for purifying C-PC generally involve chromatographic techniques (Table 13) for increasing purity by several units. Ion-exchange chromatography is the most widely used method (entries 1, 3-8, 10-11, 13, 15, 17).

Table 13. Final purity (FP) and recovery percentages (Rec.) after chromatographic treatments, as a function of chromatography type (Chrom. type), prior purification steps, column type and sample volume

#	Chrom. type	Prior steps	IP	Column type	Sample volume	Elution mode	FP	Rec. (%)	Ref.
1	IEC	SO, dialysis	3.10	DEAE-SEPHAROSE CL-6B (2.5*15 cm)	N.I.	200 mL NaCl (0-0.25 M) 1 mL/min	4.56	N.I.	[73]
2	GFC	#1	4.56	Sephadex G-100 (1.5*40 cm)	N.I.	0.002 M PB (pH 7), 0.5 mL/min	5.06	N.I.	
3	IEC	None	0.9	C10/C20 Q-Sepharose Fast Flow 8 mL (1*20 cm)	N.I.	50 mL NaCl (0.1 M then (0.1-1 M), in Tris buffer (pH 5)-40 cm/h	3.4	30.2	[85]
4	IEC	None	1	C10/C20 Q-Sepharose Fast Flow 8 mL	N.I.	0.1 M acetate buffer pH (5.6-3.4)	4.5	37.3	
5	IEC	SO, dialysis	1.81	DEAE Sepharose Fast Flow	5 mL	NaCl (0-0.35 M), 0.8 mL/min (C-PC) at NaCl concentrations between 0.25	2.5	N.I.	[21]

						and 0.30 M			
6	IEC	Charcoal, UF	2.78	DEAE Sephadex A-25 (2.6*60 mL)	3 mL 21.17 mg/mL	NaCl (0.1-0.3 M) in 10 mM PB (pH 6.8), 0.7 mL/min	4.3	42.3	[41]
7	IEC	Adsorption on different adsorbants	0.53	Q-Sepharose Fast Flow	N.I.	8 cm/h 36 mL NaCl (0-1 M) pH 7.5 , 45 cm/h	1.82	77.30	[86]
8	IEC	SO, dialysis	1.5	DEAE cellulose-11 (2*30 cm)	N.I.	Acetate buffer (pH 3.76-5.10), 20 mL/h	4.5	80%	[87]
9	HPC	SO, dialysis	0.85	Hydroxyapatite	N.I.	PB (2.5 mM – 70 mM)	2.8	N.I.	[28]
10	IEC	#9	2.8	DEAE Sephadex A-50 (1.5*20 cm)	N.I.	NaCl gradient, 0.15-0.45 M in 0.05 M PB (pH 8.0)	>4	N.I.	
11	IEC	SO, dialysis	1.45	DEAE Toyopearl 650 M (2.5x10 cm)	N.I.	NaCl (0-0.2 M)	4.42	N.I.	[88]
12	EBA	None	0.3-0.4	EBA	N.I.	$(NH_4)_2SO_4$(0-0.2 M)	2.9-3.6	35%	[32]
13	IEC	EBA, dialysis	2.9-3.6	Q-Sepharose based IEC	N.I.	NaCl (10-15 mM)	3.3-3.8	8.7%	
14	HPC	EBA, dialysis, IEC		Hydroxyapatite	N.I.	NaCl (0.2 M)	3-3.2	+5%	
15	IEC	ATPE	5.22	DEAE Sephadex (2.5*15)	N.I.	NaCl (0-0.35 M) 1 mL/min	6.6-9	N.I.	[89]
16	GFC	Activated	1.34	Sephadex G100	8 mL	0.005 M	2.77	51.9	[43]

		charcoal		(40-120 µm) (2.5 *20 cm)		NaPB, pH 7.0 2 mL/min		
17	IEC	#16	2.77	DEAE Sepharose Fast Flow (45-165 µm), (2.5*20 cm)	8 mL	NaCl (0-0.25 M), 2 mL/min	3.25	48.2
18	GFC then IEC	#16	3.51	Sephadex G100 & DEAE Sepharose	8 mL	As for #16 and #17	3.74	21.8
19	IEC then GFC	#16	3.25	DEAE Sepharose and Sephadex G100	8 mL	As for #17 and #16	3.82	25.3

IP: initial purity; FP: final purity; GFC: gel filtration column; IEC: ion exchange chromatography; EBA: extended bed adsorption; HPC: hydroxyapatite; Rec.: recovery yield

Most of the columns used are based on a positively charged diethylaminoethyl group bound to a polysaccharide matrix. Cellulose (entry 8), Sepharose (entries 1, 3-7, 10, 13, 15, 17) or a hydroxylated polymer (Toyopearl) (entry 11) are often used. For C-PC purification, a NaCl concentration gradient can be used at a neutral pH, controlling the interactions of the different proteins with the column. Near-optimal conditions were described as early as 1972 [90], based on a mixture of gel filtration and ion-exchange chromatography. These conditions were then adapted and modified [28]. Elution can be controlled by gradually changing ionic strength, but pH gradients can also be used, and both methods were tested in some studies (entries 3-4). The isoelectric point (PI) of C-PC is indicated as 4.7 [91], about 4.6 to 5.2 according to several studies [92–94], or about 4.1-6.4 [95]. The pH gradients used generally remain close to the isoelectric point, from 3.4 to 5.6 (entry 4) or from 3.8 to 5.6 (entry 8), which is almost PI-1 to PI+1, the charge of the protein being quite low, and salt gradients can remain low (<0.1 M) (entry 13), medium (0.1-0.5 M) (entries 5, 6, 10, 11, 15, 17) or extend to higher values (up to 1 M) (entry 3).

The salt concentration at which the enriched fraction is eluted depends on buffer strength and pH, and the type of column.

