Peer-reviewed version available at *Molecules* **2018**, *23*, 2065; doi:10.3390/molecules23082065

Article

Two Classes of Pigments, Carotenoids and C-Phycocyanin, in Spirulina Powder and Their Antioxidant Activities

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Running title: Two classes of pigments in spirulina

Abstract: *Arthrospira platensis* is the widely available source of spirulina and contains distinctive natural pigments including carotenoids and C-phycocyanin (C-PC). In this study, the major carotenoid and C-PC contents were determined in seven commercially available spirulina powder products and laboratory-prepared *A. platensis* trichomes (AP-1) by an LC-DAD method and a UV-Visible spectrometry, respectively. The correlation of these two pigment content levels with Hunter color coordinates and antioxidant activity was also evaluated. The *L** value failed to show a significant correlation with pigment content, but a positive correlation was observed between *a** values and the contents of total carotenoid and C-PC. As *b** values decreased, the total carotenoid and C-PC contents increased. AP-1 exhibited the highest content of total carotenoids, chlorophyll a and C-PC, and antioxidant activities among the samples. This observation could be related to degradation of these pigments during the mass production process. The carotenoid profiles suggested that the commercial spirulina powders originated from two different sources, *A. platensis* and *A. maxima*. Total carotenoid and C-PC content exhibited positive significant correlations with antioxidant activities measured by DPPH and ABTS assays. These results provide a strong scientific foundation for the establishment of standards for the commercial distribution of quality spirulina products.

Keywords: *Arthrospira platensis*; carotenoids; natural pigments; spirulina powder; C-phycocyanin; antioxidant activity

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1. Introduction

Most commercial spirulina powder is composed of dried bodies of *Arthrospira* (*Spirulina*) *platensisis* or *A. maxima* [1]. These species, also known as spirulina, are gram-negative, nontoxic species of cyanobacteria that are widely used as food supplements or natural additives. Spirulina is regarded as an ideal food and drug quality resource due to its high content of protein, lipid, vitamins, minerals, chlorophyll, β-carotene, and polysaccharides. With two distinctive natural colors, carotenoids and C-phycocyanin [2,3], spirulina use has aligned with consumer awareness regarding the importance of natural colors agents from their nutritional, pharmacological and health-related benefits. As a result, the number of applications of natural pigments and spirulina as a source of these pigments is increasing, especially in the food and cosmetic industries [4].

Carotenoids are a class of natural lipid-soluble pigments that are responsible for the red, yellow, and orange colors found in various plants and microorganisms. They function primarily as photosynthesis aids and are used in the photoprotection process. Humans and other animals are unable to synthesize carotenoids and acquire them through alimentation. Carotenoids are used in food and feed as colorants and flavorings

and in nutritional supplements as a source of provitamin A [1]. The health benefits of carotenoids to humans and animals are becoming increasingly apparent. For example, there is evidence that these pigments may protect humans from serious disorders associated with oxidative and inflammatory stress including skin degeneration and aging, cardiovascular disease, certain types of cancer, and age-related diseases of the eye, such as macular degeneration or cataracts [5–7].

C-phycocyanin (C-PC) is a hydrophilic and intense blue-colored biliprotein found in blue green algae. C-PC comprises a protein and the chromophore phycocyanobilin. The protein moiety consists of alpha and beta subunits with molecular weights near 18,000 and 20,000 Da, respectively. This colorant is highly stable in the pH range of 5-8 and exhibits a strong red fluorescence when present in its native form [4]. The biological activities of C-PC are wide-ranging and include antioxidant, antimutative, antiviral and antitumor properties. C-PC has also been shown to stimulate the immune system and exhibit hepatoprotective, antiplatelet, and neuroprotective activities [8–10]. It is believed that the various health benefits of spirulina are derived from oxidative stress reduction [11,12], and β-carotene and C-PC are important contributors to this effect [13].

Meanwhile, these pigments can be easily degraded during the mass production

process using sunlight or hot-air dry. In order to provide improved characterization of available spirulina ingredients to manufacturers and scientist, the carotenoid and C-PC content levels were determined for seven commercially available spirulina powder products and for freeze-dried *A. platensis* trichomes cultured in our laboratory which could give best condition for retaining these pigments. Additionally, the correlation of pigment levels with the Hunter Lab color parameters was assessed as was the relationship of the pigments to antioxidant activity as a method to better understand predictive quality factors for these ingredients.

