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Distribution of Chlorophylls and Carotenoids in the Different Parts of Thallus Structure from Three *Sargassum* spp. as Revealed by Multi-Chromatograms HPLC Approach

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ABSTRACT: Macroalgae such as *Sargassum* sp. has a unique morphology. Its thallus consists of three different parts: stem-like, leaf-like, and the vesicles. Each part of the thallus contains photosystems, which support its photosynthetic growth. Here we report the distribution of chlorophylls and carotenoids in the different part of thallus from three *Sargassum* spp. variants. We found that the dominant pigments are chlorophyll a and fucoxanthin. The chlorophyll c, siphonein, violaxanthin, flavoxanthin, zeaxanthin, and β-carotene were also found as accessory pigment. Prior to analysis, we develop multi-chromatograms approach by employing reversed-phase high-performance liquid chromatography (HPLC) with photodiode array (PDA) detection, which able to extract directly spectroscopic data from 350 to 700 nm wavelengths.

Keywords: *Sargassum* sp., chlorophylls and carotenoids, HPLC-PDA, multi-chromatograms

INTRODUCTION

Macroalgae (seaweeds) are one of the important marine resources in Indonesia as the largest archipelagic country in the world. It has been industrially explored as food products, source of phycocolloids (alginate, carrageenan and agar), thickening and gelling agents for biomedical and pharmaceutical applications, as well as being a material for animal feed and fertilizers.[1,2] Besides its special polysaccharides, seaweeds are also rich in varied photosynthetic pigments in almost whole of its thallus structure. The chlorophyll *c* found in seaweed has been initiated as a material for dye-sensitized solar cells.[3–5] Moreover, the carotenoids have several important biological activities such as antioxidant,[6] anticancer, antiinflammation and antiobesity.[7,8]

Unlike most macroalgae, *Sargassum* sp. is morphologically differentiated and relatively complex. It has specialized anatomical features such as leaf-like blades, stems, vesicles (air bladders), fruiting branches and a holdfast (attachment structure). Every parts of its thallus may support the photosynthesis. Previous studies have reported the functional-morphological relationship in photosynthesis as well as the variation of pigment content in the different part of thallus structure.[9–13] However, the photosynthetic pigments content of each part still need to be completely chromatographically characterized.

As noted by Nielsen et al.,[14] chromatographic data are most in studies transformed to retention time-peak area data matrices including only selected parts (wavelengths) detected in the chromatograms, which means a loss of data and introduces the problem of extracting peak data from the chromatographic profiles. In other words, the quality of the data will rely on wavelength selection and the sensitivity of peak detection for the data analysis. In the case of photosynthetic pigments, each pigment has very specific maximum absorption throughout the range of visible light and therefore the quantification of all chromatographed pigments should be more representative if we use the entire chromatographic data matrices. In the present study, we developed multi-chromatograms approach to extract the peak area data of several main detected pigments in order to study the distribution of

chlorophylls and carotenoids in the different parts of thallus structure from three *Sargassum* spp., and subsequently we also compared the result with those of the conservative method, which uses only one selected wavelength.[15]

METHODS AND EXPERIMENTAL DETAILS

Field Sampling

The three brown seaweeds, *Sargassum filipendula* C. Agardh, *Sargassum polycystum* C. Agardh and other *Sargassum* sp. (which has not yet been identified into species name) were collected from Sumenep, Madura Waters on January 4th 2010 from around 6 a.m. till 9 a.m. The seaweeds were rinsed with seawater to clean it from attached impurities or sand. Then, the samples were packed in black plastic bag to protect the seaweed from pigment deterioration induced by light. The plastic bags were deposited in the cool box, immediately transferred to the laboratory and stored in a freezer until further analysis.

