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Spirulina Phycobiliproteins as Food Components and Complements

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Abstract

Spirulina has a documented history of use as a food for more than 1000 years, and has been in production as a dietary supplement for 40 years. Among many of *Spirulina* bioactive components, blue protein C-phycoyanin and its linear tetrapyrrole chromophore phycocyanobilin occupy a special place due to broad possibilities for application in various areas of food technology. The subject of this chapter is up-to-date food applications of these *Spirulina* components, with a focus on their use as food colorants, additives, nutraceuticals, and dietary supplements. Their other actual and future food application possibilities will also be briefly presented and discussed.

Keywords: *Spirulina*, C-phycoyanin, phycocyanobilin, food components, food complements

1. Introduction: *Spirulina* as a superfood

The blue-green microalgae *Spirulina* has been used in human nutrition for centuries. It is still used as food in some places, such as the Lake Chad area, where it is sold as dried bread called "dihe" [1]. For human consumption, the commercial production of *Spirulina* dates back to the 1970s. *Spirulina* used in human nutrition is the dried commercial biomass of two cyanobacteria species traditionally called *Spirulina platensis* and *Spirulina maxima*, which belong to the genus *Arthrospira* spp. Taxonomically, these organisms are classified in kingdom Bacteria; phylum Cyanobacteria; order Oscillatoriales; family Phormidiaceae [2].

Spirulina is filamentous, helical, photosynthetic cyanobacteria naturally inhabiting alkaline brackish and saline waters in tropical and subtropical regions. Biochemical analysis has revealed its exceptional nutritive properties, so it is referred in the literature as “super food” or “food of the future” [2]. *Spirulina* is one of the richest natural sources of proteins and essential amino acids, as well as an excellent source of vitamins (primarily A, K, and vitamin B complex), macro- and micro-elements (calcium, potassium, magnesium, iron, iodine, selenium, chromium, zinc, and manganese), essential fatty acids, including γ -linoleic acid (GLA), glycolipids, lipopolysaccharides, and sulfolipids [3]. *Spirulina* is especially rich in a variety of pigments, such as chlorophylls, β -carotene, xanthophylls, and phycobilins (phycobiliproteins) (Table 1).

A huge number of *in vitro* and *in vivo* studies, published in the last few decades, have revealed potentially beneficial effects of *Spirulina* on human health. Health benefits mainly arise from the antioxidant effect of algae as a whole, or from its individual ingredients, such as phycobiliproteins (Section 2). Moreover, the presence of significant amounts of GLA, sulfated polysaccharide (calcium spirulin), and sulfolipids additionally contribute to health-promoting activities of *Spirulina* [3].

Several dried biomass products of *Spirulina* have categorized as “generally recognized as safe” (GRAS) by the Food and Drug Administration (FDA) of USA. A recommended dosage for adults is usually in the range of 3–10 g of *Spirulina* per day, while maximum daily intake should not exceed 30 g [3]. Extensive safety studies of *Spirulina* did not show the presence of cyanobacterial toxins [1]. *Spirulina* production requires the use of high quality nutrients and accurate determination of heavy metals in the culture medium, as well as in the biomass. Heavy metal analysis of commercial *Spirulina* products did not found to exceed the regulatory levels [2]. Nevertheless, it should be paid much attention during *Spirulina* cultivation to prevent contamination with heavy metals or the other cyanobacteria, capable to produce toxins.

Substance	Quantity/activity per serving (3 g')	% DV**	Substance	Quantity/activity per serving (3 g')	% DV**
Total carbohydrates	<1 g	<1	Chromium	50 μ g	41
Proteins	2 g	4	Sodium	35 mg	<2
Vitamin A (as β -carotene)	11,250 IU	230	Potassium	60 mg	2
Vitamin K	75 μ g	94	C-phycocyanin	240 mg	–
Vitamin B12	9 μ g	150	GLA	32 mg	–
Iron	7 mg	39	Chlorophyll a	30 mg	–
Magnesium	15 mg	4	Total carotenoids	15 mg	–
Manganese	0.4 mg	20	Superoxid dismutase	2500 U	–

*Recommended daily value of *Spirulina* powder.

**Percent daily values (DV) are based on a 2000 calories diet.

Table 1. Nutritional profile of commercial *Spirulina* powder (Nutrex, Hawaii, USA).

2. Phycobiliproteins

Phycobiliproteins are photosynthetic antenna pigments in the cyanobacteria, red and cryptophyte algae, that efficiently harvest light energy, which is subsequently transferred to chlorophylls during photosynthesis. Therefore, phycobiliproteins significantly contribute to the global photosynthesis. Phycobiliproteins are deeply colored, highly fluorescent, and water-soluble proteins with high propensity to form oligomers (hexamers) that constitute the building blocks of the extra-membranous antenna complex, phycobilisomes. Its intensive color arises from covalently attached linear tetrapyrrole chromophores (phycobilins) *via* thioether bonds to the cysteine residues [4].

