Comparison of different methods for extraction of phycocyanin from cyanobacterium Arthrospira maxima (Spirulina)

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Abstract

Phycocyanin is an interesting alternative for synthetic food colorants. For extraction of phycocyanin from representatives of cyanobacterial genus Arthrospira various methods have been described in literature including ultrasonication, glass bead extraction and freeze-thawing. In this work, three optimized methods for the extraction of phycocyanin from A. maxima were applied in order compare the effectiveness of the different processes. After harvesting the biomass, he extractions were carried out using ultrasonication followed by flocculation with chitosan in different organic acid solutions, glass bead extraction and freeze-thawing, both followed by centrifugation. The obtained extracts were analysed using spectrophotometry in the wavelength spectrum of 280 to 800 nm. The highest C-PC contents of 17.03 ± 0.53 % and 15.21 ± 0.41 % were achieved with the freeze-thawing and the ultrasonication method, respectively. The highest purity value of 2.02 ± 0.01 was achieved with ultrasonication and flocculation with chitosan in acetic acid. Using citric or lactic acid for flocculation with chitosan resulted in greenish extracts containing high amounts of chlorophyll. In conclusion, flocculation with chitosan in acetic acid can be an interesting alternative for centrifugation providing highly purified phycocyanin extracts.

1 Introduction

In recent years, the awareness for the potential risks caused by the use of certain food additives has risen within the consumers. This especially applies to the use of synthetic food colorants. Several of these synthetic dyes have already been demonstrated to increase the probability of developing cancer or immunological diseases (Martelli et al., 2014). Therefore, law prohibits the use of proven harmful substances for food production. However, other synthetic food dyes are still available for the industrial purposes, since they are cheap, highly effective, reliable, and chemically stable (Chen et al., 1998). Especially when it comes to blue food colorants, the industry lacks alternatives for synthetic dyes (Newsome et al., 2014).

The alternative could be Phycocyanin (PC), a protein, that can be found in cyanobacteria (C-PC) and rhodophyta (R-PC), where it plays a major role in photosynthesis as an accessory pigment of bright cobalt-blue colour (Horváth et al., 2013; Singh et al., 2015). The PC molecule consists of two subunits, α (ca. 19 kDa) and β (ca. 21 kDa). In the cell, the PC mostly occurs in its trimeric $(\alpha\beta)_3$ or hexameric $(\alpha\beta)_6$ form of three or six molecules displaying a ring-like structure of one ring (trimer) or two stacked rings (hexamer) (Abalde et al., 1998). The PC belongs to the group of phycobiliproteins (PBP) and is characterized by its hydrophilic properties. The impression of bright blue color in PC is contributed by the covalently bound chromophore phycocyanobilin (PCB), a tetrapyrrole structure attached to the apoprotein by thioether bounds at the 84th amino acid in both, the α and the β subunit. Additionally, a third PCB group is attached to the 155th amino acid of the β subunit. The amino acid sequence of the PC mostly forms helical areas, displaying a topological structure similar to the hem group in the myoglobin molecule (Stec et al., 1999). In cyanobacteria, the C-PC hexamers are part of the so called phycobilisomes (PBS). These PBS are protein structures with antenna-like protein stacks consisting of

the C-PC and phycoerythrin (PE), another PBP of reddish colour, are protruding. The antennas are attached to a third light-blue PBP, allophycocyanin (APC), which itself is attached to the photosystem II within the thylakoid membrane of cyanobacteria and eukaryotic chloroplasts. These PBS enable the utilization of light energy by electron transfer for the photosystem II, making it possible for the cyanobacterium to perform photosynthesis (Santiago-Santos et al., 2004; Singh et al., 2015).

Besides its usage as a food colorant, the PC is also known for its anti-oxidative capacity and therefore subject to research. The addition of phycocyanin in a mayonnaise product was demonstrated to enhance the anti-oxidative capacity in order to label the resulting product as a functional food (Khorsand et al., 2021). Research prospecting the influence of C-PC in the diet of European seabass on the heat stress showed, that the C-PC can actually increase the resistance of the fish to heat stress (Islam et al., 2021). Similar results are presented in another publication concerning heat stress in Nile tilapia, but the amount of C-PC per kg feed used in this experiment was much higher (El-Araby et al., 2022). For mammals (rabbits) the health beneficial effect by addition of C-PC to the feed has also been shown (Abdelnour et al., 2020).

The majority of PC for industrial demand is usually extracted from cyanobacteria *Arthrospira platensis* or *Arthrospira maxima* that are commonly referred to as *Spirulina* (Eriksen, 2008; Sekar und Chandramohan, 2008; Moraes et al., 2011). Like all cyanobacteria, Spirulina is able to perform oxygenic photosynthesis to obtain energy for the synthesis of sugar molecules. Spirulina are unicellular species. However, the individual cells form long filaments together, that mostly occur helically shaped (Tomaselli, 1997). For optimal growth, Spirulina requires a temperature between 30 and 35°C, and a pH between 9 and 11 (Usharani et al., 2012). The natural habitats of Spirulina are tropical or subtropical water bodies with high concentrations of carbonates and bicarbonates (Rajasekaran et al., 2016). The beneficial value of Spirulina for human nutrition is contributed by the high protein content of 55–70% by reference to the dry matter content (Aouir et al., 2017; Babadzhanov et al., 2004, Oliveira et al., 1999). Besides, representatives of Spirulina are rich in polysaccharides, unsaturated fatty acids, vitamins, minerals and antioxidative substances like C-PC (Rajasekaran et al., 2016; Jung et al., 2019). It is also assumed, that the intake of large amounts of intact bacteria cells has a positive impact on the competence of the immune system and displays anti-inflammatory, antioxidative, and anti-carcinogenic properties (Eriksen, 2008, Hayashi et al., 2006). That makes even the cyanobacterium itself a valuable food for human nutrition. The United States Food and Drug Administration allowed the use of Spirulina products in 2013 for various food categories like bakery products, ice cream, beverages, and chewing gums (FDA, 2013). The market volume for Spirulina products in 2016 was estimated to be 700 million US dollars and predicted to reach 2 billion US dollars by 2026 (Soni et al., 2021).

