Temperature-Programmed High Performance Liquid Chromatography Separation of Mono- and Divinyl Chlorophyll Forms From Marine Phytoplankton

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Polymeric ODS phases
Prochlorophytes
Temperature step gradients

Summary

Divinyl chlorophyll a (a_2) and divinyl chlorophyll b (b_2) are chemotaxonomic marker pigments for the marine prochlorophytes, and can be used to study their distribution in marine samples. In this paper we report a baseline resolution of mono- and divinyl forms of chlorophylls a and b employing polymeric ODS stationary phases at sub-ambient temperatures. The simultaneous resolution of mono- and divinyl forms of chlorophylls a and b, chlorophylls c_1, c_2, c_3, Mg 3,8-divinylphaeoporphyrin a_5 monomethyl ester (MgDVP), and phytol-substituted chlorophylls c was achieved when a temperature step gradient was employed during the analysis. An example is given of the utility of the protocol in oceanic field samples.

Introduction

A new type of oxygenic photosynthetic marine prokaryotes, the prochlorophytes, was discovered in the North Atlantic and Pacific Ocean, and characterized by flow cytometry, pigment analysis and electron microscopy [1]. These very small organisms (0.6 to 0.8 μm) have since been found to be ubiquitous and abundant throughout the world oceans with usually very high numerical abundances relative to other phytoplankton. Their discovery has changed our understanding of marine food webs and oceanic carbon fluxes, making its routine quantification a necessity.

HPLC pigment analysis of *Prochlorococcus marinus*, the most common cultured marine prochlorophyte [2, 3], showed a unique suite of pigments which includes 8-desethyl, 8-vinyl chlorophyll a (divinyl chl a or chl a_2) as the major light-harvesting pigment instead of monovinyl chlorophyll a (chl a_1) which is present in all other eukaryotic and prokaryotic oxygenic photoautotrophs. In addition to chl a_2 a divinyl chl b or chl b_2 (8-desethyl, 8-vinyl chlorophyll b), a chlorophyll c-like pigment MgDVP (Mg 3,8-divinylphaeoporphyrin as monomethyl ester) and the carotenoids zeaxanthin and (β,ε-carotene are present as accessory pigments.

Divinyl chlorophyll a was previously observed in the North Sea and Tropical Atlantic Ocean by Gieskes and Kraay [4] as an unidentified chlorophyll derivative (red-shifted chlorophyll a) associated with particles smaller than 1 μm.

The specific pigment composition of marine prochlorophytes allows the use of chl a_2 and chl b_2 as pigment markers in seawater samples. If HPLC analysis of algal pigments is the technique of choice for chemotaxonomic quantifications of oceanic phytoplankton populations, the method used should be able to separate monovinyl chl a and b forms from their divinyl analogues without losing the resolution of the other pigments described as occurring in marine microalgae that are of chemotaxonomic interest.

The commonly used protocols for pigment analysis are based on reversed-phase (RP) HPLC procedures, using monomeric C18 columns [5, 6, 7]. Although these methods can resolve most of the polar- and nonpolar chlorophylls and carotenoids of chemotaxonomic interest, they are not selective enough for the separation of mono- and divinyl chlorophyll a and b forms. However, a good resolution of chl a_1 and chl a_2 has been obtained with C8 columns [8, 9], although chl b_1 and chl b_2 were only partially resolved, but it has failed in the separation of chlorophylls c. A complete resolution of chl a_1 and

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chl a_2 was first achieved by normal phase (NP) HPLC [4, 10], but this procedure was unable to resolve the polar chlorophylls. Advances have, however, centered on RP-protocols because NP-systems are not suited to the routine analysis of methanol or acetone extracts.

HPLC methods based on the use of polymeric bonded phases have recently been applied to the analysis of marine algal pigments [11, 12, 13]. These methods, based on the shape recognition capacity of such phases, allowed the separation of mono- and divinyl forms of polar chlorophylls (e.g. chl c_1 and c_2) [12, 13], but failed in the separation of chl a_1 from a_2 and chl b_1 from b_2.

One of the most under-utilized parameters for controlling column selectivity in liquid chromatography is column temperature. Sander and Wise [14] pointed out that elevated temperatures enhance column efficiency whereas reduced column temperatures increase the shape selectivity of polymeric stationary phases. Applying this concept, Van Heukelem et al. [15] achieved a partial separation of chlorophylls a_1 and a_2 at 10 °C, but the chlorophyll pair b_1, b_2 remained unresolved.

