TÉCNICO LISBOA

EXTRACTION OF PHYCOCYANINS FROM SPIRULINA (*Spirulina platensis*) AND STABILITY IN EUTECTIC SOLVENTS

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Abstract

Phycocyanin (PC), a bright blue pigment derived from the cyanobacteria *Spirulina platensis*, is a promising natural pigment in the food, pharmaceutical and cosmetic industries. This work reassessed the capacity of water as a green extraction solvent for PC extraction from *Spirulina platensis* using maceration method.

The present work described a suitable method for the extraction of PC from the cyanobacteria Spirulina platensis. Distilled water was chosen as the solvent for the extraction with maceration method because it produced high concentration of PC and due to its low cost compare with the microwave method, that is more expensive even though faster. At biomass-solvent ratio of 1:4 and at the experimental conditions of 48 h, and constant stirring at room temperature, the yield obtained was 41.886 mg/g (0.1675 mg/ml) and purity 1.37, indicating that the PC was a reagent grade. In this PC extraction method, there is opportunity for a scale up due to its simplicity and low cost. Also, *Spirulina platensis* was confirmed to be a good source of PC, to be extracted for diverse purposes.

Two families of DES were used in this work, (i) based on Peg-200 as hydrogen bond donor (HBD) and (ii) based on choline chloride as hydrogen bond acceptors (HBA). Established method of heating and stirring at temperatures below 100 ºC for 30-90 mins was adopted to prepare the two groups of DES or NADES. In the preparation of Peg-200-based DES, TBPB:Peg-200 (1:2), TBAB:Peg-200 (1:2), and Aliquat-336:Peg-200 (1:2), DESs, were all formed at 70 ºC and remained as transparent and non-viscous liquid at room temperature. The second family of DES prepared include ChCl:Malic acid (1:1), ChCl:Oxalic acid (1:2), which were also formed as transparent liquid but soon became very viscous at room temperature, while ChCl:Peg-200 (1:2) formed quickly crystalized at room temperature but readily became non-viscous liquid when the component ratios was changed to 1:3. Also to be mentioned that ChCl:Citric acid (1:1) could not be formed at room temperature without addition of water. At the stoichiometric ratio with water hence, ChCl:Citric acid:Water (1:1:6), this DES was formed.

PC has the highest solubility in TBAB:Peg-200 (1:2) and ChCl:Malic acid (1:1) with apparent solubility values of 0.05538 g/g and 0.1325 g/g of DES, respectively. The color observed during solubility tests of the PC in 1 g DES are ChCl:Malic acid (green), ChCl : Oxalic acid (grey), ChCl:Peg-200 (brown) and ChCl:Citric acid (bluish green), respectively. This proved that only ChCl:Malic acid and ChCl:Citric acid have the potential to be used as color preservatives of PC.

Keywords: Natural Pigments, Spirulina, Phycocyanin, Extraction, Stabilization.

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Abbreviations

Symbols	Meaning	symbols	Meaning
A	*Frequency factor*	***IST***	*Instituto Superior Tecnico*
Aliquat 336	*Tri octyl methylammonium chloride*	k_d	*Degradation rate constant*
A_{620}	*Absorption at wavelength 620 nm*	***NADES***	*Natural Deep Eutectic Solvents*
A_{280}	*Absorption at wavelength 280 nm*	***P***	*Purity*
A_{652}	*Absorption at wavelength 652 nm*	***PC***	*PC*
A_{680}	*Absorption at wavelength 680 nm*	***PCB***	*PCBs*
APC	*Allophycocyanin*	***PBS***	*Phycobilisomes*
C	*Concentration*	***QAS***	*Quaternary Ammonium Salt*
C_R	*Relative concentration of PC*	***R***	*Gas constant*
CSMCRI		***T***	*Temperature*
DES	*Deep Eutectic Solvents*	***TBAB***	*Tetra butyl ammonium bromide*
dw	*Dry weight of PC*	***TBPB***	*Tetra-n-butyl phosphonium bromide*
E_a	*Activation Energy*	***TMAB***	*Tetra methyl ammonium bromide*
EC	*European Commission*	$t_{1/2}$	*Half-life*
E_d	*Activation Energy of degradation reaction*	t_{tr}	*Thermal transition temperature*
EFSA	*European Food Security Agency*	***USA***	*United States of America*
EU	*European Union*	***V***	*Volume of the extraction solvent*
FDA	*Food and Drugs Administration*	***V***	*Volume of the extraction solvent*
HBA	*Hydrogen Bond Acceptor*		
HBD	*Hydrogen Bond Donor*		
IBET	*Instituto de Biologia Experimental e Tecnológica*		
ILs	*Ionic liquids*		
EU	*European Union*		

CHAPTER 1

1. INTRODUCTION

Color impacts everything about our lives from the clothes we ware to the environment we live in, the furnishing in our homes and the appeal of foods. The influence of color in human perception and reality is enormous [1]. The organoleptic properties of food impact the sensory organs of the consumers by their color, taste, and smell. This means that properties like freshness, nutritional value, safety, also the aesthetic value of food directly affects the market value of colored food products [2].

Food colorants have been used to mask damaging effects of external conditions, such as light, air, temperature, moisture, and storage conditions, on food color. They are also used to homogenize foodstuffs color, through the correction of color variations and enhancement of naturally occurring food colors. Apart from their direct use as food colorants, they might also be used to contribute to the flavorful assurance, safety, quality, and organoleptic characteristics of foodstuffs as well ensuring consumers satisfaction [3].

The history of the use of food colorants as additives dates to the Egyptian civilization when candy makers added natural extracts to their candy and wine was colored as long ago as 400 BC [1]. Similarly, the use of natural colorants in food was found in Japan in a text of the Nara period (8th century) that contains references to coloring of soybean and adzuki-bean cakes [4]. In industrialized nations, synthetic dyes have been used to artificially color foods for at least a century [5]. Sir. William Henry Perkin in 1856 was the first person to apply synthetic color, mauvine, to food, which triggered the synthetic color revolution in food industry. Since then, the developing food industry made used a vast array of synthetic colors, without control over the use of these dyes regarding the level of their toxicity and other adverse effects leading to negative effects on the health and environment [1, 4].

Currently, the utilization of food colorants is highly regulated, whether the dye compounds are naturally derived or synthetically produced. The European Food and Safety Authority (EFSA), the U.S. Foods and Drugs Administration (FDA), and The World Health Organization (WHO) [2, 3] represent the most important regulatory organizations empowered to ensure the quality and security of food products, as well as to protect and promote human health. In the USA, the use of food colors is governed by the Code of Federal Regulations (CFR) (Title 21, Part 70-82) [1], while in Europe, the proposed colors and levels of use must comply with EFSA Regulation EC No 1333/2008 [6, 7]. EFSA recognizes food colorants as food additives which are used to add or restore color in food [8]. Synthetic food colorants are intensively studied in terms of safety of their use, side effects, toxicity at short and long terms as well as health impact. Consequently, a considerable amount of food colorants has been increasingly removed, even prohibited in the food industry

and their application in food products is currently regulated by FDA and EFSA, with well-established acceptable daily intake (ADI) doses.

Industrial food producers use synthetic dyes because they are cheaper, more stable, and brighter than most natural colorings. However, these synthetic compounds raise significant health concerns since they are toxicity, carcinogenic nature [3]. The growing awareness of the ingredients in the food products and the consumer demand for healthy and natural foods required the established list of permitted synthetic colors additives by the early of this century. Table 1 and 2, [9] show the European Union has authorized 43 colorants [10, 11] as food additives with each one assigned an `E number', and comparison of authorized colorants between EU and USA Table 3, [7], . 17 of which are synthetic pigments and 26 are either naturally derived, synthesized to match the naturally occurring counterparts or are inorganic pigments found in nature. In comparison, only 7 synthetic pigments, all in the form of water-soluble dyes, are permitted by USA [1]. The current established list of permitted food colorants and the regulations are already published [7].

Table 1. European Union list of food additives approved for use in foods and conditions of use Regulation EC 1333/2008 [9]

PART B

LIST OF ALL ADDITIVES

1. **Colours**

E-number	Name
E 100	Curcumin
E 101	Riboflavins
E 102	Tartrazine
E 104	Quinoline Yellow
E 110	Sunset Yellow FCF/Orange Yellow S
E 120	Cochineal, Carminic acid, Carmines
E 122	Azorubine, Carmoisine
E 123	Amaranth
E 124	Ponceau 4R, Cochineal Red A
E 127	Erythrosine
E 129	Allura Red AC
E 131	Patent Blue V
E 132	Indigotine, Indigo carmine
E 133	Brilliant Blue FCF
E 140	Chlorophylls and chlorophyllins

Table 1 (Continued). European Union list of food additives approved for use in foods and conditions of use Regulation EC 1333/2008 [9]

E-number	Name
E 141	Copper complexes of chlorophylls, chlorophyllins
E 142	Green S
E 150a	Plain caramel (1)
E 150b	Caustic sulphite caramel
E 150c	Ammonia caramel
E 150d	Sulphite ammonia caramel
E 151	Brilliant Black BN, Black PN
E 153	Vegetable carbon
E 155	Brown HT
E 160a	Carotenes
E 160b	Annatto, Bixin, Norbixin
E 160c	Paprika extract, capsanthin, capsorubin
E 160d	Lycopene
E 160e	Beta-apo-8'-carotenal (C 30)
E 161b	Lutein
E 161g	Canthaxanthin (*)
E 162	Beetroot Red, betanin
E 163	Anthocyanins
E 170	Calcium carbonate
E 171	Titanium dioxide
E 172	Iron oxides and hydroxides
E 173	Aluminium
E 174	Silver
E 175	Gold
E 180	Litholrubine BK

(1) The term caramel relates to products of a more or less intense brown colour which are intended for colouring. It does not correspond to the sugary aromatic product obtained from heating sugars and which is used for flavouring food (e.g. confectionery, pastry, alcoholic drinks).

(*) Canthaxanthin is not authorised in the food categories listed in Part D and E. The substance is in list B1 because it is used in medicinal products in accordance with Directive 2009/35/EC of the European Parliament and of the Council (OJ L 109, 30.4.2009, p. 10).

Table 2. Food categories in which the EU's group II and III color additives are permitted [7]

	Food category	Group II additives permitted[a]	Group III additives permitted[b]
1.4	Flavoured fermented milk products	Yes	Yes
1.5	Dehydrated milk	Except unflavoured products	
1.6.3	Other creams	Flavoured creams only	Flavoured creams only
1.7.1	Unripened cheese	Flavoured only	Flavoured only
1.7.3	Edible cheese rind	Yes	Yes
1.7.4	Whey cheese	Yes	
1.7.5	Processed cheese	Flavoured only	
1.7.6	Cheese products	Flavoured unripened only	Flavoured unripened only
1.8	Dairy analogues	Yes	
3.0	Edible ices	Yes	Yes
4.2.4.1	Fruit and vegetable preparations, excluding compote	Mostarda di frutta only	Mostarda di frutta only
5.2	Other confectionary	Yes	Yes
5.3	Chewing gum	Yes	Yes
5.4	Decorations, coatings and fillings	Yes	Yes
6.3	Breakfast cereals	Other than extruded, puffed and/or fruit flavoured	
6.5	Noodles	Yes	
6.6	Batters	Yes	For coating only
6.7	Precooked or processed cereal	Yes	
7.2	Fine bakery wares	Yes	Yes
8.3.3	Casings and coatings and decorations for meat	Edible external coatings of pasturmas	Only edible casings
9.2	Processed fish and fishery products	Only surimi and similar products and salmon substitutes	Only surimi and similar products and salmon substitutes
9.3	Fish roe	Except caviar	Except caviar
10.1	Eggs and egg products. All approved colorants permitted for decorative colouring of shells or stamping	Yes	Yes
10.2	Processed eggs and egg products. All approved colorants permitted for decorative colouring of shells.	Yes	Yes
12.2.2	Seasonings and condiments	Only seasonings	Only seasonings
12.4	Mustard	Yes	Yes
12.5	Soups and broths	Yes	Yes
12.6	Sauces	Not tomato based	Not tomato based
12.7	Salads and savoury-based sandwich spreads	Yes	
12.9	Protein products	Yes	Only meat and fish analogues based on vegetable proteins
13.2	Dietary foods for special medical purposes	Yes	Yes
13.3	Dietary foods for weight control	Yes	Yes
13.4	Foods suitable for people intolerant to gluten	Yes	
14.1.4	Flavoured drinks	Not chocolate milk and malt products	Not chocolate milk and malt products
14.2.3	Cider and perry	Yes	Yes
14.2.4	Fruit wine and made wine	Yes	Yes
14.2.5	Mead	Yes	
14.2.6	Spirit drinks	Yes	Yes
14.2.7.3	Aromatised wine-product cocktails	Yes	Yes
14.2.8	Other alcoholic drinks	Yes	
15.1	Potato-, cereal-, flour- or starch-based snacks	Yes	Yes
15.2	Processed nuts	Yes	Only savoury coated
16	Desserts	Yes	Yes
17.1	Food supplements, solid form	Yes	Yes
17.2	Food supplements, liquid form	Yes	Yes
17.3	Food supplements, syrup-type or chewable	Yes	Yes

Notes: [a]Group II additives (Regulation (EC) No. 1333/2008): riboflavins (E 101); chlorophylls, chlorophyllins (E 140); copper complexes of chlorophylls and chlorophyllins (E 141); caramels (E 150a–d); vegetable carbon (E 153); carotenes (E 160a); paprika extract, capsanthin, capsorbin (E 160c); beetroot red, betanin (E 162); anthocyanins (E 163); calcium carbonate (E 170); titanium oxide (E 171); iron oxides and hydroxides (E 172).

[b]Group III additives (Regulation (EC) No. 1333/2008): curcumin (E 100); tartrazine (E 102); cochineal, carminic acid, carmines (E 120); azorubine, carmoisine (E 122); Allura Red (E 129); Patent Blue V (E 131); indigotine, indigo carmine (E 133); Green S (E 142); Brilliant Black PN (E 151); Brown HT (E 155); beta-apo-8'-carotenal (E 160e); lutein (E 161b).