Pigments Extraction and Chromatography

The analysis of total pigment content in seaweed was done through several steps under dim light condition to prevent photo-oxidation of some labile pigments. The seaweeds were rinsed with distillated water, blotted dry and separated into stem, leaf, as well as vesicle. The extraction was carried out with 100% acetone.[15]

The dry extract was dissolved in acetone, micro-filtered and injected into the chromatograph with PDA detector. The Shim-pack VP-ODS C-18 column was protected with its guard column. The complete spectrum of the photosynthetic pigments in the 200-800 nm range was saved in the computer memory for later data analysis. The flow-rate was 1 mL/min, and the gradient protocol lasted approximately 80 minutes (Table 1). All these steps were carried out at room temperature.

Table 1. Elution gradient program used for the separation of seaweed pigments,[16] with modification

Time (min)	A (%)	B (%)	C (%)
0 – 10	80	10	10
10 – 25	80	16	4
25 – 45	80	20	0
45 – 65	80	20	0
65 – 80	70	30	0

A: methanol, B: acetone, C: ammonium acetate solution (1 M)

Data Analysis

The chromatographic profiles were observed for each 5-nm interval between 350 to 700 nm. The peak area data were extracted from the LC solution software for selected chlorophylls and carotenoids, and then tabulated and performed in graphical forms by using MS Excel 2010. The chromatograms and absorption spectra were plotted using Plot32 software.

RESULTS AND DISCUSSION

High-performance liquid chromatography (HPLC) with photodiode array (PDA) detection has been established for chromatographic separation with a simultaneous measurement of spectral absorption in the wavelength range 190–800 nm. As a result, each chromatographed fraction has spectral data and could be qualitatively identified without co-chromatography with an authentic marker. HPLC with photodiode array detection is a powerful method for separation and analysis of photosynthetic pigments, which exhibit typical absorption patterns.

Chlorophylls have chemical structures with aromatic tetrapyrrole macrocycle. They absorb strongly in the red and blue regions of the visible spectrum, which is generally characterized by an intense absorption in the blue spectral region (Soret or B-bands) and a moderate to intense absorption in the region above 620 nm (Qy-bands). These pigments also have an appreciably strong absorption in the visible range (Qx-bands).[17] On the other hand, carotenoids are hydrocarbons or their oxygenated derivatives, having chemical structures with conjugated chain molecules. Carotenoids absorb mainly in the blue (430 – 470 nm) region, but they also absorb in the blue-green (470 – 500 nm) and green (500 – 530 nm) regions of the spectrum. The position of the absorption maxima, usually three fine structures, is a function of the number of conjugated double bonds.[18]

The 430 nm wavelength is usually being adopted as "peak-rich" channel to identify the presence of both chlorophylls and carotenoids. In the previous study,[15] we reported twenty-six photosynthetic pigments from the three *Sargassum* spp. thalluses, which have been separated by reversed-phase HPLC with PDA detector. Furthermore, we selected eight notable pigments whose the spectral data are obviously identified (Figure 1). The chlorophyll group is represented by *a*-type, *c*-type, and Pheophytin *a*. Another distinct peak, detected in the retention time of 40.1 min, was assumed to be a chlorophyll *a* isomer because its absorption spectrum was identical to the main peak, so that it will be ignored in this paper. The carotenoid group is represented by *trans*- and *cis*-fucoxanthin, violaxanthin, flavoxanthin, and β-carotene.