In this paper we present a RP-HPLC method employing a temperature step gradient in combination with a polymeric ODS column, which allows the simultaneous separation of mono- and divinyl forms of polar- and nonpolar chlorophylls, along with most of the carotenoids occurring in oceanic samples.

Experimental

Apparatus

HPLC separations were performed with a Beckman System Gold chromatograph, which includes a model 126 solvent module, a 128 Diode Array Detector and a Rheodyne 7725i injection valve fitted with a 500 µl loop. The column was thermostated using an experimental setup (Figure 1). The inlet of the column water jacket was fitted with a 3-way solenoid valve (RS Components, 24 Vdc, i.d. 4 mm) connected with the circulating pumps (2 L min^{-1}) of a heated bath "A" and a refrigerated bath "B" (both type Neslab RTE-210). The jacket outlet was fitted with a similar valve. Both valves were interfaced with the 126 solvent module. The position of the valves controlled whether bath A or B was connected to the water jacket. A manual/programmable switch on the interface permitted manual manipulation of the valves during method development procedures. This setup permitted a change of column temperature during analysis, thus creating a temperature step gradient.

Columns

Separations were performed on two prepacked polymeric octadecylsilica analytical columns (both 250 × 4.6 i.d., particle size 5 µm): a Vydac 201-TP (Separations Group; 300 Å pore size) and a Lichrospher PAH (E. Merck; 150 Å pore size).

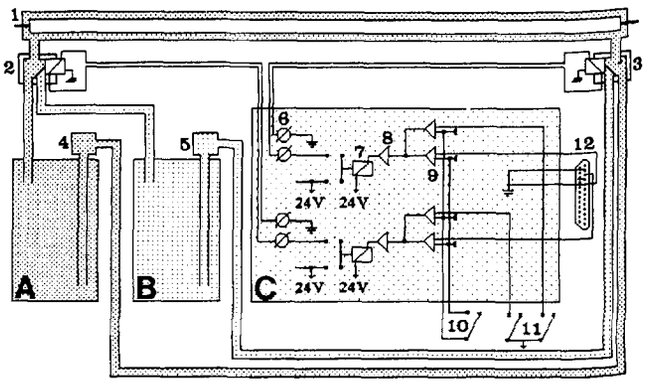

Figure 1
Schematic diagram of experimental column temperature regulation: A, heated water bath; B, refrigerated water bath; **1** = water jacket (350 mm, i.d. 25 mm) and column; **2** = 3-way outlet-valve; **3** = 3-way inlet-valve; **4** and **5** = water pumps; C, interface with relevant electronic details: **6** = valve connections; **7** = relays; **8** = drivers; **9** = triestate buffers; **10** = manual/programmable valve operation selector; **11** = manual valve manipulation switches; **12** = remote I/O connector solvent module (programmable relays: pin #9 and #11, common for both relays; pin #10: relay #1; pin 12#, relay #2).

Chemicals

HPLC grade solvents and ammonium acetate, reagent grade, were from Merck (Darmstadt, Germany) and Panreac (Barcelona, Spain). Eluents were vacuum filtered through 47 mm, 0.45 µm Nylon membranes (Alltech, Deerfield, USA or Supelco, Bellefonte, USA) and flushed with helium.

Reference Pigments

A mixture was prepared with extracts obtained from *Emiliania huxleyi* (CCAP 920/2), *Micromonas pusilla* (CCAP 1965/4) and *Phaeodactylum tricornutum* (CCAP 1052/6). The expected chlorophyll composition of such a mixture was: Chl c_1, chl c_2, two chl c_3 compounds, MgDVP, chl b_1, chl a_1 and two phytol-substituted chl c-like compounds [16]. Since no prochlorophyte cultures were available the divinyl forms of chlorophyll a and b were obtained from oceanic samples taken south of Gran Canaria island, located in the Spanish Canary Archipelago. Former analysis of samples from the area, using a normal phase setup as indicated by Kraay and Veldhuis [17], had showed the simultaneous occurrence of chlorophyll a_1 and a_2, thus revealing the presence of prochlorophytes (Van Lenning, unpublished data).

For identification purposes, pigments (chlorophylls and carotenoids) were collected at the detector outlet and their identities confirmed by determination of their spectra in various solvents as previously described [16].

Field Sampling

Water samples from the DCM (deep chlorophyll maximum) were obtained during the cruise MAST9308 (August 1993), in the area south of the Canary Islands, using 12L Niskin bottles, attached to a Rosette sampler. Seawater samples (10 L) were filtered through 47 mm glass fiber filters (Whatman GF/F) using a vacuum less than 5 cm Hg. Filters were stored in liquid nitrogen until extraction.