Table 3. comparison of authorized colorants between EU and USA [7]

EU			US		
E number	Name	Lake permitted	Name (common name)	Lake permitted	Subject to batch certification
E 100	Curcumin	Yes	Turmeric Turmeric oleoresin		
E 101	Riboflavins 5 (including riboflavin-5'-phosphate)	Yes	Riboflavin		
E 102	Tartrazine	Yes	FD&C Yellow No. 5 (Tartrazine)	Yes	Yes
E 104	Quinoline Yellow	Yes			
E 110	Sunset Yellow FCF/Orange Yellow S	Yes	FD&C Yellow No. 6 (Sunset Yellow FCF)	Yes	Yes
			Orange B		Yes
E 120	Cochineal, carminic acid, carmines	Yes	Cochineal extract, carmine	Yes	
E 122	Azorubin, carmoisine	Yes			
E 123	Amaranth	Yes			
E 124	Ponceau 4R, Cochineal Red A	Yes			
E 127	Erythrosine	Yes	FD&C Red No. 3 (Erythrosine)		Yes
E 129	Allura Red AC	Yes	FD&C Red No. 40 (Allura Red AC)	Yes	Yes
			Citrus Red No. 2		Yes
E 131	Patent Blue V	Yes			
E 132	Indigotine, Indigo carmine	Yes	FD&C Blue No. 2 (Indigotine)	Yes	Yes
E 133	Brilliant Blue FCF	Yes	FD&C Blue No. 1 (Brilliant Blue FCF)	Yes	Yes
E 140	Chlorophylls and chlorophyllins				
E 141	Copper complexes of chlorophylls, chlorophyllins	Yes	Sodium copper chlorophyllin		
E 142	Green S	Yes			
			FD&C Green No. 3 (Fast Green FCF)	Yes	Yes
E 150a–d	Plain caramel, caustic sulphite caramel, ammonia caramel, sulphite ammonia caramel		Caramel		
E 151	Brilliant Black PN	Yes			
E 153	Vegetable carbon				
E 155	Brown HT	Yes			
E 160a	Carotenes		β-Carotene Carrot oil		
E 160b	Annatto, bixin, norbixin		Annatto extract		
E 160c	Paprika extract, capsanthin, capsorubin		Paprika, paprika oleoresin		
E 160d	Lycopene		Tomato lycopene extract; tomato lycopene concentrate		
E 160e	β-Apo-8'-carotenal		β-Apo-8'-carotenal		
E 161b	Lutein				
E 161g[a]	Canthaxanthin	Yes	Canthaxanthin (not synthetic)		
E 162	Beetroot Red, betanin		Dehydrated beets (beet powder)		
E 163	Anthocyanins	Yes	Grape colour extract Grape skin extract[d]		
E 170	Calcium carbonate				
E 171	Titanium dioxide		Titanium dioxide		
E 555 and E171[b]	Potassium aluminium silicate (mica) and titanium oxide		Mica-based pearlescent pigments		
E 172	Iron oxide and hydroxides		Synthetic iron oxide		
E 173	Aluminium				
E 175	Silver				
E 174	Gold				
E 180	Litholrubin BK	Yes			
E 579[c]	Ferrous gluconate		Ferrous gluconate		
E 585[c]	Ferrous lactate		Ferrous lactate colour fixative for ripe olives		
Colouring food	Vegetable juice		Vegetable juice		
Colouring food	Fruit juice		Fruit juice		
Colouring food	Saffron		Saffron		
Colouring food	Spirulina extract		Spirulina extract		
			Toasted partially defatted cooked cottonseed flour		

Notes: [a]Only for medicinal products.
[b]Potassium aluminium silicate, i.e., mica (E 555), is an approved carrier for titanium dioxide (E 171) and iron oxides and hydroxides (E 172). Mica platelets can be coated with E 171 and/or E 172 to form pearlescent pigments. In the US, only coating with titanium dioxide is permitted.
[c]Other food additive in the EU.
[d]In the US, calcium carbonate is listed as a food substance affirmed as GRAS.

Developing new colors for the food industry is a very challenging task, as colorants need to be compatible with a foods flavor, safety, and nutritional value, and ultimately have minimal impact on the price of the product. In addition, a food colorant needs preferably to be a natural rather than synthetic compound [2]. To be mentioned that food legislation has always lagged innovation and product development, sometimes by more than a decade [5].

Natural colors are primarily derived from plants, insects, mineral ores, and microbial sources. Microbial colorants are usually preferred due to their ease of scalability and potentially lower the cost of production [2]. Natural colors are assumed to be safe if they are non-allergic, non-toxic, non-carcinogenic, and biodegradable, thereby rendering no risk to the human health and environment. Due to the lower risk advantage of natural colors and the perception of consumers to preferably consume natural products, there is an increasing interest in the discovery and research of new natural colors [2]. One of those microbial sources for potential natural pigments that has generated much interest over the years is phycocyanin, produced by the blue-green cyanobacteria *Spirulina platensis*. Table 4 presented some microbial pigments that are being used or with high potentials to be used as natural food colorants [2].

Table 4. Microbial pigments that are being used or with high potentials to be used as natural food colorants [2].

Sr.No.	Pigment	Colour	Microroganism	Bioactivity	Status	Reference
ALGAE AND MICROALGAE						
1	Astaxanthin	Pink-red	*Haematococcus pluvialis* Microalage	Antioxidantphotoprotectant, Anticancer, Antiinflammatory	IP	Reyes et al. 1996 Terao 1989;Guerinet al 2003; Dufossé, L. et al., 2005
2	β- carotene	Orange	*Dunaliella salina* Microalgae	Anticancer, Antioxidantsuppression of cholesterol synthesis	IP	Kobayashi et al. 1993; Jacobson andWasileski 1994; Fuhrman et al. 1997;Dufossé, L. et al., 2005
3	Lutein	Yellow	*Chlorella and others* Microalgae	Antioxidant	IP	Chen et al., 2016; Cao et al., 2015; Ahmed et al., 2013; Dufossé, L. et al., 2005
4	Phycoerythrin	Red	*Porphyridium cruentum and many other microalgae and cyanobacteria* Algae, Cyanobacteria	Antioxidant, Antitumor activity, Immunoregulatory	DS	Dufossé, L. (2017b); Dufossé 2018; Sonani, R. R. et al., 2014; Dufossé, L. et al., 2005
5	Phycocyanin	Blue	*Arthrospira sp. (formerly Spirulina sp.) and many other microalgae and cyanobacteria* Algae, Cyanobacteria	Antioxidant, Antitumor, Immunoregulatory	IP	Dufossé 2009;2018; Dufossé, L. et al., 2005 Cuellar-Bermudez et al., 2015; Guedes, A. C. et al., 2011
ARCHEA						
6	Canthaxanthin	Orange	*Haloferax alexandrines* Archea	Antioxidant, photoprotectant, Anticancer, Antiinflammatory	NK	Lorquin et al. 1997; Mathews-Roth 1982;Chew et al. 1998; Duffose 2006
BACTERIA						
7	Astaxanthin	Pink-red	*Agrobacterium aurantiacum* Bacteria	Antioxidant Anticancer Antiinflammatory,	RP/IP	Reyes et al. 1996 Yokoyama and Miki, 1995.

Table 4 (continued). Microbial pigments that are being used or with high potentials to be used as natural food colorants [2].

			Paracoccus zeaxanthinifaciens, Sphingobacterium multivorum Bacteria			
CYANOBACTERIA						
19	**Scytonemin**	**Reddish Brown**	***Cyanobacteria* Cyanobacteria**	**Antiinflammatory, Antiproliferative**	**NK**	**Stevenson et al. 2002**
	FUNGI					
20	Ankaflavin	Yellow	*Monascus sp.*	Antitumor, Antiinflammatory	IP	Hsu et al. 2011; Dufossé 2017a
21	Anthraquinones	Red and other hues Known as Arpink red or Natural Red	*Penicillium oxalicum (and many other fungi)*	Antifungal, Virucidal	IP	Andersen et al. 1991; Agarwal et al. 2000; Venil and Lakshmanaperumalsamy 2009
22	Azaphilones	Red	*Talaromyces atroroseus Penicillium purpurogenum*	Antioxidant, Anticancer Antioxidant	DS	Dufossé 2017b,Gao, J.M. et al., 2013; Frisvad, J.C. et al 2013;Venkatachala,M. etal., 2016 Dufossé 2017b;; PatilS. et al., 2015;Padmapriya, C. et al., 2016
23	β- carotene	Yellow-orange	*Blakeslea trispora, Fusarium sporotrichioides, Mucor, circinelloides, Neurospora crassa, Phycomyces, Blakesleeanus*	Anticancer, Antioxidant, suppression of cholesterol synthesis	IP	Dufossé 2017a;Costa et al. 2005;Dufossé 2009; Lopes et al. 2009;Cerdá-Olmedo 2001; Terao 1989
24	Canthaxanthin	Orange, pink	*Monascus spp*	Antioxidant, Anticancer	NK	Mathews-Roth 1982; Chew et al. 1998;Cooney et al. 1966; Dufossé 2009, 2018
25	Cycloprodigiosin	Red	*Pseudoalteromonas denitrificans*	Antiplasmodial, Anticancer	DS	Kim et al. 1999;Yamamoto et al. 1999
			Paracoccus carotinifaciens Bacteria	Antioxidant Anticancer		
8	Canthaxanthin	Orange	*Bradyrhizobium spp. Lactobacillus pluvalis.*	Antioxidant, Anticancer	RP	Lorquin et al. 1997; Mathews-Roth 1982;Chew et al. 1998; Duffose 2006
9	Granadaene	Orange-red	*Streptococcus agalactiae*	Antioxidant, detoxify ROS	DS	George and Nizet 2009;Rosa-Fraile 2006
10	Heptyl prodigiosin	Red	*α-Proteobacteria*	Antiplasmodial	DS	Lazaro et al.2002
11	Prodigiosin	Red	*Serratia marcescens Pseudoalteromonas rubra*	Anticancer, DNA Cleavage, Immunosuppressant	IP	Feher et al. 2008; Deorukhkar et al. 2007;Melvin et al. 2000; Tsuji et al. 1990
12	Phycocyanin	Blue, green	*Pseudomonas Spp.*	Cytotoxicity, Neutrophil apoptosis, Ciliary dysmotility, Proinflammatory	IP	Baron and Rowe 1981
13	Rubrolone	Red	*Streptomyces echinoruber*	Antimicrobial	DS	Iacobucci and Sweeney 1981;Schüep et al. 1978 Dharmaraj. et al, 2009
14	Staphyloxanthin	Golden	*Staphylococcus aureus*	Antioxidant, detoxify ROS	NK	Liu et al. 2005a, 2005b;Clauditz et al. 2006
15	Tryptanthrin	Light-dark Yellow	*Cytophaga/Flexibacteria AM13,1 Strain*	Antioxidant, Anticancer	NK	Wagner-D¨obler et al. 1996
16	Undecylprodigiosin	Red	*Streptomyces sp.*	Antibacterial, Antioxidative, UV-protective, Anticancer	NK	Gerber1975; Stankovic et al. 2012; Liu et al. 2005a, 2005b
17	Violacein	Purple	*Janthinobacterium lividum, Pseudoalteromonas tunicate, Pseudoalteromonas spp. Chromobacterium violaceum*	Antioxidant, detoxify ROS	NK	Duran et al. 2012; Matz et al. 2004; Konzen et al. 2006
18	Zeaxanthin	Yellow	*Staphylococcus aureus, Flavobacterium spp.,*	Photoprotectant, Antioxidant	DS	Hammond and White 1970

Table 4 (continued). Microbial pigments that are being used or with high potentials to be used as natural food colorants [2]

26	Lycopene	Red	*Fusarium Sporotrichioides, Blakeslea trispora*	Antioxidant, Anticancer	RP/DS	Di Mascio et al. 1989; Giovannucci et al. 2002
27	Monascorubramin	Red	*Monascus spp*	Antioxidant, Anticancer	IP	Dufossé, 2018; Blanc et al. 1994
28	Naphtoquinone	Deep blood red	*Cordyceps unilateralis*	Anticancer, Antibacterial, Trypanocidal	RP	Prathumpai et al. 2006; Nematollahi et al. 2012; Venturaet al. 2009; Dufossé 2017b
29	Riboflavin	Yellow	*Ashbya gossypi*	Anticancer, Antioxidant, protection against cardiovascular diseases, in vision	IP	Unagul et al. 2005; Hong et al. 2008;Powers 2003;Dufossé 2018
30	Rubropunctatin	Orange	*Monascus spp*	Anticancer	IP	Blanc et al. 1994; Zheng et al. 2010
31	Xanthomonadin	Yellow	*Xanthomonas oryzae*	protection against photo damage	NK	Raja gopal et al. 1997,Dufossé 2009
YEAST						
32	Astaxanthin	Pink-red	*Xanthophyllomyces dendrorhous formerly Phaffia rhodozyma*	Antioxidant, photoprotectant, Anticancer, Antiinflammatory	DS	Ramirez et al. 2000;Florencio et al. 1998; Flores-Cotera and Sanchez 2001
33	Melanin	Black	*Saccharomyces, Neoformans*	Antimicrobial, Antibiofilm and antioxidant	NK	Vinarov et al. 2003
34	Torularhodin	Orange-red	*Rhodotorula spp.*	Antioxidant, Antimicrobial	DS	Kot et al., 2016; Zoz et al., 2015; Yadav, K. S. et al., 2017

*industrial status adopted from Dufossé (2006; 2017; 2018)

DS Development stage; *IP* Industrial production; *RP* Research project; NK- Not Known

1.1 Motivation

The ban in trading and manufacturing of synthetic color products in international markets by the European Union and Japanese governments [12], [13], has led to a continuous demand for natural food pigments in the global market over the last two decades, from 2400 million tons in 2000, 3000 million tons in 2005, to 8000 million tons in 2010 and potential for further increase [13]. These numbers indicate a viable opportunity and motivation for development of natural pigment production business.

Pigment additives in food play a significant role in the buying decision process of the prospective consumers. It is an important attribute of food and several studies suggest that color of food or beverage do play profound roles in flavor and taste perception [13] and this knowledge has generated strong commercial interest in the development of new safer technologies to modulate the properties of natural food pigments that leverage their introduction in the food market.

Several vegetable matrices are already used to obtain a range of colorants. For example, annatto, an orange-red condiment and food coloring derived from the seeds of the achiote tree (*Bixa orellana*) native to tropical regions from Mexico to Brazil, is used to extract colors ranging from yellow to red. This range of yellow-orange colors can also be obtained from curcuminoids present in turmeric, a flowering plant (*Curcuma Longa)* native in Indian subcontinent and Southeast Asia. Jabuticaba (*Myrciaria cauliflora*), a

native tree from Brazil, Argentina, Paraguay, Peru and Bolivia, is known for its purplish-black, white-pulped fruits which grow directly on the trunk, which are used to prepare extracts rich in anthocyanins, phenolic compounds responsible for blue, purple, and red colors [10]. However, one of the factors that severely limits the use of natural colorants is their stability. In general, natural additives are less stable than synthetic ones. This instability has encouraged researchers from around the world to search for new technologies applicable to the food and beverage market to obtain new non-toxic colorants that can be used in food or to develop new ways to stabilize the natural colorants in use [2, 14].

From this perspective, the cyanobacteria *Spirulina platensis*, a blue-green alga due to the presence of phycocyanin, a pigment-protein complex, is an alternative for obtaining a natural blue pigment. Depending on the purity, the market price for phycocyanin is approximately USD$500 per kg, for food-grade, to USD$100,000 per kg, for analytical grade [15].

