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Separation, identification and quantification of photosynthetic pigments from three Red Sea seaweeds using reversed-phase high-performance liquid chromatography

Muhammad M. I. Hegazi

Marine Science Department, Faculty of Science, Suez Canal University, Ismailia, Egypt.

ABSTRACT

Thirty one photosynthetic pigments (chlorophylls, carotenoids and degradation products) from the seaweeds, *Codium dwarkense* (Chlorophyta), *Laurencia obtusa* (Rhodophyta) and *Lobophora variegata* (Phaeophyta), were separated in a single-step procedure by reversed phase high-performance liquid chromatography. An elution gradient of methanol, acetone and ammonium acetate solution, and a program time of 65 min, was used to obtain high resolution peaks. Eighteen photosynthetic pigments were separated from *C. dwarkense*, sixteen from *L. obtusa* and fourteen from *L. variegata*. Chlorophyll *b*, micronone, microxanthin, neoxanthin, siphonein and siphonoxanthin were the most typical and characteristic pigments of *C. dwarkense*, while chlorophyll c_1, c_2, fucoxanthin, fucoxanthol, flavoxanthin, diatoxanthin were the most typical pigments in *L. variegata*. In *L. obtusa,* chlorophyll *d*, α-cryptoxanthin, β-cryptoxanthin and fucoxanthin were the most abundant pigments.

KEYWORDS: *Codium dwarkense, Laurencia obtuse, Lobophora variegata,* Chlorophylls, Carotenoids, HPLC

INTRODUCTION

One of the most urgent requirements of biological oceanography is the acquisition of data on marine primary productivity, to enable maps of the most productive areas of the world's oceans to be prepared. The determination of photosynthetic pigments and their degradation products is one of most reliable methods of estimating phytoplankton, seaweed and other marine plant biomass in the marine environment.

Codium dwarkense, Laurencia obtusa and Lobophora variegata are widely distributed seaweed species at Ras Muhammad, Red Sea. They have successfully colonized a great part of the reef flat of the Ras Muhammad area, determining the productivity of the reef flat community in this area. *C. dwarkense, L. obtusa and L. variegata* like all other species of algae are characterized by specific sets of pigment, with chlorophyll *a* being the most abundant; other photosynthetic pigments are considered accessory pigments. Variations of colour in these species are related to varying amounts of pigment (chlorophylls, carotenoids and their breakdown products), with changes in colour during growth and reproduction caused by the accumulation of secondary carotenoids (Burczyk 1987). Although many methods involving column and thin-layer chromatography have been described for the separation and identification of seaweed pigments (Jeffrey 1969; Kleining 1969; Czeczuga 1986; Burczyk 1987; Palermo *et al.* 1991), it is difficult to separate all the photosynthetic pigments in a single-step procedure. While great efforts have been made to separate the photosynthetic pigments of phytoplankton (Mantoura & Llewellyn 1983; Wright & Shearer 1984; Kleppel *et al.* 1988; Kohata *et al.* 1991; Kraay *et al.* 1992; Van Heukelem *et al.* 1992,1994; Jeffrey *et al.* 1997), less effort has been dedicated to those of seaweeds. The work done by Hegazi *et al*. (1998) has led to a wide spread use of reversed phase high performance liquid chromatography (HPLC) techniques for the separation, determination and identification of seaweed pigments.

The aim of this work is to determine the concentrations of individual photosynthetic pigments in *C. dwarkense*, *L. obtusa* and *L. variegata* using a HPLC analysis. Moreover, the present research would ascertain relevant information on the primary productivity of the reef

flat of Ras Muhammad and to map up the relationship between colour change and concentrations of photosynthetic pigments.

MATERIALS AND METHODS

C. dwarkense, *L. obtusa* and *L. variegata* were collected from the reef flat from Ras Muhammad, northern Red Sea. Each algal thallus was gently washed by sea water and immediately transferred to the laboratory in an ice box and stored at - 80 ºC until chromatographic analysis was performed. The algal thallus was dried gently with absorbent Whatman filter papers for a few seconds and weighed. The thallus was ground manually in a porcelain mortar in a cold and darkened fume cupboard to prevent photo-oxidative breakdown of labile pigments. 100 % acetone and small amount of magnesium carbonate were added in order to prevent the accidental formation of chlorophyll metabolites. This procedure was repeated until the algal thallus became colorless, 5 ml of ethyl ether were added and the extract was filtered. The extract was concentrated by a low pressure rotary evaporator at 25ºC. The dry extract was dissolved in 1 ml of acetone, microfiltered at 0.45 μm and 20 μl before being injected into the chromatograph.