Sample Preparation

As a precaution to avoid pigment degradation, each sample was extracted immediately before its analysis and all operations were performed under subdued light. Samples were extracted with a CO_2-cooled Braun Melsungen homogenizer for 20 s, using 0.25–0.30 mm glass pearls. Methanol (95 %) was chosen as the extraction solvent for field samples since 90 % acetone extracts would need additional dilution steps to avoid the peak distortion effect which occurs in RP-HPLC systems using methanolic mobile phases [18]. Unialgal cultures were extracted in acetone (90 %) [18] to avoid the formation of chlorophillide a, due to the activity of chlorophyllase present in *P. tricornutum*. The extracts were filtered through a 25 mm GF/F glass fiber filter to remove cell debris and filter fragments. Volumes of 200 to 500 µL were injected directly (methanol extracts) into the liquid chromatograph. Acetone extracts were diluted to 64 % acetone prior to injection.

Chromatographic Procedure

Chromatography was performed using eluent A (methanol: 1 M ammonium acetate, 8:2, v/v), as previously described [19] and eluent B1 (acetone: acetonitrile, 1:1, v/v) or B2 (acetone). Solvents, gradients, column temperature and flow rates are given in Table I. The columns were conditioned by passage of initial eluent composition and flow rate for at least 1 h. Eluting peaks were recorded by absorption at 430 and 450 nm.

Results and Discussion

Figure 2 shows the separation of pigments present in an oceanic sample using a Vydac 201 TP column thermostated at 10 °C. The mobile phases employed were based on those previously proposed for the separation of polar chlorophylls [13]. These polar chlorophylls, formerly well resolved at 27 °C, partially coeluted at 10 °C (Rs 0.8). However, a baseline resolution of chlorophylls a_1 and b_1 from their divinyl counterparts was achieved (Rs > 1.6). This result contrasts with the partial resolution of chlorophylls a_1 and a_2, and the coelution of chlorophyll b_1 and b_2, obtained by Van Heukelem et al. [15] using the same column and temperature. These differences could be explained by the higher selectivity of acetonitrile-based mobile phases towards these compounds [13] in comparison with methanol-based phases [15].

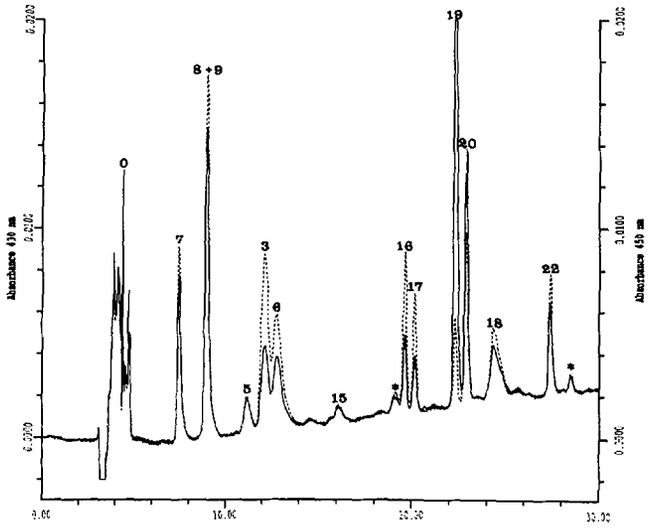

Figure 2
Absorbance chromatogram obtained from an oceanic field sample on a wide pore polymeric column (Vydac 201 TP) thermostated at 10 °C using A (methanol-1 M ammonium acetate, 8:2, v/v) and B (acetonitrile-acetone, 7:3, v/v) as the eluents employed in the gradient (Table I): detection, 430 nm (solid line) and 450 nm (dotted line). Numbering of peaks correspond to those in Table II. (*) Unidentified pigments.

Table I. HPLC solvent gradients used for isothermic (Vydac 201-TP) and step temperature gradient (Lichrospher PAH) analysis. Solvent A was methanol:1 M ammonium acetate, 80:20, v/v; Solvent B 1 was acetonitrile:acetone, 1:1, v/v; B2 was acetone.