Phycobilins are produced by heme metabolism. Heme is synthesized from protoheme IX by ferrochelatase. Then, heme oxygenase cleaves heme and biliverdin IX α is obtained. Biliverdin IX α is reduced by ferredoxin-dependent bilin reductases to obtain phycobilins. Final step in phycobiliproteins biosynthesis is the covalent attachment of bilin chromophores to the apoproteins, catalyzed by phycobiliprotein lyases. Slow spontaneous *in vitro* attachment of tetrapyrrole chromophores to the apoproteins has low fidelity and mixture of oxidation products is obtained [5].

Spirulina produces two phycobiliproteins: C-phycoerythrin (C-PC) as the major pigment and allophycoerythrin (APC), which is present in much smaller quantities, approximately at an 10:1 ratio [3]. C-phycoerythrin level varies based on growing conditions, and may constitute up to 20% of the dry weight of *Spirulina* [6]. C-phycoerythrin and APC are homologous proteins and both bind phycoerythrin (PCB) chromophore [7, 8]. The presence of the third phycobiliprotein, red phycoerythrin, in *Arthrospira platensis* is the subject of debate. While some studies have found that *Spirulina* produces small amounts of phycoerythrin, the other ones did not detect phycoerythrin in *Spirulina* [9].

2.1. Structure and physicochemical properties of C-phycoerythrin and phycoerythrin

C-phycoerythrin (CAS registry number 11016-15-2) is water-soluble, intensive blue protein with strong fluorescence. It is the heterodimer consisting of α - (~18 kDa) and β -subunits (~19 kDa), which form $\alpha\beta$ monomers, further aggregating to trimers ($\alpha\beta$)₃ and hexamers ($\alpha\beta$)₆. Hexamer form represents a functional unit of phycobilisomes. C-phycoerythrin is α -helical protein, with one well-defined domain (similar to the globins) observed within the 3D structure of both chains. Color and intensive fluorescence of C-PC arises from PCB, covalently attached to Cys-84 of α -subunits, while β -subunit binds two PCB molecules *via* Cys-82 and Cys-153 residues [8]. Allophycoerythrin has similar structure and physicochemical properties as C-PC. Unlike C-PC, β -subunit of APC binds only one PCB molecule (at Cys-84) [7–8]. Amino acid variation of phycoerythrins between cyanobacteria and red algae species are very minor [10].

The VIS absorption spectrum of the native C-PC has pronounced specific peak at 620 nm, arising from bound PCB. Phycoerythrin has a molecular weight of 586.7 g/mol and characteristic fluorescence spectrum with an emission peak at 640 nm. Spectra of free PCB differ from

spectra of native protein, in sense of intensity and shape of absorption and emission bands [11]. Bilin chromophore is a very sensitive indicator of the conformational state of the protein, enabling monitoring of C-PC denaturation/renaturation by standard spectroscopic methods. Thermal denaturation of C-PC induces shift of absorption maximum from 620 to 600 nm with significant decrease in protein absorbance (color intensity) and fluorescence [12]. Changes of PCB conformation upon denaturation induce these phenomena: chromophore in native protein has stretched conformation, while denaturation changes PCB conformation to the cyclic, similar to the free chromophore [13].

2.1.1. Production, isolation, and purification of C-phycoerythrin and phycoerythrin

Thanks to the high protein (C-PC) content, as well as large availability, *Arthrospira platensis* is culture of choice for C-PC production. *Spirulina* growth requires dry, hot, and sunny climatic conditions [14]. Photoautotrophic *Spirulina* production is outdoor method, used for commercial production of C-PC at tropical and subtropicals regions, in open ponds and raceways. In the mixotrophic production, *Spirulina* cultivation is performed in an enclosed reactor with the addition of glucose, yielding a higher amount of C-PC. *Spirulina* can grow even heterotrophically, but in this case small yield of pigments is obtained [10]. Presence of covalently attached chromophore makes recombinant production of C-PC more complicated in comparison to other proteins. Complete synthesis of C-PC depends not only on co-expression of α - and β -chains, but also on parallel synthesis of PCB and its covalent attachment to protein [15].

Crucial parameters for C-PC production are lighting conditions (light spectrum, quality, intensity, and cycle), climatic conditions (pH and temperature), and media type. Their optimization strategies are reviewed in [16], with higher productivity in closed bioreactor systems than open ponds. Utilization of agricultural waste to replace the synthetic chemicals in algae cultivation media could also have enviro-economical impact.