The blue pigments from *Spirulina platensis* have been shown to have promising applications in food and non-food products [16]. The food industry, for example, utilizes blue coloring in several products, especially baby foods, and to obtain other colors, such as purple and violet. It has also been used as a colorant in healthy drinks, beverages, confectionary and cosmetics. Furthermore, phycocyanin has proven to possess therapeutic properties including antioxidant, anti-inflammatory and anti-cancer activities. Small quantities are also used as biochemical tracers in immunoassays due to its fluorescent properties [14, 16].

1.2 Phycocyanin Extraction

Different methods have been used to extract PC from *Spirulina platensis* biomass with different level of yields. Also, several factors such as solvent type, solvent to biomass ratio, duration of extraction and temperature are responsible for the release of PC from the cell wall of the biomass and the consequent extraction yield [17]. Some of these methods Some of these several methods of extraction of phycocyanin are categorized into two, the conventional and the green methods [18]. The conventional methods include (i.) maceration (soaking in organic solvents) and (ii.) Freezing-Thawing. This category of the extraction method is basically applied to dry biomass, followed by thermal extraction with organic or aqueous solvent depending on the polarity of the component to be extracted. Slow operation, high consumption of solvents and moderate yield and efficiency constitute setbacks for this class of method.

On the other hand, the green method includes (i.) Pressurized Liquid Extraction, (ii.) Microwave Extraction, (iii.) Ultrasound Extraction, (iv.) High-pressure Homogenization, etc. The advantages of these category of phycocyanin extraction methods are that they show better results for extraction process, they do not use organic solvents or European Union prohibited [21] chemical harmful to the environment and the rate of operation is faster than the conventional methods [18].

The choice of extraction methods and solvents used in this study was motivated by the already published result of Ruiz-Dominguez *et al.* [18] which proved that water is a good extraction solvent, combined with maceration and the microwave extraction methods.

However, several publications [19]], [31], [32],] have cited the use of Deep Eutectic Solvents (DES) in particular, Natural Deep Eutectic Solvents (NADES) as promising solvents for the extraction of bioactive ingredients from biomass. This class of new solvents are characterized by being produced from natural resources, eco-friendly, biodegradable, with low or negligible toxicity, low cost, and of simple preparation [19]. These properties of the DES and NADES qualify them to meet environmental, technological, and economical demands of a green chemistry [19] and in this case provide good benefits for their use in the food and pharmaceutical industries, which must meet tight regulations.

1.3 Objective of the Study

The objective of the work was (i) to carry out literature search on PC, focusing on the traditional extraction methods used to extract this pigment from natural resources and also the experimental color stability conditions for PC; (ii) to select and prepare DES, in particular NADES, with different types of primary metabolites that are suitable for the simultaneous extraction and stabilization of PC; (iii) to determine solubility and stability PC in prepared DES or NADES and understand the solvent effects.

1.4 Limitations of the Study

The limitations experienced on this work spring from the outbreak of corona virus, alias covid-19. This pandemic hampered the progress of laboratory works that needed to be carried out for the completion of this work.

CHAPTER 2

2. PHCOCYANIN EXTRACTED FROM SPIRULINA PLATENSIS

2.1 Spirulina platensis

Spirulina platensis is blue-green microalga considered important due to its economical, ecological, and nutritional benefits [[20]. This nitrogen-fixing cyanobacterium plays an important role in the production of valuable phycobiliproteins pigments, like phycocyanin, since they are capable to grow in nitrogen-free media, thus decreasing contamination risks and production costs [21]. One of *Spirulina platensis* benefits is its ability to produce large quantity of brilliantly colored phycobiliproteins that are water-soluble proteins, bearing covalently attached open chain tetrapyrroles, known as phycobilins [22]. There are two classes of photosynthetic pigments in cyanobacteria: the phycobiliproteins, that contains the water-soluble proteins, and the carotenoids and chlorophyll, that contain the insoluble in water and small molecular weight proteins [23]. Based on the visible maximum absorption wavelength properties, phycobiliproteins can be categorized into four spectroscopic classes as phycoerythrocyanin (λ_{Amax} = 575 nm), phycoerythrin (Red) (λ_{Amax} = 565-575 nm), phycocyanin (Blue), (λ_{Amax} = 615-640 nm) and allophycocyanin (Cyan), (λ_{Amax} = 650-655 nm) [22], [23]. However, in the Spirulina algae, phycocyanin provides its characteristic green–blue color [23].

2.2 Phycocyanin

Phycocyanin is a protein composed of two kinds of polypeptide subunits – α (20.5 kDa) and β (23.5 kDa), polymerized to form $\alpha_3\beta_3$. There are nine Phycocyanobilins moieties in phycocyanin, one in each α-chain and two in each β-chain, that act as chromophores. A scheme of phycocyanin protein and the chemical structure of the chromophore groups, open chain tetrapyrroles, is depicted in Figure 1 [23]. These tetrapyrrole structures are responsible for the typical blue color of phycocyanin, with absorption maximum at 614 nm, while the protein part confers the stability with respect to pH and temperature.

Phycocyanin is covalently linked to the phycobiliproteins by thioether linkage [23]. Phycobiliproteins arrange in Phycobilisomes, that are assembled in a fashion that enables highly efficient transfer of energy unidirectionally to the center reaction, Figure 2.

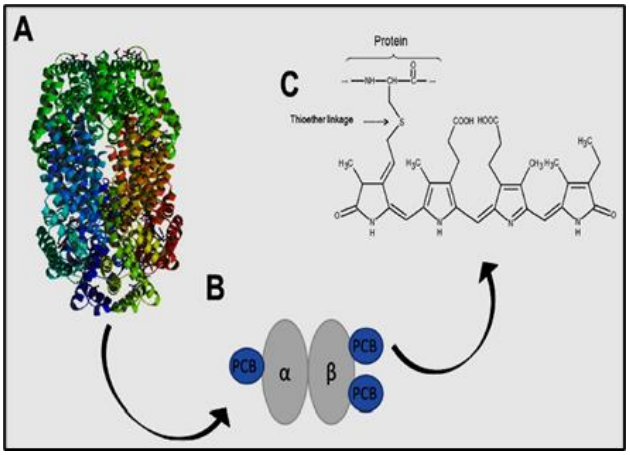


Figure 1. (A) Crystal structure of Phycocyanin from cyanobacterium S. platensis. (B) Schematic representation of PC assembly composing of two protein subunits, α and β chains, forming $\alpha_3\beta_3$ complex. (C) Chemical structure of PCB, the chromophore group responsible for blue color of PC [23].

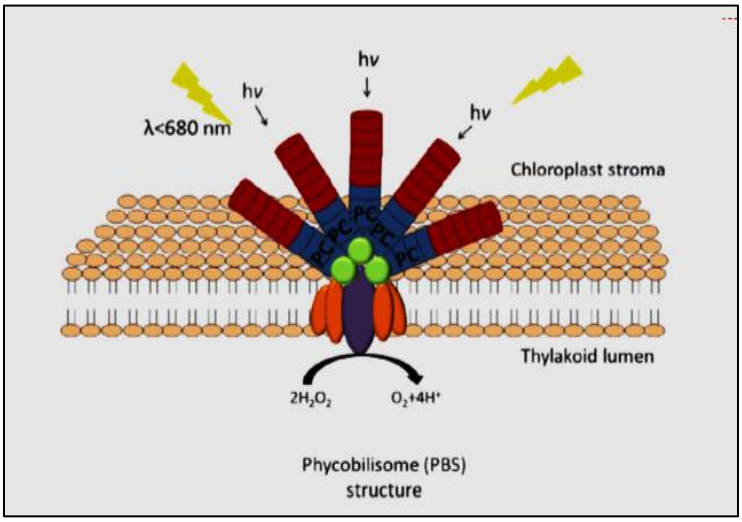


Figure 2: Schematic structure of phycobilisomes, major complex light collection in cyanobacteria and red algae [23].

The major limitation for the extensive application of PC in the food and feed industry is its high sensitivity to heat, which leads to high degradation in protein content and a drastic blue color reduction [25]. In Figure 3, above 40ºC there is a considerable reduction in the relative concentration of PC in solution, for all the pH considered in the study [16].

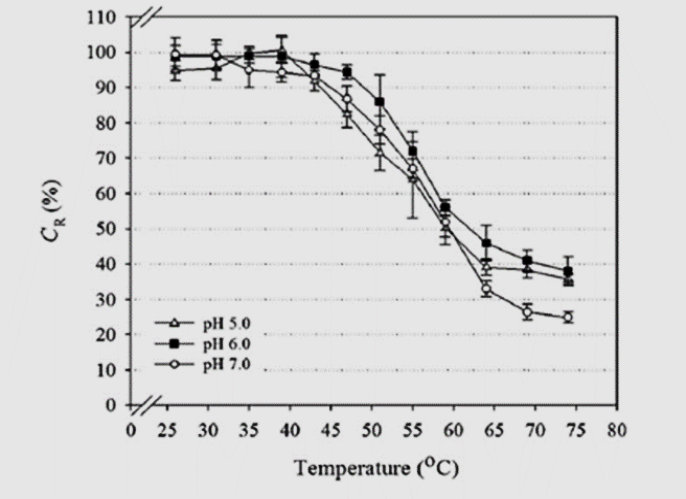


Figure 3: Relative concentration (C_R) of phycocyanin after 30 min incubation at each temperature [16]

Stability of phycocyanin is pH and temperature dependent. Phycocyanin is stable over a pH range between 5 and 7.5 at 25°C. However, it has been demonstrated that at lower temperatures (4°C), it remains stable over longer periods. Moreover, PC withstands high temperatures (up to 72°C for short exposures) without being damaged.

CHAPTER 3

DETERMINATION OF PC EXTRACT PROPERTIES

The extraction of proteins from natural sources can be achieved using several extraction technologies, to obtain protein extracts. The properties of those extracts highly depend on purity of the desired protein in the extract, or in other words, the presence to some stabilizing agents that help the keeping or even enhancing the properties of the protein.

3.1 Concentration of PC

According to Chaiklahan *et. Al.* [16], the total concentration of PC is calculated considering the total amount of PC, that absorb light at 620 nm, and allophycocyanin, that absorb light at 652 nm. Bennette and Bogorad [18] used the following equations 1, 2 and 3 to calculate the total concentration of PC.

$$C_{PC}\left(\frac{mg}{mL}\right) = \frac{A_{620} - 0.474A_{652}}{5.34} \qquad Equation\ (1)$$

$$C_{APC}\left(\frac{mg}{mL}\right) = \frac{A_{652} - 0.208\,A_{620}}{5.09} \qquad Equation\ (2)$$

$$C_{PC}\left(\frac{mg}{g}\right) = \frac{\left(C_{PC}\left(\frac{mg}{mL}\right)\right) \times V}{dw} \qquad Equation\ (3)$$

where C_{PC} is the concentration of phycocyanin in mg/mL, C_{APC} the concentration of allophycocyanin in mg/mL, A_{620} and A_{652} represent the absorbance at 620 and 652 nm, V is the volume of solvent used in mL and dw represents the dry biomass used in g.

3.2 Purity of PC

The purity of extracted PC was calculated by the ratio of the absorbances at 620 nm (A_{620}) and 280 nm (A_{280}). PC is considered food grade when A_{620}/A_{280} is ≤ 0.7, reagent grade when A_{620}/A_{280} is between 0.7 and 3.9 and analytical grade when A_{620}/A_{280} is ≥ 4.0 [26], [27], [23]].

$$Purity\ (P) = \frac{A_{620}}{A_{280}} \qquad Equation\ (4)$$

3.3 Stability of PC

The use of extracted PC is limited in the food industry due to the precipitation of the protein part of the PC, which causes major loss of the bright blue color, [[28]]. This unstable nature of PC is very prevalent at certain experimental conditions and thus dependent on temperature, pH, light intensity, and extraction solvents. For example, the best color clarity and solubility of PC occurs at 47°C and pH = 5.5 – 6.0, while PC loses its color at temperature between 50 – 60°C and is irreversibly denatured at 65°C [28].

Several studies have been made to select chemical compounds that can act as preservatives (carbohydrates, salts etc.) and the experimental optimal conditions to achieve protection of the PC chromophore group in natural state in the extract. This knowledge is crucial to extend limits of PC application in food industry. These effects are categorized into two subheadings, viz: experimental conditions and addition of preservatives. Two publications were found and will be subsequently discussed to better understand the stability of PC.

The relative concentration of PC (C_R %) is the remaining concentration of PC as a percentage of the initial concentration C_o is calculated as in equation 5 [16]:

$$C_R\,(\%) = \frac{C}{C_o} \times 100 \qquad Equation\ (5)$$

3.3.1 Two relevant publications on the stability of PC

3.3.1.1 Article 1: Thermal degradation kinetics of the PC from *Spirulina platensis* [29]*]*

Phycocyanin is the main pigment produced by the cyanobacterium *S. platensis* and may reach 20% in dry weight of the cell protein and it is an important pigment in phycobiliprotein which is not only used as a natural nutritious and coloring pigment for foods and cosmetics but also as a potential therapeutic agent in the treatment of oxidative diseases and as a fluorescent marker in biomedical research. The current tendency to use natural pigments has turned phycocyanin into an attractive bioproduct. In Europe, the search for natural dyes is growing since the artificial dyes are generally considered to be toxic or somehow dangerous.

Due to the possible denaturation of the protein fraction of the phycocyanin and consequent loss of color, it is of interest to use stabilizing agents to maximize the shelf life of the protein solutions of greater biotechnical and pharmaceutical interest.

This article describes the study of the thermal degradation kinetics and stability of the aqueous extract of PC from *Spirulina platensis,* in the temperature range from 50 to 65 ºC at pH values of 5, 6 and 7, in the presence of a diverse concentration of a stabilizing agent, sorbitol.

3.3.1.2 Culture conditions of Spirulina platensis

The *Spirulina platensis* used was cultivated in a 450 L open outdoor photo-bioreactor under uncontrolled conditions with 20% Zarrouk synthetic medium and initial biomass concentration of 0.3 g/dm^3 in south Brazil [29]. Samples were drawn every 24 h and absorbance measurements were carried out at 670 nm in a spectrophotometer (FEMTO Espectrofotometro 700 Plus) to determine its concentration. The biomass was recovered by filtration, pressing and extrusion, dried at 50 ºC for 6 h, frozen at -18 ºC, ground in a ball mill and sieved with 150 mesh sieves.

3.3.1.3 Extraction of PC

PC was extracted according to Silveira et. al [[30]. 2 g of dried biomass was mixed with 50 ml of aqueous solution of sorbitol at concentrations of 10, 20, 30, 40 and 50 (w/w), and 0.01% (p/v) of sodium azide in a rotary shaker at 30 ºC. The samples were collected at 24, 48 and 72 h. After extraction, the suspension was centrifuged, vacuum filtered, and the supernatant collected for absorbance measurement at 615 and 652 nm. The concentration of PC was determined according to equation (1), but the absorbance was read not at 620 nm but at 615 nm, in agreement with the obtained PC spectra. The relative concentration of PC was calculated using equation (5) above.