Hydroxyapatite columns are generally used in combination with ion-exchange chromatography (entries 9 and 14) to increase C-PC purity further through the complexation of the calcium and phosphate ions of this mineral matrix with the amino and carboxyl groups of proteins. The proteins are then eluted in different concentrations of competitive ions or at different pH values. Some authors have also used an extended bed adsorption system to adsorb proteins separately from cell debris and contaminants (entry 12). With complementary anion-exchange chromatography and hydroxyapatite purification processes for less pure fractions, it is possible to achieve purities >3 [32]. Proteins can also be purified on the basis of molecular size, by gel filtration (entries 2 and 16). The molecular weight of C-PC was initially estimated at about 30 kDa ([96]) in various microalgae, but it seems to be much larger, with a molecular weight of about 91 kDa, in *Arthrospira platensis*, as recently shown ([97]) with the use of large-volume polyglucoside beads (Sephadex G100 to Sephadex G150). Gel filtration can also be associated with ion-exchange chromatography, which can be performed before or after this method, with similar results (entries 18 and 19). Other purification techniques involve filtration, including nanofiltration in particular, to separate molecules of different molecular weights [42, 43, 85]. Some authors have also used activated charcoal or chitosan to absorb impurities [56, 85, 98]. In another, similar micoralga (*Spirulina fusiformis*), an aqueous ethacridine lactate (Rivanol) solution has been used for purification [6]. In association with salting out and gel chromatography, a very high protein purity (4.3) was obtained.

All these purification techniques can be used alone or as successive steps, and combinations are generally used, to increase purity. However, such approaches are likely to result in a decrease in recovery yield, albeit one less marked than that observed with ATPE and salting out. In a recent

study [43], an activated charcoal column gave a recovery yield of 89%, which was decreased to 51.9% by one-step gel filtration, and to 48.2% by IEC chromatography. The combination of these two methods resulted in the recovery of only 22 to 25% of C-PC (entries 16-19). Even high-grade C-PC obtained with a high recovery value by ATPE, such as that with a final purity of 5.21 and a recovery value exceeding 97% obtained in one study (table 12, entry 7), cannot be readily exploited if it is not packaged in an environment ensuring an appropriate shelf life for its field of application.

C-PC stability

Like most natural dyes and pigments, C-PC is stable only in very specific conditions. Most studies of the stability of C-PC have investigated the influence of temperature and pH on the persistence of the dye [33, 34, 39, 40, 52, 99–104]. Depending on the type of buffer salt and its concentration, differences in stability may be observed at any given pH and temperature, but there is a homogeneous tendency (Table 14). Below 25°C, the dye remains stable for at least five days if stored at a pH between 5 and 6. However, this stability gradually decreases with increases in temperature from 25°C to 45°C, resulting in a half-life ($t_{½}$) of less than one day, with severe decreases at higher temperatures [40, 100, 102]. At neutral pH, the dye seems to be slightly less stable [33, 103, 104] than at pH 6, and at pH>8, the dye is unstable. Below pH 5, the dye is rapidly degraded, with color completely disappearing within one day at pH 3, even at low temperature [104]. The chromophore of C-PC is phycocyanobilin, a linear tetrapyrrole covalently bound to phycobiliproteins via a cysteine residue with a thioether linkage [105]. It contains two carboxylic acids, with pKa values of 2.4 and 9.6 according to molecular modeling. Hence, at pH values between 5 and 7, the highest stability observed corresponds to a monoanion form in which the hydrogen atom binds to the two carboxylate groups [106].

In terms of light stability, the exposure of C-PC to intense illumination (3.28 x10^5 lux) for a period of 24 h leads to about 70% degradation of the pigment at pH 5 and 80% degradation at pH 7 [107]. At pH 7, C-PC exposed to white fluorescent light from a 20 W lamp is gradually degraded over a period of two months, with a loss of ~10% at 4°C, ~30% at 25°C and ~50% at 40°C [108]. Attempts have been made to increase the stability of the dye through the use of different preservatives. No effect of egg or whey protein, or of maltodextrin alone or in combination with xanthan gum, gum arabic or carrageenan was observed [103]. Beet pectin slightly improved thermal stability [109]. Sugars have also been tested. Honey is potentially a good preservative, but stability appears to be more closely linked to sugar concentration than to sugar type. Hence, with its high concentration at saturation, fructose would be expected to have a better effect than sucrose, maltose or glucose, whereas lactose would have no effect [110]. NaCl may have a positive effect even at 60°C, and may be a better preservative than sucrose and glucose [100, 111]. Sucrose can also stabilize C-PC at low temperature, as can calcium chloride, whereas citric acid at a low concentration (4 mg/mL) can keep C-PC stable, even at 35°C for 45 days [112]. Another study showed calcium chloride and ascorbic acid to be less effective preservatives than sucrose and citric acid, whereas benzoic acid stabilized the dye very effectively. Sodium azide and sodium citrate can also improve stability at 4°C and 25°C [98]. Another possible method of stabilization is the incorporation of C-PC into a matrix that protects it from oxidation. For example, C-PC is well-stabilized when solubilized in micelles [113, 114], in chitosan or alginate/chitosan microcapsules [115, 116], or in polyethylene oxide nanofibers [117].