Isolation of C-PC in high yield requires efficient extraction process. There are several effective approaches used for C-PC extraction: freezing and thawing, homogenization with mortar and pestle, sonication, high pressure homogenization, osmotic shock (using distilled water), acid treatment, enzymatic treatment (by lysozyme), organic solvent extraction, etc. [17]. Potential applications of C-PC in medicine or for research purposes (as fluorescent tag) require its high purity. The purity of C-PC is evaluated using ratio between absorbance at 620 and 280 nm (A_{620}/A_{280}). C-PC preparations with A_{620}/A_{280} greater than 0.7 is considered as food grade, while preparations with A_{620}/A_{280} more than 3.9 and 4 have reactive and analytical grade of purity, respectively [14]. C-phycoerythrin price strongly depends on its purity, ranging from \$200 to \$2.2 million per kilogram. Numerous different procedures for C-PC purification (usually after protein precipitation with ammonium sulfate) use one or more chromatographic steps (ion-exchange chromatography, hydrophobic chromatography, gel filtration, hydroxyapatite chromatography, and expanded bed adsorption chromatography) or two-phase aqueous extraction [10]. Changing light conditions during cultivation of *Spirulina* (blue and red light *vs.* normal) could increase yield and purity of C-PC [18].

Phycoerythrin (CAS 20298-86-6) isolation requires cleavage of thioether bond between apoprotein and bilin chromophore, by acid hydrolysis, enzymatic cleavage, or alcohol reflux. The most common procedure for the cleavage of PCB from C-PC is still conventional reflux in methanol,

lasting up to 16 hours [10]. Performing ethanolysis in the sealed vessel at 120°C decreases reaction time to 30 minutes, and obtained PCB has higher purity in comparison to conventional reflux method [17]. Phycocyanobilin can be produced in mammalian cells by metabolic engineering, introducing genes for heme oxygenase-1 and PCB:ferredoxin oxidoreductase, with simultaneous knock-down of biliverdin reductase A to prevent PCB reduction to phycocyanorubin [19].

3. Food applications of C-phycocyanin and phycocyanobilin

3.1. Stability and technologies to improve stability

Natural food colorants are often sensitive to heat, light, oxygen, acidic conditions, and exposure to oxidants, such as ascorbic acid and trace metal ions. Generally speaking, natural C-PC is not a particularly stable protein. It was found to be unstable to heat and light in aqueous solution. The presence of photosensitive PCB makes C-PC sensitive to light and prone to free-radical oxidation [20]. The optimum pH range for C-PC was found to be 5.0–6.0 [21] and it is insoluble in acidic solution (pH 3) [22]. The critical temperature for C-PC stability is 47°C, with a sharp drop in the protein half-life values above this temperature. At 50°C, the C-PC solution showed maximum stability at pH 6.0, while at 60°C the maximum protein stability was at pH 5.5 [23]. Exposure to light of 3×10^5 lux for 24 hours in aqueous solution at pH 5 and 7 caused ~80% of its degradation [22]. Therefore, although C-PC has high potential for applications in food industry, biotechnology, and medicine, stability issue is one of the limiting factors for its successful application.

There are an increasing number of studies dealing with development of methods to increase C-PC/PCB stability and expand their application to different food systems. Addition of 20% glucose, 20% sucrose, or 2.5% sodium chloride was considered suitable for prolonging the stability of the C-PC extract [23]. The natural protein cross-linker methylglyoxal does not significantly stabilize C-PC, whereas addition of honey or high concentration of sugars greatly diminishes thermal degradation of protein. After sterilization (80 and 100°C) of fructose syrups with mixture of C-PC and yellow pigment of *Carthamus tinctorius*, the syrups remain clear, with only partial blue color degradation even after 2 months of storage [6]. The rate of C-PC thermal degradation was decreased in the presence of benzoic acid, followed by citric acid and sucrose, while calcium chloride and ascorbic acid supported the least protein stability in comparison to the other food preservatives studied [24]. After solubilization into reverse micelles, C-PC embedded into the structured interfacial water layer was protected from the bleaching processes, reflecting in stable protein spectral parameters as long as the microemulsion was stable [25]. Incorporation of C-PC into polyethylene oxide nanofibers, or addition of sorbitol (50%) and glucose (20%), increased protein thermostability, considering its almost twice extended half-life [26]. C-phycocyanin incorporated into polysaccharide beads such as alginate/chitosan microcapsules and alginate microspheres, showed greater antioxidant activity and thermal stability. These beads are resistant in simulated gastric fluid, while rapidly release C-PC in simulated intestinal fluid [27]. The addition of anionic and ferulated beet pectin enhanced the color stability of the C-PC extract upon heating (65°C) and slowed down its degradation and color lost by proteases, such as Alcalase 2.4 L, papain, and bromelain [28]. C-phycocyanin stabilized by cross-linking of its subunits with formaldehyde exhibited

similar spectroscopic (absorption/fluorescence) properties as native protein, and showed adequate energy coupling after glutaraldehyde-mediated conjugation with R-phycoerythrin [29].

3.2. Safety and bioavailability

Numerous toxicological studies, such as acute, sub-chronic, chronic, mutagenic, teratogenic/developmental toxicity, carcinogenic, and multiple generational/reproduction tests, have confirmed excellent safety profile of *Arthrospira platensis* and *Arthrospira maxima* (Class A rating by the dietary supplements information expert committee of the US pharmacopeial convention). They were of paramount importance in the determination that water extracts of *Spirulina* or C-PC are safe as well. Only very rare, single-case events of adverse incidences associated with consumption of *Spirulina* have been reported [30].