2. Results

2.1. Colorimetric evaluation of spirulina powder

Because carotenoids and C-PC are pigments, a colorimetric evaluation was performed on the spirulina powders obtained from *Athrospira platensis* cultivated in our laboratory (AP-1) or purchased from on-line and local stores (C1–C7). All of the powders were dark green and differed slightly from each other in the Hunter color coordinates of brightness (L^*), redness (a^*) and yellowness (b^*). The highest L^* and

102 *b** values were obtained in C6, with C7 displaying the second highest *L** value (Table
103 1). All samples showed negative *a** values due to the green color, and the mean *b**
104 values were between 6.3 and 13.3.

105

106 *2.2. Carotenoid and C-PC content in spirulina powder*

107

108 Calibration curves were constructed by analyzing a mixture containing five
109 carotenoids at various concentration levels and plotting peak area against the
110 concentration of each reference standard (Table 2). The curves showed good linearity,
111 and the correlation coefficients were between 0.997 and 0.999 for all of the compounds
112 over the concentration ranges of quantification. The recovery of four carotenoids
113 excepting diatoxanthin was assessed by spiking samples with high and low
114 concentrations of each reference compound, 1,000 and 30 ng, respectively. Spiking
115 with 19.2 and 2.8 ng was done for diatoxanthin. The average recoveries were between
116 85.6% and 107.4% (n = 3). The limits of detection (LOD) were determined by serial
117 dilution based on a signal-to-noise (S/N) ratio of 3:1 (Table 2). The peak purity was
118 determined by the photodiode array detector and the corresponding computer software
119 that confirmed the singularity of each peak. In addition, the absorption spectrum of

120

121 **Table 1.** Hunter values and pigment contents in spirulina samples, and their antioxidant activities.