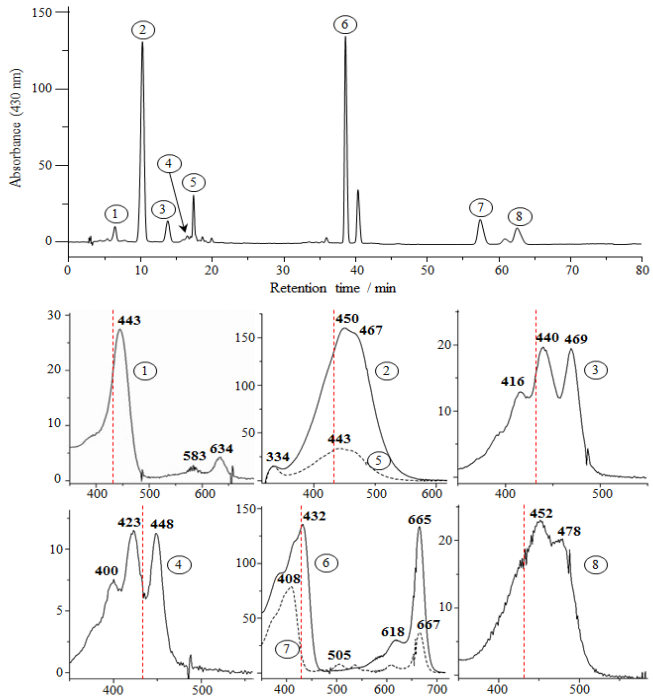

Figure 1. HPLC elution profile of the stem of *Sargassum polycystum* C. Agardh detected at 430 nm and absorption spectra of eight selected fractions. The vertical short dashed lines represent the position of 430 nm wavelength in the correspond spectra. Details are given in Table 2.

Each pigment exhibits distinctive maxima of its absorption spectrum as detected by the photodiode array. The 430 nm wavelength is almost in the maximum absorption of Soret band of chlorophyll *a*, but the structural variation strongly affects their spectral characteristic. For instance, the Soret band of pheophytin *a* (7), the magnesium-free derivatives of the chlorophyll *a* (6), undergo a hypsochromic shift and the maximum absorption becomes around 410 nm. The c-type chlorophylls (1), constructed by fully unsaturated phytoporphyrin macrocycle, has a maximum band intensity in about 10 – 20 nm higher than chlorophyll *a* which is arranged by phytochlorin system. Moreover, the carotenoids have rather varied molecular structures, and hence the 430 nm wavelength is occasionally near to the maximum absorption. This situation could be clearly recognized in the spectral comparison of fucoxanthin (2 and 5), violaxanthin (3) and β-carotene (8) towards flavoxanthin (4). The isomerization of carotenoids itself causes an alteration from *trans* to *cis* conformation, causing a significant hypsochromic shift (2 – 5 nm).

Table 2. Details of selected pigments depicted in Figure 1

No. of Peak	Retention Time (min)	Pigment
1	6.4	chlorophyll c_1
2	10.1	*trans*-fucoxanthin
3	13.6	violaxanthin
4	16.4	flavoxanthin
5	17.3	*cis*-fucoxanthin
6	38.3	chlorophyll *a*
7	57.0	pheophytin *a*
8	62.2	β-carotene

In order to obtain a better quantification method, we develop multi-chromatograms approach to extract the peak area data of those pigments in the wider range throughout the 350 – 700 nm regions. The peak area data were collected from the post run analysis of LC Solution software, tabulated and screened for the eight designated fractions. Interestingly, the bar chart (Figure 2) shows that the peak area data establish 'the spectral pattern' of each pigment fractions as well as it reveals the dominance of chlorophyll *a* and fucoxanthin.

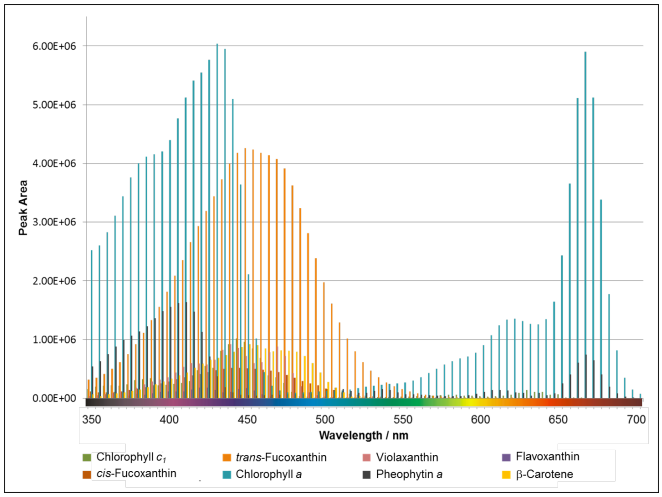

Figure 2. Peak area data of several pigment fractions observed in each 5-nm interval for 350 to 700 nm. (The chromatographic data was taken from the vesicle of *Sargassum polycystum*)