Desert Lake Technologies, LLC got GRAS notification in 2012 for its CyaninPlus™ product, consistent with section 201(s) of the Federal Food, Drug and Cosmetic Act. It is a water extract of the *Spirulina platensis* or *Spirulina maxima* intended for use as an ingredient in food at levels of up to 250 mg per serving, equivalent to approximately 125 mg of C-PC.

In animal models, C-PC possesses low toxicity and lack of adverse effects. For example, in acute oral toxicity study, the measured LD₅₀ values were estimated to be greater than 3 g/kg for rats and mice, without mortality even at the highest dose of C-PC from *Arthrospira maxima* tested (3 g/kg o.p.). No changes in behavior or histopathology, or effect on body weight were observed [31]. Furthermore, acute and sub-chronic oral toxicity study revealed that C-PC (isolated from *Spirulina platensis*) at high concentrations [0.25–5.0 g/kg body weight (w/w)] did not induce any symptoms of toxicity nor mortality of the albino rats [32]. In human randomized, double-blind, placebo-controlled study, high dose of C-PC-enriched aqueous extract from *Spirulina platensis*, equivalent to ~1 g phycocyanin per day (the highest dose generally recognized as safe by the US FDA), after 2 weeks showed safety regarding anticoagulant activity and platelet activation status markers, but reduced levels of aspartate transaminase and alanine transaminase in conjunction with rapid and robust relief of chronic pain [33]. Unlike cancer cells, C-PC is non-toxic to normal cells, for example, platelets and erythrocytes [34]. Although *Spirulina* is not regarded as source of allergens, there is one case report describing anaphylaxis caused by C-PC [35]. To conclude, animal and clinical scientific studies support that *Spirulina* and C-PC, its most abundant organic component, are safe for human consumption, in agreement by their more than 1000 years use in diet.

Bioavailability is the term used to describe how much of the nutrient are easily absorbed into the body and so is able to have an active effect. *Spirulina* is extremely digestible, high energy but low calorie and low fat natural food. Many studies demonstrated *in vivo* effects of orally administered *Spirulina* or C-PC [1–3]. Our research group has shown that C-PC is rapidly digested by pepsin in simulated gastric fluid, releasing chromopeptides varying in size 2–13 amino acid residues. Released chromopeptides had significant antioxidant activity and metal-chelating property, with cytotoxic effect on cancer cell lines positively correlating their antioxidative capacity, with chromophore portion being most responsible for these effects [36]. There is no literature data related to transport of PC-derived peptides or PCB from gastrointestinal tract to circulation. Our previous studies demonstrated that PCB binds to human serum albumin (HSA) with high affinity ($2.2 \times 10^6 \text{ M}^{-1}$) [37], and stabilizes protein structure

[38], suggesting that in circulation HSA most likely transports PCB to tissues, similar to other bioactive food-derived substances. Many studies observed *in vitro* effects of C-PC in cell culture, but the location of protein inside cells is controversial and it is still unknown whether C-PC requires a transport protein carrier to enter cells. For skin delivery and protection from oxidative stress damage, C-PC is characterized by a reduced bioavailability, due to its high molecular weight, and therefore it is encapsulated in hyalurosomes as carrier [39].

3.3. Interactions with food matrix components

In addition to their sensitivity to light, heat, and oxidants, natural food colors are prone to interact with other food ingredients, especially if they are carrying proteinous component, such as C-PC. Several studies have found that both C-PC and PCB binds to food matrix components, such as proteins, lectins, saccharides, lipids, and polyphenols [40–47].

C-phycoyanin non-covalently binds to bovine serum albumin (BSA), with binding constant $6.8 \times 10^5 \text{ M}^{-1}$ and $n = 1.2$, as determined by fluorescence quenching of BSA. FT-IR, and synchronous fluorescence spectroscopy confirmed the conformation of BSA has been affected the interaction with C-PC [40]. In the recent study, we found that PCB also interact with BSA, showing high affinity binding ($K_a = 2 \times 10^6 \text{ M}^{-1}$), determined by protein fluorescence quenching and microscale thermophoresis. Two binding sites were detected on BSA, at the inter-domain cleft and at subdomain IB, with stereo-selective binding of the *P* pigment conformer to the protein. Although complex formation partly masked the antioxidant properties of PCB and BSA, a mutually protective effect against free radical-induced oxidation was found [41]. Additionally, PCB binding to major whey protein β -lactoglobulin changes its secondary and tertiary structure, causing higher resistance to digestion by pepsin and pancreatin [42].