*Table 14. Stability of C-PC with pH and temperature**

	<10°C	25°C	>40°C	>60°C
pH 3	Very unstable, $t_{1/2}$<1 day 0% after 1 day	Very unstable, $t_{1/2}$<1 day 0-72% after 2 days		

pH 4	Unstable 24% after 5 days	Unstable 32% after 5 days		
pH 5	Stable 82-100% after 5 days 99% after 30 days	Stable 90-92% after 2 days 76-100% after 5 days 88% after 10 days	$t_{1/2}$ = 2-19 h (47-50°C) $t_{1/2}$ =20 min (59°C)	$t_{1/2}$=11 min (60°C)
pH 6	Stable 93-100% after 5 days 98% after 30 days	Stable 91-100% after 2 days 92% after 4 days 84-100% after 5 days 84% after 10 days	70% after 1 hour (95% with glucose) $t_{1/2}$=3 h – 24 h (47-50°C), $t_{1/2}$=45 min (59°C)	30% after 1 hour (50% with glucose) $t_{1/2}$=3 min (60°C)
pH 7	Stable 84%-100% after 5 days 91% after 5 days 98% after 30 days	Stable 64-92% after 2 days 67% after 4 days 85-88% after 5 days 79% after 10 days	$t_{1/2}$=2 h – 10 h (47-50°C) $t_{1/2}$=25 min (59°C) $t_{1/2}$ =3 days (35°C)	$t_{1/2}$=1 min (80°C) $t_{1/2}$=6 min (60°C)
pH 8	Unstable 58% after 5 days 87.5% after 5 days	Unstable 67% after 2 days 49% after 4 days 34-68% after 5 days		
pH 9	Unstable 24% after	Unstable		

	5 days	40% after 5 days
pH 10	Unstable 4% after 5 days	Unstable 42% after 2 days 32% after 5 days

*Note: either half-life $t_{1/2}$ of C-PC or amount of C-PC remaining after a fixed time is indicated.

Unfortunately, C-PC stability does not seem to be associated with purity. The possible link between stability and the presence of contaminating molecules in the C-PC extract has not really been studied. An understanding of the dependence of C-PC stability on several factors, such as the presence of carbohydrates or other potential preservatives, a favorable pH, temperature, illuminating conditions, and so on, could also be used to improve the culture and extraction procedures for spirulina, to prevent C-PC denaturation during its formation and extraction.

Conclusion

The determination of C-PC concentration in *Arthrospira platensis* extracts remains challenging and merits further research, as simple spectrophotometric methods provide only approximate values, and fail to take into account several parameters, including the influence of co-products and the substrate used. However, such methods may be sufficient for comparisons of different extraction methods. Based on the extensive studies of C-PC extraction already published, it seems to be possible to obtain quantitative amounts of this protein at a high grad through the use of several methods, either alone or together. Various parameters, including the duration of incubation, temperature, pH, buffer molarity, source and level of shearing, may affect the extraction yield. Freeze-thaw methods appear to be an efficient pretreatment, as they weaken cell membranes, making it possible to achieve high C-PC extraction yields with low-energy treatments. Innovative methods are gradually being developed (US probes, pulsed electric field) to

facilitate the rapid release of C-PC, and US treatment with probes is now a widely used, effective method for shortening extraction time and obtaining high yields. These high yields are often associated with the release of multiple contaminants, but simple purification methods (salting out, aqueous two-phase purification) can be used to achieve food-grade quality, whereas more complex methods, often based on successive steps of preparative chromatography, are required to achieve higher grades. High yields and purities can make it possible to decrease the amount of culture required to obtain a given amount of pigment; achieving this goal is important, to decrease the environmental impact of C-PC extraction [118]. However, the environmental cost of such technological requirements must be examined in detail at the industrial scale, as the simple incubation of frozen or freeze-dried spirulina in a suitable buffer may also provide high extraction yields. This pigment can persist for more than a month at an appropriate temperature, pH and with appropriate packaging, but further studies may be required to extend its stability further, for its incorporation into alimentary formulations with a long shelf life.

Acknowledgments

This work received financial support from the FEDER Greenalg ESR_R&S_DI-000173 project.

Declarations

Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

References

1. Ullah K, Ahmad M, Sofia, et al (2014) Algal biomass as a global source of transport fuels: Overview and development perspectives. Prog Nat Sci Mater Int 24:329–339. https://doi.org/10.1016/j.pnsc.2014.06.008

2. Borowitzka MA (2013) High-value products from microalgae—their development and commercialisation. J Appl Phycol 25:743–756. https://doi.org/10.1007/s10811-013-9983-9

3. Ciferri O (1983) Spirulina, the edible microorganism. Microbiol Rev 47:551–578

4. Farrar WV (1966) Tecuitlatl; A Glimpse of Aztec Food Technology. Nature 211:341–342. https://doi.org/10.1038/211341a0

5. Hoseini SM, Khosravi-Darani K, Mozafari MR (2013) Nutritional and medical applications of spirulina microalgae. Mini-Rev Med Chem 13:1231–1237. https://doi.org/10.2174/1389557511313080009

6. Minkova KM, Tchernov AA, Tchorbadjieva MI, et al (2003) Purification of C-phycocyanin from *Spirulina (Arthrospira) fusiformis*. J Biotechnol 102:55–59. https://doi.org/10.1016/S0168-1656(03)00004-X

7. Cisneros M, Rito-Palomares M (2004) A simplified strategy for the release and primary recovery of C-phycocyanin produced by *Spirulina maxima*. Chem Biochem Eng Q 18:385–390

8. Chamorro-Cevallos G (2016) Methods for extraction, isolation and purification of C-phycocyanin: 50 years of research in review. Int J Food Nutr Sci 3:1–10. https://doi.org/10.15436/2377-0619.16.946

9. Eriksen NT (2008) Production of phycocyanin—a pigment with applications in biology, biotechnology, foods and medicine. Appl Microbiol Biotechnol 80:1–14. https://doi.org/10.1007/s00253-008-1542-y

10. Khan M, Varadharaj S, Shobha JC, et al (2006) C-phycocyanin ameliorates doxorubicin-induced oxidative stress and apoptosis in adult rat cardiomyocytes: J Cardiovasc Pharmacol 47:9–20. https://doi.org/10.1097/01.fjc.0000191520.48404.27