Items	Samples							
	AP-1	C1	C2	C3	C4	C5	C6	C7
Hunter Values								
*L**	19.2 ± 0.7^{c}	18.8 ± 0.5^{c}	16.9 ± 0.9^{d}	17.4 ± 0.9^{d}	16.3 ± 0.1^{d}	14.1 ± 0.6^{e}	28.0 ± 1.2^{a}	24.4 ± 0.1^{b}
*a**	-7.4 ± 0.1^{b}	-7.2 ± 0.1^{b}	-6.8 ± 0.2^{a}	-9.0 ± 0.1^{d}	-8.1 ± 0.3^{c}	-7.5 ± 0.2^{b}	-9.5 ± 0.0^{e}	-10.0 ± 0.0^{f}
*b**	6.9 ± 0.1^{d}	8.4 ± 0.1^{b}	7.0 ± 0.2^{d}	8.7 ± 0.4^{b}	8.7 ± 0.4^{b}	6.3 ± 0.1^{e}	13.3 ± 0.3^{a}	8.0 ± 0.1^{c}
Carotenoids (μg/g)								
All-*trans*-zeaxanthin	1266.7 ± 118.1^{a}	535.3 ± 84.8^{b}	694.0 ± 113.9^{b}	132.7 ± 16.8^{e}	216.4 ± 34.4^{d}	89.5 ± 15.4^{f}	307.5 ± 13.8^{c}	115.1 ± 12.5^{e}
Diatoxanthin	263.2 ± 40.8^{a}	136.3 ± 24.5^{b}	166.5 ± 30.9^{b}	50.7 ± 9.8^{d}	59.5 ± 3.0^{d}	49.4 ± 5.9^{d}	79.8 ± 5.4^{c}	87.5 ± 10.1^{c}
13–*cis*-*β*-Carotene	55.7 ± 9.6^{a}	21.7 ± 0.3^{c}	36.5 ± 3.9^{b}	8.3 ±1.2^{d}	5.7 ± 0.3^{e}	9.7 ± 1.4^{d}	18.4 ± 3.4^{c}	6.7 ± 0.7^{e}
All-*trans*-*β*-carotene	2296.5 ± 51.3^{a}	716.2 ± 25.3^{b}	923.1 ± 174.4^{b}	83.1 ± 14.0^{d}	162.2 ± 16.7^{c}	155.4 ± 23.7^{c}	189.6 ± 16.2^{c}	23.6 ± 3.2^{e}
9–*cis*-*β*-Carotene	381.6 ± 49.0^{a}	165.5 ± 20.3^{c}	249.6 ± 31.2^{b}	50.7 ± 5.2^{e}	69.2 ± 13.4^{e}	64.7 ± 10.8^{e}	89.4 ± 4.9^{d}	3.7 ± 0.3^{f}
Others	165.2 ± 13.2^{a}	124.2 ± 9.8^{b}	161.2 ± 4.1^{a}	56.2 ± 1.8^{d}	69.0 ± 9.2^{c}	46.3 ± 3.7^{e}	78.3 ± 2.5^{c}	46.7 ± 6.5^{e}
Total carotenoids (mg/g)	4.43 ± 0.03^{a}	1.70 ± 0.15^{c}	2.23 ± 0.35^{b}	0.38 ± 0.03^{f}	0.58 ± 0.05^{e}	0.41 ± 0.05^{f}	0.76 ± 0.03^{d}	0.28 ± 0.02^{g}
Chlorophyll a (mg/g)	10.8 ± 1.1^{a}	3.4 ± 0.3^{c}	4.7 ± 0.6^{b}	3.5 ± 0.6^{c}	2.7 ± 0.2^{d}	3.3 ± 0.4^{c}	2.6 ± 0.0^{d}	3.6 ± 0.5^{c}
C-Phycocyanin (mg/g)	251.2 ± 11.2^{a}	100.2 ± 1.1^{d}	153.3 ± 2.3^{b}	106.5 ± 1.5^{c}	113.4 ± 9.1^{c}	144.8 ± 4.1^{b}	94.9 ± 6.3^{d}	108.6 ± 0.7^{c}
Anti-oxidant activity of carotenoid extracts								
Total phenolics (μmol GAE/g)	1.3 ± 0.1^{d}	2.3 ± 0.4^{c}	1.5 ± 0.2^{d}	4.6 ± 0.6^{b}	2.9 ± 0.5^{c}	6.4 ± 0.6^{a}	2.9 ± 0.4^{c}	2.5 ± 0.0^{c}
Total flavonoids (μmol QE/g)	26.6 ± 2.4^{a}	22.8 ± 3.4^{a}	24.0 ± 1.0^{a}	15.6 ± 0.9^{b}	15.0 ± 2.4^{b}	12.9 ± 0.4^{c}	18.5 ± 1.9^{b}	24.0 ± 2.9^{a}
DPPH (μmol TE/g)	18.5 ± 0.5^{a}	8.4 ± 1.3^{c}	10.3 ± 1.1^{b}	6.1 ± 0.8^{d}	5.8 ± 1.2^{d}	5.2 ± 0.8^{d}	7.3 ± 0.4^{c}	6.4 ± 0.8^{d}
ABTS (μmol TE/g)	33.7 ± 5.0^{a}	18.7 ± 3.9^{b}	23.6 ± 1.6^{b}	24.8 ± 4.2^{b}	21.4 ± 3.6^{b}	18.1 ± 2.0^{b}	19.3 ± 3.1^{b}	19.3 ± 3.2^{b}
Antioxidant activity of C-PC extracts								

Total phenolics (μmol GAE/g)	82.1 ± 4.8^{a}	66.2 ± 4.5^{bc}	70.6 ± 2.6^{b}	47.0 ± 5.4^{c}	53.9 ± 8.8^{c}	62.4 ± 4.8^{bc}	64.3 ± 6.2^{bc}	47.6 ± 0.8^{c}
Total flavonoids (μmol QE/g)	ND	ND	ND	ND	ND	ND	ND	ND
DPPH (μmol TE/g)	18.7 ± 0.2^{a}	14.8 ± 1.4^{b}	19.1 ± 1.5^{a}	18.1 ± 1.6^{a}	15.5 ± 2.3^{b}	16.2 ± 1.2^{b}	16.5 ± 1.1^{b}	14.4 ± 0.9^{b}
ABTS (μmol TE/g)	108.3 ± 10.2^{a}	66.6 ± 3.3^{c}	83.4 ± 5.1^{b}	42.7 ± 4.4^{e}	49.4 ± 1.5^{d}	51.7 ± 2.7^{d}	47.3 ± 6.1^{de}	42.5 ± 6.2^{e}

*L**, lightness; *a**, + red – green; *b**, + yellow – blue (CR-400, Minolta); GAE, gallic acid equivalent; QE, quercetin equivalent; TE, trolox equivalent.
Data are expressed as the mean (the average value of content for dry weight) and SD (the standard deviation value) of three independent experiments. Different letters in the same row mean significantly different (p<0.05). ND, not detected.