C-phycoyanin also interacts with food-derived lectins. Jacalin, tumor-specific lectin from cempedak, binds C-PC specifically in a carbohydrate-independent manner, and with affinities better than that for porphyrins. The binding pattern involves both ionic and hydrophobic interactions and more than one contact site [43]. Concanavalin A and peanut agglutinin can also interact with C-PC, although the nature of the interaction is distinctly different from that for jacalin. The legume lectins bind C-PC *via* two distinct sites, and the binding is weaker in the presence of their specific carbohydrate ligands. Therefore, lectins are proposed as useful carrier for targeted delivery of C-PC in photodynamic therapy [44].

Well-known cryoprotecting disaccharide trehalose interacts with C-PC and decreases the internal protein dynamics, slowing down molecular motions responsible for its unfolding and denaturation [45]. Although it was found that C-PC interacts with lipids at the air-water interface, the oxidation of monogalactosyldiacylglycerol could not be prevented by the introduction of C-PC molecules at the lipid-water interface [46]. Formation of complex between C-PC fragments and polyphenols, in order to obtain more stable blue color for application in food, feed, cosmetic, and pharmaceutical products, was recently patented [47].

3.4. Health-promoting effects

A good part of the bioactivity properties of *Spirulina* are assigned to the pronounced antioxidant capacity of C-PC, mainly attributed to its chromophore (PCB) moiety (see below). This

phycobiliprotein has proven (in)dependent therapeutic effects, such as anticancer, antiinflammatory, and antimicrobial effects, immune enhancement function, liver, and kidney protection, among others. These benefits were subject of many recent excellent review articles (e.g. [48]). As potential safe and non-toxic compounds, C-PC and PCB become a new hot spot in the medicine. The complex mechanisms of its pharmacological actions begin to be fully understood at the molecular level. C-phycocyanin is currently not in clinical use, because positive health-related reports are not integrated deeply and accurately enough, putting limitations to its application as a drug. Otherwise, C-PC-encapsulated chitosomes, capable of preserving the protein stability in the gastrointestinal tract and with enhancing efficacy are in development [49].

Phycocyanobilin is potent inhibitor of certain NADPH oxidase isoforms, likely because in mammalian cells it is rapidly reduced to phycocyanorubin, a close homolog of bilirubin. Over-activity of NADPH oxidase causes oxidative stress, and is known to mediate and/or exacerbate numerous pathological conditions [50]. *In vitro*, PCB is capable to modulate other important markers of oxidative stress and endothelial dysfunction, such as eNOS and/or VCAM-1, and to markedly up-regulate heme oxygenase-1, a key enzyme responsible for generation of a potent antioxidant bilirubin [51].

The suitable clinical dose of PCB remains to be defined. Without mass-produced pigment derived from commercially available PCB-enriched *Spirulina* extracts, bioengineered organisms, or chemically synthesized pigment, ingestion of whole *Spirulina* is still the least expensive way to benefit from this phytonutrient. A tablespoon of *Spirulina* powder (about 15 g) contains approximately 100 mg of PCB, daily dose that might be effective [50]. Interestingly, no relevant data about relative absorption and bioefficacy of free PCB or *Spirulina*-bound pigment exist for either rodents or humans.

3.4.1. Antioxidant properties

Proteins bearing colored prosthetic groups, such as a highly conjugated linear tetrapyrrole chromophore in C-PC, can be both the source and target of reactive species in biological systems. An extremely high antioxidant capacity of C-PC was unambiguously established, based on experiments carried out both *in vivo* and *in vitro*. It not only scavenges, for instance, peroxy, hydroxyl, and superoxide radicals, but also inhibits the lipid peroxidation mediated by reactive oxygen species. The bilin group seems to be the main target, since the *in vitro* radical assisted bleaching of PCB color in protein clearly indicates its involvement in the scavenging of reactive species [52]. A key contribution of the structural components and various modulating factors on the antioxidant activity of C-PC will be briefly mentioned here, as they can influence protein utility as a food supplement and therapeutic agent.

C-phycocyanin is a more efficient peroxy nitrite scavenger than free PCB due to (additional) interactions with tyrosine and tryptophan residues of the apoprotein [53]. Differences in the amino acid composition affect C-PC antioxidant capacity. Selenium-C-phycocyanin purified from Se-enriched *Spirulina platensis* exhibits stronger antioxidant free radicals scavenging activity than standard protein, attributed to the incorporation of selenoamino acids into the polypeptide chains of protein [54]. C-phycocyanin (from *Spirulina fusiformis*) exposed to blue light shows better *in vitro* antioxidant property than protein exposed to normal light, due to

marginal changes in the apoprotein cysteine content [55]. Interestingly, bilin group is not the main target of C-PC reaction with hypochlorous acid and singlet oxygen [56].