The analytical HPLC separation of seaweed pigments was carried out on a Hewlett Packard Series 1100 chromatograph equipped with a G1315 diode-array detector. Absorbance was registered at 430 nm (carotene detection) and 660 nm (chlorophyll detection). The complete spectrum of the photosynthetic pigments in the 400 to 700 nm range was saved in computer memory for later interpretation. The injection loop size was 20 μl. The method used consists of an elution gradient of methanol, acetone and ammonium acetate solution (1 M) (Hegazi *et al.* 1998). Semi-preparative HPLC separation of the authentic standard pigments was carried out using a Shimadzu chromatographic system (LC-6A series) equipped with SPD-M6A photodiode array detector. The column was a Spherisorb ODS-2, using 5 μm spherical particles (250 mm x 10 mm I.D.). The gradient program was similar to that used in the analytical column with a flow of 4 ml/min. The pigments were collected at the outlet of the detector, and the solvent was evaporated immediately under N_2 flow. The dry pigment was re-dissolved in acetone, benzene, diethyl ether, ethanol, or hexane. All the separated photosynthetic pigments were identified according to their spectral characteristics and compared with the published data in different types of solvent (Foppen 1971; Van Heukelem *et al.* 1992,1994; Jeffrey *et al.* 1997). The HPLC peaks were identified by comparing the retention times and spectral data with those of the authentic standards. The quantifications were done according to the methods used by Hegazi (1999).

RESULTS AND DISCUSSION

In *C. dwarkense* (Chlorophyta), eighteen photosynthetic pigments were separated and detected: chlorophyllide *a*, siphonein, neoxanthin, neoxanthin-like, violaxanthin, microxanthin, micronone, micronone-like, lutein-5,6-epoxide, siphonoxanthin, lutein, chlorophyll *b*, chlorophyll *b*′, chlorophyll *a*, chlorophyll *á*, *α*-carotene, *β*-carotene, and phaeophytin *a*. Siphonoxanthin was the most typical and distinct carotene in caulerpales. Chlorophylls *a* and *b* were the most common pigments in the green alga studied, responsible for the green colour of this group of algae. In *C. dawarkense* chlorophyll *b* have a great value than chlorophyll *a* with 0.262 mg/g and 0.054 mg/g respectively. The carotenoids siphonein have 0.035 mg/g, followed by neoxanthin and α carotene with 0.31 mg/g and 0.026 mg/g respectively.

The *L. obtusa* chromatogram showed sixteen individual photosynthetic pigments: chlorophyllide *a*, chlorophyll *d*, fucoxanthin , violaxanthin, fucoxanthol, *α*-cryptoxanthin-like, *α*-cryptoxanthin, *β*-cryptoxanthin, zeaxanthin, lutein-5.6-epoxide, lutein, chlorophyll *a*,

chlorophyll *á*, *α*-carotene, *β*-carotene, and phaeophytin *a*. The chromatogram confirms that fucoxanthin is a naturally occurring material in the red alga *L. obtusa.* This is not due to the presence of epiphytic diatom since all the species investigated were washed carefully in sea water and examined under the microscope before being subjected to the extraction procedure, and the spectral data agrees with previous published data. On the other hand, BjØrnland & Aguilar-Martinez (1976) reported the absence of chlorophyll *d* in eight species of red algae, while our results showed it to be one of the most typical and characteristic pigments of Rhodophyta. In *L. obtusa* there is a lot of chlorophyll *a* (0.24 mg/g) , much more than chlorophyll *d* (0.0008 mg/g). The carotenoids are dominated by fucoxanthin (0.14mg/g) and β carotene (0.09 mg/g).