3.3.1.4 Determination of the PC degradation kinetics with addition of stabilizing agent

The study of the effects of stabilizing agents on the PC extract is crucial to prevent the loss of color and degradation of the protein fraction of the PC, since this is the fraction that determines the color.

3.3.1.5 Materials and Methods

Aqueous solution of sorbitol was added to the extract at concentrations of 10, 20, 30, 40 and 50 (w/w)%, incubated at 62 ºC and triplicate samples removed periodically, for up to 30 min. The degradation rate constant and the half-life of the PC extract were calculated by equations (7) and (8), for each sorbitol concentration added to the crude extract, under the established conditions.

The first order degradation kinetic model was calculated by equation 6.

$$\frac{dC_F}{dt} = -kdC \qquad Equation\ (6)$$

And the half-life ($t_{1/2}$) in second (s)

$$t_{1/2} = \frac{ln\,2}{K_d} \qquad Equation\ (7)$$

where CF is the PC concentration (mg/ml), t is the time (s) and K_d is a degradation rate constant (s^{-1}).

The Arrhenius equation which relates temperature, speed of elementary reactions and allows for the determination of the activation energy and the frequency factor for protein degradation reactions, was used to calculate K_d

$$K_d = Ae^{-E_d/RT} \qquad Equation\ (8)$$

where K_d is a degradation rate constant (s^{-1}), *A* is the frequency factor (s^{-1}), E_d is the activation energy degradation reaction (cal.g.mol^{-1}), *T* is the temperature (K) and *R* is the gas constant (cal. g.mol^{-1} K^{-1}).

3.3.1.6 Results and Discussions

The addition of different concentrations of aqueous solutions of sorbitol in the range between 10 and 50 (w/w)%, to crude PC, yield a first order PC degradation kinetics model, with high correlation factors between 0.92 and 0.97 in the kinetic constant determination. Also, it was verified that the PC was denatured more quickly at higher temperature, since the half-life (the time taken for the initial phycocyanin concentration to be reduced by half) values decreases as the working temperature increases. Using Equation (6) and (7), the K_d and $t_{1/2}$ values were obtained for the different *T* and pH values, as presented in Table 1.

Table 1. Half-life and k_d of the PC at 62 ºC for sorbitol concentrations added to the aqueous extract [29]

	pH 5		
Sorbitol concentration (%, w/w)	Kd (s^{-1})	r^2	$t_{1/2}$ (s)
10	3.9×10^{-4}	0.94	1,732
20	2.3×10^{-4}	0.88	3,465
30	2.9×10^{-4}	0.95	2,310
40	2.2×10^{-4}	0.93	3,465
50	6.0×10^{-5}	0.94	11,552
	pH 6		
Sorbitol concentration (%, w/w)	Kd (s^{-1})	r^2	$t^{1/2}$ (s)
10	7.7×10^{-4}	0.96	866
20	5.0×10^{-4}	0.95	1,386
30	1.2×10^{-4}	0.98	6,931
40	1.1×10^{-4}	0.98	6,931
50	6.0×10^{-5}	0.95	11,552
	pH 7		
Sorbitol concentration (%, w/w)	Kd (s^{-1})	r^2	$t_{1/2}$ (s)
10	6.7×10^{-4}	0.92	990
20	4.0×10^{-4}	0.90	1,733
30	3.0×10^{-4}	0.92	2,310
40	2.1×10^{-4}	0.94	3,466
50	6.0×10^{-5}	0.92	11,552

From Table 1, it is verified that the half-life of PC increases with increase in the concentration of sorbitol, hence, showing sorbitol to be a good preservative agent for PC at pH = 5, 6 and 7. It was also observed that between 50 and 65 ^{o}C the aqueous crude phycocyanin extract was less stable at pH 7. In addition to the temperature dependence of phycocyanin degradation and consequently of color loss, a first order kinetics law was obtained for protein heat degradation, at each pH value. Using Eq. (8), the values for *A* and E_d were determined for each pH value, as shown in Table 2.

Table 2: Frequency factor (A), activation energy (Ed) and the respective correlation coefficients (r^2) to each pH values studied, 5, 6 and 7 [29]

pH	$A\ (s^{-1})$	Ed (kcal gmol^{-1})	r^2
5	1.86×10^{54}	87.36	0.94
6	2.45×10^{86}	135.57	0.96
7	2.03×10^{70}	111.14	0.90

Figure 4 shows evolution of the relative concentration of PC, at a fixed period of (30 min) for the 3 different pH analyzed in this work. An increment in the sorbitol concentration increases the relative concentration of PC for all the pH conditions studied.

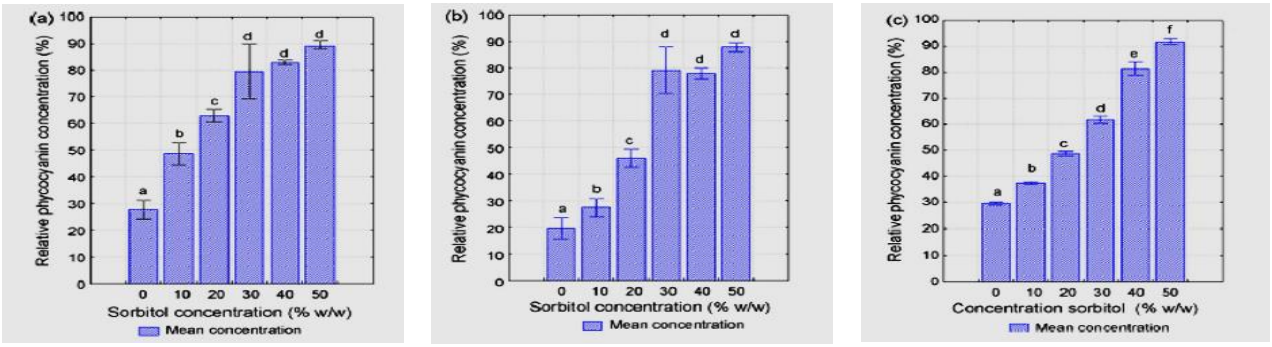


Figure 4 Analysis of the effect of the different sorbitol concentration (w/w)% on the relative PC concentration %) of the extracts at a) pH = 5, b) pH=6, c) pH=7, after 30 min immersed in a water bath [29].

Figure 5 shows the evolution of PC relative concentration at a different concentration of sorbitol and at different experiment times, from 30 s to 1800 s, for the 3 pH conditions studied in this work. Again, it can be

observed that sorbitol causes an increment in the relative PC concentration at all the times analyzed as the treatment time increased, higher percentages of added sorbitol resulted in larger relative PC concentrations.

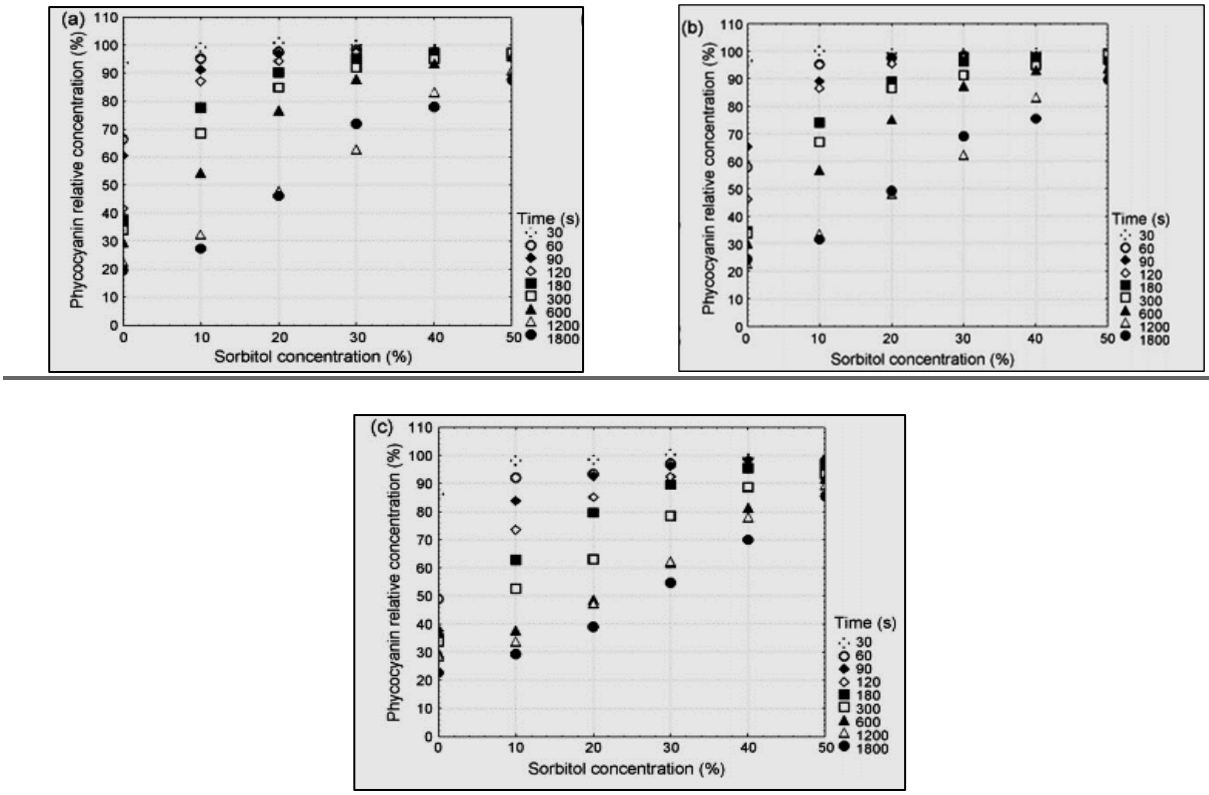


Figure 5. Relative PC concentration (w/w)% for treatment times of 30–2400 s with sorbitol concentrations between 10 and 50% (w/w), for aqueous crude extract at a) pH = 5, b) pH = 6 and c) pH = 7 [29].

3.3.1.7 Conclusion

The kinetic model for the thermal degradation of the aqueous crude PC extract was validated as being of the first order according to the correlation obtained. At temperatures between 50 and 55 ºC, the aqueous crude phycocyanin extract was more stable at pH = 6 and between 57 and 65 ºC at pH = 5. It was shown to be least stable between 50 and 65 ºC at pH = 7.

Sorbitol was found to be an efficient stabilizer due to its ability to delay degradation process of PC molecules at concentration starting from 10% (w/w). This can be better appreciated through the analysis of the half-life of PC solution with and without sorbitol at pH = 5.0 and 65 ºC, which were of 29 min and 5 min, respectively.

Addition of 10 to 50% (w/w) sorbitol, at 62 ºC, increased the half-life of the aqueous PC crude extracts from 277 s to between 1732 s and 11,552 s at pH 5, from 89 s to between 866 and 11,552 s at pH = 6 and to between 990 and 11,552 s at pH = 7, showing that PC de-colorization is probably related to protein-chain structure. Also, the half-life was found to be proportionally related with the sorbitol concentration (%, w/w) added.

Furthermore, at the end of 30 min of heat treatment, the mean relative concentration of PC was maintained at 80% and 79% with addition of 30% (w/w) sorbitol to the PC extract at pH = 5.0 and pH = 6.0, respectively. At pH = 7.0 and with addition of 50% (w/w) sorbitol, the value of C_R of PC was maintained at 85% after 30 min of heat treatment.

3.3.2.1 Article 2: Effect of preservatives for food grade PC from Spirulina Platensis [31]

Food additives have been used by mankind for centuries, for example salt, sugar and vinegar were among the first to be used as preservatives in food. Preservatives are required to ensure that manufactured foods or additives remain safe and unspoiled for a long time (shelf life). The present study describes the effect of selected food grade preservatives for PC in order to develop a stable product with long shelf life.

Several methods have been developed for the separation and purification of PC from cyanobacteria. Nevertheless, the purity and recovery are relatively low because of its highly sensitive to light, oxygen, and moisture, and consequently the search for efficient preservatives is mandatory. Sodium azide (NaN_3) and dithiothreitol (DTT) are commonly used as preservatives of PC for analytical purpose, but they are toxic and cannot be used in food applications. So, for the development of food grade PC only edible preservatives, with specific properties can be used. It is, therefore, desired to develop a simple, but more efficient method for the separation, purification, and stabilization of the food grade PC from cyanobacteria.

3.3.2.2 Efficacy of preservatives

Preservatives are essential to ensure that the quality of manufactured foods or additives are preserved from degradation or de-colorization that could occur during long time storage and exposure to other environmental factors like sunlight, heat etc. This paper studied the effect of selected food grade preservatives, such as sucrose, calcium chloride and citric acid, calcium chloride + sucrose, calcium chloride + sucrose to develop a stable PC product with long shelf life. Furthermore, the denaturation of PC with urea as a denaturant and thermal unfolding studies through differential scanning calorimetry (DSC) was carried out to select a stabilizing agent having Hofmeister series behavior acting on hydrophobic interactions.

3.3.2.3 Materials and Methods

Sucrose, calcium chloride, citric acid, sodium phosphate buffer (0.1 M, pH = 7.0), Ammonium sulphate (25% and 50%), and urea used were of analytical grade available from commercial sources (source not provided in the article) and Spirulina powder (food grade, from CSMCRI). CARY 500 Scan UV-vis, NIR spectrophotometer, Mettler Toledo DSC822 instrument with stare software.

3.3.2.4 Extraction of C-phycocyanin

One gram of shade-dried Spirulina powder was suspended in 25 ml of sodium phosphate buffer (0.1 M, pH = 7.0) and was freeze at -20 ºC and thawing at room temperature 25 ± 5 ºC in the dark was done repeatedly. The mixture was subsequently centrifuged at 10,000 x g for 30 min at 0 ± 5 ºC and a phycobiliprotein-containing clear supernatant was collected and precipitated by ammonium sulphate (25% and 50%), followed by dialysis against distilled water at 0 ± 5 ºC for 48 h in dark, by intermittent changing of distilled water at regular intervals and freeze-dried. The complete process for production of stable C-phycocyanin is shown in Figure 6.

3.3.2.5 Stability of C-Phycocyanin

For checking efficacy of the chosen preservatives, sucrose (0.1 g), calcium chloride (0.1 g), citric acid (0.1 g), calcium chloride (0.1 g) + sucrose (0.1 g) and calcium chloride (0.05 g) + sucrose (0.05 g) was added in 25 ml PC solution (10 mg of freeze-dried PC was dissolved in 25 ml of sodium phosphate buffer (0.1 M, pH 7.0)) separately and kept at 0 ± 5 ºC and 35 ± 5 ºC for 15 days.

3.3.2.6 Estimation of PC

The absorbance of PC and preservative mixtures were measured every alternate day for 15 days on a CARY 500 UV-vis, NIR spectrophotometer at wavelength 250-700 nm. Concentration and purity of extracted PC were evaluated through equations (3).