For the extraction of phycocyanin, various methods like freeze-thawing (Prabhath et al., 2019; Doke et al., 2005; Tan et al., 2003), glass bead extraction (Moraes et al., 2011), and ultrasonication (Furuki et al., 2003) have been described. A problem in C-PC extraction that often occurs, is the presence of chlorophyll in the extract (Günerken et al., 2015; Doke, 2005; Li et al., 2020). In this work, three different methods (ultrasonication followed by flocculation with chitosan in different organic acids, glass bead

extraction followed by centrifugation, and freeze-thawing followed by centrifugation) for the extraction of C-PC were carried out in order to compare the C-PC yield, purity and selectivity obtained by these methods.

2 Materials and Methods

2.1 Spirulina cultivation

The cyanobacterium used for the experiments was *A. maxima* UTEX 2342 (purchased from Culture Collection of Algae, University of Texas, Austin, USA). It was cultivated in a 10 L algabag (algatec GbR, Sottrum, Germany) in half-concentrated Spirulina medium (by Culture Collection of Algae Göttingen, Germany, version of march 2007) at 25°C for 33 days. The culture was aerated with air. The light intensity was 63 µmol photons s^{-1} m^{-2} and the light came from VALOYA C75 DIM spectrum AP67 (Valoya Ltd, Helsinki, Finland). Increase in biomass was measured using photometric absorption measurement at 800 nm (OD_{800}).

2.2 Biomass harvesting

In exponential phase at OD_{800} = 1.32, the biomass was harvested and concentrated by filtering through a 40 µl mesh tissue. The concentrated biomass was then washed twice by adding deionised water in 50 ml tubes (1:2 w/v), thoroughly shaken, centrifuged (3,500 rpm, 10 min), and the supernatant was discarded. The washed biomass was stored at 4°C for 18 hours. Hereafter, the dry matter content was measured thermo-gravimetrically and the biomass was used for the C-PC extraction.

2.3 Extraction with ultrasonication

For extraction by ultrasonication, 15 g of the biomass were filled with 135 g of deionised water (1:10, w/w) for adjusting to a dry matter content of 1,25% and attached to a ultrasonicator

(0,8 s interval, 100% intensity) with flow-through cell by a peristaltic pump (100 mg mL^{-1}) and ultrasonicated for 27 minutes (equivalent to 18 flow-through cycles). After confirming the successful cell disruption by microscopy, the cell suspension was divided onto 3 approaches with 50 ml volume of cell suspension each and stored at 4°C for 1 hour. Every approach was then added 5 g of a 1% chitosan solution in either acetic acid, citric acid, or lactic acid (10% acid concentration each). After adding the different chitosan solutions (5 g), all three approaches were stirred for 10 min (80 rpm) and then filtered by a 60 µm plankton sieve. The three resulting filtrates were considered the C-PC extracts and the pH and absorption spectrum was measured.

2.4 Freeze-thawing

The remaining biomass (10 g) was mixed with 40 ml of $CaCl_2$ solution (10 g L^{-1}). An aliquot was used for glass bead extraction (see next part), the rest was divided onto several micro reaction tubes and frozen at -80°C. After 18 h, the cell suspension was thawed at room temperature for 4 hours and then frozen at -80°C again. After another 20 hours, the cell suspension was thawed for 4 h and then centrifuged (10,000 rpm, 30 min, 4°C), the supernatants were measured photometrically.

2.5 Glass bead extraction

An aliquot of the cell suspension in $CaCl_2$ solution (10 g L^{-1}), as mentioned, before was used for glass bead extraction. Therefore, 500 µl of the cell suspension were pipetted into micro reaction tubes already filled with 500 mg of glass beads (Ø 0.25–0.5 mm; Verder Scientific GmbH & Co. KG, Haan, Germany). The micro reaction tubes were applied to a bead mill (Retsch bead mill MM301; Verder Scientific GmbH & Co. KG, Haan, Germany) and underwent cell disruption using 4 disruption cycles with 30 Hz for 25 s each with 30 s of cooling phase in between. After disruption the samples where kept on ice and centrifuged (10,000 rpm, 30 min, 4°C). The bluish supernatants were measured photometrically.

2.6 Photometric analysis

The calculation of the C-PC concentration and purity was carried out measuring the absorption spectrum from 280 to 800 nm with a Genesys 50 UV/VIS spectrophotometer (Thermo-Fisher Scientific Inc., Waltham, USA). The concentration and purity were then calculated using the following equations first postulated by Bennett and Bogorad (1973):

$$(1)\ c_{C-PC}\left[mg \bullet ml^{-1}\right] = \frac{A_{620} - 0{,}474 * A_{650}}{5{,}34}$$

$$(2)\ purity_{C-PC}[-] = \frac{A_{620}}{A_{280}}$$

Where is C_{C-PC} = C-PC concentration in the extract; A_x = absorption of the final extract at the wavelength x; $purity_{C-PC}$ = the purity of the C-PC in the extract measured as the ratio of absorption at 620 nm to 280 nm. The selectivity as the ratio of the absorption at 620 nm and 438 nm was chosen as a value to assess the abundance of undesired chlorophyll a within the extract.

$$(3)\ selectivity[-] = \frac{A_{620}}{A_{438}}$$

Where is A_X = absorption of the final extract at the wavelength X.

The mean absorption spectra of all five approaches were formed by calculating the arithmetic mean of every measured wavelength for each approach. Hereafter, the five mean absorption spectra were

normalized by defining the absorption at 620 nm as 1 (corresponding to 100%) and assigning all other absorptions a value relative to the absorption at 620 nm. Therefore, the lowest measured absorption was subtracted from every absorption in the spectrum. Then, each of these values was multiplied by the reciprocal of the absorption at 620 nm (whom the the lowest measured absorption was subtracted before).

2.7 Statistical Analysis

The glass bead extraction as well as the freeze-thawing extraction were carried out in biological triplicates (n = 3). After measurement, the three results were used to calculate the arithmetic mean ($\bar{x}$) and standard deviation (SD). The ultrasonicated approaches were carried out just once each. The resulting extracts were then measured as technical triplicates (n = 3). After measurement, the three results of each approach were used to calculate the arithmetic mean ($\bar{x}$) and standard deviation (SD).