Temp (°C)	Time (min)	Flow rate (ml min^{-1})	% A	% B/Type	Gradient system
a: isothermic					
10	0.0	0.9	60	40/ B1	Injection
	22.0	0.9	0	100	Linear
	28.0	0.9	0	100	Linear
	30.0	0.9	60	40	Linear
b: step temperature gradient					
31	0.0	0.8	80	20/ B2	Injection
31	15.0	0.8	67	33	Linear
8	17.0	0.8	50	50	Linear
8	35.0	0.8	20	80	Linear
8	37.0	0.8	0	100	Linear
8	42.0	0.8	0	100	Linear
8	45.0	0.8	80	20	Linear
8	48.0	0.8	80	20	Linear

Oceanic samples and mixed unialgal extracts were then chromatographed on the more efficient Lichrospher PAH column under isothermal conditions ranging from 5 to 40 °C. The acetonitrile based eluent B1 used for analysis employing the Vydac 201 TP column had to be substituted by B2 (acetone) to avoid excessive retention times of the peaks.

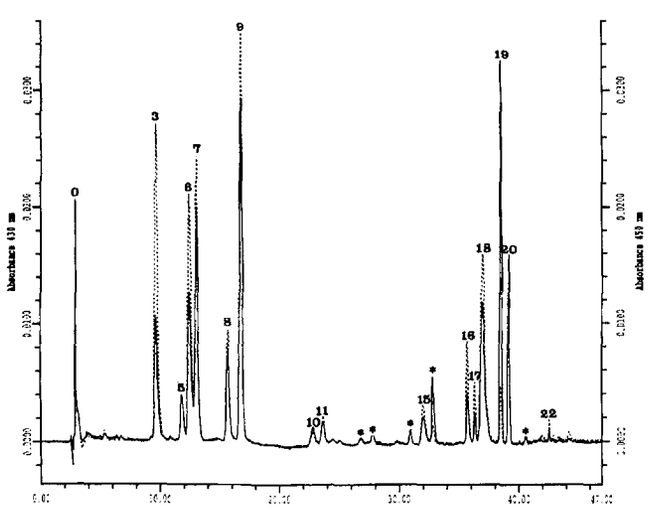

Figure 3

Absorbance chromatogram obtained from an oceanic field sample on a polymeric column (Lichrospher PAH), employing a temperature step gradient (0–17 min: 31 °C; 17–end: 8 °C), using A (methanol-1 M ammonium acetate, 8:2, v/v) and B (acetone) as the eluents employed in the gradient (Table I); detection and peak numbering as in Figure 2.

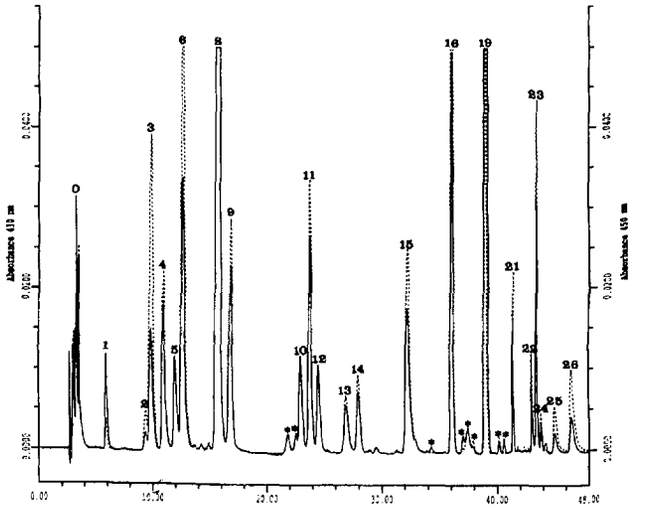

Figure 4

Absorbance chromatogram obtained from mixed extracts of *E. huxleyi, P. tricornutum, and M. pussilla;* chromatographic conditions and peak numbering as in Figure 3.

Table II. Peak identification table

Peak nr.	Pigment	Source*	Peak nr.	Pigment	Source*
1	Chlorophillide	D	14	Micromonal	C
2	1° Chlorophyll c_3	A	15	Diadinoxanthin	A,B,D
3	2° Chlorophyll c_3	A	16	Chlorophyll b_1	B,C
4	Chlorophyll c_1	D	17	Chlorophyll b_2	B
5	MgDVP	C	18	Zeaxanthin	B
6	Chlorophyll c_2	A,D	19	Chlorophyll a_1	A,B,C,D
7	19'-Butanoyloxyfucoxanthin	B	20	Chlorophyll a_2	B
8	Fucoxanthin	A,B,D	21	Unidentified carotene	C
9	19'-Hexanoyloxyfucoxanthin	A,B	22	β,ε-Carotene	B,C
10	Neoxanthin	B,C	23	β,β-Carotene	A,C,D
11	Prasinoxanthin	B,C	24	Unidentified carotene	A,C,D
12	Unidentified carotene	C	25	1° Phytol-substituted Chl c	A
13	Unidentified carotene	C	26	2° Phytol-substituted Chl c	A
	* Sources:				
A:	*Emiliania huxleyi*		C:	*Micromonas pusilla*	
B:	Field sample		D:	*Phaeodactylum tricornutum*	