11. González R, Rodríguez S, Romay, C, et al (1999) Anti-inflammatory activity of phycocyanin extract in acetic acid-induced colitis in rats. Pharmacol Res 39:55–59. https://doi.org/10.1006/phrs.1998.0409

12. Romay C, Ledón N, González R (1998) Further studies on anti-inflammatory activity of phycocyanin in some animal models of inflammation. Inflamm Res 47:334–338. https://doi.org/10.1007/s000110050338

13. Nagaraj S, Arulmurugan P, Rajaram MG, et al (2012) Hepatoprotective and antioxidative effects of C-phycocyanin from *Arthrospira maxima* SAG 25780 in CCl4-induced hepatic damage rats. Biomed Prev Nutr 2:81–85. https://doi.org/10.1016/j.bionut.2011.12.001

14. Xia D, Liu B, Xin W, et al (2016) Protective effects of C-phycocyanin on alcohol-induced subacute liver injury in mice. J Appl Phycol 28:765–772. https://doi.org/10.1007/s10811-015-0677-3

15. Ou Y, Lin L, Yang X, et al (2013) Antidiabetic potential of phycocyanin: Effects on KKAy mice. Pharm Biol 51:539–544. https://doi.org/10.3109/13880209.2012.747545

16. Liu Y, Xu L, Cheng N, et al (2000) Inhibitory effect of phycocyanin from *Spirulina platensis* on the growth of human leukemia K562 cells. J Appl Phycol 12:125–130. https://doi.org/10.1023/A:1008132210772

17. McCarty MF (2007) "'Iatrogenic Gilbert syndrome"'– A strategy for reducing vascular and cancer risk by increasing plasma unconjugated bilirubin. Med Hypotheses 69:974–994. https://doi.org/10.1016/j.mehy.2006.12.069

18. Fernandes e Silva E, Figueira F da S, Lettnin AP, et al (2018) C-phycocyanin: Cellular targets, mechanisms of action and multi drug resistance in cancer. Pharmacol Rep 70:75–80. https://doi.org/10.1016/j.pharep.2017.07.018

19. Fernández-Rojas B, Hernández-Juárez J, Pedraza-Chaverri J (2014) Nutraceutical properties of phycocyanin. J Funct Foods 11:375–392. https://doi.org/10.1016/j.jff.2014.10.011

20. Kannaujiya VK, Sinha RP (2016) Thermokinetic stability of phycocyanin and phycoerythrin in food-grade preservatives. J Appl Phycol 28:1063–1070. https://doi.org/10.1007/s10811-015-0638-x

21. Kissoudi M, Sarakatsianos I, Samanidou V (2018) Isolation and purification of food-grade C-phycocyanin from *Arthrospira platensis* and its determination in confectionery by HPLC with diode array detection. J Sep Sci 41:975–981. https://doi.org/10.1002/jssc.201701151

22. Liu Q, Huang Y, Zhang R, et al (2016) Medical Application of *Spirulina platensis* Derived C-phycocyanin. Evid Based Complement Alternat Med 2016:1–14. https://doi.org/10.1155/2016/7803846

23. P. Singh, M. Kuddus, G. Thomas (2010) An efficient method for extraction of C-phycocyanin from Spirulina sp. and its binding affinity to blood cells, nuclei and genomic DNA. Int Res J Biotechnol 1:80–85

24. Sobiechowska-Sasim M, Stoń-Egiert J, Kosakowska A (2014) Quantitative analysis of extracted phycobilin pigments in cyanobacteria—an assessment of spectrophotometric and

spectrofluorometric methods. J Appl Phycol 26:2065–2074. https://doi.org/10.1007/s10811-014-0244-3

25. Safi C, Ursu AV, Laroche C, et al (2014) Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods. Algal Res 3:61–65. https://doi.org/10.1016/j.algal.2013.12.004

26. Auer GK, Weibel DB (2017) Bacterial Cell Mechanics. Biochemistry 56:3710–3724. https://doi.org/10.1021/acs.biochem.7b00346

27. Bennett A, Bogorad L (1973) Complementary chromatic adaptation in a filamentous blue-green alga. J Cell Biol 58:419–435. https://doi.org/10.1083/jcb.58.2.419

28. Boussiba S, Richmond AE (1979) Isolation and characterization of phycocyanins from the blue-green alga *Spirulina platensis.* Arch Microbiol 120:155–159. https://doi.org/10.1007/BF00409102

29. Lauceri R, Bresciani M, Lami A, Morabito G (2017) Chlorophyll a interference in phycocyanin and allophycocyanin spectrophotometric quantification. J Limnol. https://doi.org/10.4081/jlimnol.2017.1691

30. Yacobi YZ, Köhler J, Leunert F, Gitelson A (2015) Phycocyanin-specific absorption coefficient: Eliminating the effect of chlorophylls absorption: Phycocyanin-specific absorption coefficient. Limnol Oceanogr Methods 13:e10015. https://doi.org/10.1002/lom3.10015

31. Wachda, Hadiyanto H, Harjanto GD, et al (2019) Production of antioxidant C-phycocyanin using extraction process of *Spirulina platensis* in large scale industry. IOP Conf Ser Mater Sci Eng 633:012025. https://doi.org/10.1088/1757-899X/633/1/012025

32. Niu J-F, Wang G-C, Lin X, Zhou B-C (2007) Large-scale recovery of C-phycocyanin from *Spirulina platensis* using expanded bed adsorption chromatography. J Chromatogr B 850:267–276. https://doi.org/10.1016/j.jchromb.2006.11.043

33. Purohit A, Kumar V, Chownk M, Yadav SK (2019) Processing-independent extracellular production of high purity C-phycocyanin from *Spirulina platensis*. ACS Biomater Sci Eng 5:3237–3245. https://doi.org/10.1021/acsbiomaterials.9b00370