127 **Table 2**. Linear ranges and correlation coefficients of calibration curves.

Compounds	Range (μg/mL)	Slope (a)[a]	Intercept (b)[b]	Regression (r^2)	LOD (ng)
All-*trans*-zeaxanthin (**1**)	0.40-25.0	132.4	14.2	0.9991	~2
Chlorophyll a (**2**)	2.00-250	27.0	-55.6	0.9993	~20
Diatoxanthin (**3**)	0.14-0.69	141.4	0.80	0.9990	~1
13-*cis*-β-Carotene (**4**)	0.08-12.5	128.5	15.0	0.9996	~1
All-*trans*-β-carotene (**5**)	0.14-100	122.6	4.10	0.9997	~1
9-*cis*-β-Carotene (**6**)	0.08-12.5	124.4	6.00	0.9968	~1

128 [a,b] Slope and intercept represent a and b in $Y = ax + b$ linear model. Y means peak area and x,
129 concentration.

each peak was compared with the characteristics of each standard compound.

All-*trans*-*β*-carotene, all-*trans*-zeaxanthin, 9-*cis*-*β*-carotene and diatoxanthin were found to be the major carotenoids present in spirulina (Figure 1). 13-*cis*-*β*-Carotene was also detected. The content of all-*trans*-*β*-carotene was highest among the four major carotenoids in AP-1, C1, C2 and C5 while that of all-*trans*-zeaxanthin was highest in the remaining samples. AP-1, C1 and C2 contained more than 1.6 mg/g dry weight of total carotenoids while the other samples contained less than 0.8 mg/g total carotenoid content. AP-1 showed the highest total carotenoid content of 4.43 ± 0.03 mg/g.

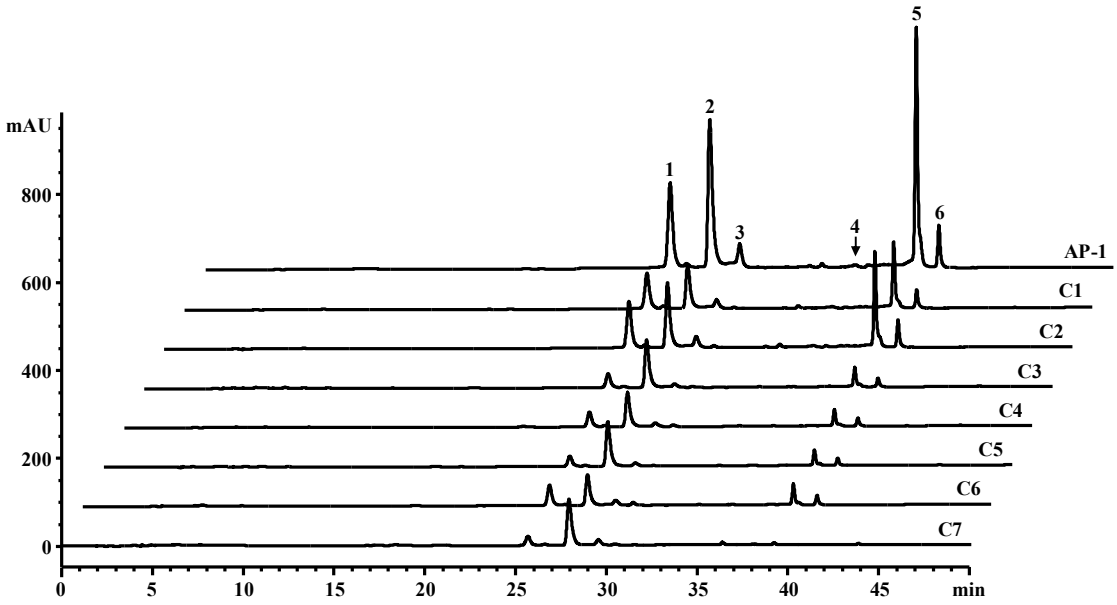

Figure 1. LC chromatogram of carotenoid extract from spirulina samples (450 nm). Peaks **1**-**6** correspond to all-*trans*-zeaxanthin, chlorophyll a, diatoxanthin, 13-*cis*-*β*-carotene, all-*trans*-*β*-carotene and 9-*cis*- *β*-carotene, respectively.

