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Separation and identification of chlorophylls and carotenoids from *Caulerpa prolifera*, *Jania rubens* and *Padina pavonica* by reversed-phase high-performance liquid chromatography

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Abstract

Thirty-one photosynthetic pigments (chlorophylls, carotenoids and degradation products) from the seaweed, *Caulerpa prolifera* (chlorophyta), *Jania rubens* (rhodophyta) and *Padina pavonica* (phaeophyta), were separated in a single-step procedure by reversed-phase high-performance liquid chromatography, using an elution gradient of methanol, acetone and ammonium acetate solution, and a program time of 65 min, to obtain high resolution peaks of the separated photosynthetic pigments. We consider that the program times used make it possible to inject a mixed extract of the different seaweed groups. Eighteen photosynthetic pigments were separated from *Caulerpa prolifera*, 16 from *Jania rubens* and 14 from *Padina pavonica*. Chlorophyll *b*, micronone, microxanthin, neoxanthin, siphonein and siphonoxanthin were the most typical and characteristic pigments of *Caulerpa prolifera*, while chlorophyll c_1, c_2, fucoxanthin, fucoxanthol, flavoxanthin, diatoxanthin were the most typical pigments in *Padina pavonica*. In *Jania rubens*, on the other hand, chlorophyll *d*, α-cryptoxanthin, β-cryptoxanthin and fucoxanthin were the most common pigments.

Keywords: *Caulerpa prolifera*; *Jania rubens*; *Padina pavonica*; Seaweed; Chlorophylls; Carotenoids

1. Introduction

Seaweeds and other marine plants are the primary producers in the marine environment. They form the standing crop and determine the productivity of all communities. Seaweeds have been classified according to thallus colour for more than 200 years. Different seaweed groups are characterised by specific sets of pigment, chlorophyll *a* being the most abundant, while the other photosynthetic pigments are considered as accessory [1]. Variations in seaweed thallus colour are related to varying amounts of pigment (chlorophylls, carotenoids and their breakdown products), with changes in colour during the growth curve being caused by the accumulation of secondary carotenoids [2]. Although many methods involving column and thin-layer chromatography have been described for the separation and identification of seaweed pigments [2–9], it is difficult to separate all the photosynthetic pigments in a single-step procedure. Chromatographic analysis of photosynthetic pigments is a powerful

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tool for characterising the seaweed population, with the concentration of chlorophyll *a* being used to estimate biomass and productivity while the degradation products are diagnostic indicators of the physiological status and grazing processes [10].

In recent years, the literature has described high-performance liquid chromatography as a sensitive method for separating photosynthetic pigments. However, while great efforts have been made to separate the photosynthetic pigments of phytoplankton, little time has been dedicated to those of seaweeds [10–19]. Most of these methods depend on conventional reversed-phase HPLC which, given the wide difference of polarity between photosynthetic pigments and the acidic character of some chlorophyll catabolites (chlorophyllides, phaeophorbides), requires complex solvent programs that originate large band widths in the tailing peaks. In order to resolve this problem, systems involving ion-suppression or ion-pairing techniques [10,20,21] have been developed to obtain good resolution. The aim of this work was to develop a single-step procedure, using reversed-phase HPLC and diode array detection, that would be suitable for the analysis and identification of the wide number of individual photosynthetic pigments present in different seaweed groups.

2. Experimental

2.1. Field sampling

The seaweeds were collected from different depths (0–5 m) of the Mar Menor lagoon (SE Spain). The samples were washed carefully by sea water and immediately transferred to the laboratory in an ice box and stored at −80°C until the moment of chromatographic analysis. The three species chosen represented the three different groups of seaweeds: *Caulerpa prolifera* (green algae), *Jania rubens* (red algae) and *Padina pavonica* (brown algae).

2.2. Pigment extraction

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 the labile pigments [22] with 100% acetone, and a small amount of magnesium carbonate in order to prevent the accidental formation of chlorophyll metabolites. This procedure was repeated until the algal thallus became colorless, at which point 5 ml of diethyl ether were added and the extract was filtered. The extract was concentrated using a low-pressure rotary evaporator at 25°C. The dry extract was dissolved in 1 ml of acetone, microfiltered at 0.45 μm (Lida, Kenosha, WI, USA) and 20 μl were injected into the chromatograph.