3 Results

The five resulting C-PC extracts (ultrasonication with chitosan-acetic acid, chitosan-citric acid, and chitosan-lactic acid flocculant, glass bead extraction, and freeze thawing) were analysed using spectrophotometry. The dry matter content of the initial washed and concentrated biomass was 12.58 ± 0.84%. For the ultrasonication extraction, the dry matter content was adjusted to 1.26% with deionised water. For both, the glass bead extraction and the freeze-thawing, dry matter content was adjusted to ca. 2.5% with $CaCl_2$ solution.

The C-PC concentrations of the five different extracts were: 2.23 ± 0.06 mg mL^{-1} (ultrasonication and acetic acid), 1.17 ± 0.08 mg mL^{-1} (ultrasonication and citric acid), 1.48 ± 0.02 mg mL^{-1} (ultrasonication and lactic acid), 2.98 ± 0.20 mg mL^{-1} (glass bead extraction), and 5.11 ± 0.16 mg mL^{-1} (freeze-thawing). The highest purity of C-PC was observed in the ultrasonicated with acetic acid (2.02 ± 0.01) and the freeze-thawed sample (1.94 ± 0.01). The lowest purity was found in the ultrasonicated samples with citric and lactic acid (1.00 ± 0.06 and 1.28 ± 0.06, respectively). The extract obtained by glass bead extraction had a purity of 1.58 ± 0.01. C-PC concentration and purity of the five final extracts are displayed in Fig. 1. Pertaining to the dry matter content used for the different extraction methods, the highest mass ratio of C-PC to dry matter used was 17.03 ± 0.53% for the freeze-thawed samples, followed by ultrasonication with acetic acid (15.21 ± 0.41%). C-PC to dry matter mass ratio was lower for glass bead extraction (10.92 ± 0.74%) and for the ultrasonication with citric acid (10.11 ± 0.13%) and lactic acid (8.02 ± 0.58%). For C-PC contents of the five final extracts see also Fig. 2.

The normalized absorption spectra of the five different extracts all showed a distinct peak with maximum absorption at 620 nm (ultrasonication) or 616 nm (glass bead extraction and freeze-thawing). The ultrasonicated samples with citric and lactic acid additionally showed higher absorption in the area from 280 nm to 480 nm and around 680 nm. The glass bead extracted and the freeze-thawed samples

displayed a marginal higher absorbance at around 650 nm. The normalized absorption spectra are shown in Fig. 3.

The ratio of the absorption of 620 nm to 438 nm used as a measuring unit for the selectivity of the extraction method was highest for freeze-thawing (23.80 ± 0.40) and glass bead extraction (16.17 ± 0.36). The bluish extract of the ultrasonicated sample with acetic acid had a ratio of 8.43 ± 0.20. The ultrasonicated samples with citric and lactic acid had values of 2.94 ± 0,21 and 4.62 ± 0.29, respectively. The values for the selectivity of all five extracts are illustrated in Fig. 4.

The final pH of the ultrasonicated extracts were 4.03 (for acetic acid), 3.14 (for citric acid), 3.02 (for lactic acid). For the extract by bead mill extraction, the pH was found to be 7.14. The extract resulted from freeze-thawing had a pH of 6.49. The freeze-thawed extract, the extract form glass bead extraction and the one from ultrasonication using acetic acid were of an intense bluish colour (‘cobalt blue’), while the ultrasonicated samples with citric and lactic acid displayed a more greenish proportion (‘aqua green’) in the colour composition. See also Fig. 5.

4 Discussion

While *A. platensis* is well known for its C-PC content and has been the subject of various scientific works pertaining extractions methods, literature about extraction of C-PC from *A. maxima* can barely be found. However, on the cellular level, both, *A. platensis* and *A. maxima* are very similar, though small differences in the morphology of the cells and the microfilaments or trichomes were described (Tomaselli, 1997). The chemical composition of both species is also very similar anent the protein, carbohydrate, and lipid content, as well as the fatty acid composition. Contrarily, *A. maxima* demonstrated a better growth when culturing temperature was chosen to be between 20 and 40°C. Besides, at optimal culturing temperature of 30°C the protein content in *A. maxima* was slightly higher than in *A. platensis*, while not varying significantly at other temperatures (Oliveira et al., 1999).

4.1 CPC concentration and content

The freeze-thawing method, that was is described as one of the most effective and easiest methods for C-PC extraction (Tan et al., 2020), provided the highest C-PC concentration (5.11 mg mL^{-1}) in this work. This value is higher than most values for C-PC concentration reported in literature for C-PC extracts from *A. maxima* (Nisticò et al., 2022)d *platensis* (Aoki et al., 2021; Silveira et al., 2007; Moraes et al., 2011). For the glass bead extraction, that was carried out with the same biomass concentration as the freeze-thawing extraction, the C-PC concentration was found to be 2.98 mg mL^{-1}. The lower C-PC concentration in the glass bead extracts can be explained by a less effective cell disruption. The ultrasonication method, that used lower biomass concentrations lead to extracts with a C-PC concentration between 2.23 mg mL^{-1} and 1.17 mg mL^{-1}.

The C-PC concentration is strongly influenced by the used dry matter concentration, while the C-PC yield per dry matter is not. However, one publication stated, that in freeze-thawing extraction even a dry matter contents of 4% can result in reduced C-PC contents compared to 0.5 and 2%, which obtained higher C-PC contents not significantly differing among each other (Tan et al., 2020). Initial dry matter contents of more than 8% were shown to lead to high concentrated cell suspensions in which extractant efficiency is reduced (Silveira et al., 2007). Therefore, publications mostly show the amount of C-PC yielded per gram of dry matter making the results of different extraction methods more comparable.