Column temperature influenced both retention times and elution order of the components. The fucoxanthin-like pigments eluted before the polar chlorophylls when using low column temperatures. This elution order gradually inverted with increasing column temperatures. The polar chlorophylls, fucoxanthin, 19'-hexanoyloxy- and 19'-butanoyloxyfizcoxanthin present in the oceanic samples were baseline resolved at 25 °C. However, chlorophyll c, present in the mixed algal extract, was not resolved at this temperature. Best results for the polar chlorophylls and fucoxanthin-like pigments were obtained at 31 °C (initial 17 min. of Figures 3 and 4). Using column temperatures of 24 °C or higher, zeaxanthin eluted before the chlorophyll b derivatives. The chlorophylls pairs b_1, b_2 and a_1, a_2 were partially resolved at 23 °C. Reducing column temperatures improved the separation of these chlorophylls, but the elution order of zeaxanthin relative to chlorophyll b forms gradually inverted, causing coelution of the three components under consideration. At column temperatures below 12 °C zeaxanthin eluted after chlorophyll b_2, reaching baseline resolution at 10 °C.

Since no isothermal conditions were found to resolve all chlorophylls and carotenoids present in the field sample, the use of a temperature gradient during analy-

sis was investigated. Although constant temperature bath circulators are standard items in most laboratories, equipment capable of producing quick and reproducible temperature gradients is not. We therefore used an experimental setup (Figure 1), capable of creating a temperature step gradient, employing two standard bath circulators. An oceanic sample was injected (500 µl) into the chromatograph, using the mobile phase gradient as indicated in Table I. Column temperature was maintained isothermal at 31 °C during the first 17 minutes of the analysis. Then bath B was connected to the column jacket, by activating the inlet and outlet valves, until the end of the analysis. Best results were obtained when bath "B" was set at 8 °C. 'The results of this analysis are shown in Figure 3. The resolution of polar chlorophylls and fucoxanthin-like pigments during the initial 17 min of the chromatogram was good. Although the initial part of the analysis was performed at a high column temperature, a baseline resolution of mono- and divinyl chlorophylls a and b forms was still achieved during the remaining analysis time using a column temperature of 8 °C. Temperature was a critical parameter in the separation of chlorophyll c_2 from 19'-butanoyloxyfucoxanthin and MgDVP. Variations of only 1 °C would decrease the resolution of two of these pigments. The resolution of peaks present in the latter part of the analysis was less sensitive to variations in temperature.

Using column temperatures ≤ 8 °C always resolved mono- and divinyl chlorophyll a and b forms. Lower column temperatures only increased retention times of the compounds.

We applied the above methodology to the analysis of a mixed sample, prepared from extracts obtained from *E. huxleyi, M. pusilla* and *P. tricornutum*. The results are shown in Figure 4. Two chlorophyll c_3 compounds of *E. huxleyi* were partially separated (Rs 0.9) and the two phytol-substituted chlorophyll c-like pigments of the same culture [16] were baseline resolved. Chl c_1 was well resolved from c_3 and MgDVP.

It must be borne in mind that the protocol herein presented was designed for the analysis of polar and non-polar chlorophylls, to be used in combination with fluorimetric detection modules which are, at the present time, standard items with most HPLC systems used for pigment analysis. Although the resolution of the chlorophylls present in field- and mixed culture samples was good enough using only absorbance detection, more than 100 carotenoids have been described as occurring in algae [20], and the ability of the method to resolve more complicated mixtures of carotenoids needs further investigation.

Conclusions

The use of polymeric ODS stationary phases at reduced column temperatures allows the separation of mono- and divinyl forms of chlorophylls a and b. Good separations could be achieved when the proper mobile phases were employed, irrespective of the pore size of the stationary phase used in this study. The use of temperature step gradients during analysis facilitates the combination of qualities achieved at individual isothermic conditions. Large volumes (500 µL) of field sample can be injected without losing the desired resolution of the carotenoids and chlorophylls present in the sample. Good results are obtained for both acetone and methanol extracts when the sample is injected immediately after extraction. Mobile phases from Merck or Panreac gave the same results.

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