The chlorophyll *a* is the primary and the most abundant photosynthetic pigments of seaweeds. As a specialized chlorophyll-protein complex (P700), it is directly involved in the Photosystem I light reaction. It is responsible for red- and blue-light absorption. Additionally, brown alga produce accessory chlorophylls, chlorophyll(ide)s c_1 and c_2 which may be derived from an intermediate in the biosynthesis of chlorophyll *a*. Brown seaweeds also accumulate β-carotene, fucoxanthin, and violaxanthin.[19] Closer inspection of Figure 2 portrays the function of carotenoids as accessory pigments in the light harvesting antenna, which are able to absorb light energy in the blue to green regions (420 – 550 nm). A number of carotenoid also have photoprotective role and its existence depends on the variety of responses towards irradiation level.[20,21]

Furthermore, we simulated the ratio of peak area data of the seven pigment fractions to those of the chlorophyll *a*

and then compared the result. The chlorophyll *a* fraction was chosen because it has a maximum absorption of Soret band which is very close to the 430 nm channels (i.e. 432 nm). The averages of peak area data collected by multi-chromatogram approach tend to be lower than the single peak area data observed in the 430 nm channel (0.30 – 68.31% lower). Conversely, the ratios of peak area data tend to be higher, that is 55.2, 38.8, 74.9, 120.2, 39.6, 203.0, and 95.6%, on average, for chlorophyll c_1, *trans*-fucoxanthin, violaxanthin, flavoxanthin, cis-fucoxanthin, pheophytin a, and β-carotene in the three *Sargassum* spp., respectively (see Table 3 for representative data). This great variation proves the importance of considering the whole of chromatographic data in the quantification of pigments distribution.

Table 3. The comparison of peak area data and ratio of several pigment fractions in the vesicle of *Sargassum polycystum*, extracted in 430 nm wavelength and 350 – 700 nm

Pigment	430 nm		350 – 700 nm	
	Peak Area	Ratio*	Peak Area (average)	Ratio*
chlorophyll c_1	514973	0.09	200080	0.10
trans-fucoxanthin	3443153	0.57	1561010	0.79
violaxanthin	654781	0.11	343923	0.18
flavoxanthin	137401	0.02	107054	0.05
cis-fucoxanthin	477314	0.08	220711	0.11
chlorophyll *a*	6041375	1.00	1965085	1.00
pheophytin *a*	377300	0.06	368837	0.19
β-carotene	686258	0.11	434082	0.22

* Ratio = peak area of corresponding fraction/peak area of Chlorophyll a

Finally, we could perform the distribution chart of several main chlorophylls and carotenoids in the different parts of thallus structure from three *Sargassum* spp. as revealed by multi-chromatogram approach (Figure 3). The chlorophyll *a* content is widely varied, but in this case the presence of Pheophytin *a* should be noticed. Pheophytin is usually marked as a sensitive indicator of pigment degradation. There is only little variation in the content of minor carotenoids, such as β–carotene, violaxanthin, and flavoxanthin, as predicted by the peak area data. The amount of *trans*-fucoxanthin is commonly higher in both stem and vesicle parts. Carotenoid molecules are naturally present in *trans* configuration, hence the occurrence of cis-type indicates partial isomerization of *trans*-fucoxanthin. Optimization of extraction method is being important to minimize the degradation and isomerization of highly labile pigments.