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The green photosynthetic pigment chlorophyll a which is essential for photosynthesis in cyanobacteria as primary electron donor was also determined at the same LC conditions used for carotenoid analysis with a different wavelength. AP-1 showed the highest level of chlorophyll a, 10.8±1.1 mg/g, while the mean values in the other samples were between 2.6-4.7 mg/g.

C-PC, a major biliprotein of spirulina, was extracted by grinding the sample powder with sea sand and sonication at 4°C. The extraction efficiency observed at pH 7 was higher than at pH 4 and 10 (data not shown). AP-1 showed the greatest amount of C-PC, 251.2±11.2 mg/g, and the mean values in the commercial spirulina samples were between 94.9-153.3 mg/g.

Among the three major pigments in spirulina, the content of C-PC was highest with a value of 10-25% (w/w). The average percentages of chlorophyll a and carotenoids were 0.26-1.1% and 0.03-0.38%, respectively. The total carotenoid content varied by up to eight-fold among the commercial samples, while the content variations of chlorophyll a and C-PC were 1.8 and 1.6-fold, respectively.

2.3. Antioxidant activity of carotenoid and C-PC extracts

Carotenoids, phycocyanins and spirulina are known to have potent antioxidant activity. DPPH and ABTS methods were applied to determine antioxidant activity of carotenoid and C-PC extracts obtained from spirulina samples. The C-PC extract showed higher antioxidant activities than the carotenoid extract in the DPPH and ABTS assays. The carotenoid extract of AP-1 with the highest content of total carotenoids and chlorophyll a exhibited the highest DPPH and ABTS radical scavenging activities among the tested carotenoid extracts. AP-1 with the highest content of C-PC also showed the highest antioxidant activities among the C-PC extract

174 samples. DPPH and ABTS assays displayed slightly different antioxidant activity
175 pattern in carotenoid and C-PC extracts, respectively.

176 The total phenol and flavonoid contents were evaluated to investigate the effect of
177 phenolic and flavonoid compounds on the antioxidant activities of these extracts. The
178 results showed that the mean value of total phenol content in the carotenoid extract was
179 in the range of 1.3-6.4 µmol gallic acid equivalents (GAE)/g dry weight, which was
180 much lower than that of 47.0-82.1 µmol GAE/g observed in the C-PC extract (Table 1).
181 Additionally, significant negative correlation were observed between total phenolic
182 content in the carotenoid extract with the content of carotenoids, chlorophyll a and
183 flavonoids, and DPPH radical scavenging activity of the extract (Table 3). Meanwhile,
184 the mean flavonoid content in the carotenoid extract was in the range of 12.9-26.6
185 quercetin equivalents (QE)/g and displayed significant positive correlations with
186 carotenoid and chlorophyll a contents and DPPH radical scavenging activity of the
187 extract. Flavonoids were not detected in the C-PC extract. Total phenol content of the
188 C-PC extract exhibited significant positive correlations with the content level of three
189 pigments and antioxidant activities of the extract.

Table 3. Pearson's correlation coefficients of Hunter values, pigment contents and antioxidant activities.

Traits	TC	CA	PC	TP-C	TF-C	DPPH-C	ABTS-C	TP-P	DPPH-P	ABTS-P
*L**	-0.12	-0.11	-0.28	-0.29	0.25	-0.01	-0.12	-0.09	-0.24	-0.25
*a**	0.58**	0.39	0.50*	-0.12	0.13	0.42*	0.22	0.66**	0.39	0.68**
*b**	-0.32	-.041*	-0.54**	-0.10	-0.15	-0.26	-0.24	-0.19	-0.21	-0.42*
TC		0.92**	0.86**	-0.63**	0.66**	0.97**	0.71**	0.83**	0.50*	0.95**
CA			0.94**	-0.45*	0.55**	0.94**	0.80**	0.68**	0.50*	0.86**
PC				-0.30	0.42*	0.87**	0.74**	0.71**	0.53**	0.86**
TP-C					-0.82**	-0.63**	-0.37	-0.40	-0.12	-0.57**
TF-C						0.67**	0.31	0.44*	0.07	0.60**
DPPH-C							0.77**	0.80**	0.52**	0.90**
ABTS-C								0.43*	0.53**	0.65**
TP-P									0.55**	0.83**
DPPH-P										0.49*

TC, total carotenoid content; CA, chlorophyll a content; PC, phycocyanin content; TP-C, total phenolic content in carotenoid extract; TF-C, total flavonoid content in carotenoid extract; DPPH-C, antioxidant activity of carotenoid extract on DPPH assay; ABTS-C, antioxidant activity of carotenoid extract on ABTS assay;; TP-P, total phenolic content in C-PC extract; TF-P, total flavonoid content in C-PC extract; DPPH-P, antioxidant activity of C-PC extract on DPPH assay; ABTS-P, antioxidant activity of C-PC extract on ABTS assay.