34. Doke JM (2005) An improved and efficient method for the extraction of phycocyanin from Spirulina sp. Int J Food Eng 1:. https://doi.org/10.2202/1556-3758.1037

35. Silveira ST, Burkert JFM, Costa JAV, et al (2007) Optimization of phycocyanin extraction from *Spirulina platensis* using factorial design. Bioresour Technol 98:1629–1634. https://doi.org/10.1016/j.biortech.2006.05.050

36. Sala L, Ores J da C, Moraes CC, Kalil SJ (2018) Simultaneous production of phycobiliproteins and carbonic anhydrase by *Spirulina platensis* LEB-52. Can J Chem Eng 96:1896–1902. https://doi.org/10.1002/cjce.23131

37. Colla LM, Oliveira Reinehr C, Reichert C, Costa JAV (2007) Production of biomass and nutraceutical compounds by *Spirulina platensis* under different temperature and nitrogen regimes. Bioresour Technol 98:1489–1493. https://doi.org/10.1016/j.biortech.2005.09.030

38. Moraes CC, Sala L, Cerveira GP, Kalil SJ (2011) C-phycocyanin extraction from *Spirulina platensis* wet biomass. Braz J Chem Eng 28:45–49. https://doi.org/10.1590/S0104-66322011000100006

39. Sarada R, Pillai MG, Ravishankar GA (1999) Phycocyanin from Spirulina sp: influence of processing of biomass on phycocyanin yield, analysis of efficacy of extraction methods and

stability studies on phycocyanin. Process Biochem 34:795–801. https://doi.org/10.1016/S0032-9592(98)00153-8

40. Pan-utai W, Kahapana W, Iamtham S (2018) Extraction of C-phycocyanin from *Arthrospira* (Spirulina) and its thermal stability with citric acid. J Appl Phycol 30:231–242. https://doi.org/10.1007/s10811-017-1155-x

41. Liao X, Zhang B, Wang X, et al (2011) Purification of C-phycocyanin from *Spirulina platensis* by single-step ion-exchange chromatography. Chromatographia 73:291–296. https://doi.org/10.1007/s10337-010-1874-5

42. Chaiklahan R, Chirasuwan N, Loha V, et al (2011) Separation and purification of phycocyanin from Spirulina sp. using a membrane process. Bioresour Technol 102:7159–7164. https://doi.org/10.1016/j.biortech.2011.04.067

43. Chaiklahan R, Chirasuwan N, Bunnag B, et al (2018) Stepwise extraction of high-value chemicals from *Arthrospira* (Spirulina) and an economic feasibility study. Biotechnol Rep Amst Neth 20:e00280

44. Tavanandi HA, Raghavarao KSMS (2020) Ultrasound-assisted enzymatic extraction of natural food colorant C-Phycocyanin from dry biomass of *Arthrospira platensis*. LWT 118:108802. https://doi.org/10.1016/j.lwt.2019.108802

45. Shehadul Islam M, Aryasomayajula A, Selvaganapathy P (2017) A Review on macroscale and microscale cell lysis methods. Micromachines 8:83. https://doi.org/10.3390/mi8030083

46. Seddon AM, Curnow P, Booth PJ (2004) Membrane proteins, lipids and detergents: not just a soap opera. Biochim Biophys Acta BBA - Biomembr 1666:105–117. https://doi.org/10.1016/j.bbamem.2004.04.011

47. Primo ED, Otero LH, Ruiz F, et al (2018) The disruptive effect of lysozyme on the bacterial cell wall explored by an *in-silico* structural outlook: Effect of Lysozyme on the Bacterial Cell Wall. Biochem Mol Biol Educ 46:83–90. https://doi.org/10.1002/bmb.21092

48. Sala L, Moraes CC, Kalil SJ (2018) Cell pretreatment with ethylenediaminetetraacetic acid for selective extraction of C-phycocyanin with food grade purity. Biotechnol Prog 34:1261–1268. https://doi.org/10.1002/btpr.2713

49. Sharma R, Bhunia B, Mondal A, et al (2020) Statistical optimization of process parameters for improvement of phycobiliproteins (PBPs) yield using ultrasound-assisted extraction and its kinetic study. Ultrason Sonochem 60:104762. https://doi.org/10.1016/j.ultsonch.2019.104762

50. Ores J da C, Amarante MCA de, Kalil SJ (2016) Co-production of carbonic anhydrase and phycobiliproteins by Spirulina sp. and *Synechococcus nidulans*. Bioresour Technol 219:219–227. https://doi.org/10.1016/j.biortech.2016.07.133

51. P. Prabuthas, S. Majumdar, P. P. Srivastav, H. N. Mishra (2009) Standardization of rapid and economical method for neutraceutical extraction from algae. https://doi.org/10.13140/2.1.2627.0082

52. Chen K-H, Wang SS-S, Show P-L, et al (2019) Rapid and efficient recovery of C-phycocyanin from highly turbid *Spirulina platensis* algae using stirred fluidized bed ion exchange chromatography. Sep Purif Technol 209:636–645. https://doi.org/10.1016/j.seppur.2018.08.057

53. Bachchhav MB, Kulkarni MV, Ingale AG (2020) Process-intensified extraction of phycocyanin followed by β-carotene from *Spirulina platensis* using ultrasound-assisted extraction. Sep Sci Technol 55:932–944. https://doi.org/10.1080/01496395.2019.1580293

54. Tavanandi HA, Chandralekha Devi A, Raghavarao Ksms (2018) A newer approach for the primary extraction of allophycocyanin with high purity and yield from dry biomass of *Arthrospira platensis*. Sep Purif Technol 204:162–174. https://doi.org/10.1016/j.seppur.2018.04.057