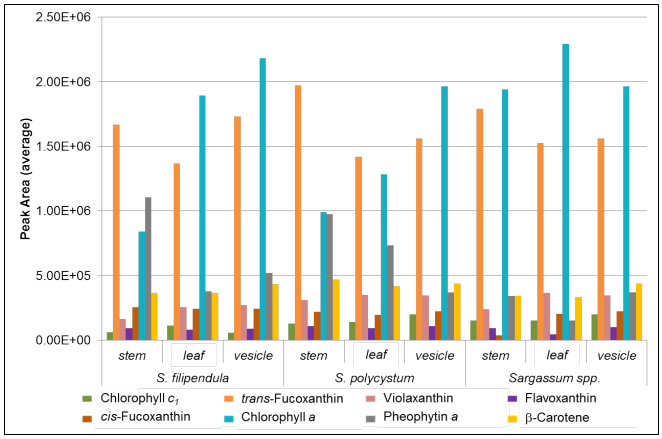

Figure 3. The distribution of several main chlorophylls and carotenoid in the different parts of thallus structure from three *Sargassum* spp. as revealed by multi-chromatogram approach.

CONCLUSION

The multi-chromatogram approach is better as the matter of fact: (i) to detect the whole of photosynthetic pigments which were present at the sample, (ii) to determine the distribution ratio of pigment fractions. Nevertheless, in this study the peak area data were still extracted and selected manually from the three-dimensional chromatographic data by using the LC Solution software. In order to increase the efficacy of data analysis, a mathematical programming is necessary. Further studies are now in progress.

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REFERENCES

1. P. Burtin, Nutritional Value of Seaweeds, *Bot. Mar.*, **2003**, *2*, 498–503, doi: 10.1515/botm.1988.31.3.231.
2. R. J. Radmer, Algal Diversity and Commercial Algal Products, *BioScience*, **1996**, *46*, 263–270, doi: 10.2307/1312833.
3. X.-F. Wang, C.-H. Zhan, T. Maoka, Y. Wada, and Y. Koyama, Fabrication of Dye-Sensitized Solar Cells Using Chlorophylls c_1 And c_2 and Their Oxidized Forms c_1' and c_2' from Undaria pinnatifida (Wakame), *Chem. Phys. Lett.*, **2007**, *447*, 79–85, doi: 10.1016/j.cplett.2007.08.097.
4. X.-F. Wang, Y. Koyama, O. Kitao, Y. Wada, S. Sasaki, H. Tamiaki, and H. Zhou, Significant Enhancement in the Power-Conversion Efficiency of Chlorophyll Co-Sensitized Solar Cells by Mimicking the Principles of Natural Photosynthetic Light-Harvesting Complexes, *Biosensors and Bioelectronics*, **2010**, *25*, 1970–1976.
5. C.-G. Kuo and B.-J. Sheen, Seaweed Chlorophyll on the Light-electron Efficiency of DSSC, *J. Chin. Chem. Soc.*, **2011**, *58*, 186–190, doi: 10.1002/jccs.201190075.