2.3. High-performance liquid chromatography

The analytical HPLC separation of seaweed pigments was carried out on a Hewlett-Packard Series 1100 chromatograph (Waldbronn, Germany) and 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 400–700 nm range was saved in the computer memory for later interpretation. The analytical column was a LiChroCART 250×4 mm I.D. packed with LiChrospher 100, RP-18 e (5-μm spherical particles). The precolumn was an ODS-Hypersil (C_{18}), 5 μm, 20×4.0 mm (Hewlett-Packard). The injection loop size was 20 μl. The method used consists of an elution gradient of methanol, acetone and ammonium acetate solution (1 *M*), similar to the method used by Zapata [23] and Van Heukelem et al. [17] with the slight modification shown in Table 1. The flow-rate was 1 ml/min, and the gradient protocol lasted approximately 65 min. All these steps were carried out at room temperature.

Semi-preparative HPLC separation of the authentic standard pigments was carried out using a

Table 1
Gradient program used for the separation of seaweed pigments

Time (min)	Flow rate (ml/min)	A (%)	B (%)	C (%)
0–10	1	80	10	10
10–25	1	80	16	4
25–45	1	80	20	0
45–65	1	80	20	0

A, methanol; B, acetone; and C, ammonium acetate (1 *M*).

Shimadzu chromatographic system (LC-6A series) equipped with SPD-M6A photodiode array detector (Shimadzu, Kyoto, Japan). The column was a Spherisorb ODS-2, using 5-μm spherical particles (250×10 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.

2.4. Chemical

The solvents used were of HPLC grade (Scharlau, Barcelona, Spain). Ammonium acetate was analytical-reagent grade (Panreac, Barcelona, Spain) and the solutions were filtered through 0.45-μm membrane filters (Millipore, Bedford, MA, USA) and degassed prior to use.

2.5. Preparation of seaweed pigments

Both chlorophylls *a* and *b* were separated from *Caulerpa prolifera* (chlorophyta) and terrestrial plant leaves; phaeophytin *a* was obtained by acidification of the chlorophyll *a* solution with 13% (v/v) hydrochloric acid [24]; chlorophylls c_1 and c_2 were separated from phaeophyta; chlorophyllide *a* was prepared by enzymic de-esterification of chlorophyll *a* [25]. α-Carotene, β-carotene, lutein, lutein-5,6-epoxide, zeaxanthin, neoxanthin, violaxanthin, siphonein, siphonoxanthin, β-cryptoxanthin, antheraxanthin and fucoxanthin were separated from *Caulerpa prolifera*, *Jania rubens*, *Padina pavonica*, terrestrial plant leaves and some fruits.

2.6. Identification of pigments

All the separated photosynthetic pigments were identified according to their spectral characteristics and compared with the published data [2–9,26,27] in different types of solvent. The HPLC peaks were identified by comparing the retention times and spectral data with those of the authentic standards.

3. Results and discussion

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 suggest that there is interaction with residual silanol groups of the non-capped surface of silica support [20].

Pigment dissociation can be eliminated by ion-suppression in acidic mobile phase or ion-pairing reagents buffered at neutral pH [10,20]. Acidic mobile phase may produce degradation products, while ion-pairing reagents do not dissolve well in organic solvent or have high reaction times [20]. The use of ammonium acetate as buffering agent of the mobile phase has provided good results in the separation of ionogenic chlorophyll derivatives [17,28]. The ammonium acetate accelerates proton equilibrium after the elution of an acidic compound, diminishing the peak tailing and masking silanol-free groups [18,23,28]. For the chromatographic separation we used an elution gradient program of methanol, acetone and ammonium acetate solution (1 *M*). The protocol took 65 min to provide good resolution.

Fig. 1 illustrates a well-resolved chromatogram corresponding to the separation of photosynthetic pigments from the red alga *Jania rubens*. The procedure permitted analysis of chlorophylls, carotenes and xanthophylls in a single-step and at a fixed temperature.

Table 2 shows the retention times of all the photosynthetic pigments identified in the three representative seaweed groups. We consider that the program times used make it possible to inject a mixed extract of the different seaweed groups, since the separated factor (α) and peak resolution (R_s) in the table are higher than 1. This indicates the

