

HPLC determination of phytoplankton and microphytobenthos pigments: comparing resolution and sensitivity of a C₁₈ and a C₈ method

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Abstract

The effectiveness of two HPLC methods (a monomeric C₁₈ column with a high ion strength solvent gradient and a monomeric C₈ column with a pyridine-containing mobile phase) in the separation, identification, and quantification of pigments are compared, using phytoplankton, microphytobenthos, and algal cultures. Although the two studied methods showed good analytical resolution, the C₁₈ method presents a higher discrimination for several carotenoid pigments. On the other hand, the C₈ method allows the separation of chlorophylls *c*₁, *c*₂, and Mg-2, 4-divinyl phaeoporphyrin *a*₅ monomethyl ester as well as the pair chlorophyll *a*/divinyl Chl *a*. The C₁₈ method had a shorter elution program and a lower solvent flow rate, making it cheaper and faster than the C₈ method. Furthermore, the C₁₈ method showed significantly lower detection and quantification limits, a particularly important advantage for pigments present in trace amounts in both phytoplankton and microphytobenthic samples. Differences in the sensitivity of the two studied methods were due mainly to differences in flow rate, with significantly higher peak areas for slower flow rates. No method is ideal for all pigments and sample types and the choice of method should be made in accordance with the objective of each study, taking into consideration the advantages and disadvantages identified for each method.

Since the 1980s, high-performance liquid chromatography (HPLC) has been used commonly for the separation, identification, and quantification of chlorophylls and carotenoids in phytoplankton (Mantoura and Llewellyn 1983; Wright et al. 1991; Zapata et al. 2000). The importance of HPLC-based phytoplankton pigment analysis in oceanographic studies has led to the publication of a comprehensive monograph in which both modern analytical methods and their application to biological oceanography were reviewed exhaustively (Jeffrey et al. 1997b). The photosynthetic pigments detected and quantified by HPLC can supply information about the biomass, taxonomy, and physiological state of microalgae (Letelier et al. 1993; Schlüter et al. 2000; Rodríguez et al. 2002; Zapata et al. 2004; Latasa 2007). The widespread use of remote sensing to study oceanic primary production has led to an increase in papers where HPLC analyses are used to calibrate satellite ocean color measurements in terms of both biomass and phytoplankton groups (Dandonneau et al. 2004; Alvain et al. 2005).

HPLC also has been applied, although to a lesser extent, to microphytobenthic pigment analysis (Klein and Riaux-Gobin 1991; Brotas and Plante-Cuny 1998; Cartaxana et al. 2006). In the case of microphytobenthos, a complex pool of pigments and degraded forms coexist which originate from autochthonous microphytobenthos populations, allochthonous plant material, grazing, and diagenic processes (Cariou-Le Gall and Blanchard 1995; Brotas and Plante-Cuny 1998; Cartaxana et al. 2003). Therefore, the separation and quantification of pigments and their degraded forms is particularly complex in sediment samples. For microphytobenthos, the use of optical techniques, such as PAM (Pulse Amplitude Modulated) fluorescence and spectroradiometry, has pushed the need to validate optical data with HPLC (Jesus et al. 2006).

HPLC methods described in the literature differ in resolution, sensitivity, and rapidity on the basis of the type of column (stationary phase) and solvent gradient (mobile phase) used. Up to now, different columns have been tested, including octadecylsilica (ODS) C₁₈ and octylsilica (OS) C₈ columns, with different diameters, lengths, particle sizes, and different degrees of effectiveness in the resolution of key pigments (Kraay et al. 1992; Schmid and Stich 1995; Rodríguez et al. 1998; Zapata et al. 2000). In fact, the comparison of the different existing HPLC methods is still a subject of discussion.

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An important aspect is the sensitivity of the methods (detection limit), essential in the analysis of algal samples with low biomass. The various methods yield different results regarding the detection of minor pigments which might be essential for chemotaxonomic purposes.