6. X. Yan, Y. Chuda, M. Suzuki, and T. Nagata, Fucoxanthin as the Major Antioxidant in Hijikia fusiformis, a Common Edible Seaweed, *Biosci., Biotechnol., Biochem.*, **1999**, *63*, 605–607, doi: 10.1271/bbb.63.605.
7. H. Maeda, M. Hosokawa, T. Sashima, K. Funayama, and K. Miyashita, Fucoxanthin from Edible Seaweed, Undaria pinnatifida, Shows Antiobesity Effect through UCP1 Expression in White Adipose Tissues, *Biochem. Biophys. Res. Commun.*, **2005**, *332*, 392–397, doi: 10.1016/j.bbrc.2005.05.002.
8. H. Maeda, T. Tsukui, T. Sashima, M. H. PhD, and K. Miyashita, Seaweed Carotenoid, Fucoxanthin, as a Multi-Functional Nutrient, *Asia Pac. J. Clin. Nutr.*, **2008**, *17*, 196–199, doi: 10.6133/apjcn.2008.17.s1.47.
9. J. A. Kilar, M. M. Littler, and D. S. Littler, Functional-Morphological Relationships in *Sargassum Polyceratium* (phaeophyta): Phenotypic and Ontogenetic Variability in Apparent Photosynthesis and Dark Respiration1, *J. Phycol.*, **1989**, *25*, 713–720, doi: 10.1111/j.0022-3646.1989.00713.x.
10. K. Gao and I. Umezaki, Comparative Photosynthetic Capacities of the Leaves of Upper and Lower Parts of Sargassum Plants, *Botanica Marina*, **2009**, *31*, 231–236, doi: 10.1515/botm.1988.31.3.231.
11. K. Gao, Comparative Studies of Photosynthesis in Different Parts of *Sargassum rhunbergii*, *Jpn. J. Phycol.*, **1989**, *37*, 7–16.
12. D. J. Garbary and K. Y. Kim, Anatomical Differentiation and Photosynthetic Adaptation in Brown Algae, *Algae*, **2005**, *20*, 233–238, doi: 2005.20.3.233.
13. M. Terasaki, A. Hirose, B. Narayan, Y. Baba, C. Kawagoe, H. Yasui, N. Saga, M. Hosokawa, and K. Miyashita, Evaluation of Recoverable Functional Lipid Components of Several Brown Seaweeds (phaeophyta) from Japan with Special Reference to Fucoxanthin and Fucosterol Contents, *J. Phycol.*, **2009**, *45*, 974–980, doi: 10.1111/j.1529-8817.2009.00706.x.
14. N.-P. V. Nielsen, J. M. Carstensen, and J. Smedsgaard, Aligning of Single and Multiple Wavelength Chromatographic Profiles for Chemometric Data Analysis Using Correlation Optimised Warping, *J. Chromatogr., A*, **1998**, *805*, 17–35, doi: 10.1016/S0021-9673(98)00021-1.
15. R. Indrawati, Heriyanto, L. Limantara, and A. B. Susanto, Study of Pigments Distribution in the Stem, Leaf and Vesicle of *Sargassum filipendula* C. Agardh, *Sargassum polycystum* C. Agardh and Other *Sargassum* spp. from Madura Waters by High Performance Liquid Chromatography, in: Proceedings of Natural Pigments Conference for South-East Asia (NP-SEA), Ma Chung University, Malang, Indonesia, **2010**, 225–230.
16. M. M. Hegazi, A. Pérez-Ruzafa, L. Almela, and María-Emilia Candela, Separation and Identification of Chlorophylls and Carotenoids from Caulerpa Prolifera, Jania Rubens and Padina Pavonica by Reversed-Phase High-Performance Liquid Chromatography, *J. Chromatogr., A*, **1998**, *829*, 153–159, doi: 10.1016/S0021-9673(98)00803-6.
17. H. Scheer, An Overview of Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications, in:*Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications*, ed. Bernhard Grimm, Robert J. Porra, Wolfhart Rüdiger, and Hugo Scheer, Springer Netherlands, Dordrecht, **2006**, 1–26, doi: 10.1007/1-4020-4516-6_1.
18. J. Gross, *Pigments in Vegetables: Chlorophylls and Carotenoids*, Van Nostrand Reinhold, **1991**, isbn: 978-0-442-00657-0.
19. A. V. S. S. Sambamurty, *A Textbook of Algae*, I.K. International, India, **2005**, isbn: 978-81-88237-44-9.
20. N. K. Choudhury and R. K. Behera, Photoinhibition of Photosynthesis: Role of Carotenoids in Photoprotection of Chloroplast Constituents, *Photosynthetica*, **2001**, *39*, 481–488, doi: 10.1023/A:1015647708360.
21. S. W. Wright and S. W. Jeffrey, Pigment Markers for Phytoplankton Production, in: *Marine Organic Matter: Biomarkers, Isotopes and DNA*, ed. John K. Volkman, Springer Berlin Heidelberg, Berlin, Heidelberg, **2006**, 71–104, doi: 10.1007/698_2_003.