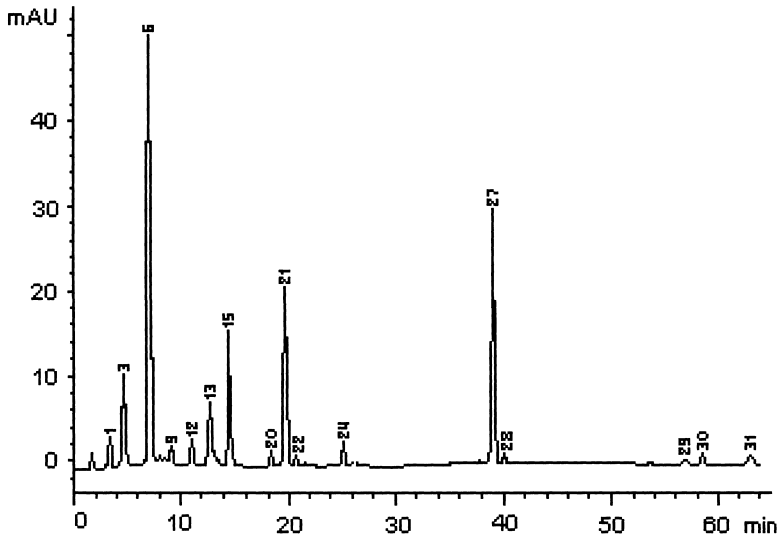

Fig. 1. High-performance liquid chromatogram of acetone extract from *Jania rubens* (rhodophyta). The peak numbers correspond to the individual photosynthetic pigments listed in Table 2.

absence of overlapping between peaks, while the R_s values demonstrate that resolution between adjacent bands is greater than 98% [29].

Table 3 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 solvent. All the absorption maxima of the spectral data coincide with the previous published data.

Our results are an improvement over those of Barlow et al. [19], 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, e.g., those of chlorophyll c_1 and c_2. They are also better than those of Van Heukelem et al. [17], who used more than one run with different temperatures to separate the photosynthetic pigments of phytoplankton.

The *Jania rubens* chromatogram showed 16 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 *Jania rubens*. However, 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 coincided with the previous published data. On the other hand, Bjørnland and Aguilar-Martinez [6] described 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 *Caulerpa prolifera* (chlorophyta), 18 separate photosynthetic pigments were detected: chlorophyllide *a*, siphonein, neoxanthin, neoxanthin-like, violaxanthin, microxanthin, micronone, micronone-like, lutein-5,6-epoxide, siphonoxanthin, lutein, chlorophyll *b*, chlorophyll *b́*, chlorophyll *a*, chlorophyll *á*, α-carotene, β-carotene, and phaeophytin *a*. Siphonoxanthin was seen to be as the most typical and distinct carotene in caulerpales. Chlorophylls *a* and *b* are the most common pigments in the green alga studied and are in fact responsible for the green color of this group of algae.

In *Padina pavonica* 14 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*.

Table 2
Retention times, separation factor (α) and resolution value (R_s) of photosynthetic pigment peaks in the three representive seaweed groups

No. of peak	*Caulerpa prolifera*	*Jania rubens*	*Padina pavonica*	$w/2$	t'_R	α	R_s
1	+	+	−	0.14	2.13	—	—
2	−	−	+	0.24	2.86	1.34	1.92
3	−	+	−	0.18	3.43	1.2	1.36
4	−	−	+	0.23	3.99	1.16	1.37
5	+	−	−	0.37	4.96	1.24	1.62
6	−	+	+	0.19	5.85	1.18	1.59
7	+	−	−	0.22	6.47	1.11	1.51
8	+	−	−	0.32	7.53	1.16	1.96
9	+	+	+	0.24	8.69	1.15	2.07
10	+	−	−	0.27	9.61	1.11	1.80
11	−	−	+	0.27	10.21	1.06	1.11
12	−	+	+	0.23	10.95	1.07	1.48
13	−	+	−	0.26	11.84	1.08	1.82
14	+	−	−	0.33	12.75	1.08	1.54
15	−	+	−	0.18	13.35	1.05	1.18
16	−	−	+	0.28	13.94	1.04	1.28
17	+	−	−	0.27	14.69	1.05	1.36
18	−	−	+	0.25	15.36	1.05	1.29
19	−	−	+	0.28	16.03	1.04	1.26
20	−	+	−	0.22	17.09	1.07	2.12
21	−	+	+	0.25	18.62	1.09	3.26
22	+	+	−	0.21	19.54	1.05	2.00
23	+	−	−	0.26	23.06	1.18	7.49
24	+	+	−	0.29	23.99	1.04	1.69
25	+	−	−	0.21	30.88	1.29	13.78
26	+	−	−	0.27	32.26	1.04	2.88
27	+	+	+	0.20	38.01	1.18	12.23
28	+	+	+	0.28	39.09	1.03	2.25
29	+	+	−	0.31	55.81	1.43	28.34
30	+	+	+	0.31	57.14	1.02	2.15
31	+	+	+	0.53	62.55	1.09	6.44

w, band width; $t'_R = t'_{Ri} - t_0$ (where t'_R is the real retention time, t'_{Ri} is the uncorrected retention time from HPLC of the peak and t_0 is the dead time); $\alpha = t'_{Ri}/t'_{Ri-1}$ (where α is the separated factor, t'_{Ri} is the corrected retention time of the peak and t'_{Ri-1} is the retention time of the previous peak); $R_s = [(t'_{Ri} - t'_{Ri-1})]/[1/2(w_i + w_{i-1})]$ (where R_s is the resolution value, w_i is the band width of the peak and w_{i-1} is the band width of the previous peak); w/2 is peak width at half height.