The main objective of this study was to compare the resolution and sensitivity of two HPLC methods in the separation and quantification of chlorophylls, carotenoids, and degradation products: a method based on a monomeric C₁₈ column and a mobile phase composed of methanol, acetonitrile, and ethyl acetate (Kraay et al. 1992), and a method using a monomeric C₈ column and a pyridine-containing mobile phase (Zapata et al. 2000). For simplicity, these two methodological approaches will be referred as the C₁₈ and C₈ methods. Methods were tested using a wide variety of sample types: phytoplankton, microphytobenthos, and algal cultures.

Materials and procedures

Phytoplankton samples—Seawater samples were obtained from different regions of the Portuguese coast: near Figueira da Foz (40°04'N, 09°52'W), Aveiro (40°18'N, 09°00'W), Nazaré (39°34'N, 9°43'W) and Cascais (38°41'N, 09°25'W). Water samples (5 L) were filtered onto a Whatman GF/F filter (nominal pore size 0.7 µm and 47 mm diameter). The filters were kept frozen at -80°C before extraction. Photosynthetic pigments were extracted with 5 mL of 95% cold-buffered methanol (2% ammonium acetate) for 30 min at -20°C, in the dark. Samples were sonicated (Bransonic, model 1210) for 30 s at the beginning of the extraction period. The samples were centrifuged at 1100 g for 5 min, at 4°C. Extracts were filtered (Fluoropore PTFE filter membranes, 0.2 µm pore size) and immediately injected in the HPLC.

Microphytobenthic samples—Sediment samples were collected in mudflats of the Tagus estuary (38°44'N, 09°09'W) at low tide using contact corers (Honeywill et al. 2002). The sediment disc (0–2 mm) was wrapped in foil and stored in liquid nitrogen. In the laboratory, samples were transferred to -80°C and later freeze-dried. A sub-sample of approximately 0.2 g of freeze-dried sediment was extracted with 2 mL of 95% cold-buffered methanol (2% ammonium acetate) for 15 min at -20°C, in the dark. Samples were sonicated (Bransonic, model 1210) for 30 s at the beginning of the extraction period. Extracts were filtered (Fluoropore PTFE filter membranes, 0.2 µm pore size) and immediately injected in the HPLC.

Algal cultures—Pigments were extracted from exponentially growing cultures of a variety of marine algal species that were selected for the diversity in their pigment composition. Phy-

toplankton cultures isolated from the Tagus estuary and adjacent coastal ocean were obtained from the Institute of Oceanography (IO) Algae Collection (ALISU), University of Lisbon. The cultures are listed with their designation indicated in parentheses: (1) *Coelastrum reticulatum* (IO 41-01), (2) *Emiliania huxleyi* (IO 30-01), (3) *Rhodomonas* sp. (IO 55-01), (4) *Cylindrotheca closterium* (IO 19-01), and (5) *Scrippsiella trochoidea* (IO 14-01). All cultures except *Coelastrum reticulatum* were grown on f/2 enrichment seawater medium (Guillard and Ryther 1962) with a salinity of 28. *Coelastrum reticulatum* was grown on BB medium (Bold's Basal Medium) as described by Nichols and Bold (1965). The cultures were grown at 15.5°C in glass Erlenmeyer flasks exposed to ca. 30 µmol photons m⁻² s⁻¹. Of each culture, 10 mL were filtered onto 25 mm Whatman GF/F filters (nominal pore size 0.7 µm). The filters were frozen immediately at -80°C and later extracted with 4 mL of 95% cold-buffered methanol (2% ammonium acetate) for 30 min at -20°C, in the dark. Samples were sonicated (Bransonic, model 1210) for 30 s at the beginning of the extraction period. Extracts were centrifuged at 1100 g for 5 min, at 4°C, filtered (Fluoropore PTFE filter membranes, 0.2 µm pore size), and immediately injected in the HPLC.