3.3.2.7 Purity assay

The assay to assess the purity of the extracted PC was done through the following steps in figure 6, and by applying equation (4) to PC absorbance at 620 nm and 280 nm measured using a UV-vis spectrophotometer.

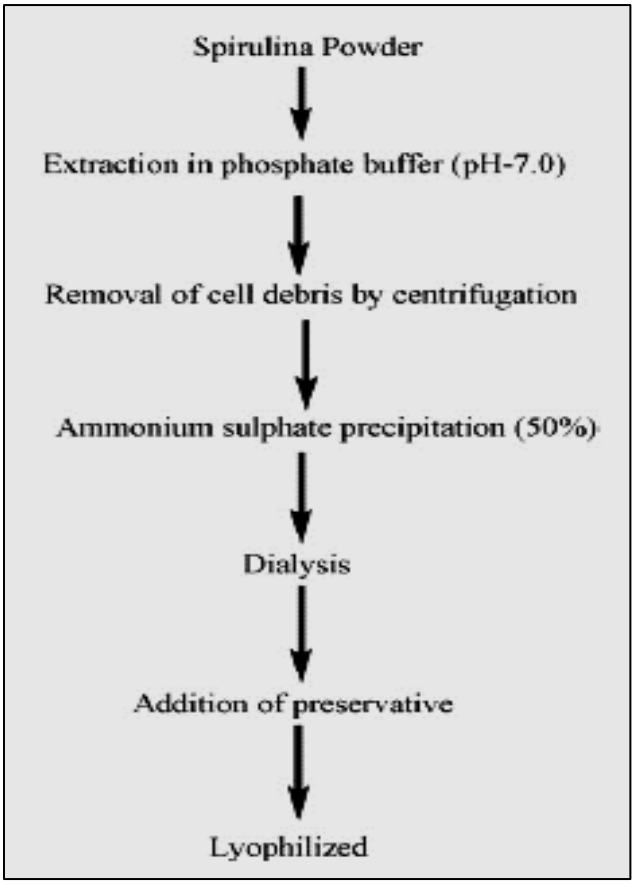


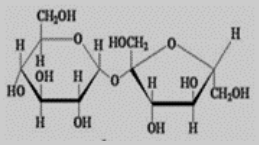
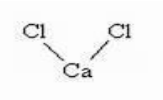
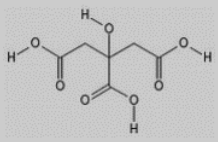
Figure 6: Flow chart to produce stable food grade PC from Spirulina platensis [31]

3.3.2.8 Stability of PC Extract

The stability of aqueous PC extract was evaluated by adding the three different selected preservatives, sucrose, calcium chloride, citric acid, and the combination of sucrose and calcium chloride. Two different experimental conditions were tested: 1) at 0 ± 5 ºC in the refrigerator for 15 days; 2) at room temperature 35 ± 5 ºC for 15 days.

These preservatives were separately added to 25 ml PC solution (10 mg of freeze-dried PC were dissolved in 25 mL of sodium phosphate buffer (0.1 M, pH 7.0)) according to the two experimental conditions described above. The chemical structure of the preservatives is shown in Table 3.

Table 3: Food grade preservatives used in this work to stabilize PC.

S/N	Preservatives and Amount (g)	Chemical formula	Chemical structure
a.	Sucrose (0.1g)	$C_{12}H_{22}O_{11}$	
b.	Calcium chloride (0.1g)	$CaCl_2$	
c.	Citric acid (0.1g)	$C_6H_8O_7$	
d.	Calcium chloride (0.1g) + sucrose (0.1g)		
e.	Calcium chloride (0.05g) + sucrose (0.05g)		

3.3.2.9 Visible absorption spectrophotometry of the denatured C-PC

The absorption spectra of PC were measured in the range 450–700 nm using a CARY 500 Scan UV–vis, NIR spectrophotometer at room temperature as a function of urea concentration (0–10 M). For the spectral measurement PC (0.2 mg/ml) samples were prepared in 0.1 M sodium phosphate buffer (pH = 6.0) with addition of appropriate urea concentration in the range of 0–10 M. With the addition of urea, the absorbance at 620 nm decreases as time increases and then, levels off. To ensure that the denaturation process is completed, the mixed C-PC–urea solutions were kept overnight at 4 ºC before spectral measurements.

3.3.2.10 Differential scanning calorimetry

Thermal unfolding of C-PC was determined using a Mettler Toledo DSC822 instrument with Star software. The calorimeter had one sample cell and one reference cell. C-PC samples (40 ml, 10 mg/ml) in the absence and presence of an appropriate urea concentration (0–4 M) were scanned from 20 ºC to 80 ºC with a heating rate of 40 ºC/h. For control measurements, buffer solution with and without urea were used.

3.3.2.11 Results and Discussions

The purity of the PC determined by taking the ratio of the spectrophotometer absorbance measured at 620 nm and 280 nm (A620 nm/A280 nm) was greater than 0.7 (Purity = 2.25), signifying that the PC was of reagent grade since it is between 0.7 and 3.9.

In Table 4. the PC preservative ability of the selected compounds is shown. Comparing the effect of calcium chloride to that of citric acid at 35 ± 5 ºC after 15 days it can be concluded that calcium chloride is a poor stabilizer for PC at high temperature. Also, about 16% and 10% PC contents remaining (i.e., about 84% and 90% PC content loss) with sucrose and calcium chloride preservatives, respectively. Citric acid was probably able to maintain the stability of PC in aqueous solution due to its chelating ability, because is a triacid, and is also able to reduce pH value by 1.5 units [18] with less discoloration after 45 days.

Table 4: Effect of preservatives or stabilizers on PC percentage at regular time intervals [31]].

	1st day	3rd day	5th day	7th day	9th day	11th day	13th day	15th day	45th day
Control 0 ± 5 °C	100	98.92	98.65	98.24	97.75	97.73	96.27	93.29	86.28
Control 35 ± 5 °C	100	88.54	69.97	47.55	31.38	21.11	12.46	5.09	2.55
Calcium chloride (.1 g) 0 ± 5 °C	100	99.67	98.9	98.8	97.01	95.71	92.88	92.3	88.28
Calcium chloride (.1 g) 35 ± 5 °C	100	94	84.88	67.46	51.82	35.79	15.17	9.02	3.29
Sucrose (0.1 g) 0 ± 5 °C	100	95.69	94.35	93.54	91.24	91.03	90.77	90.57	87.88
Sucrose (0.1 g) 35 ± 5 °C	100	99.32	96.21	88.67	77.95	46.6	26.26	16.47	1.07
Citric acid 0 ± 5 °C	100	99.64	99.34	97.5	93.97	93.95	93.94	91.42	87.61
Citric acid 35 ± 5 °C	100	95.39	94.27	90.04	89.78	88.15	87.95	84.24	67.90
$CaCl_2$ and sucrose (.1) 0 ± 5 °C	100	97.85	97.22	96.84	95.19	93.81	93.73	91.86	89.83
$CaCl_2$ and sucrose (.1) 35 ± 5 °C	100	97.37	90.08	89.14	79.11	57.34	33.77	22.88	3.47
$CaCl_2$ and sucrose (.05) 0 ± 5 °C	100	94.79	93.82	92.27	92.01	90.7	89.69	88.55	84.43
$CaCl_2$ and sucrose (.05) 35 ± 5 °C	100	97.25	88.83	81.01	61.49	43.73	26.23	18.58	7.30

In Figure 8, the experimental results in terms of PC remaining concentration during the 15 days of assays are presented. The loss of PC content in aqueous solution (i.e., buffer of pH = 7.0) at 0 ± 5 ºC is less than that of 35 ± 5 ºC which proved that high temperature affects the stability of PC.

Previous studies show that PC is sensitive towards light, pH, temperature, and oxygen, which together might lead to up to 90% loss of PC content and discoloration [20]. PC is insoluble in acidic solutions (pH = 3.0) but highly soluble at pH = 7.0 accounting for its efficient extraction in buffer of pH = 7.0.

Through the analysis of the UV-vis spectra in Figure 9, it could be concluded that all the assays carried out at 0 ± 5 ºC are much more stable than those carried out at 35 ± 5 ºC, except for the assay in which citric acid (4

mg/ml) was added, which show good PC stability at both temperatures. Thus, it can be concluded that citric acid was found to be a more efficient preservative at 35 ± 5 ºC than both sucrose and calcium chloride.

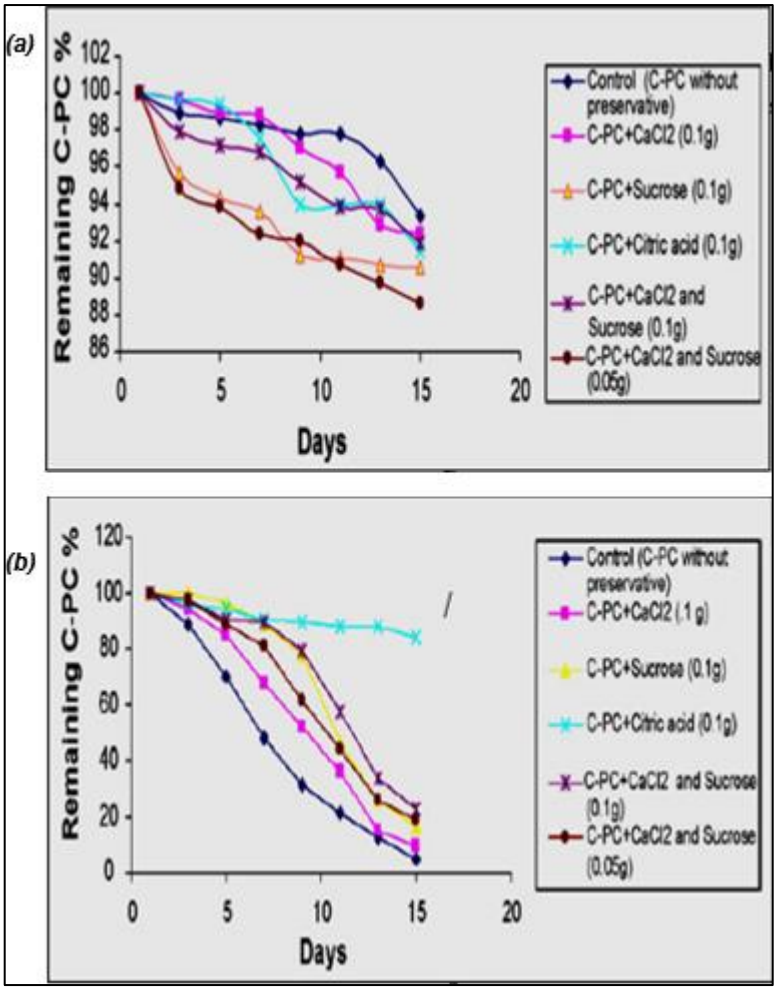


Figure 8. Effect of preservatives on PC concentration (% remaining) througout 15 days at a) 0 ± 5 ºC and b) 35 ± 5 ºC [31]

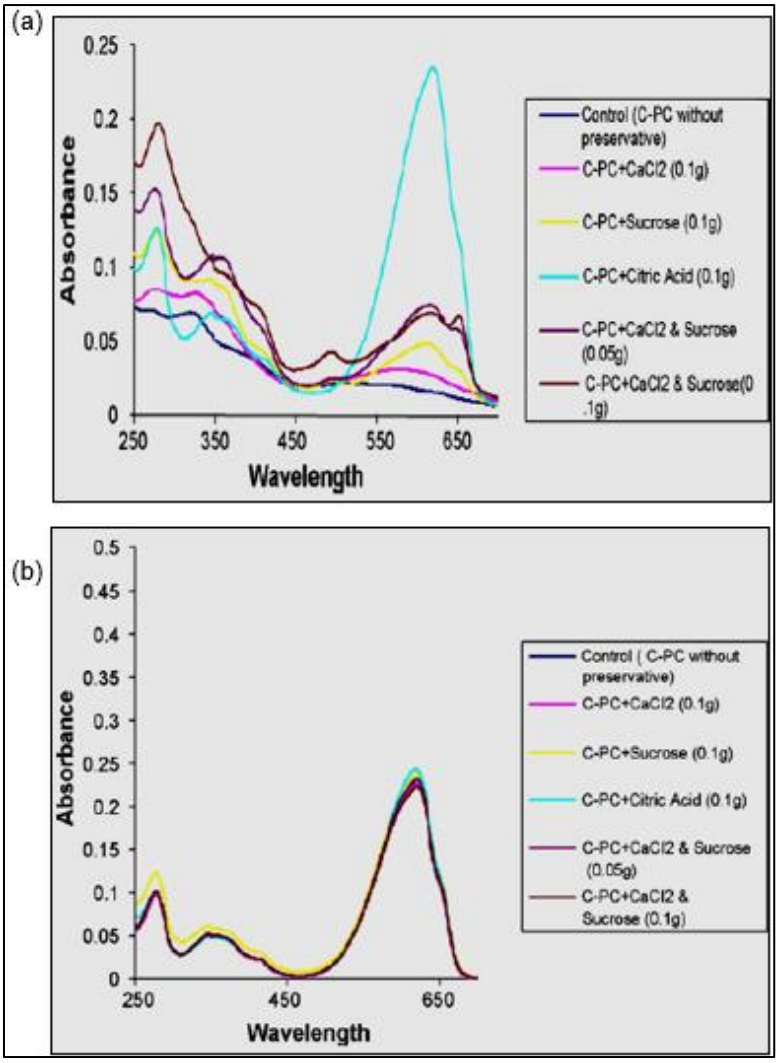


Figure 9. Effect of preservatives on spectra of C-PC after 15 days at a) 0 ± 5 ºC and b) 35 ± 5ºC. [31]

3.3.2.13 Conclusion

It can be concluded that citric acid (4 mg/ml) has the most effective preservative capacity for maintaining the stability of PC in aqueous phase at 35 ± 5ºC even for 45 days with minimum loss of PC content and improvement on its shelf life. Further studies should be carried out since citric acid has the potential of acting as stabilizer of other phycobiliproteins.

CHAPTER 4

NOVEL SOLVENTS

4.1 Introduction

In food, pharmaceutical, cosmetic, and chemical industries, organic solvents are used for extracting, purifying, separation and administration of flavors, fragrances, medicines, and dyes from plants [32]. The goals of undertaking extraction process could be to extract and isolate a single compound or group of compounds present in a plant for metabolomic analysis and industrial production.

Each of these goals requires different solvent characteristics and since a single solvent possessing all extraction qualities does not exist, solvents used in these processes are different and their properties need to be tailored to suit each compound extraction and purification purposes, since metabolites also have interactions with other cellular components with different polarities, stabilities, and boiling points [32]

These solvents adjustable properties include high penetration ability, broad or specific solubilizing capacity, polarity, and cell wall break-down ability. Solvents, such as alcohols, chloroform, ethyl acetate, etc. are generally applied for this purpose [32]. However, organic solvents have been found not suitable in many applications due to its toxic nature, flammability, explosive, and poor biodegradability. The need to extract natural pigment safe for consumption results lead to the application of green natural solvents, which are not inherently toxic to human and are environment friendly. The development of green extraction technologies not only seeks the total replacement of common organic, toxic, and highly volatile compounds, which evaporate into the atmosphere, but also the use of nontoxic solvents using generally regarded as safe compounds.