*Significant at $P<0.05$. **Significant at $P<0.01$.

3. Discussion

Spirulina has been used as an additive in a variety of health foods and animal feeds, and has been produced commercially for the last 30 years for these purposes. Commercially grown spirulina is normally produced in large outdoor ponds under controlled conditions or harvested directly from lakes [1]. It contains substantial amount of two distinctive antioxidant pigmented antioxidants: the yellow-to-red carotenoids and blue C-phycocyanin (C-PC). In this study, the major carotenoid and C-PC content levels were determined using an LC-DAD method and a UV spectrometer, respectively, for seven commercially available spirulina powder products (C1-C7) with freeze-dried *A. platensis* trichomes cultured in our laboratory (AP-1). The correlation of the content level of these two pigments with a visible parameter and antioxidant activity was also evaluated.

The L^* value failed to show a significant correlation with pigment content or antioxidant activities (Table 3). As the a^* values (redness) increased, the total carotenoid and C-PC content increased. The redness value displayed a non-significant correlation with chlorophyll a content. As the b^* values (+ yellow – blue) decreased, the total carotenoid and C-PC content of the extracts increased.

There were substantial differences in carotenoid, chlorophyll a and C-PC content between the lyophilized sample AP-1 and commercially available spirulina powders (C1-C7). AP-1 was cultivated and freshly prepared in our laboratory. The total carotenoid, chlorophyll a and C-PC contents observed in AP-1 were the highest seen among the eight samples. AP-1 also exhibited the highest antioxidant activities among the samples. The overall lower amounts of carotenoid, chlorophyll a and C-PC contents found in commercial spirulina powder compared to AP-1 could stem from pigment degradation during the mass production process, as the natural pigments are sensitive to light, heat and oxygen. It has been reported that the carotenoid composition differences of dried commercial spirulina preparations are most likely due to their thermolability [14]. While reportedly stable over a pH range of 5-7.5 at 9 ± 1°C, thermal instability of C-PC obtained from *A. platensis* above 40°C has been observed [15]. Chlorophyll degradation is known to occur during the roasting process [16]. A solar or hot-air based drying process is utilized in the mass production of most commercial spirulina powders, which could decrease the pigment content of the powder. Additional food processing techniques and storage conditions can have a similar effect. The antioxidant potential of *A. platensis* powder was easily degraded after exposure of the biomass to heat and light [17].

The content of all-*trans*-*β*-carotene was highest among the four major carotenoid ingredients of the spirulina powder samples in AP-1, C1, C2 and C5 while that of all-*trans*-zeaxanthin was highest in the other samples. Spirulina are multicellular and

filamentous blue-green microalgae belonging to the two separate genera *Spirulina* and *Arthrospira*, which consist of approximately 15 species. Of these, *Arthrospira platensis* is the most common and widely available source of spirulina and most of the published research and public health decisions refer to this specific species [18]. Therefore, *A. plantensis* was chosen and cultivated in this study as a control. However, two *Arthrospira* species, *A. platensis* and *A. maxima*, have been used as a food source, dietary supplement, and feed supplement [1]. It has been reported that the content of zeaxanthin was higher than that of *β*-carotene in a dried *A. maxima* sample [14]. Therefore, the difference in carotenoid profiles between the two groups might result from the fact that the group including AP-1 belonged to *A. platensis* and the other group belonged to *A. maxima*. Contrary to previous findings, *β*-cryptoxanthin was not detected in the spirulina powder used in this study [2,14]. Instead of *β*-cryptoxanthin, diatoxanthin was detected along with 9-*cis*-*β*-carotene and 13-*cis*-*β*-carotene (Fig. 1) [1,18].