55. Pan-utai W, Iamtham S (2019) Physical extraction and extrusion entrapment of C-phycocyanin from *Arthrospira platensis*. J King Saud Univ - Sci 31:1535–1542. https://doi.org/10.1016/j.jksus.2018.05.026

56. Pan-utai W, Iamtham S (2019) Extraction, purification and antioxidant activity of phycobiliprotein from *Arthrospira platensis*. Process Biochem 82:189–198. https://doi.org/10.1016/j.procbio.2019.04.014

57. Furuki T, Maeda S, Imajo S, et al (2003) Rapid and selective extraction of phycocyanin from *Spirulina platensis* with ultrasonic cell disruption. J Appl Phycol 15:319–324

58. Stramarkou M, Papadaki S, Kyriakopoulou K, Krokida M (2016) Recovery of functional pigments from four different species of microalgae. IOSR J Environ Sci Toxicol Food Technol 10:26–30. https://doi.org/10.9790/2402-1009022630

59. Hadiyanto, Suttrisnorhadi, Sutanto H, et al (2015) The effects of temperature and frequencies in ultrasound assisted extraction of phycocyanin from microalgae Spirulina sp. International conference of chemical and material engineering (ICCME) 2015: Green Technology for Sustainable Chemical Products and Processes, Semarang, Indonesia, p 030009

60. Dianursanti, Indraputri CM, Taurina Z (2018) Optimization of phycocyanin extraction from microalgae *Spirulina platensis* by sonication as antioxidant. Proceedings of the International Symposium of Biomedical Engineering (ISBE) 2017, Bali, Indonesia, p 030013

61. Minchev I, Petkova N, Milkova-Tomova I (2020) Ultrasound-assisted extraction of chlorophylls and phycocyanin from *Spirulina platensis*. Biointerface Res Appl Chem 11:9296–9304. https://doi.org/10.33263/BRIAC112.92969304

62. Tavanandi HA, Mittal R, Chandrasekhar J, Raghavarao KSMS (2018) Simple and efficient method for extraction of C-Phycocyanin from dry biomass of *Arthospira platensis*. Algal Res 31:239–251. https://doi.org/10.1016/j.algal.2018.02.008

63. Manirafasha E, Murwanashyaka T, Ndikubwimana T, et al (2017) Ammonium chloride: a novel effective and inexpensive salt solution for phycocyanin extraction from *Arthrospira (Spirulina) platensis*. J Appl Phycol 29:1261–1270. https://doi.org/10.1007/s10811-016-0989-y

64. Yu J (2017) Application of an ultrafine shearing method for the extraction of C-phycocyanin from *Spirulina platensis*. Molecules 22:2023. https://doi.org/10.3390/molecules22112023

65. Jaeschke DP, Mercali GD, Marczak LDF, et al (2019) Extraction of valuable compounds from *Arthrospira platensis* using pulsed electric field treatment. Bioresour Technol 283:207–212. https://doi.org/10.1016/j.biortech.2019.03.035

66. Moraes CC, De Medeiros Burkert JF, Kalil SJ (2010) C-phycocyanin extraction process for large scale use : C-phycocyanin extraction process. J Food Biochem 34:133–148. https://doi.org/10.1111/j.1745-4514.2009.00317.x

67. Raso J, Frey W, Ferrari G, et al (2016) Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes. Innov Food Sci Emerg Technol 37:312–321. https://doi.org/10.1016/j.ifset.2016.08.003

68. Martínez JM, Luengo E, Saldaña G, et al (2017) C-phycocyanin extraction assisted by pulsed electric field from *Artrosphira platensis*. Food Res Int 99:1042–1047. https://doi.org/10.1016/j.foodres.2016.09.029

69. Viana Carlos TA, dos Santos Pires Cavalcante KM, de Cássia Evangelista de Oliveira F, et al (2021) Pressurized extraction of phycobiliproteins from *Arthrospira platensis* and evaluation of its effect on antioxidant and anticancer activities of these biomolecules. J Appl Phycol 33:929–938. https://doi.org/10.1007/s10811-020-02358-z

70. Herrera A, Boussiba S, Napoleone V, Hohlberg A (1989) Recovery of c-phycocyanin from the cyanobacterium *Spirulina maxima*. J Appl Phycol 1:325–331. https://doi.org/10.1007/BF00003469

71. Rito-Palomares M, Nuñez L, Amador D (2001) Practical application of aqueous two-phase systems for the development of a prototype process for c-phycocyanin recovery from *Spirulina maxima*: Aqueous two-phase process for recovery of c-phycocyanin. J Chem Technol Biotechnol 76:1273–1280. https://doi.org/10.1002/jctb.507

72. Chen K-H, Wang SS-S, Show P-L, et al (2018) A rapid and efficient technique for direct extraction of C-phycocyanin from highly turbid *Spirulina platensis* algae using hydrophobic interaction chromatography in stirred fluidized bed. Biochem Eng J 140:47–56. https://doi.org/10.1016/j.bej.2018.09.005

73. Zhang Y-M, Chen F (1999) A simple method for efficient separation and purification of c-phycocyanin and allophycocyanin from *Spirulina platensis*. Biotechnol Tech 13:601–603. https://doi.org/10.1023/A:1008914405302

74. Marzorati S, Schievano A, Idà A, Verotta L (2020) Carotenoids, chlorophylls and phycocyanin from Spirulina: supercritical CO_2 and water extraction methods for added value products cascade. Green Chem 22:187–196. https://doi.org/10.1039/C9GC03292D

75. Wang WJ, Zhang XL, Xu CB, Cheng HY (2011) Purification and concentration of C-phycocyanin from *Spirulina platensis* Using Aqueous Two-Phase System. Appl Mech Mater 138–139:995–1001. https://doi.org/10.4028/www.scientific.net/AMM.138-139.995