4.1.1 Ionic liquids

Ionic liquids (ILs) are low melting point organic salts (<100 ºC), composed of an organic cation and an organic or inorganic anion. Compared with conventional organic solvents, ILs are in fact the only class of solvents composed exclusively of ions in the liquid state. ILs are a class of solvents obtained by chemical synthesis, that have been classified as environmentally friendly due to their negligible vapor pressure at room temperature. ILs have many attractive physicochemical properties, such as chemical and thermal stability, non-flammability, high conductivity, and good solubility of various compounds [32]. They have also become very popular for the possibility of easy tailoring their properties, such as polarity, hydrogen bonding, hydrophobicity, viscosity, density, surface tension etc., for different applications [[32]. This is possible through the judicious combination of different cations and anions [33]. These fluids have found a huge number of applications, such as biocatalytic processes, electrochemical applications, extraction solvents

[33] dissolution of a wide range of compounds from metallic salts to polymers, in medical and pharmaceutical fields, for gas separation, to mention a few [34].

Although ILs overcome some of the major problems of organic solvents, their use in food, pharmaceutical and biomedical applications is much delayed in comparison with other areas not only due to their high cost of production and purification but also due to their toxicity and sometimes even the lack of proper toxicity data that severely hinder their wide use [[34]. On the other hand, the application of ILs in green extraction processes is also greatly challenged by their poor biodegradability, sustainability, and biocompatibility [34]. Also, high viscosity is one of the greatest obstacles for the application of ILs in natural products extractions [[32].

4.1.2 Deep Eutectic Solvents

Deep Eutectic Solvents (DES) are another class of alternative solvents that have similar physical properties to ILs [32]. Unlike the classical ILs, composed of only ions, DES are composed of one hydrogen bond donor (HBD), typically a salt, and an organic hydrogen bond acceptor (HBA), typically, an acid, an alcohol, an amide. By definition, a eutectic solvent is a mixture of two or more compounds to yield a mixture that melts at much lower temperature than the individual pure compounds. One of the most significant DES melting temperature depression was observed for a mixture of choline chloride and urea in a (1:2) mole ratio. The resulting mixture has a melting point of 12 °C (far less than the melting point of choline chloride, 302 °C and urea, 133 °C), which makes it liquid at room temperature [33]. The most common DES are based on choline chloride (ChCl), carboxylic acids, and other hydrogen-bond donors, e.g., urea, citric acid, succinic acid, and glycerol. DES have similar characteristics to ILs but are cheaper to produce (lower cost of the raw materials), less toxic, and often biodegradable 33]. Some features of DES give it advantages over ILs because they are easier to prepare with high purity at low cost. Also, higher melting points of many DES, however, can hamper their application as a green solvent at room temperature [32].

The strength of the hydrogen bonds is documented to be correlated with the temperature of phase transition, stability and dissolving properties of a given eutectic solvent. It is a rule of thumb that the stronger the ability to form hydrogen bond, the higher decrease in melting temperature [35]. A scheme of the solid-liquid phase diagram of a DES is shown in Figure 9. Although many authors defend that DES are only attained at the eutectic composition, DES are in fact mixtures of two compounds and all the available compositions can be used if they are located in the homogeneous one liquid phase area of the phase diagram.

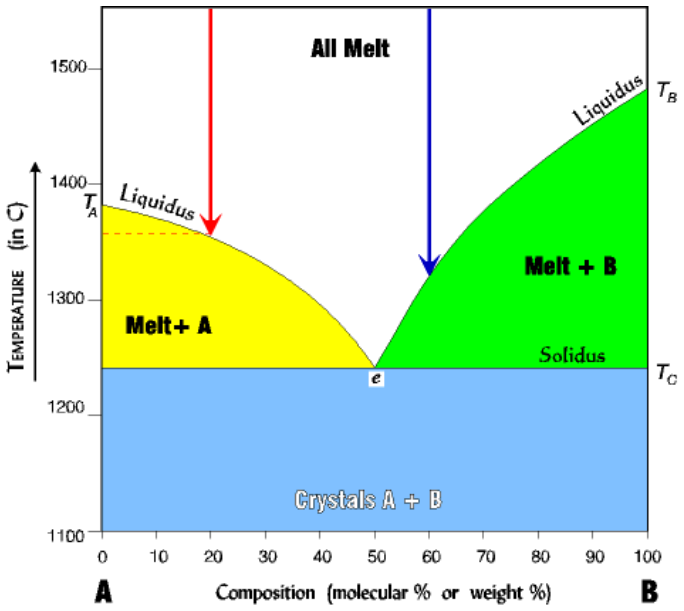


Figure 9: Schematic solid-liquid phase diagram of a eutectic mixture [38].

A significant number of the DES proposed in literature is formed by mixing a quaternary ammonium salt, especially choline chloride, and a carboxylic acid. The physical properties and phase behavior of which are significantly affected by the structure and mole fraction of the components. Typically, DES are more viscous than molecular liquids due to the complex hydrogen bonding network established between the DES components. [37].

Eutectic solvents have been used to decrease the melting temperature of molten salt, enabling their application in an enormous variety of technologies [37]. Unlike the room-temperature ILs, DES are easy to prepare in a pure state (i.e., high purity at low cost), low toxicity and low cost, depending on the starting compounds properties [34] and are nonreactive with water [33, 35]. These solvents have been used in organic reactions, enzyme reactions, and electrochemistry etc. According to Abbot *et al.* [37], DESs are generally grouped into four categories:

1. Quaternary ammonium salt and metal salt
2. Quaternary ammonium salt and metal salt hydrate
3. Quaternary ammonium salt and HBD
4. Metal salt and HBD

The 3rd class is the most chemically stable and is the most used as solvents.

4.1.3 Natural Deep Eutectic Solvents

Choi *et al.* [32], discovered that many plant metabolites in solid state became liquid when they mixed in a certain condition and these liquids that formed play a role as alternative media to water in living organisms. As alternative media, they also function to solubilize, store and transport non-water-soluble metabolites in living cell and organisms. These liquids were termed NADES therefore extending the composition of DES to natural products, such as citric acid and proline [32].

In Table 4 is presented the over 100 combinations of NADES or DES formed strictly of natural primary metabolites (e.g., sugars, sugar alcohols, organic acids, amino acids, and amines) bound together by strong intermolecular interactions, particularly hydrogen bonding [30].

NADES can be regarded as new, eco-friendly, non-toxic solvent that can be used in green extractions [19] since they fully respect the green chemistry principles and offer many advantages including readily available components, biodegradability, very attractive physicochemical properties that are tunable, low toxicity profile, sustainability, low cost, with most of their components come from natural sources, they are non-toxic to the environment and the humans, truly simple to. This versatility overcame some of the limitations of ILs and DES [32, 35].

NADES are formed by the complexation between a hydrogen bond acceptor and a hydrogen bond donor [35]. The charge delocalization occurring is hereafter responsible for the decrease in melting point of the mixture relative to the melting point of the raw materials as shown in (Figure 10).

4.13.1 Application of ILs and DES in liquid-liquid extraction of natural products

The use of solvents for liquid-liquid extraction depends on their physical properties, such as viscosity, density, and miscibility. It is convenient to select solvents with low viscosity to facilitate mixing but with a large density difference for the separation process. The hydrophilicity of ILs is an important factor determining its water miscibility and extraction efficiency. ILs can be hydrophobic or hydrophilic depending on the structure of cations and anions, though the anion seems to be more important. Those based on lower H-bond accepting strength anions ([PF6], [Tf2N] and [BF4]) are normally water immiscible, therefore, they are the solvents of choice for forming biphasic systems in most IL extraction. However, hydrophilic ILs such as with [Cl] or [I], can be induced to form aqueous biphasic systems when in contact with concentrated solutions of the water-structuring salt, K3PO4. This new system can be utilized to recycle hydrophilic ILs from aqueous solutions. The viscosity and hydrophobicity of ILs also increases with the length of the alkyl chain. ILs-based aqueous two-phase systems are great candidates for the replacement of volatile solutions in typical liquid-liquid extraction due to their high extraction efficiency for different kinds of compounds.

Since the phycocyanin to extract is a protein, this paper will only focus on the application of DES to amino acids and proteins [32].

4.1.3.1.1 Amino acids

The pH value of the aqueous solution has a great effect on the characteristics of amino acids, as their charge state changes with increasing pH of the medium from cations to zwitterions and anions successively. The higher extraction efficiency corresponds with the range in which the cationic form of the respective amino acids dominates. Arg and Lys can form cationic and dicationic species, why the pH range of efficient extraction of these amino acids is much broader. Crown ethers play an important role as a complexing reagent and H-bonding occurs between the ammonium center of cationic amino acid and the polyether. Unlike conventional organic solvents, it is not necessary to add counter-ions in the presence of a crown ether with [Bmim][PF6] as a partitioning solvent from aqueous solution. The complexes of AAILs with copper enabled the separation of racemic amino acids, which was achieved through a chiral ligand-exchange mechanism [32].

4.1.3.1.2 Proteins

For the extraction of proteins, pH, extraction time, and the volume of the ILs affect the extraction efficiency. The hydrophobic interactions, the electrostatic interaction and salting-out effects are important driving factors for the extraction of proteins from aqueous solution. Different types of ILs can be used for the extraction of proteins from aqueous solution, such as imidazolium based ILs with [Cl], [Br], [PF6] anions [32]

Table 4: Different combinations of natural ionic liquids or deep eutectic solvents from natural products made through vacuum evaporating method. [32].

components			mole ratio
component 1	component 2	component 3	
choline chloride	lactic acid		1:1
choline chloride	malonic acid		1:1[a]
choline chloride	maleic acid		1:1, 2:1[a],
choline chloride	DL-malic acid		1:1, 1.5:1,
choline chloride	citric acid		1:1, 2:1,
choline chloride	aconitic acid		1:1
choline chloride	L-(+)-tartaric acid		2:1
choline chloride	glycol		1:1,1:2
choline chloride	1,2-propanediol		1:1, 1:1.5, 1:2, 1:3
choline chloride	1,2-propanediol		2:1 [a]
choline chloride	glycerol		1:1, 3:2
choline chloride	*meso*-erythritol		2:1 [a]
choline chloride	xylitol		5:2
choline chloride	ribitol (adonitol)		5:2

choline chloride	D-sorbitol		3:1, 5:2
choline chloride	D-xylose		2:1, 3:1
choline chloride	A-L-rhamnose		2:1
choline chloride	D-(+)-glucose		1:1, 2:1[a]
choline chloride	D-(+)-glucose		5:2
choline chloride	D-(-)-fructose		1:1, 1:1.5, 1:2[a]
choline chloride	D-(-)-fructose		5:2
choline chloride	sorbose		5:2, 1:1
choline chloride	D-mannose		5:2
choline chloride	D-(+)-galactose		5:2
choline chloride	sucrose		4:1, 1:1
choline chloride	D-(+)-trehalose		4:1
choline chloride	maltose		4:1
choline chloride	raffinose		11:2
choline chloride	proline	DL-malic acid	1:1:1[a]
choline chloride	xylitol	DL-malic acid	1:1:1
choline bitartrate	D-(+)-glucose		1:1
betaine	D-(+)-glucose		5:2[a]
betaine	sucrose		4:1, 1:1[a]
betaine	sucrose		2:1
betaine	D-(+)-trehalose		4:1
betaine	D-sorbitol		3:1[a]
betaine	DL-malic acid		1:1
betaine	L-(+)-tartaric acid		2:1
betaine	D-mannose		5:2
betaine	inositol	raffinose	9:1:1[a]
betaine	sucrose	proline	1:1:1
betaine	sucrose	proline	5:2:2
betaine	D-(+)-glucose	proline	1:1:1
betaine	DL-malic acid	D-(+)-glucose	1:1:1
betaine	DL-malic acid	proline	1:1:1
betaine	DL-malic acid	inositol	1:1:1[a]
betaine	oxalic acid	D-(+)-glucose	1:1:1
betaine	citric acid		1:1
lactic acid	D-(+)-glucose		5:1
lactic acid	β-alanine		1:1
DL-malic acid	D-xylose		1:1[a]
DL-malic acid	D-(+)-glucose		1:1, 1:2[a]
DL-malic acid	sucrose		1:1
DL-malic acid	D-(-)-fructose		1:1[a]
DL-malic acid	D-mannose		1:1

DL-malic acid	sucrose		1:1, 2:1
DL-malic acid	maltose		2:1[a]
DL-malic acid	D-(+)-trehalose		2:1[a]
DL-malic acid	lactose		2:1, 1:1
DL-malic acid	raffinose		3:1[a]
DL-malic acid	xylitol		1:1[a]
DL-malic acid	adonitol		1:1[a]
DL-malic acid	D-sorbitol		1:1
DL-malic acid	D-(+)-glucose	D-(-)-fructose	1:1:1
DL-malic acid	D-(+)-glucose	glycerol	1:1:1
DL-malic acid	sucrose	glycerol	1:1:2
DL-malic acid	L-proline	choline chloride	1:1:1
citric acid	D-xylose		1:1[a]
citric acid	D-(+)-glucose		2:1[a]
citric acid	D-(-)-fructose		1:1
citric acid	sorbose		1:1[a]
citric acid	D-mannose		1:1
citric acid	D-(+)-galactose		1:1[a]
citric acid	sucrose		1:1
citric acid	maltose		2:1
citric acid	D-(+)-trehalose		2:1[a]
citric acid	raffinose		3:1
citric acid	D-sorbitol		1:1
citric acid	ribitol		1:1
citric acid	xylitol		1:1
citric acid	adonitol		1:1
citric acid	L-proline		1:1, 1:2, 1:3
citric acid	DL-malic acid		1:1[a]
phytic acid sodium	betaine		1:6
phytic acid sodium	DL-malic acid		1:6
phytic acid sodium	glycerol		1:6
phytic acid sodium	L-proline		1:6
phytic acid sodium	D-(+)-glucose		1:6
phytic acid sodium	choline chloride		1:3
D/L-proline	sucrose		2:1, 3:1
D/L-proline	sucrose		4:1,1:1[a]
D/L-proline	D-sorbitol		1:1
D/L-proline	D-(+)-glucose		1:1, 5:3

D/L-proline	lactic acid		1:1
D/L-proline	DL-malic acid		1:1
D/L-proline	citric acid		1:1, 2:1
D/L-proline	malonic acid		1:1[a]
D-proline	D-(+)-glucose		5:3
L-proline	D-(+)-glucose		5:3
L-serine	DL-malic acid		3:2, 1:1
L-serine	D-(+)-glucose		5:4[a]
L-glutamic salt	sucrose		2:1
L-glutamic salt	D-(+)-glucose		1:1
D-(+)-glucose	DL-malic acid		1:1[a]
D-(+)-glucose	citric acid		1:1
D-(+)-glucose	L-(+)-tartaric acid		1:1
D-(+)-glucose	D-(-)-fructose	sucrose	1:1:1[a]
D-(-)-fructose	sucrose		1:1
β-alanine	DL-malic acid		3:2, 1:1
β-alanine	citric acid		1:1

[a] Not stable; solid precipitate within 7 days.