The most abundant and typical pigments of this brown group are fucoxanthin, flavoxanthin, diatoxanthin and zeaxanthin. While chlorophyll c_1 and chlorophyll c_2 are the characteristic chlorophylls of phaeophyta.

Although 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, 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, do not agree with those of Zapata et al. [23], who used algae dried in a desiccator at 4°C for pigment extraction or Henley and Ramus [30], who analysed pigments extracted over a period of 24 h from *Ulva rotundata* in *N*,*N*-dimethylformamide (DMF) at room temperature. In our opinion, when dried algae are used or the extracts are kept at in room temperature for 24 h, the photosynthetic pigments are broken down and incorrect analytical results ensue. The breakdown of pigments and variations in algal color were also observed in our study during the reproduction period and under severe environmental conditions in the intertidal zone.

Table 3
List of photosynthetic pigments and spectral data in the mobile phase and in different solvents

No.	Pigment	Acetone	Benzene	Diethyl ether	Ethanol	Hexane	Ref.	Eluent
1	Chlorophyllide *a*	428, 616, 652		428, 662			[21]	408, 432, 508, 536, 580, 608, 664
2	Chlorophyll c_1	443, 580, 630		444, 478, 628			[31]	444, 584, 632
3	Chlorophyll *d*	446, 596, 688		447, 512, 595, 643, 688			[26]	448, 512, 548, 596, 632, 644, 688
4	Chlorophyll c_2	444, 580, 630		448, 582, 629			[31]	448, 584, 632
5	Siphonein				455		[27]	452
6	Fucoxanthin		443, 461, 485		448	427, 450, 476	[7,27]	452
7	Neoxanthin		423, 448, 478		415, 438, 467	416, 437, 466	[7,27]	416, 440, 468
8	Neoxanthin-like		423, 448, 478		415, 438, 467	416, 437, 466	[7,27]	416, 436, 464
9	Violaxanthin		428, 454, 483		419, 441, 471	443, 472	[7,27]	416, 440, 472
10	Microxanthin				396, 420, 447	396, 418, 446	[5]	424, 448
11	Flavoxanthin		432, 481		400, 421, 448	400, 420, 446	[7,27]	424, 448
12	Fucoxanthol					400, 425, 450	[7]	448
13	α-Cryptoxanthin		433, 457, 488		428, 449, 473	422, 445, 475	[27]	420, 448, 476
14	Micronone				445	419, 440, 467	[5,27]	440, 468
15	α-Cryptoxanthin-like		433, 457, 488		428, 449, 473	422, 445, 475	[27]	420, 448, 476
16	Antheraxanthin				421, 443, 473	421, 443, 470	[27]	420, 444, 468
17	Micronone-like				445	419, 440, 467	[5,27]	440, 464
18	9-*cis*-Neoxanthin				414, 437, 466		[32]	412, 436, 464
19	Diatoxanthin				428, 452, 479	450, 479	[7,27]	456, 480
20	β-Cryptoxanthin					425, 447, 476	[33]	424, 452, 480
21	Zeaxanthin		440, 463, 492			425, 450, 479	[7,33]	452, 480
22	Lutein-5,6-epoxide				424, 444, 483		[7]	420, 440, 468
23	Siphonoxanthin				448		[5,27]	448, 464
24	Lutein				420, 445, 475		[7]	424, 448, 472
25	Chlorophyll *b*	454, 596, 644		453, 593, 642			[21]	468, 548, 596, 652
26	Chlorophyll *b́*	454, 596, 644		453, 592, 642			[21]	468, 548, 596, 652
27	Chlorophyll *a*	428, 616, 662		430, 615, 661			[21]	412, 432, 532, 580, 616, 664
28	Chlorophyll *á*	428, 616, 662		428, 614, 661			[21]	412, 432, 532, 580, 616, 664
29	α-Carotene					420, 442, 472	[7]	420, 448, 476
30	β-Carotene					425, 449, 477	[33]	428, 452, 476
31	Phaeophytin *a*	410, 468, 668		408, 503, 667			21	412, 448, 472, 508, 536, 560, 608, 668

All the photosynthetic pigments of seaweeds listed here have been reported in previous studies [2–9,34].

The advantage of the chromatographic separation technique used by us compared with the other published works [10–19] 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. Note, for example, the clearly defined peaks for chlorophylls c_1 and c_2 as well as those for lutein and zeaxanthin.

Finally the HPLC protocol proposed for seaweed pigments analysis is suitable for the identification and quantification of seasonal variations in the pigment content of different seaweed species.

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