76. Böcker L, Hostettler T, Diener M, et al (2020) Time-temperature-resolved functional and structural changes of phycocyanin extracted from *Arthrospira platensis*/Spirulina. Food Chem 316:126374. https://doi.org/10.1016/j.foodchem.2020.126374

77. Patil G, Raghavarao KSMS (2007) Aqueous two phase extraction for purification of C-phycocyanin. Biochem Eng J 34:156–164. https://doi.org/10.1016/j.bej.2006.11.026

78. Antelo FS, Anschau A, Costa JAV, Kalil SJ (2010) Extraction and purification of C-phycocyanin from *Spirulina platensis* in conventional and integrated aqueous two-phase systems. J Braz Chem Soc 21:921–926. https://doi.org/10.1590/S0103-50532010000500022

79. Chew KW, Chia SR, Krishnamoorthy R, et al (2019) Liquid biphasic flotation for the purification of C-phycocyanin from *Spirulina platensis* microalga. Bioresour Technol 288:121519. https://doi.org/10.1016/j.biortech.2019.121519

80. Zhang X, Zhang F, Luo G, et al (2014) Extraction and separation of phycocyanin from *Spirulina* using aqueous two-phase systems of ionic liquid and salt. J Food Nutr Res 3:15–19. https://doi.org/10.12691/jfnr-3-1-3

81. Zhao L, Peng Y, Gao J, Cai W (2014) Bioprocess intensification: an aqueous two-phase process for the purification of C-phycocyanin from dry *Spirulina platensis*. Eur Food Res Technol 238:451–457. https://doi.org/10.1007/s00217-013-2124-5

82. Wang F, Liu Y-H, Ma Y, et al (2017) Application of TMA-PEG to promote C-phycocyanin extraction from *S. platensis* in the PEG ATPS. Process Biochem 52:283–294. https://doi.org/10.1016/j.procbio.2016.11.006

83. Seo Y, Choi W, Park J, et al (2013) Stable isolation of phycocyanin from *Spirulina platensis* associated with high-pressure extraction process. Int J Mol Sci 14:1778–1787. https://doi.org/10.3390/ijms14011778

84. Prabakaran G, Sampathkumar P, Kavisri M, Moovendhan M (2020) Extraction and characterization of phycocyanin from *Spirulina platensis* and evaluation of its anticancer, antidiabetic and antiinflammatory effect. Int J Biol Macromol 153:256–263. https://doi.org/10.1016/j.ijbiomac.2020.03.009

85. Amarante MCA de, Corrêa Júnior LCS, Sala L, Kalil SJ (2019) Analytical grade C-phycocyanin obtained by a single-step purification process. Process Biochem S1359511319305756. https://doi.org/10.1016/j.procbio.2019.11.020

86. Silveira ST, de Menezes Quines LK, Burkert CAV, Kalil SJ (2008) Separation of phycocyanin from *Spirulina platensis* using ion exchange chromatography. Bioprocess Biosyst Eng 31:477–482. https://doi.org/10.1007/s00449-007-0185-1

87. Kumar D, Dhar DW, Pabbi S, et al (2014) Extraction and purification of C-phycocyanin from *Spirulina platensis* (CCC540). Indian J Plant Physiol 19:184–188

88. Telegina TA, Biryukov MV, Terekhova IV, et al (2018) Isolation and characterization of water-soluble chromoproteins from *Arthrospira platensis* cyanobacteria: C-Phycocyanin, allophycocyanin, and carotenoid- and chlorophyll-binding proteins. Appl Biochem Microbiol 54:631–638. https://doi.org/10.1134/S0003683818060145

89. Patil G, Chethana S, Sridevi AS, Raghavarao KSMS (2006) Method to obtain C-phycocyanin of high purity. J Chromatogr A 1127:76–81. https://doi.org/10.1016/j.chroma.2006.05.073

90. Binder A, Wilson K, Zuber H (1972) *C* -phycocyanin from the thermophilic blue-green alga *Mastigocladus laminosus* , isolation, characterization and subunit composition. FEBS Lett 20:111–116. https://doi.org/10.1016/0014-5793(72)80030-9

91. Baskin EM, Bukshpan S, Zilberstein GV (2006) pH-induced intracellular protein transport. Phys Biol 3:101–106. https://doi.org/10.1088/1478-3975/3/2/002

92. Abalde J, Betancourt L, Torres E, et al (1998) Purification and characterization of phycocyanin from the marine cyanobacterium *Synechococcus* sp. IO9201. Plant Sci 136:109–120. https://doi.org/10.1016/S0168-9452(98)00113-7

93. Santiago-Santos MaC, Ponce-Noyola T, Olvera-Ramírez R, et al (2004) Extraction and purification of phycocyanin from *Calothrix* sp. Process Biochem 39:2047–2052. https://doi.org/10.1016/j.procbio.2003.10.007

94. Sala L, Figueira FS, Cerveira GP, et al (2014) Kinetics and adsorption isotherm of C-phycocyanin from *Spirulina platensis* on ion-exchange resins. Braz J Chem Eng 31:1013–1022. https://doi.org/10.1590/0104-6632.20140314s00002443

95. Jiang L, Wang Y, Yin Q, et al (2017) Phycocyanin: A potential drug for cancer treatment. J Cancer 8:3416–3429. https://doi.org/10.7150/jca.21058

96. Kao O, Berns DS, Maccoll R (1971) C-Phycocyanin monomer molecular weight. Eur J Biochem 19:595–599. https://doi.org/10.1111/j.1432-1033.1971.tb01353.x

98. Mogany T, Kumari S, Swalaha FM, Bux F (2019) Extraction and characterisation of analytical grade C-phycocyanin from *Euhalothece* sp. J Appl Phycol 31:1661–1674. https://doi.org/10.1007/s10811-018-1661-5