Total carotenoid content showed high correlation coefficients of 0.92 and 0.86 to chlorophyll a and C-PC content, respectively. The total carotenoid content also exhibited greater positive correlations with antioxidant activities than the total flavonoid contents of the carotenoid extract. These strong correlations suggest that the major antioxidant compounds in the carotenoid extract are carotenoids, not phenols or flavonoids, although phenols and flavonoids are also known antioxidants, and the flavonoid content level contributed in portion to the antioxidant activity of the samples with low content of total carotenoids. Chlorophyll a and C-PC contents also showed significant positive correlations, respectively, with the other two pigment contents and antioxidant activities. Compared to the C-PC content in C-PC extract, the total phenol content showed similar positive correlations with antioxidant activities of this extract. This result suggests that the phenol compounds in the C-PC extract also contributed to

the antioxidant activity in portion, although C-PC is the highest abundant and major antioxidant ingredient of this extract.

The antioxidant activities against DPPH and ABTS radicals displayed overall significant positive correlations to each other in both carotenoid and C-PC extracts. While in the carotenoid extract DPPH assay exhibited greater correlations with the lipophilic total carotenoid, chlorophyll a and flavonoid contents than ABTS assay, in the C-PC extract ABTS assay showed higher positive correlations with the hydrophilic C-PC and total phenolic contents than DPPH assay. These results provide a scientific foundation for proper identification and establishment of standards for distribution of quality commercial spirulina products. Future studies will evaluate the stability of these natural pigments in spirulina powder during storage under various conditions.

4. Materials and Methods

4.1. Sample preparation

Athrospira platensis (Nordstedt) Gomont (P_PS_00001194) was provided by the Korea Institute of Ocean Science and Technology (Geoje, Korea). Cultivation was conducted for a period of 10 days in 500 ml baffled flasks containing 300 ml of Zarrouk's medium (Sigma, St. Louis, MO, USA) with stirring at 120 rpm at 25°C under 130 μmol/m^2 s illumination. Cells were harvested by centrifugation (Vision Scientific Co., Ltd., VS-24SMTi, Daejeon, Korea) at 3,000 ×g for 5 min at 4°C and freeze-dried (ilShin BioBase Co., Ltd., TFD 5503, Korea, -40°C). The lyophilized sample (AP) was stored at -80°C until analysis was completed. The seven commercially available spirulina powder samples (C1-C7) were purchased from various online and in-store sources. The cultivation regions of these products included

Australia, China, India, New Zealand and USA.

4.2. Colorimetric evaluation

Color attributes of samples were measured with a colorimeter (Konica Minolta CR-400, Osaka, Japan). Prior to testing, the colorimeter was calibrated using a Minolta standard white reflector plate. Color measurements were taken between three and five times per sample, depending on the portion of each powder. The data were presented as L^* (lightness), a^* (redness), and b^* (yellowness) values according to the Hunter color system.

4.3. Carotenoid analysis

All extraction procedures were performed under subdued light to avoid pigment degradation. Two hundred and fifty milligrams of the lyophilized samples were homogenized using a pestle in a prechilled mortar with 1 mL of acetone (stabilized with 0.01% butylated hydroxytoluene, BHT), sea sand, Na_2SO_4 and $NaHCO_3$. The solution was transferred to a 10 mL conical tube and sonicated three times for 10 min. The extract was centrifuged at 5,700 *g* at 4°C for 10 min (Eppendorf, 5430R, Hamburg, Germany), and 5 mL of the supernatant was dried under a stream of N_2 gas and dissolved in 500 µL of a CH_2Cl_2 and acetone mixture (1:1, v/v). This sample solution was filtered through a 0.45 µm membrane filter (Whatman, PTFE, 13 mm) prior to LC analysis.

Carotenoid analysis was conducted according to our previously reported method [19] using an Agilent 1260 HPLC system (Hewlett-Packard, Waldbronn, Germany). Chlorophyll a was also quantified using the same LC conditions, with UV detection set