Since the value for the C-PC content (mass ratio of C-PC per dry matter) can also be transformed into a percentage, and in all approaches of this work, the same biomass was processed, the C-PC content could be used as a measurand of extraction efficiency. In this work, the freeze-thawing method obtained the highest C-PC content (17.03%) indicating the most effective extraction. This is followed by the ultrasonication method combined by flocculation with chitosan in acetic acid (15.21%). This is more than in most other publications for C-PC extraction with various methods form *A. platensis* and for extraction from *A. maxima* using stirring for 24 hours (Nisticò et al., 2022). A comparison of the results from this work with various results from other publications can be found in Table 1. In all these publications, deionised water or sodium phosphate buffers were used for C-PC extraction. In this work, a calcium chloride solution was used, because previous experiments (not shown) showed the superiority over other extractants (deionised water, phosphate buffers). Therefore, the high C-PC contents obtained by freeze thawing can be partially attributed to the choice of extractant. But this suggestion is conflicting the results on the efficiency of different extractants, that could not find significant differences between sodium chloride solution, calcium chloride solution, deionised water, and phosphate buffer (pH 7) anent the C-PC concentration obtained (Silveira et al., 2007). Interestingly, in a publication, that also performed the freeze-thawing extraction, a similar C-PC content of 17.28% (and also a similar purity) was measured (Tan et al., 2020). However, another publication demonstrated, that less common cell disruption methods like high pressure homogenization or microwaving could result in even higher C-PC contents of more than 20% (corresponding to 200 mg g^{-1}) using *A. maxima* as source (Ruiz-Domínguez et al., 2019). For *A. platensis*, even simple cell lysis in deionised water could result in a higher C-PC content of 21.1% (Aoki et al., 2021).

The bright variety of results can hardly be explained by the different extraction methods alone, but rather by contribution of varying protein contents in the cells. The protein content of *A. platensis* is around 55–70% by reference to the dry matter (Aouir et al., 2017; Babadzhanov et al., 2004, Oliveira et al., 1999). The C-PC content in *A. platensis* was stated to lie between 14% (Ali and Saleh, 2012) and 20% (Vernès et al., 2015) of its dry matter, and therefore, more than 20% of the whole proteome in this cyanobacterium is contributed by C-PC. On the other hand, the chemical composition of Spirulina is strongly depending on the culturing conditions used to grow the biomass as shown by various publications before (Oliveira et al., 1999; Marrez et al., 2014; Olguín et al., 2001; Markou et al., 2012). The protein content in *A. maxima* has been demonstrated to be higher when low temperatures (20–30°C) were applied for cultivation. Whereas low temperatures contrarily increase the carbohydrate content of the cells (Oliveira et al., 1999). The culture media also has an influence on the protein content. BG-11 medium was found to lead to

higher amounts of protein in the biomass when compared to modified BG-11 and Zarrouk's Medium (Marrez et al., 2014). Standardized media often show higher protein contents in final biomass than experimental waste stream media. The reason for this is assumed to be the lack in accessible nitrogen leading to a nitrogen deficiency in the cells when cultivated with non-standardized media (Marrez et al., 2014; Olguín et al., 2001). Also, the various available strains display a bright variety in chemical composition which can be attributed to their natural habitat (Aouir et al., 2017). The protein content of the Spirulina cells is also depending on the light intensity applied for cultivation. The authors of another work found out, that less lumination contributed to higher protein contents in *A. platensis* (Olguín et al., 2001; Markou et al., 2012). All these findings make it hard to compare different extract methods published in literature.

Table 1
Overview of various results for C-PC extraction in other publications compared to the results from this work. Shown are the used organism, the extraction method, the C-PC concentration ($c_{C\text{-}PC}$), the purity, and the C-PC content. HP = high pressure

organism	extraction method	c_{C-PC} [mg mL^{-1}]	Purity [-]	C-PC content [%]	Source
A. maxima	HP homogenization	-	-	29.1	Ruiz-Domínguez et al. (2019)
A. maxima	microwaving	-	-	22.6	Ruiz-Domínguez et al. (2019)
A. maxima	Freeze-thawing	5.11	1,94	17.03	this work
A. maxima	Sonication and flocculation	2.23	2,02	15.21	this work
A. maxima	Glass bead extraction	2.98	1,58	10.92	this work
A. maxima	Stirring for 24 h (before UF)	0.23	0,74	11.6	Nisticò et al. (2022)
A. platensis	Lysis in deionized water	0.16	1.76	21.1	Aoki et al. 2021
A. sp	Freeze-thawing	-	1.95	17.28	Tan et al. (2020)
A. platensis	Pulsed electric fields	-	0.51	15.19	Martínez et al. (2017)
A. platensis	Freeze-thawing	-	1.06	13.19	Prabhath et al. (2019)
A. platensis	Stirring at 35°C for 48 h	3.97	0.80	9.93	Minchev et al. (2021)
A. platensis	Pulsed electric fields	-	-	8.52	Jaeschke et al. (2019)
A. platensis	ultrasonication	-	-	6.00	Furuki et al. (2003)
A. sp	Freeze-thawing	-	1.34	8.63	Doke (2005)
A. sp	Air drying	-	1.80	8.00	Doke (2005)
A. platensis	Rotary shaker for 4 h	3.68	0.46	4.60	Silveira et al. (2007)
A. platensis	sonication + glass beads	0.21	-	4.38	Moraes et al. (2011)

4.2 Purity

The extract obtained by ultrasonication followed by flocculation with chitosan in acetic acid displayed the highest purity with 2.02. Second highest purity was found in the freeze-thawed extract (1.94). These values are higher than in most other publications for C-PC from *A. maxima* (Nisticò et al., 2022)d *platensis* (Aoki et al., 2021; Martínez et al., 2017; Prabhath et al., 2019; Doke, 2005; Silveira et al., 2007). Higher values for the purity can be achieved by further purification. In another publication, ion exchange chromatography with pH gradient elution was used to obtain C-PC extracts with purities of 4.2 and 3.5 (Amarante et al., 2020). In general, purity values of higher than 0.7 are considered food grade and a purity of more than 4.0 are considered analytical grade (Rito-Palomares et al., 2001). Therefore, all the extracts obtained in this work are food grade.