4.1.4 Methods of Preparation of DES and NADES

DES and NADES are commonly prepared using three most methods. After carefully weighing the components inside a capped vial, one of the following methods of preparation can be applied.

4.1.4.1 Heating and Stirring method

Stir the component mixture and heat it in a water bath below 100 ºC, between 30-90 min until a clear liquid is formed. Cool down slowly.

4.1.4.2 Vacuum and Evaporating

Dissolve components in water and evaporate the water using rotary evaporator. Put the obtained liquid in a desiccator with silica gel, until constant weight is reached.

4.1.4.3 Freeze Drying method

Mix the components and water and freeze the obtained aqueous solution. Then perform a series of freeze-dry cycles to achieve a clear viscous liquid.

CHAPTER 5

EXPERIMENTS CARRIED OUT IN THIS THESIS

5.1 Extraction of PC from *Spirulina Platensis*

Several methods **[37**, **38]** have been used for extraction of biochemicals from microalgae and the process involves disruption of microalgal cell wall with appropriate methods depending on the rigidity of the cell wall and compounds of interest. The ideal extraction method should be selective towards extraction of the target microalgal products and simultaneously minimize the co-extraction of contaminants **[37]**. In Table 5, the main 5 classes of cell disruption methods are presented. Also, some of the advantages and disadvantages of these methods of cell wall disruption can be found in ***Annexes 1***.

Table 5. Summary of cell wall disruption methods used for microalgae [37].

Cell disruption methods			
(A) Mechanical	**(B) Chemical**	**(C) Thermal/thermo-chemical**	**(E) Biological**
1. Solid shear • Bead milling/bead beating • Grinding (with/without cryogens) • Homogenisers (high speed) • Mechanical cell press **2. Liquid shear** • High pressure homogeniser (microfluidiser)	• Solvent extractions • Acidic/alkaline • Ionic liquids • Chelating agents • Detergents • Osmosis • Oxidation • Nanoparticles • Supercritical fluid extraction	• Autoclave • Steam explosion • Hydrothermal liquefaction • Freeze drying **(D) Electromagnetic** • Microwaves (with/without solvents) • Ultrasound	• Antibiotics • Enzymes (lytic, autolysis) • Phage **(F) Current** • Pulsed electric field

Two methods were used in this work to disrupt the cell wall of Spirulina Platensis: i) Chemical method (Maceration - solvent extraction) and ii) Electromagnetic method (microwave extraction).

5.1.1 General Materials and Preparations

Spirulina (*Arthrospira platensis*) was the cyanobacteria selected for this research work. A sachet of dried Spirulina powder, ISWARI brand (100% biological), was bought at Celeiro. A microwave (Discover CEM SP-D QLab O and Sorvall centrifuge GS-3 rotor), a spectrophotometer (Thermo Scientific Genesys 10uv), a magnetic stirrer with thermometer, 200 ml capped bottle and a measuring scale were used.

5.1.2 Microwave Assisted Extraction Method

This is an alternative green method for cell disruption and extraction of compounds from natural matrices, in this case microalgae. It provides a possibility of extraction of biochemical components including lipids, pigments, carbohydrates, vitamins, and proteins, individually or as an extract. Some of the advantages of application of microwave-assisted extraction are the reduction of working time and higher yield and purity of the extracted products [39]. It has been critically evaluated for industrial-scale applications, revealing effective cell wall disruption with relatively low energy input, a rapid treatment time and the avoidance of the utilization of hazardous substances [39].

5.1.2.1 Materials and Experimental parameters

Microwave (Discover CEM, SP-D, Q Lab O) capable of operating at input power of 1000 W and frequency of 2450 MHz, and a centrifuge (Hettich Zentrifigen Mikro 220R) were used.

5.1.2.2 Methods

20 mg of *Spirulina platensis* was weighed into the 20 mL test tube plus 5 mL of distilled water. The mixture was then placed inside a microwave for 30 s, at 100 ºC and a power input of 100 W. The resulting mixture was divided into two samples and centrifuged at 3140xg for 5 min [18]] to separate the biomass residue and the supernatant. The biomass residue was discarded. The supernatant was then analyzed to determine the concentration and purity of PC through absorbance measurements with the spectrophotometer. The results are shown in Table 6.

Table 6. Absorbance of PC extract from Spirulina Platensis using the microwave method.

Absorbance					
	Wavelength (nm)	Sample 1	Sample 2	Average	Standard Deviation
1.	620	0.695	0.774	0.74	0.06
2.	652	0.542	0.591	0.57	0.04
3.	280	0.666	0.716	0.69	0.04

5.1.3 Maceration Extraction Method

Maceration is a solvent extraction method that is applied by soaking the dried biomass materials in solvent (distilled water). This method has some drawback like use of large amount of solvent, long extraction time and generally low yield, and is being replaced by more environmentally friendly, functional, fast methods.

Two sets of extractions were made, the first using an equal amount of PC powder (20 mg) to that used in in the microwave method to compare the two methods; and the second one of 600 mg of PC powder, to produce larger quantity of extracted PC for further solubility experiments.

5.1.3.1 Materials

Three 200 mL bottles with caps, magnetic stirrers, clamps, spatulas, measuring scale balance, Petri-dish, Aluminum foil paper, dried spirulina powder and stop-watch.

5.1.3.2 Method

20 gram of dried powdered *Spirulina platensis* was weighed inside 200 mL test tube and 5 mL of the distilled water were added. Then, the mixture was put inside a rotary shaker for one cycle of 48 h at room temperature. The mixture was centrifuged at 6000 rpm for 10 mins and the supernatant UV-vis spectra was drawn. The spectrophotometer readings are presented in Table 7. The samples presented in the Table 7 for maceration method I were from the same cycle but was only divided for the sake of centrifuging. The case was same for maceration II.

5.1.4 Maceration Extraction Method II

600 mg of dried powdered spirulina platensis was weighed each inside three 200 ml capped bottles and 150 ml of distilled water was added and magnetically mixed for 48 hr. The bottles were shielded with aluminum foil paper to prevent oxidation by sunlight. After 48 h of continuous stirring, the mixtures were centrifuged at 6000 rpm for 10 min and the solid residue was separated from the supernatant. Then, concentrations, yields, and purities of PC in the samples were determined by measuring the absorbance of PC with the spectrophotometer between the range 620-680 nm. The supernatants were then freeze-dried.

The supernatants obtained from the maceration extraction II was placed inside refrigerator at -20 ºC for 24 h and at -50 ºC for 1 h before being freeze in Nitrogen at about -200 ºC. Finally, it was freeze-dried for 72 h in the apparatus of Figure 10. The results of the absorbance measurements are shown in Table 7.

Table 7. Absorbance for PC extract from Spirulina Platensis using the maceration method a) Method I and b) Method II.

a)	**Absorbance**					
	wavelength (nm)	Sample 1	Sample 2	Average	Standard Deviation	
1.	620	1.426	1.418	**1.422**	**0.006**	
2.	652	1.115	1.110	**1.113**	**0.004**	
3.	280	1.156	1.153	**1.155**	**0.002**	
b)	**Absorbance**					
	wavelength (nm)	Sample 1	Sample 2	Sample 3	Average	Standard Deviation
1.	620	2.275	2.278	1.662	**2.0**	**0.4**
2.	652	1.532	1.445	1.332	**1.4**	**0.1**
3.	280	1.685	1.369	1.498	**1.5**	**0.2**

Figure 10. The plate dryer with condenser for freeze-drying of extracted PC.

It can be observed that a somehow higher values were obtained for the absorbance in the second maceration procedure than for the first. In fact, comparing the results obtained from both maceration methods, and neglecting the highest (maceration II) and the lowest values (maceration I), the concentration of PC in the extracts are in good agreement.

5.1.5 Comparison of the Extraction Methods

Using Bennette and Bogorad [8] equations, equations (1), (2) and (4) above, the concentration of PC (in mg/mL and in mg/g) and the purity of extracted PC using both the Microwave and the Maceration Methods were calculated and are listed in Table 8. The values used for these calculations are the average value reported in Table 6 and 7.

Table 8. PC concentration and purity of the two extraction methods used in this work.

Method	C_{PC} (mg/mL)	C_{PC} (mg/g)	P
Microwave	0.087	21.815	1.063
Maceration I	0.168	41.886	1.232
Maceration II	0.260	65.115	1.375

It can be observed that a higher concentration of PC in the extract and a higher purity was observed for the maceration method (48 h) in comparison with the microwave method (30 s). Thus, apart from the length of time required, maceration method is preferred when PC yield , purity and set-up cost is considered. The extracted PC is reagent grade since value of purity obtained from both methods is between 0.7 and 3.9. It is also interesting to notice that in the 2nd maceration procedure a higher concentration and purity of PC was obtained in comparison to the 1st extraction performed using the same methodology and biomass-solvent ratios. This can be because a higher mass of Spirulina was used and the fact that no heating was involved in the extraction process. Also, the experimental procedure was not fully established in the lab and two experiments were not enough to obtain fully reproducible results.

5.2 DES for stabilizing PC from Spirulina platensis

5.2.1 Preparation of Deep Eutectic Solvents

Two different sets of DES or NADES were prepared: in the first set the same HBD, Polyethylene glycol-200 (Peg-200), was used with four different HBA; in the second set the same HBA, Choline Chloride (ChCl), was used with another four different set of HBD. To be mentioned that a chemical compound can act as HBD or HBA depending on the other DES component. Stirring and heating was the preferred method for DES preparation.

5.2.1.1 Materials and Methods

The HBA and HBD chemicals (The purity and suppliers are as shown in Tables 8 and 9), distilled water, capped vials, spatula, magnetic stirrer with temperature measuring devices, and measuring

5.2.2.2 Peg-200 based DES

Peg-200 based DES were prepared by mixing and heating at stoichiometric ratios, previously determined, and published in the literature by other authors, of Peg-200 and each one of the HBA. These ratios were carefully chosen so that a homogeneous liquid would be obtained from the mixture of the two compounds. The composition of the DES prepared and the HBA are listed in Table 8.

Table 8. Stoichiometric ratios of the Peg-200 based DES prepared in this work

	HBD component	HBA components	Supplier	Stoichiometric ratios
1.	Peg-200 99% purity Supplier: Sigma Aldrich	Tetramethylammonium bromide (TMAB - $C_4H_{12}BrN$ – 99% purity)	Sigma Aldrich Germany	1:2
2.		Tetra-n-Phosphonium bromide (TBPB - $C_{16}H_{36}BrP$ – 99% purity)	Sigma Aldrich Germany	1:2
3.		Tetrabutylammonium bromide, (TBAB - $C_{16}H_{36}BrN$ – 99% purity)	Sigma Aldrich Germany	1:2
4.		Aliquat 336 ($C_{25}H_{54}ClN$ – 99% purity)	Sigma Aldrich Germany	1:2

The actual amount in grams (g) of each component was calculated and weighed out with the use of scale type OHAUS (*e = 0.01 g, d = 0.001 g*). These results are listed in ***Annex 2***. Then, the mixtures were heated at 70 ºC and magnetically stirred at 405 rpm for about 30-90 minutes until uniform liquids were formed as shown in Figure 11.

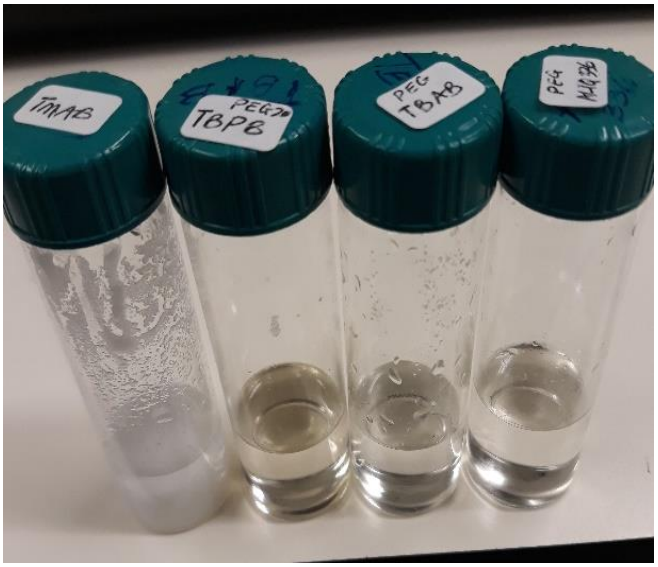


Figure 11. Photograpgh of the Peg-200 based DES prepared in this work.

As it can be seen from Figure 11, all the DES are liquid at room temperature, except for TMAB:Peg-200 DES which was obtained as a liquid with a solid in suspension. A possible explanation for this fact is that the amount weighted was not accurate enough and the composition prepared fall inside this region of the phase diagram.

5.2.2.3 Choline Chloride based DES

The preparation of this set of choline chloride-based DES followed the same procedure as those based on Peg-200, using the mixing and heating procedure. Choline chloride was mixed with each one of the HBD listed in Table 9. The calculations and the actual ratios of the DES components can be found in ***Annex 3***. Again. the stoichiometric ratios were taken from literature. DES prepared are presented in Figure 12.

Table 9. Stoichiometric ratios of the ChCl based DES prepared in this work.

	HBA component	HBD components	Supplier	Stoichiometric ratios
1.	Choline Chloride Purity – 99+% Supplier: Sigma-Aldrich Germany	Oxalic acid - $(COOH)_2$ purity 99+%	Sigma-Aldrich Germany	1:2
2.		Malic acid - $C_4H_6O_5$ Purity – 99+%	ACROS New Jersey, USA	1:1
3.		Peg-200 - $H(OCH_2CH_2)_nOH$ Purity – 99%	Sigma-Aldrich Germany	1:2
				1:3
4.		Citric acid - $C_6H_8O_7$ Purity – 99.5%	Sigma-Aldrich Germany	1:1
5		Citric acid + water		1:1:6

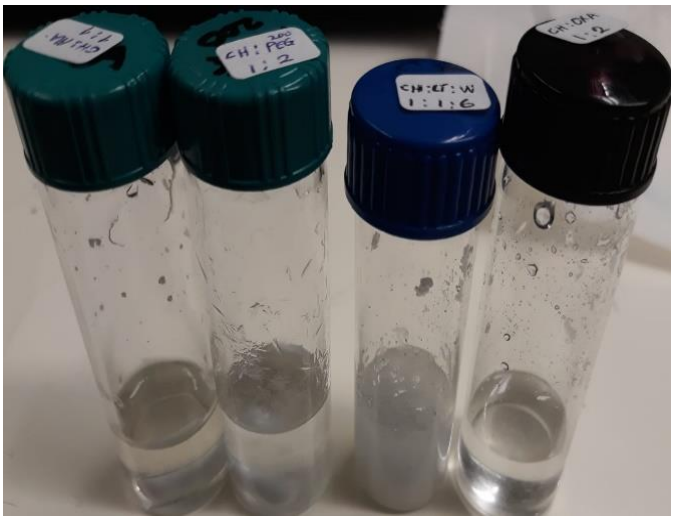


Figure 12. Choline Chloride-based DES prepared in this work.