C-phycoyanin generates hydroxyl radicals in the light, while scavenging them in the dark. Radical generation ability disappears, but scavenging greatly increases in denaturated protein, confirming the role of phycobilin moiety in scavenging. Trypsin hydrolysis of C-PC demonstrated the apoprotein portion also made a significant contribution to the antioxidant activity [57]. The heat denatured (spray-dried) C-PC shows the same level of activity as the intact protein, finding important for preparation and utilization of C-PC [58]. C-phycoyanin can be cloned and expressed in *Escherichia coli*, to reduce the cost and time for protein production. Recombinant holoprotein (α -subunit) not only retained the spectroscopic characteristics of the native protein, but also its bioactive properties, including powerful radical scavenging activity [59]. Although less potent, recombinant apo-C-PC β -subunit acts as an antioxidant on human erythrocytes as well [60]. Other bioactivities of recombinant biliproteins should be further studied to provide additional health benefits.

3.5. Food colorants

In the last decades, consumers are becoming more educated and aware of what they eat, demanding for clean labeling of the food/beverage products and making the pressure to food industry to switch from artificial to natural ingredients and additives. The main consumers of vividly colored food products are children. Due to their low weight, they are at constant risk to exceed recommended daily intake (mg/kg weight) of artificial colorants. Nowadays, the leading confectioners switched to natural colors to avoid obligatory label warning for acceptable daily intake levels of the colorings. Consequently, market of natural food colors is in prominent expansion, expecting to reach \$2.5 billion by 2025.

Compared with other natural pigments, natural blue pigments are rare, because a complex combination of molecular features (such as π -bond conjugation, aromatic ring systems, heteroatoms, and ionic charges) is required to absorb red light (~600 nm region) [61]. Anthocyanins are the primary source of blue color in plants, but their color is pH dependent. On the other hand, fungi and microorganisms produce many blue compounds in response to stress or predators and therefore their unpredictable biological activities make their safety for food use questionable. None of discovered natural blue pigments cannot reach shade, brilliance, vividness, molar absorptivity, and stability of Brilliant Blue FCF (Blue 1 or E133), the most used of approved synthetic blue food colorants, and concomitantly to be safe and cost-effective [61]. In this moment, the only permitted natural blue food colorants are gardenia blue (in Japan), blue anthocyanins and *Spirulina* color (composed mainly of C-PC). Although gardenia blue and blue anthocyanins have better stability to heat and light than C-PC [22], only C-PC can offer brightness, brilliance, and shade most similar to Brilliant Blue FCF, making this protein much more acceptable and ensuring seamless switches from artificial to natural food colors for existing food products. Trichotomine, indole alkaloid from kusagi berries (native in China and Japan), is the most promising natural bright blue colorant due to molar absorptivity ($70,000 \text{ M}^{-1} \text{ cm}^{-1}$) similar to that Brilliant Blue FCF ($134,000 \text{ M}^{-1} \text{ cm}^{-1}$). Limited supply of kusagi berries and the low concentration of pigment make this option economically unjustified [61].

In contrast, although PCB have relatively low molar absorptivity ($37,900 \text{ M}^{-1} \text{ cm}^{-1}$) [37], *Spirulina* can be sustainably produced in huge, almost unlimited amounts, and the high pigment concentration provide its extraction in cost-effective way.

Demand for C-PC as a natural blue food colorant has experienced exponential growth in the past 5 years, especially after FDA approval of *Spirulina* extract as a food colorant for gum and candy in 2013, with market estimated at more than \$50 million. In 2014, its application was expanded to frosting, ice cream and frozen desserts, dessert coatings and toppings, dry beverage mixes and powders, yogurts, custards, puddings, cottage cheese, gelatin, breadcrumb, and ready-to-eat cereals. In 2015, coatings in dietary supplements and pharmaceuticals were also approved and, in this moment, C-PC is the only approved natural blue colorant in the US, Europe, and Asia. In US FDA Code of Federal Regulations, *Spirulina* extract is approved as color additive exempt from certification, prepared by the filtered aqueous extraction of the dried biomass of *Spirulina platensis* and containing phycocyanins as the principal coloring components [62].

Commercial powder formulations of C-PC, such as Linablue® (DIC Corporation, Japan), are declared as completely soluble in cold and warm water and <20% ethanol, making a homogeneous transparent solution, with stable color shade in the pH range 4.5–8.0 (except at C-PC pI value around pH 4.2), which can be improved by the presence of protein-containing ingredients; low thermal stability, which can be improved in high density sucrose solutions; with low light stability, which can be improved in the presence of antioxidant like ascorbate; and with no tongue dyeing effect. In combination of C-PC with red, yellow, and other natural colorants, it is possible to obtain vibrant green, purple, and other natural colors. As FDA is still considering the petition for copper chlorophyllin, natural green food color often involves C-PC or *Spirulina* extract mixed with safflower or turmeric extract (curcumin).

Due to its refreshing ice cool color, C-PC is also increasingly promoted as natural color for alcoholic beverages, such as FIRKIN Blue gin.

In comparison to artificial colors, natural colorings are less vivid, and interactions with food matrix components can result in further decrease in their vibrancy, or unwanted change in color and flavor. For example, our research group observed an instant clear color change from blue to green when PCB interacts with BSA [41]. Therefore, switching from artificial to natural colorings in existing food products can be challenging and complex.