Since the purity is defined as the ratio between phycocyanin (A_{620}) and aromatic amino acids (A_{280}), a lower purity indicates a relatively increased protein concentration in the extract. This can be the consequence of a higher degree of cell disruption corresponding to a lower selectivity of the whole extraction method due to the division of particles that otherwise would be easily separated from the extract (Furuki et al., 2003). Compared to incubation in deionised water, the extraction of C-PC by ultrasonication was demonstrated to rapidly decrease the purity value of the resulting extract. The purity also decreased when ultrasonication lasted for longer than 20 min which is assumed to be the consequence of the release of proteins from cell organelles (Tavakoli et al., 2021). Besides, the pH influencing the water-solubility of disintegrated cell proteins can contribute to a varying C-PC purity. In fact, protein isolates from *A. platensis* were shown to have their lowest solubility at a pH of 3 corresponding to the isoelectric point of the protein isolate (Devi et al., 1981). The solubility of the proteins is increasing when pH is decreased (pH 2) and also when the pH is increased (pH 4–10) (Benelhadj et al., 2016). This is, more or less, in accordance with the purities found in this work, where the ultrasonicated extract in acetic acid (pH 4.03) had the highest purity, followed by the freeze-thawed extract (pH 6.49), and the bead milled extract (pH 7.14) showing the lowest purity of the three bluish extracts. The two ultrasonicated extracts with citric acid and lactic acid had pH values close to the isoelectric point of spirulina protein isolate (3.14 and 3.02, respectively), but lower purities. Since the C-PC concentration was also lower compared the acetic acid sample, the pH of the final extract apparently affected the C-PC stability causing it to denaturize. The maximum stability for C-PC lies between pH 5 and 7.5 (Sarada et al., 1999; Chaiklahan et al., 2012). At pH 4, a slight and at pH 3, a massive decrease of C-PC concentration could be observed. This is attributed to unfolding of the protein structure leading to precipitation (Wu et al., 2016). A change in the protein conformation by proteolytic digestion of C-PC was demonstrated to decrease the absorption at 620 nm while simultaneously increasing the absorption in the UV spectrum (with a peak at 350 nm) which is associated with the folding of the PCB chromophores (Debreczeny et al., 1989). This can explain the higher absorption in the area of 280 to 380 nm for the ultrasonicated samples as a result of degradation due to the pH.

The method to separate the extract from the cell debris can also contribute to different purity values of the extract. The ultrasonicated samples were processed by chitosan flocculation instead of

centrifugation. Since chitosan is a well-known coagulating agent due to its high number of charged amino acid groups, it can bind proteins and flocculate them out of the extract (Li et al., 1992). This has maybe furtherly contributed to the high purity of the ultrasonicated extract with acetic acid. In this case, the low purity for the ultrasonicated samples containing citric acid and lactic acid is rather implicated by the decrease in C-PC concentration due to the low pH than the increase of protein concentration.

4.3 Selectivity

In previous extraction experiments, the extracts' quality was often depreciated by the presence of chlorophyll a in the final C-PC solution resulting in greenish or greyish extract colour. Therefore, in this work a measurand to quantify the ratio of C-PC and chlorophyll a using the ratio of absorptions at 620 nm (absorption maximum of C-PC) and at 438 nm (one absorption maximum of chlorophyll a) was established. This A_{620}/A_{438}-ratio, that was considered the *selectivity*, was highest for the freeze-thawed and glass bead extracts (20.28 and 14.56, respectively). The selectivity for the ultrasonicated extracts was lower than 10 in all three samples (with acetic acid, citric acid and lactic acid).

Excessive cell disruption can cause higher proportions of unwanted substances in the final extract, since the increased degree of cell constituent destruction leads to the solution of substances that are supposed to be separated from the extract with the solid parts (Furuki et al., 2003). There are extraction methods that had already been shown to reduce the abundance of chlorophyll a in C-PC extracts from *A. platensis*, e.g. pulsed electric fields treatment (PEF) (Jaeschke et al., 2019; Li et al., 2020) or high-pressure processing (HPP) (Li et al., 2020) when compared to ultrasonication. In comparison to homogenisation of the biomass with mortar and pestle, the freeze-thawing method yielded C-PC extracts with lower chlorophyll contents (Sarada et al., 1999), indicating, that rough physical methods show lower selectivity. On the other hand, bead milling is associated with a reduced selectivity, due to the formation of small cell debris particles (Günerken et al., 2015). Contrary to this, the glass bead extracts in this work, showed a high selectivity compared to the ultrasonicated samples, which can best be observed looking at the normalized absorption spectres. But regarding the low

C-PC content of 10,92% (per dry matter) obtained in this experiment, a non-completed cell disruption can contribute to a selectivity higher than expectable. Acid extraction also was described to result in C-PC extracts with significant amounts of chlorophyll (Doke, 2005). This is supported by the fact, that the ultrasonicated extracts with low pH values (ranging from 3–4) showed the highest chlorophyll a contamination in this work. On the other hand, the extraction solution has an influence on the chlorophyll concentration in the final extract. Sodium chloride (NaCl) solutions with concentrations of more than 5 g L^{-1} were shown to significantly reduce the chlorophyll in C-PC extracts compared to less concentrated NaCl-solutions and deionised water (Li et al., 2020). In this work, freeze-thawing and glass bead extraction were carried out using calcium chloride (10 g L^{-1}), which is in accordance to the findings mentioned before. The ultrasonicated samples had lower selectivity values than the extracts yielded with freeze-thawing and glass beads. Because of the flocculation with chitosan as part of the extraction

methodology, calcium chloride solution was not suitable as the extractant. Instead, deionised water was used for the ultrasonication, and can therefore explain the lower selectivity. The separation technique can also contribute to lower sensitivity, since the ultrasonication was carried out accompanied by flocculation and filtration, while for freeze-thawing and glass bead extraction centrifugation was applied. However, previous experiments, that are not shown in this work, showed, that centrifugation of the ultrasonicated samples resulted in green-brownish extracts indicating a failed separation of extract and cell debris.

Actually, the contamination of C-PC extracts with high amounts of chlorophyll can bias the calculation of C-PC concentration, because the absorption of chlorophyll can interfere with the absorption of phycocyanin and allophycocyanin (Yacobi et al., 2015). Therefore, some publications already suggested the adaption of the common formula for the calculation of the C-PC concentration taking the potential presence of chlorophyll a in the final extract into account. (Fabre et al., 2022; Lauceri et al., 2018). However, this bias only applies for less concentrated C-PC extracts (Yacobi et al., 2015; Lauceri et al., 2018). In literature, the adaption of the formula apparently could not prevail by now.