Again, two of the DESs, ChCl:Citric Acid, (1:1) and ChCl:Peg-200 (1:2) did not yield a homogeneous liquid solution and thus were discarded. Nevertheless, there are some authors in literature that add water to DES, to make them a completely homogeneous solution. This was done to ChCl:Citric Acid DES with the addition of water at molar ratio (1:1:6), while ChCl:Peg-200 NADES at (1:3) was prepared and since it is a homogeneous liquid it was used in the PC apparent solubility tests as reported in Table 9.

5.2.2 Apparent Solubility determination

To better evaluate which DES should be used in the extraction of PC from *Spirulina platensis,* the solubilities of PC in the prepared DES could yield valuable information about the interactions between PC and the solvent. Since it was not possible to prepare a calibration curve, the apparent solubility was determined. Apparent solubilities of PC in the prepared DES were determined by adding a known small quantity of PC to 1 g of DES in a capped vial with continuous stirring at 50 ºC, until PC precipitation was observed. The last addition of PC that is still soluble in DES is taken as the apparent solubility. Due to the high viscosity of the prepared DES at room temperature, the apparent solubility was measured at 50ºC.

5.2.2.1 Solubility of PC in Peg-200 based DES

The apparent solubility of PC in 1 g of Peg-200-based DES is listed in Table 10. The extracted PC was found to be much less soluble in the ALIQ366:Peg-200 DES than in the other two DESs, which are based on TBAB and TBPB salts. Also, the PC solubility in these other two DES is very similar which is probably due to the similarity between the salts, just changing the ammonium core center for a phosphonium core center. Aliquat 366 is a more hydrophobic salts than these other two salts, with longer hydrocarbon chains (methyltrioctyl ammonium chloride) and this is probably the explanation for the low PC solubility in DES containing this salt.

Table 10. Apparent solubility of PC in Peg-200 based DES (g/g).

HBA	PC Solubility (g/g)
TBAB	0.0554
ALIQ. 336	0.0380
TBPB	0.0516

5.2.2.2 Apparent Solubility of PC in ChCl-based DES

The total amount of PC that dissolved in 1 g of ChCl-based DES is listed in Table 11. The extracted PC was found to be more soluble in ChCl : Malic acid DES than the other two DESs as shown in Figure 13.

Table 11. Apparent solubility of PC in ChCl based DES (g/g).

HBD	PC Solubility (g/g)
Malic acid	0.1325
Oxalic acid	0.0859
Peg-200	0.0808
Citric acid + water	0.0202

From the results of Table 11, it can be concluded that PC has the highest solubility in the ChCl:Malic Acid DES, followed by ChCl:Oxalic Acid and ChCl:Peg-200 and finally ChCl:Citric Acid presents the lowest solubility. A comparison between the apparent solubilities for the two classes of DES indicates that there is not much of a difference in the PC apparent solubility when ChCl, TBAB and TBPB are used, in combination with small chain organic acids or alcohols, such as Peg-200. The very low apparent PC solubility for ChCl:Citric Acid:Water DES is probably due to the water presence and more stronger hydrogen bond. It is also interesting to observe the PC color in these DES: green in ChCl:Malic Acid DES, greenish brownish in ChCl:Peg-200 DES and blueish in ChCl:Citric Acid:Water NADES. Also, to be mentioned that PC precipitated in ChCl:Oxalic Acid DES with a grey color as shown in Figure 16.

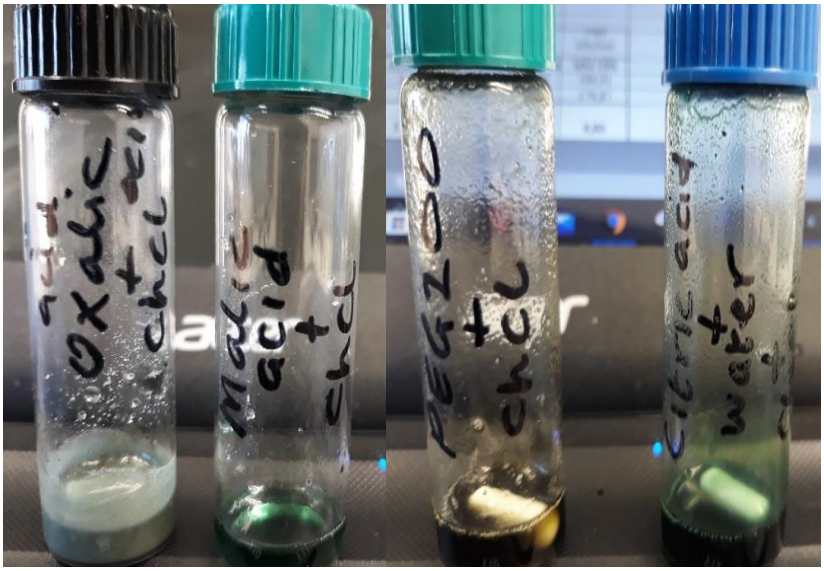


Figure 13. PC solubilized in ChCl based DES

5.2.3 Results and Discussions

Both the spirulina powder and the extracted phycocyanin were carefully shielded with aluminum foil paper to prevent them from sunlight oxidation since phycocyanin is known to be highly sensitive to environmental conditions like light, temperature, etc., [18]. Microwave method though operates faster than the maceration method yields lower concentration of PC than the maceration method. Besides, microwave method tends to be more expensive than the maceration method. The concentration obtained with maceration method doubles that of the microwave and this confirms what was described by Mari Carmen Ruiz-Domínguez [18] being that the overexposure to high heat (temperature or power) could damage the PC content in the extraction. The purity of the PC extracted also indicates that it was of a reagent grade being a value between 0.7 and 3.9. It is also observed that possibility of combining the two methods could present a higher yield giving that maceration in solvent first could soften the cyanobacteria cell wall to reduce overexposure during microwave extraction method.

The color observed during solubility of the PC in the prepared NADES proved that only ChCl:Malic Acid and ChCl:Citric Acid have potential to be used for maintaining the blue-green color of the PC. The bluish green color produced when extracted PC was dissolved in ChCl:Citric Acid can be attributed to chelating effect of citric acid [16] and its ability to stabilize protein fraction conformation of PC solution. The other two DESs. ChCl:Peg-200 and ChCl:Oxalic acid did not show any potential as stabilizing agent for PC.

CHAPTER 6

CONCLUSIONS

The present work described a suitable method for the extraction of PC from the cyanobacteria Spirulina platensis. Distilled water was chosen as the solvent for the extraction with maceration method because it produced high concentration of PC and due to its low cost compare with the microwave method, that is more expensive even though faster. At biomass-solvent ratio of 1:4 and at the experimental conditions of 48 h, and constant stirring at room temperature, the yield obtained was 41.886 mg/g (0.1675 mg/ml) and purity 1.37, indicating that the PC was a reagent grade. In this PC extraction method, there is opportunity for a scale up due to its simplicity and low cost. Also, *Spirulina platensis* was confirmed to be a good source of PC, to be extracted for diverse purposes.

This work also describes the heating and stirring method of preparation of DES or NADES, from the induvial pure compounds. Established method of heating and stirring at temperatures below 100 ºC for 30-90 mins was adopted to prepare two groups of DES or NADES using (i) Peg-200 as HBD plus four HBA compounds and (ii) ChCl as HBA and four HBD compounds, respectively. Apparent solubility of PC in the prepared DES was determined and both TBAB:Peg-200 and ChCl:Malic Acid have the highest apparent solubilities of 0.0554 g/g of DES and 0.1326 g/g of NADES, respectively.

Although the apparent solubility of PC in ChCl:Citric Acid was low, PC showed a blueish green color when in solution with this solvent, while in ChCl:Malic Acid it acquired a green color. This shows that the two NADES possess the potential to be used as PC stabilizers. The low solubility of PC in these DES or NADES is probably due to their high viscosity. Consequently, this property needs to be further tuned for better results.

PC high solubility in distilled water and its subsequent color stabilization in edible preservatives like citric acid and malic acid, confirm PC as a suitable pigment of high economic consideration and safety for food industry.

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Annex 1 Advantages and disadvantages of current cell disruption techniques for microalgal biotechnology

Method	Operates at Industrial Scale	Suitability for Commercial Application	Advantages	Disadvantages
High pressure homogeniser	√	-	Destruction of cell walls at room temperature, effective for neutral lipid extraction	High energy input, not effective for extraction of high molecular weight proteins
Mechanical cell press	√	-	Industry standard for oil recovery from oilseeds	Inefficient cell disruption, high energy input
Hydrodynamic cavitation	√	-	Relatively low energy input	Cavitation area limited
Horn sonication	√	++	Effective cell wall disruption, low maintenance cost, relatively rapid process, hazardous chemicals are not required	Multiple units required, cavitation area limited, high operational costs and energy input
Bath sonication	x	+++	Effective cell wall disruption, minimal maintenance cost, relatively rapid, no hazardous substances required	High operational costs and energy input
Microwaves	x	++++	Effective cell wall disruption and excellent recovery of bioactives, relatively low energy input, fast heating and short reaction time, reduced solvent usage	Generates heat, high maintenance cost
Bead milling/beat beating	√	++	Effective cell wall disruption, rapid extraction	Varied efficiency across species, additional step required to remove beads, high maintenance costs and energy input
Osmotic shock	x	-	Low energy input, easier to scale-up	Inefficient cell disruption, generation of waste saltwater, time consuming
Acid/alkali	√	-	Low energy input	Requires disposal of acid/alkali after extraction, carotenoid degradation
Enzymatic hydrolysis	√	++	Effective cell wall hydrolysis, high selectivity, mild treatment, carotenoid bioactivity not affected	High cost of enzymes, longer treatment time, enzymes must be disposed of after use
Autoclave	x	+	Low maintenance cost	High energy input, not suitable for pigments
Steam explosion	√	+++++	Effective cell wall disruption, low maintenance costs, relatively low energy input	Varied efficiency across species
Freeze drying	√	+	Mild operating conditions, drying and extraction can be incorporated in one step, does not affect cellular components	Cell disruption variable and often the integrity of the cell wall is weakened but not disrupted, cost associated with pump maintenance, time consuming, expensive, high energy input
Nanoparticles	x	-	Non-toxic	Expensive, additional step required to remove nanoparticles, technology in its infancy
Supercritical fluid extraction	√	+	Polarity of solvent is tunable, fast process, uses non-toxic solvents such CO_2, effective for carotenoid extraction	Expensive, not suitable for scale-up
Grinding (with/without cryogens)	x	-	Quick and efficient at a laboratory-scale	Time consuming, degradation of some of the bioactives

Method	Operates at Industrial Scale	Suitability for Commercial Application	Advantages	Disadvantages
Pulse electric field	√	+	High selectivity, mild treatment, carotenoid bioactivity not affected, relatively low energy input	Still in its infancy
Hydrothermal liquefaction	x	-	Uses a wet feedstock	High variability in recovery, high energy input and temperature, requires expensive catalyst
Ionic liquids	x	-	Low cost	Still in their infancy, issues over toxicity
Soxhlet extraction	√	+	Cost-effective, easy to scale-up	Long extraction time, uses large amounts of solvents (often toxic)

√: Yes; x: No; -: Not suitable; +: Weak; ++: Moderate; +++: High; ++++: Higher; +++++: Very high.

Annex 2 **Molar mass, stoichiometric ratios and weighed amount of the Peg-200 DES components**

Preparation of Deep Eutectic Solvents - (Peg-200 based DES)								
	HBD	MM	HBA	MM	MM DES	Molar Ratio HBD	Molar Ratios HBA	
1	Peg200	200	TMAB	154,0500	184,6833	2	1	
2			TBPB	339,3500	246,4500	2	1	
3			TBAB	332,3700	244,1233	2	1	
4			Aliquat 336	446,1000	282,0333	2	1	
	To prepare		Mass to weighed (g)		Weighed Mass (g)		Total mass weighed (g)	
	1	g	HBD	HBA	HBD	HBA	HBD + HBA	
1	0,0054	mol	0,7220	0,2780	0,7456	0,2784	1,0240	TMAB
2	0,0041	mol	0,5410	0,4590	0,5419	0,4586	1,0005	TBPB
3	0,0041	mol	0,5462	0,4538	0,5484	0,4539	1,0022	TBAB
4	0,0035	mol	0,4728	0,5272	0,4786	0,5272	1,0058	Aliquat 336

Annex 3 **Molar mass, stoichiometric ratios and weighed amount of the Choline Chloride DES components**

Preparation of Natural Deep Eutectic Solvents (NADES)														
	HBA	MM	HBD	MM	Distilled Water	MM	MM DES	MM DES with water	Molar Ratio HBA	Molar Ratios HBD	Distilled Water	MM DES (ChCl:Peg 200) (1:3)
1	ChCl	139,6	Peg200	200,0000			179,8733		1	2		184,9050
2		x	Citric acid	192,1240	H_2O	18,0000	165,8720	54,9680	1	1	6	
3			Malic acid	134,0874			136,8537		1	1		
4			Oxalic acid	90,0300			106,5600		1	2		

	To prepare		Mass to weighed (g)			Weighed Mass (g)			Total mass weighed (g)		
	1	g	HBA	HBD	Distilled Water	HBA	HBD	Distilled Water	HBA + HBD	HBD	
1	0,0056		0,2587	0,7413		0,2600	0,7420		1,0020	Peg 200	
2	0,0060		0,4209	0,5791	0,3256	0,4220	0,5800	0,3256	**1,3276**	Citric acid	
3	0,0073		0,5101	0,4899		0,5120	0,4890		1,0010	Malic acid	
4	0,0094		0,4367	0,5633		0,4370	0,5630		1,0000	Oxalic acid	

ChCl (HBA) : Peg-200 (HBD) NADES (1:3)											
To prepare (g)	Mole (mol)		Mass to weighed (g)		Mass weighed (g)		Molar ratio	Molar Ratio	Total mass weighed	MM DES	
	ChCl:Peg-200	g	HBA	HBD	HBA	HBD	HBA	HBD			
1,3706	0,0074		0,2587	1,1119	0,2600	1,1164	1,0000	3,0000	1,3764	184,9050	
1,0000	0,0056		0,2587	0,7413	0,2600	0,7420	1,0000	2,0000	1,0020	179,8733	
Peg 200 Make-up	0,0019		0,0000	0,3706	0,0000	0,3744	1,0000	1,0000	0,3744	5,0317	