3.6. Functional food additives, nutraceuticals, and dietary supplements

In last decade, there is an increase in chronic diseases and increasing costs of health care due to busy lifestyles and unhealthy nutrition. On the other hand, people are more health conscious and more interested in health-promoting products to improve their health quality. This imposed a demand for functional food ingredients and additives, nutraceuticals and dietary supplements of natural origin.

There are several studies dealing with incorporation of *Spirulina* or its proteins into different food, such as biscuits, pasta, milk-based products, various breads, and crisps, in order to create protein-enriched functional food products. In all these studies the food was fortified by whole *Spirulina* powder or biomass, except in the study where isolated C-PC was used [63]. *Spirulina* was incorporated into pasta (e.g. [64–66]), biscuits and cookies (e.g. [67–69]),

extruded products (e.g. [70, 71]), ice cream [72], yoghurt and acidophilus milk (e.g. [73–74], baby food formulas [75], and bread [76]. However, all these food products were thermally processed leading to destruction of C-PC and resulting in green-yellowish color of product due to partially retained carotenoids and chlorophyll. This fact was ignored in almost all studies, except in the study monitoring C-PC degradation at 615 nm [71].

Although protein component of C-PC added nutritive value to these products, bioactivity of sensitive PCB component cannot be exploited. The only way to take full advantage of health-promoting effects of bilin component, is addition of *Spirulina* biomass/powder, PCB-enriched *Spirulina* or C-PC alone, after all thermal food-processing steps. Similarly, although *Spirulina* fortification of milk have positive influence on viability of milk fermenting microbiota [74], their activity also decrease content of precious bilins. In the most of the studies dealing with *Spirulina* biomass-food enrichment, the limiting factor for quantity of added *Spirulina* was consumer acceptance due to sensory characteristics related to flavor and taste. The use of C-PC or *Spirulina* protein isolate/concentrate fraction would reduce undesirable fishy off-flavor of whole algae biomass and in that way notably improve consumer acceptance.

Possible advantages of joint administration of flavanol-rich cocoa powder and *Spirulina*, or PCB-enriched *Spirulina* extracts was proposed [77]. As inhibitor of NADPH oxidase, PCB would minimize NADPH oxidase-derived oxidative stress, while flavanols would promote vasodilation by up-regulation of $\cdot\text{NO}$ production. Cocoa-*Spirulina* powder blended with milk (cow's, soy, and rice) can yield a drink with a tasty rich chocolate flavor, as cocoa can mask the unpleasant flavor and odor of *Spirulina*/C-PC. These two nutraceuticals could complement each other actions in prevention of senile dementia by optimizing cerebrovascular perfusion, and by suppressing cerebral oxidant stress. Combined supplementation with PCB, citrulline, taurine, and supranutritional doses of folic acid and biotin could help in slowing the progression of diabetic complications, based on their complementary action on the oxidative stress and the associated loss of $\cdot\text{NO}$ bioactivity [78].

Besides being component of many dietary supplements, C-PC also becomes popular component of different wellness bioactive drinks, providing attractive blue color and nutraceutical properties [e.g. Ocean Mist by Allgalio Biotech, B Blue bioactive drink by B blue, Bloo tonic by Cidererie Nicol, Holy water by Juice Generation, Natura blue by Natura4Ever, Smart chimp by Smart chimp and many other drinks based on Blue Majik (C-PC-enriched organic extract of *Spirulina platensis* by E3Live) made by other producers].

Purified PCB is still not available as a nutraceutical supplement, but new research turned toward methods for efficient cleavage of PCB from the C-PC [17]. Further stabilization will enable commercially available PCB as food colorant and dietary supplement.

3.7. Future carriers of bioactive substances and food additives with promising techno-functional and food-preserving properties

In recent years, the food industry is in increasing search for new sources of inexpensive food protein having nutritional and techno-functional characteristics similar to high-cost animal proteins. In addition to plant one, proteins extracted from microalgae are becoming favorable alternative due to availability and sustainability of their production on one hand, and due to their extraordinary nutritional and bioactive properties, as well as suitable functional properties on the other hand.

In this moment, there are only few studies investigating the functional properties of *Spirulina* protein concentrate/isolate, with phycobilins being the main functional protein component. Proteins isolated from *Spirulina* are quite capable of reducing the interfacial tension at the aqueous-air interface at relatively lower bulk concentrations than common food proteins [79]. In comparison with soy protein isolates, *Spirulina* protein isolate (SPI) demonstrates lower water, but higher oil absorption capacity. SPI showed good emulsifying and foaming capacity, and ability to form protein films and gels [80]. This study demonstrated that emulsifying capacity, the emulsion aging stability, the emulsion microstructure and opacity as well as the foaming capacity and the foam stability were pH dependent. Also, emulsifying and foaming capacities have shown to be positively correlated to the protein solubility [80]. *Spirulina* protein isolate forms gels after heating (90°C) and cooling, showing fairly low minimum critical gelling concentrations (1.5 wt% in aqueous solution) compared to other food (soy) proteins [81]. *Spirulina* protein concentrate (SPC) have shown higher emulsifying and similar foaming capacity, when compared with soybean meal [82]. The rheological and textural parameters increased linearly with increased C-PC addition (0.25–1.25% w/w) in oil-in-water emulsions, suggesting C-PC emulsion stabilizing role [83]. From food technology point of view, these studies imply that C-PC or *Spirulina* proteins are promising food ingredients and additives, and that further studies are needed to fully exploit their most likely excellent functional properties.