Declarations

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Authors' contribution: Conceptualisation by Jan Kuhnholz; Investigation by Jan Kuhnholz and Anja Noke; Methodology by Jan Kuhnholz, Till Glockow, Verena Siebecke, Thu Le Anh, Long-Dinh Tran; Writing by Jan Kuhnholz; Supervision by Anja Noke

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Figures

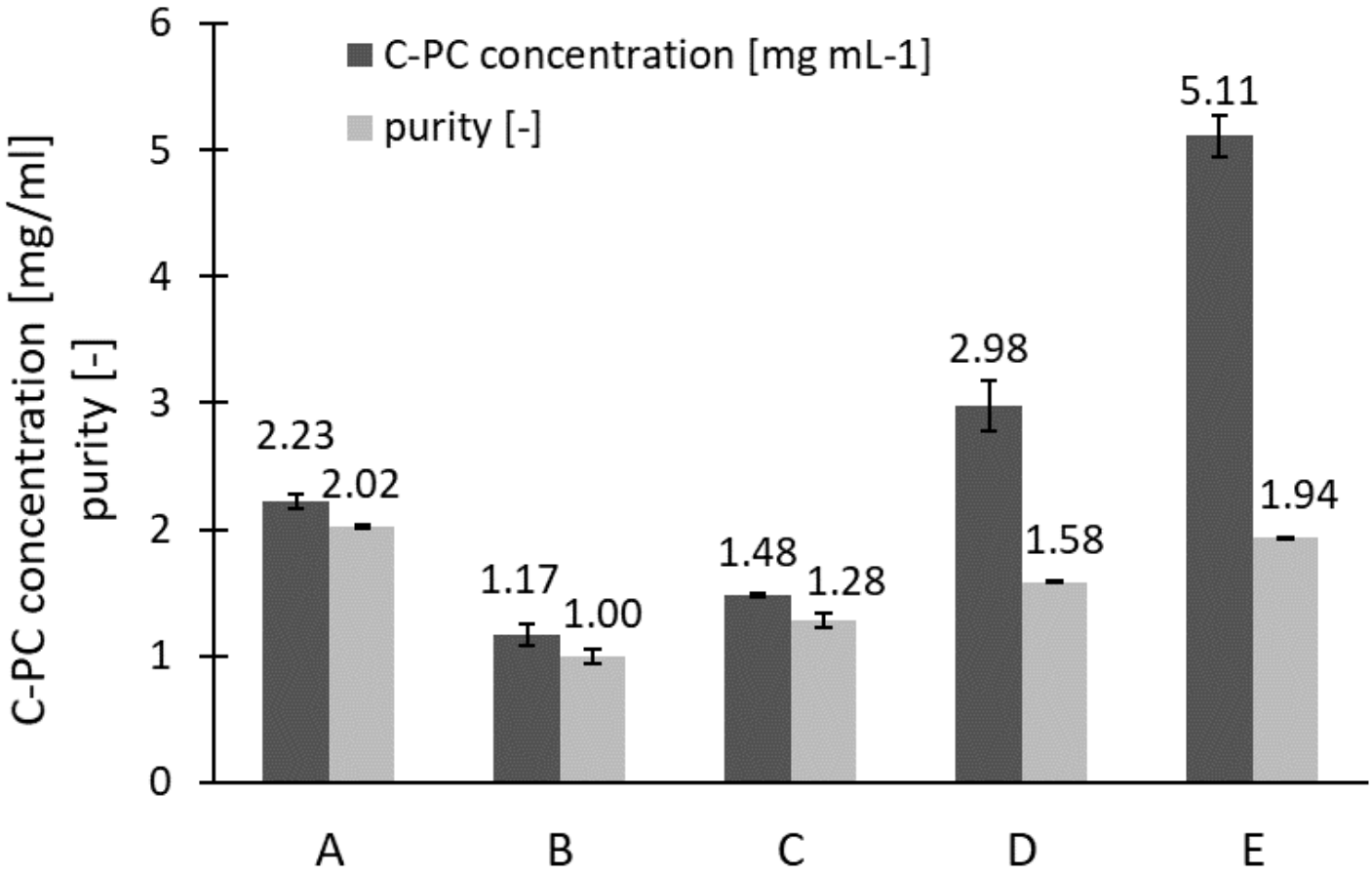

Figure 1

C-PC concentration and purity of the 5 different approaches: ultrasonication followed by flocculation with chitosan in acetic acid (A), citric acid (B), lactic acid (C); glass bead extraction followed by centrifugation (D); freeze-thawing followed by centrifugation (E); values display the arithmetic mean of the triplicates (n=3); error bars show the standard deviations.