In *L. variegata* fourteen pigments were reported: chlorophyll c_1, chlorophyll c_2, fucoxanthin, violaxanthin, flavoxanthin, fucoxanthol, antheraxanthin, 9 cis.neoxanthin, diatoxanthin, zeaxanthin, chlorophyll *a*, chlorophyll *á*, *β*-carotene and phaeophytin *a*. The typical pigments of this brown group were fucoxanthin, flavoxanthin, diatoxanthin and zeaxanthin, while chlorophyll c_1 and chlorophyll c_2 are the characteristic chlorophylls of the Phaeophyta. In *L. variegata* chlorophyll *a* is the most important (0.27 mg/g), followed by c_1 and c_2 (0.001 mg/g each). The carotenoid fucoxanthin was the dominant pigment (0.12 mg/g), followed by β carotene (0.06 mg/g) and violaxanthin (0.04 mg/g).

The individual photosynthetic pigments of the species of seaweed studied had a broad-spectrum polarity, ranging from the low polarity of carotenes to the very high polarity of chlorophyllides, which are dissociated at neutral pH. Because of this, the anionic character of the carboxylic acid group of non-esterified chlorophylls ($pK_a < 4$) originate a hydrophobic interaction, with the non-polar stationary phase being the most important retention mechanism on reversed-phase columns. Thus, the polar pigments are rapidly eluted in reversed-phase columns using methanol-water or acetonitrile-water as mobile-phase. However, peak tailing or even disappearance of the peak suggests that there is an interaction with residual silanol groups of the non-capped surface of silica support (Shioi *et al.* 1984).

Pigment dissociation can be eliminated by ion-suppression in acidic mobile-phase or ion-pairing reagents buffered at neutral pH (Mantoura & Llewellyn 1983; Shioi *et al.* 1984). Acidic mobile-phase may produce degradation products, while ion-pairing reagents do not dissolved well in organic solvent or have high reaction times (Shioi *et al.* 1984). The use of ammonium acetate as buffering agent of the mobile phase has provided good results in the separation of ionogenic chlorophyll derivatives (Van Heukelem *et al.* 1994). The ammonium acetate accelerates proton equilibrium after the elution of an acidic compound, diminishing the peak tailing and masking silanol free groups (Lim & Peters 1984).

The procedure used allowed the analysis of chlorophylls, carotenes and xanthophylls in a single-step at a fixed temperature. Fig. 1 illustrates a well-resolved three dimensional chromatogram corresponding to the separation of photosynthetic pigments from the red alga *L. obtusa*. In this chromatogram the separation factor (α) and peak resolution (R_s) were higher than 1. This indicates the absence of overlapping between peaks, while the R_s values demonstrate that resolution between adjacent bands was greater than 98 %.

All the steps of the procedure were carried out rapidly using fresh algae in cool, dark fume cupboards to prevent breakdown of the photosynthetic pigments, and only small quantities of phaeophytin *a* were detected, while no phaeophytins were detected from other chloropigments or magnesium-free metabolites such as phaeophorbides. The results, therefore, contrast with those of Zapata *et al*. (1987), who used algae dried in a dessicator at 4 ºC for pigment extraction, or Henley & Ramus (1989), who analysed pigments extracted over a period of 24 h from *Ulva rotundata* in N, N-dimethyl formamide (DMF) at room temperature. When dried algae are used or the extracts are kept at room temperature for 24 h, it seems to me that the photosynthetic pigments are broken down and incorrect analytical results may ensue. The breakdown of pigments and variations in algal color were also

observed in our study during the reproductive period and under severe environmental conditions in the intertidal zone.