318 at 430 nm. Under these conditions, standard compound peaks eluted at the following t_R
319 (min): 25.0 for all-*trans*-zeaxanthin, 27.2 for chlorophyll a, 28.9 for diatoxanthin, 36.3
320 for 13-*cis*-*β*-carotene, 39.0 for all-*trans*-*β*-carotene and 40.3 for 9-*cis*-*β*-carotene.
321 Methanol, water and methyl-*tert*-butyl ether used in the HPLC system were all of
322 HPLC grade, and all other chemicals were extra grade. A stock solution of diatoxanthin
323 (0.691 mg/L) was purchased from DHI (Hoersholm, Denmark), and the other
324 carotenoids and chlorophyll a were purchased from *CaroteNature* GmBH (Switzerland)
325 and Sigma (St. Louis, MO, USA), respectively.
326
327 *4.4. C-PC analysis*
328
329 C-PC was quantified by UV-Visible electronic absorption using an external
330 calibration method [4]. Briefly, one hundred milligrams of the lyophilized samples
331 were ground with seasand and extracted by four times sonication for 30 min each with
332 30 mL of PBS (pH 7.0) buffer solution. The extract was centrifuged at 20,000 *g* at 4°C
333 for 6 hours and the supernatant was filtered through a 0.45 μm membrane filter
334 (Whatman, PTFE, 13 mm). Two hundred microliters of each extract was transferred to
335 a 96 well plate, where a microplate reader (BioTek, Synergy H1, Winooski, VT, USA)
336 was used to detect the absorbance at 620 nm. Working calibration solutions in the range
337 of 0.5-5 mg/mL were prepared by diluting the stock solution of C-phycocyanin (Sigma,
338 St. Louis, MO, USA) with PBS buffer. All of the procedures were performed under
339 subdued light to avoid pigment degradation as described above.
340
341 *4.5. Total phenol and total flavonoid contents*
342
343 The total phenol content of the extract was determined spectrophotometrically

according to our previously described method [20], which is a slightly modified form of the Folin-Ciocalteu colorimetric method [21]. One hundred microliters of the extract was mixed with 500 µL of Folin-Ciocalteu solution (Merck, Darmstadt, Germany) and 400 µL of a 200 mM sodium carbonate solution. After mixing, the solution was centrifuged at 3,000 *g* for 5 min. Two hundred microliters of the upper layer were transferred to a 96-well plate and the absorbance was measured at 765 nm with a microplate reader (BioTek, Synergy H1, Winooski, VT, USA). Using a gallic acid (Sigma, Hong Kong, China) calibration standard, the results were expressed as µmol gallic acid equivalents per g (µmol GAE/g).

The total flavonoid content was determined using a diethylene glycol colorimetric method [21] using quercetin as the standard. Sample extracts (20 µL) were added to 170 µL of 90% diethylene glycol and 10 µl of a 4 M NaOH solution. The absorbance was measured at 420 nm after 10 min. The results were expressed as µmol quercetin (Sigma-Aldrich, Munich, Germany) equivalents per gram (µmol QE/g).

4.6. Antioxidant activity test with DPPH radical

The scavenging activities of the samples on 1,1-diphenyl-2-picrylhydrazyl (DPPH) were measured using a slightly modified form of our previously reported method [20]. The radical scavenging activity was expressed as Trolox (Sigma, St. Louis, MO, USA) equivalents per gram (µmol TE/g).

4.7. Antioxidant activity test with ABTS radical

The ABTS radical was generated using a previously reported method [22]. Each extract (20 µL) was reacted with 180 µL of the $ABTS^{\bullet+}$ solution at room temperature,

and the absorbance was measured at 734 nm after 10 min. The antioxidant activity of each sample was expressed as Trolox (Sigma, USA) equivalents per gram (μmol TE/g).

4.8. Statistical analysis

All of the contents and the antioxidant activities are expressed as the means ± standard deviations (SD) of triplicate determinations. The differences among samples were statistically evaluated via one-way analysis of variance (ANOVA). The values were evaluated at the 5% significance level using two-sided tests. Pearson's correlation coefficients were obtained using IBM SPSS Statistics 24.0 software (Armonk, NY, USA).

Author Contributions: Conceptualization, S.-S.K., M.G.F. and M.-J.A.; Data curation, D.H.L.; Funding acquisition, J.K., S.-S.K., C.M.K. and M.-J.A.; Investigation, W.S.P. and M.L.; Methodology, W.S.P., H.-J.K., and M.G.F.; Resources, J.K. and C.M.K.; Validation, H.-J.K. and D.H.L.; Writing–original draft, W.S.P. and H.-J.K.; Writing–review and editing, M.L., M.G.F. and M.-J.A.

Funding: This study was supported by the Korea Institute of Toxicology (Grant KK-1709) and the Next-Generation BioGreen 21 Program, Rural Development Administration, Republic of Korea (SSAC, Grant# no. PJ01318402).

Conflicts of Interest: The authors declare no conflicts of interest.

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