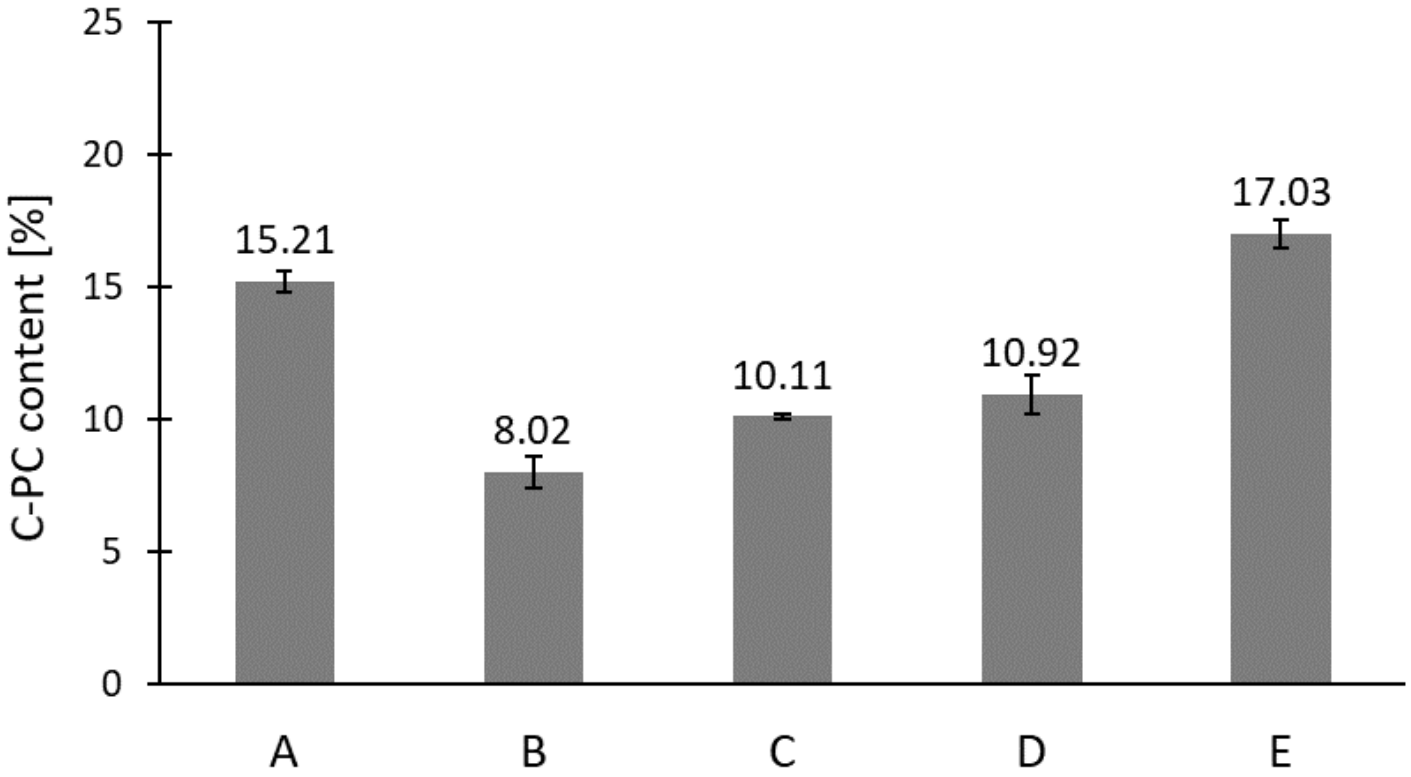

Figure 2

C-PC content of the 5 different approaches: ultrasonication followed by flocculation with chitosan in acetic acid (A), citric acid (B), lactic acid (C); glass bead extraction followed by centrifugation (D); freeze-thawing followed by centrifugation (E); values display the arithmetic mean of the triplicates (n=3); error bars show the standard deviations.

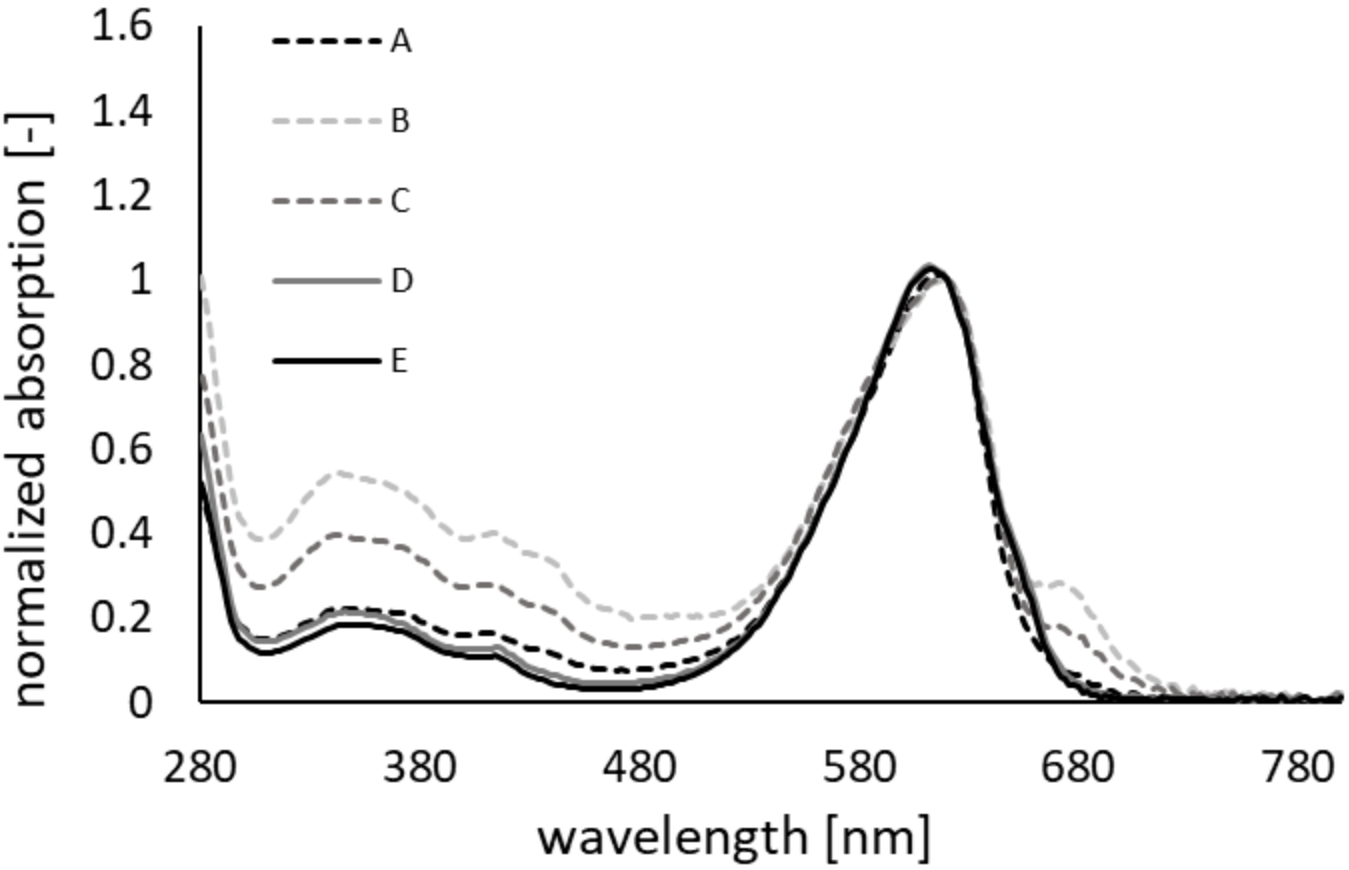

Figure 3

Normalized absorption spectra of the 5 different approaches: ultrasonication followed by flocculation with chitosan in acetic acid (A), citric acid (B), lactic acid (C); glass bead extraction followed by centrifugation (D); freeze-thawing followed by centrifugation (E)