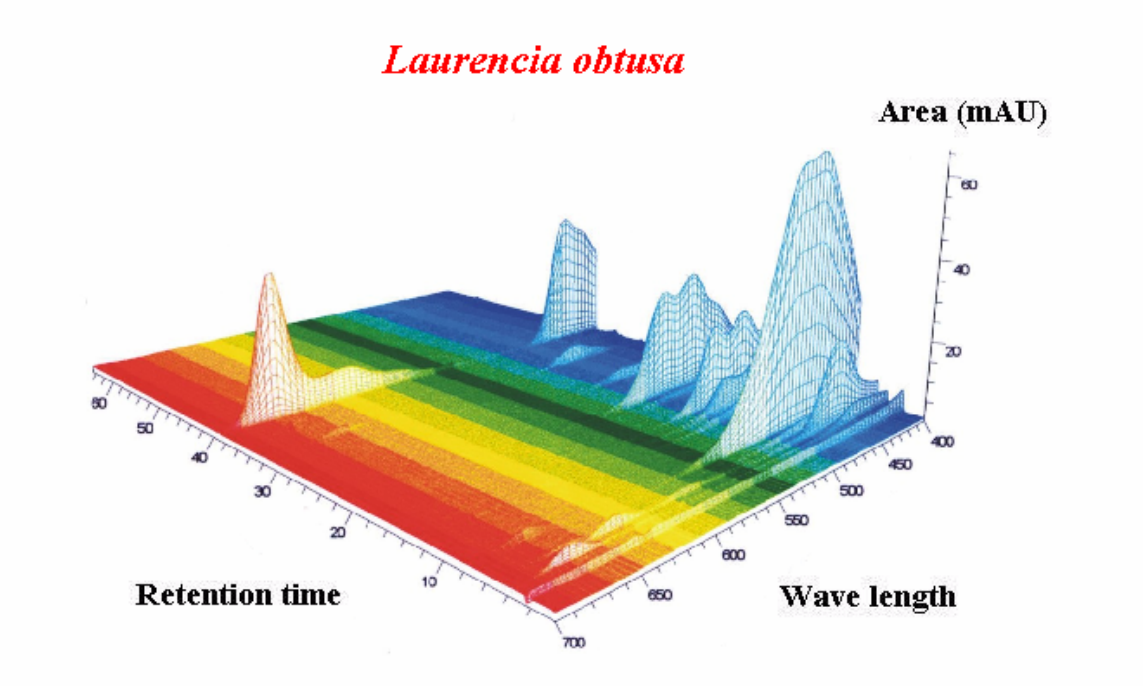

Figure (1): Three-dimensional chromatogram of the individual photosynthetic pigments of *L. obtusa*.

Table 1 identifies the photosynthetic pigments according to the absorption maxima (nm) of each peak in the mobile phase. These are compared with spectral data in different types of solvents. All the absorption maxima of the spectral data coincide with the previous published data (Foppen 1971; Burczyk 1987; Hegazi *et al*. 1998).

The present results are probably more representative than that of Barlow *et al.* (1997), who used a C_8 column of greater polarity than C_{18}, which would explain the low resolution for polar pigments and reduced retention times. They are also more reliable than those of Van Heukelem *et al.* (1994), who used more than one run with different temperatures to separate the photosynthetic pigments of phytoplankton.

It is generally agreed that all taxonomic groups of algae have specific sets of pigments, which vary in concentration from one species to another. The balance between pigment values occurred within narrow margins in each group and the variability in concentration must be related to the quantity and quality of the light in the marine environment (Dring 1982). Nevertheless, Nakayama *et al.* (1983) reported that there is a fixed relationship, in brown algae, between fucoxanthin and total carotenoids (70-90%). The present results confirmed that *C. dwarkense, L. obtusa and L. variegata* have unique suites of photosynthetic pigments, and the variation of their concentration values depends on a multiple factors such as depth, temperature, seasonality, habitat, availability of light, etc,. With all these factors we can calculate the efficiency of photosynthetic process and no fixed proportion between all pigments during the work period.

The advantage of the chromatographic separation technique reported in this study, compared with the other published works (Mantoura & Llewellyn 1983; Wright & Shearer, 1984; Hegazi *et al.* 1998) is that we used elution gradients which varied with program times and a well capped octadecyl C_{18} column, which permits high resolution of the separated photosynthetic pigments with small differences in polarity provided pigments are not highly polar. Note, for example, the clearly defined peaks for chlorophyll c_1 and c_2 as well as those for lutein and zeaxanthin.

Table 1: List of photosynthetic pigments, spectral data in the mobile phase and in different solvents.