98. Mogany T, Kumari S, Swalaha FM, Bux F (2019) Extraction and characterisation of analytical grade C-phycocyanin from *Euhalothece* sp. J Appl Phycol 31:1661–1674. https://doi.org/10.1007/s10811-018-1661-5

99. Antelo FS, Costa JAV, Kalil SJ (2008) Thermal degradation kinetics of the phycocyanin from *Spirulina platensis*. Biochem Eng J 41:43–47. https://doi.org/10.1016/j.bej.2008.03.012

100. Chaiklahan R, Chirasuwan N, Bunnag B (2012) Stability of phycocyanin extracted from Spirulina sp.: Influence of temperature, pH and preservatives. Process Biochem 47:659–664. https://doi.org/10.1016/j.procbio.2012.01.010

101. Duangsi R, Phoopat N, Ningsanond S (2009) Phycocyanin extraction from *Spirulina platensis* and extract stability under various pH and temperature. 2:

102. Hadiyanto, Christwardana M, Sutanto H, et al (2018) Kinetic study on the effects of sugar addition on the thermal degradation of phycocyanin from Spirulina sp. Food Biosci 22:85–90. https://doi.org/10.1016/j.fbio.2018.01.007

103. Li Y, Zhang Z, Paciulli M, Abbaspourrad A (2020) Extraction of phycocyanin—A natural blue colorant from dried spirulina biomass: Influence of processing parameters and extraction techniques. J Food Sci 1750-3841.14842. https://doi.org/10.1111/1750-3841.14842

104. Silva LA, Kuhn KR, Moraes CC, et al (2009) Experimental design as a tool for optimization of C-phycocyanin purification by precipitation from *Spirulina platensis*. J Braz Chem Soc 20:5–12. https://doi.org/10.1590/S0103-50532009000100003

105. Padyana AK, Bhat VB, Madyastha KM, et al (2001) Crystal structure of a light-harvesting protein C-phycocyanin from *Spirulina platensis*. Biochem Biophys Res Commun 282:893–898. https://doi.org/10.1006/bbrc.2001.4663

106. Minic SL, Milcic M, Stanic-Vucinic D, et al (2015) Phycocyanobilin, a bioactive tetrapyrrolic compound of blue-green alga Spirulina, binds with high affinity and competes with bilirubin for binding on human serum albumin. RSC Adv 5:61787–61798. https://doi.org/10.1039/C5RA05534B

107. Jespersen L, Strømdahl LD, Olsen K, Skibsted LH (2005) Heat and light stability of three natural blue colorants for use in confectionery and beverages. Eur Food Res Technol 220:261–266. https://doi.org/10.1007/s00217-004-1062-7

108. Choi WY, Lee HY (2018) Kinetic analysis of stabilizing C-phycocyanin in the *Spirulina platensis* extracts from ultrasonic process associated with effects of light and temperature. Appl Sci 8:1662. https://doi.org/10.3390/app8091662

109. Selig MJ, Malchione NM, Gamaleldin S, et al (2018) Protection of blue color in a spirulina derived phycocyanin extract from proteolytic and thermal degradation via complexation with beet-pectin. Food Hydrocoll 74:46–52. https://doi.org/10.1016/j.foodhyd.2017.07.027

110. Martelli G, Folli C, Visai L, et al (2014) Thermal stability improvement of blue colorant C-Phycocyanin from *Spirulina platensis* for food industry applications. Process Biochem 49:154–159. https://doi.org/10.1016/j.procbio.2013.10.008

111. Wu H-L, Wang G-H, Xiang W-Z, et al (2016) Stability and antioxidant activity of food-grade phycocyanin isolated from *Spirulina platensis*. Int J Food Prop 19:2349–2362. https://doi.org/10.1080/10942912.2015.1038564

112. Mishra SK, Shrivastav A, Mishra S (2008) Effect of preservatives for food grade C-PC from *Spirulina platensis*. Process Biochem 43:339–345. https://doi.org/10.1016/j.procbio.2007.12.012

113. Bermejo R, Talavera EM, delValle C, Alvarez-Pez JM (2000) C-phycocyanin incorporated into reverse micelles: a fluorescence study. Colloids Surf B Biointerfaces 18:51–59. https://doi.org/10.1016/S0927-7765(99)00129-0

114. Falkeborg MF, Roda-Serrat MC, Burnæs KL, Nielsen ALD (2018) Stabilising phycocyanin by anionic micelles. Food Chem 239:771–780. https://doi.org/10.1016/j.foodchem.2017.07.007

115. Yan M, Liu B, Jiao X, Qin S (2014) Preparation of phycocyanin microcapsules and its properties. Food Bioprod Process 92:89–97. https://doi.org/10.1016/j.fbp.2013.07.008

116. Gustiningtyas A, Setyaningsih I, Hardiningtyas SD, Susila AAR (2020) Improvement stability of phycocyanin from *Spirulina platensis* encapsulated by water soluble chitosan nanoparticles. IOP Conf Ser Earth Environ Sci 414:012005. https://doi.org/10.1088/1755-1315/414/1/012005

117. Braga ARC, Figueira F da S, Silveira JT da, et al (2016) Improvement of thermal stability of C-phycocyanin by nanofiber and preservative agents: C-phycocyanin stability : preservative and nanofiber. J Food Process Preserv 40:1264–1269. https://doi.org/10.1111/jfpp.12711

118. Papadaki S, Kyriakopoulou K, Tzovenis I, Krokida M (2017) Environmental impact of phycocyanin recovery from *Spirulina platensis* cyanobacterium. Innov Food Sci Emerg Technol 44:217–223. https://doi.org/10.1016/j.ifset.2017.02.014