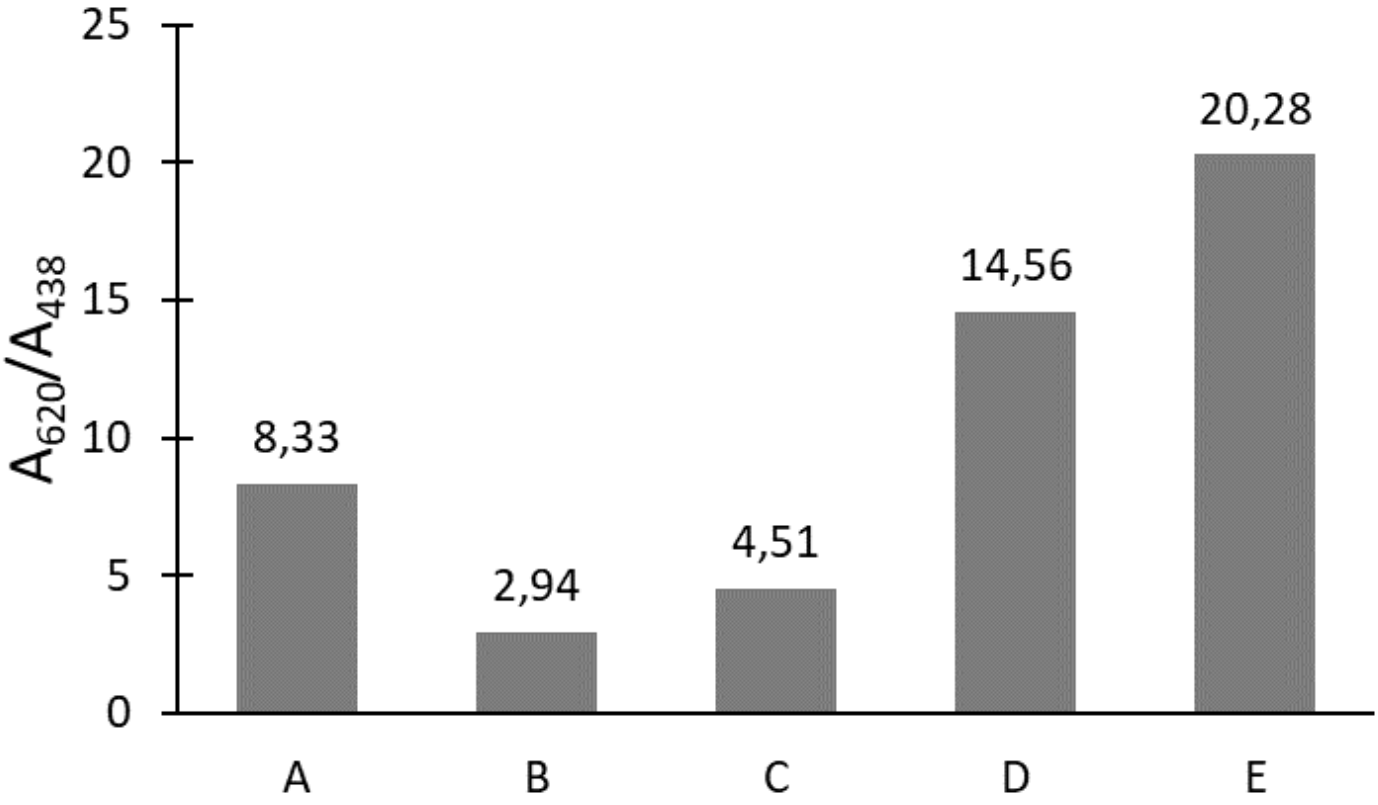

Figure 4

Ratio of absorption at 620 nm (A_{620}) to absorption at 438 nm (A_{438}) as a measurand for the selectivity of the 5 different approaches: ultrasonication followed by flocculation with chitosan in acetic acid (A), citric acid (B), lactic acid (C); glass bead extraction followed by centrifugation (D); freeze-thawing followed by centrifugation (E); values display the A_{620}/ A_{438}-values of the normalized spectra.

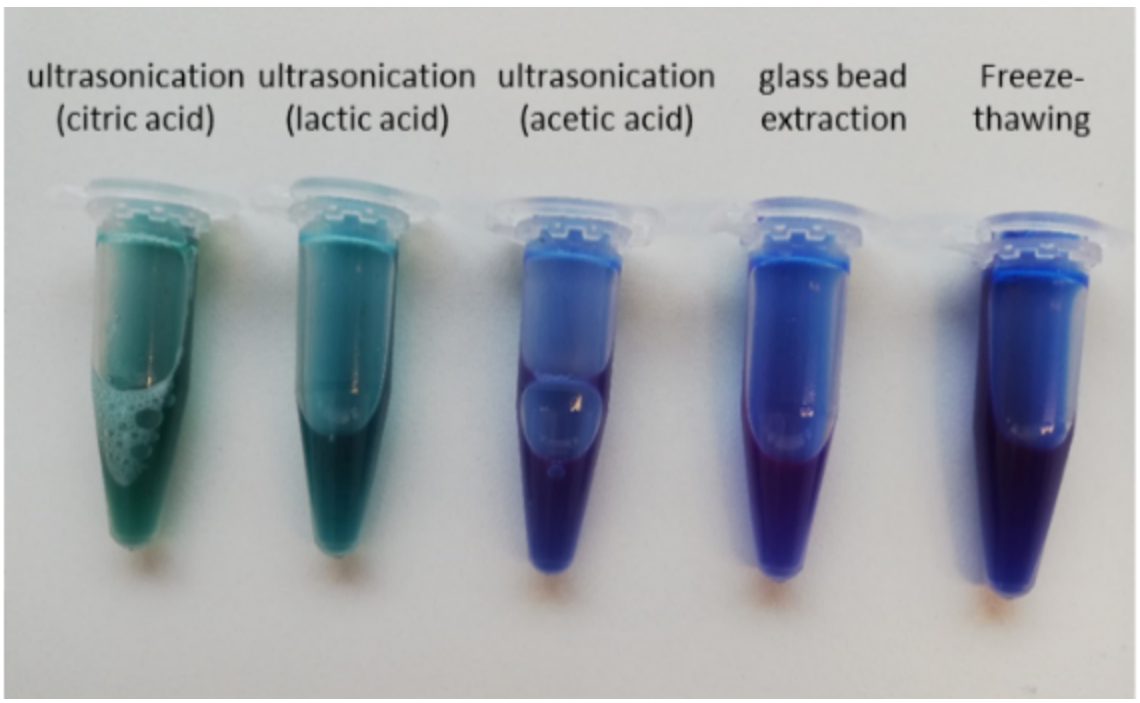

Figure 5

Photography of the 5 resulting C-PC-extracts. Extracts are not diluted.