No.	Pigment	Acetone	Benzene	Diethyl ether	Ethanol	Hexane	Eluent
1	Chlorophyllide a	428,616,652		428,662			408,432,508,536,580, 608,664
2	Chlorophyll c_1	443,580,630		444,478,628			444,584,632
3	Chlorophyll d	446,596,688		447,512,595,643, 688			448,512,548,596,632, 644,688
4	Chlorophyll c_2	444,580,630		448,582,629			448,584,632
5	Siphonein				455		452
6	Fucoxanthin		443,461, 485		448	427,450,476	452
7	Neoxanthin		423,448, 478		415,438,467	416,437,466	416,440,468
8	Neoxanthin-like		423,448, 478		415,438,467	416,437,466	416,436,464
9	Violaxanthin		428,454, 483		419,441,471	443,472	416,440,472
10	Microxanthin				396,420,447	396,418,446	424,448
11	Flavoxanthin		432,481		400,421,448	400,420,446	424,448
12	Fucoxanthol					400,425,450	448
13	α-Cryptoxanthin		433,457, 488		428,449,473	422,445,475	420,448,476
14	Micronone				445	419,440,467	440,468
15	α-Cryptoxanthin-Like		433,457, 488		428,449,473	422,445,475	420,448,476
16	Antheraxanthin				421,443,473	421,443,470	420,444,468
17	Micronone-like				445	419,440,467	440,464
18	9 cis-neoxanthin				414,437,466		412,436,464
19	Diatoxanthin				428,452,479	450,479	456,480
20	β-Cryptoxanthin					425,447,476	424,452,480
21	Zeaxanthin		440,463, 492			425,450,479	452,480
22	Lutein-5,6-epoxide				424,444,483		420,440,468
23	Siphonoxanthin				448		448,464
24	Lutein				420,445,475		424,448,472
25	Chlorophyll b	454,596,644		453,593,642			468,548,596,652
26	Chlorophyll ´b	454,596,644		453,592,642			468,548,596,652
27	Chlorophyll a	428,616,662		430,615,661			412,432,532,580, 616,664
28	Chlorophyll ´ a	428,616,662		428,614,661			412,432,532,580, 616,664
29	α-Carotene					420,442,472	420,448,476
30	β-Carotene					425,449,477	428,452,476
31	Phaeophytin a	410,468,668		408,503,667			412,448,472,508,536 560,608,668

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الملخص العربى

فصل وتعريف والتقدير الكمى لأصباغ التمثيل الضوئى لثلاثـــة أنواع من أعشاب البحـر الأحمـر باســـــتخدام جهاز كروماتوجرافـــــيا الســـــــــوائل ذو الكفاءة العاليـــــة وذى الحالة العكسـية

محمـد مسـعد إبراهيم حجـازى

قسم علوم البحار - كليـــة العلوم – جامعـــة قناة الســـويس - الإسماعيلية - مصر

تم فصل 31 من الأصباغ المســـئولة عن البنـــاء الضـــوئى (الكلوروفيلات و الكاروتيينات ومشتقاتها) فـى خطـــوة واحـــدة وتقـديرها كميـــا باســـــــتخدام جهـاز كروماتوجرافـــــيا الســـــــوائل ذو الكفاءة العالـيـــــــــة ذى الحالة العكســـــية بإستخدام تركيبة من محلول مـن الأسيتون ، المثانول ، ومحلول أسيتات الأمونيوم خلال برنامج من 65 دقيقة ، للحصول على فصل مثـالى للأصـباغ المسئولة عن عملية البنـاء الضوئى ، أيضـا تم فصل 18 نوع من الأصباغ المسئولة عن عملية البنـاء الضوئى من طحاب الكوديوم دواركنيز ، 16 نوع من اللورانســيا أبتيوســا ، 14 نوع من اللويوفرا فاريجاتا.
وقد وجد أن الكلوروفيـــل (ب) ، الميكرونونى ، الميكرواكسانتين ، النيواكسانثين ، السيفونين، السيفونواكسانثين من أكثر الصبغات تمييزا لطحاب الكوديوم دواركنيز ، بينمـــا وجد أن الكلوروفيل ج 1 ، ج 2 ، الفوكواكسانثين ، الفوكواكسانثول ، والفيلافواكسانثين ، الديناتواكسانثين هم أكثر الأنواع تميزا لطحاب اللويوفرا فاريجاتا . ومـن ناحيـة أخرى وجد أن الكلوروفيل د ، ألفا كريبتواكسانثين ، بيتا كريبتواكسانثين ، الفوكواكسانثين هم الأكثـر تميـزا لطحـاب اللورانســيا أبتيوثـا.