In contrast to other natural food colors, but similar to other food-derived proteins, C-PC can be used to modify techno-functional properties of food matrices or as carrier of bioactive substances. As a biodegradable, biocompatible, and poor immunogenic protein molecule, C-PC is suitable as carrier for preparation of protein-based nanoparticles. Drug delivery *via* their loading into C-PC nanoparticles have shown to be more effective and safer [84, 85]. By analogy, C-PC-based nanoparticles can be used for food applications in the future as carrier for other active substances, acting together in synergistic manner and complementing mutual benefits. In order to fully utilize all benefits of valued bilin component, C-PC should be added only after all thermal pretreatments.

The natural food colorings are often associated with functional properties. C-Phycocyanin/PCB with their extraordinary antioxidative activities could have role in maintaining of the lipid oxidative stability, especially in food products with high lipid contents. Addition of C-PC was found to inhibit linoleic acid peroxidation and decrease TBARS value in liposome-meat system [86]. Some studies demonstrated that C-PC exhibited antibacterial and antifungal potential [87, 88], suggesting that C-PC/PCB can also serve as antimicrobial agent. Silver nanoparticles-based antimicrobial packaging is a promising form of active food packaging, and C-PC was used for synthesis of bio-silver nanoparticles [89]. Incorporation of *Spirulina* powder in strudels can significantly retard lipid oxidation and reduced the number of yeast and mold resulting in prolonged shelf life [68]. Therefore, C-PC/PCB in addition to their role as food colors can contribute to food preservation and improvement of food shelf life and/or to the reduce addition of non-natural food preservatives.

4. Other applications of C-phycoyanin and phycocyanobilin

As we have seen, C-PC and PCB have excellent antioxidant properties. Irradiation of these molecules with visible light produces reactive oxygen species, making them good candidates for application in photodynamic therapy (PTD). Indeed, it was shown that anticancer activities of C-PC against breast cancer MCF-7 cells increases upon exposure to He-Ne laser (632.8 nm wavelength) [90]. C-phycoyanin has specific affinity for tumor-associated macrophages (TAM), which have been proposed to be a “target for cancer therapy”. Formation of non-covalent conjugate between Zn-phthalocyanine and C-PC resulted in an enhanced photodynamic effect with selective accumulation in the tumor site, probably through the specific binding of C-PC to TAMs [91].

In comparison to other fluorophores, phycobiliproteins have a high molar extinction coefficient and fluorescence quantum yield, as well as a large Stokes shift. Therefore, C-PC could be a good candidate for applications as fluorescent marker. When C-PC is extracted by low ionic strength buffers monomers will be the dominant form, inducing decrease of protein fluorescence. Therefore, in order to obtain stabilized highly fluorescent oligomers cross-linking of C-PC is needed. Chemically stabilized C-PC, fused to the biospecific domains such as streptavidin, is used as a biospecific fluorescent assay. Further, C-PC fluorescence can be also used for *in vivo* monitoring of cyanobacterial growth and detection of toxic cyanobacteria in drinking water [15]. Furthermore, strong quenching of C-PC fluorescence Hg^{2+} ions implies its potential application as biosensor for heavy metals in aquatic systems [92]. Interestingly, PCB synthesized in mammalian cells through metabolic engineering could be useful optogenetic tool for regulation of cell processes by light [19].

Artificial photosynthesis is currently a hot topic in science and technology. Consequently, there are growing demands for designing photoelectron-chemical (PEC) cells, capable to perform artificial photosynthesis. In PEC devices, light-harvesting proteins (such as C-PC) are used to “sensitize” metal and semiconductor surfaces. BioPEC solar hydrogen generator with a hematite-phycoyanin hybrid photo anode was designed. In order to obtain PEC cells with higher performances, the stability of immobilized C-PC needs to be improved [93].

Beside the application for food and drink coloring, C-PC is also used as a cosmetics colorant in lipsticks, eyeliners, and eye shadows preparations [94].

5. Conclusion

The vast majority of studies regarding *Spirulina* bioactive components used whole algal (dried) mass or its aqueous extracts. Major *Spirulina* deep blue color protein C-phycoyanin and its bilin chromophore have remarkable potential for use in food technology, as safe food colorant, functional food additive, nutraceutical and/or dietary supplement, given their excellent health-related properties, and opportunity for sustainable and relatively inexpensive mass production.

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