HPLC pigment analysis—Pigment extracts were analyzed using a Shimadzu HPLC comprised of a solvent delivery module (LC-10ADVP) with system controller (SCL-10AVP), a photodiode array (SPD-M10ADVP), and a fluorescence detector (RF-10AXL). The chromatographic separation of pigments was achieved using two methods: a method using a monomeric ODS C₁₈ column (Kraay et al. 1992) and a method using a monomeric OS C₈ column (Zapata et al. 2000). The characteristics of the different columns used are shown in Table 1. The solvent gradient used with the C₁₈ method followed Kraay et al. (1992) adapted by Brotas and Plante-Cuny (1996) with a flow rate of 0.6 mL min⁻¹, an injection volume of 100 µL, and a run duration of 35 min. The solvent gradient used with the C₈ method followed Zapata et al. (2000) with a flow rate of 1 mL min⁻¹, an injection volume of 100 µL, and a run duration of 40 min. Details on gradient profiles and mobile phase compositions are shown in Table 2. Each sample was diluted in 10% water (HPLC-grade), to prevent distortion of early eluting peaks (Zapata and Garrido 1991).

Pigments were identified from absorbance spectra and retention times and concentrations calculated from the signals in the photodiode array detector or fluorescence detector (Ex. 430 nm; Em. 670 nm). Calibration of the HPLC peaks was performed using commercial standards, namely, Chl *a*, Chl *b*,

Table 1. Characteristics of the chromatographic columns used in this study.

Columns	Bonded phase	Dimensions (mm)	Pore size (Å)	Particle size (µm)	Surface area (m ² g ⁻¹)	Carbon load (%)
Supelcosil LC-18	Monomeric octadecylsilica	250 × 4.6	100	5	170	11
Symmetry C8	Monomeric octylsilica	150 × 4.6	100	3.5	337	12.27

Table 2. Gradient profiles and mobile phase compositions employed with the two HPLC methods (C₁₈ and C₈ method).

		Solvent		
		A	B	C
		Methanol:water [*]	Acetonitrile:water	Ethyl acetate
		(85:15 v/v)	(90:10 v/v)	
		%	%	%
C18 method	Time (min)			
	0	60	40	0
	2	0	100	0
	7	0	80	20
	17	0	50	50
	21	0	30	70
	28.5	0	30	70
	29.5	0	100	0
	30.5	60	40	0
	35	60	40	0
C8 method	Time (min)			
		A	B	
		Methanol:acetonitrile:aqueous pyridine [†]	Methanol:acetonitrile:acetone	
		(50:25:25 v/v/v)	(20:60:20 v/v/v)	
		%	%	
	0	100	0	
	20	60	40	
	26	5	95	
	38	5	95	
	40	100	0	

*Buffered with 0.5 M ammonium acetate (final concentration)

†The aqueous pyridine solution (0.25 M) was adjusted to pH 5.0 with acetic acid addition

and β , β -carotene standards from Sigma, Chl c_2 , Chl c_3 , peridinin, 19'-butanoyloxyfucoxanthin, fucoxanthin, 19'-hexanoyloxyfucoxanthin, neoxanthin, prasinoxanthin, violaxanthin, diadinoxanthin, antheraxanthin, alloxanthin, diatoxanthin, lutein, zeaxanthin, crocoxanthin, divinyl Chl a , chlorophyllide a , pheophorbide a , and pheophytin a standards from DHI (Institute for Water and Environment). The absorbance spectra of other algal pigments and their elution order were compared with literature data (Mantoura and Llewellyn 1983; Jeffrey et al. 1997a; Brotas and Plante-Cuny 1998; Brotas and Plante-Cuny 2003; Méléder et al. 2003). Resolution (R_s) between a peak and the preceding one was calculated (for critical pigment pairs) using the following equation: $R_s = 2(Rt_2 - Rt_1)/W$, where Rt_2 and Rt_1 are the retention times of two adjacent peaks, and W is the sum of peak widths at baseline. Calibration curves were established for each pigment standard and the pigment response factor (f_p , mg.(mAU.s)⁻¹) derived from the calibration slope as expressed by the equation: $f_p = \Delta W_p / \Delta A_p$, where A_p is the peak area and W_p is the weight of pigment injected. Limits of detection (LOD) and quantification (LOQ) were calculated as the amount of pigment (in nanograms) resulting in a signal-to-noise ratio (SNR) of three and ten, respectively, as described by Hooker et al. (2005). The SNR, as used in this study, was the signal amplitude (or peak height) divided by the observed noise (characterized

using a $2 \times$ standard deviation formulation) at the same wavelength from which the pigment signal was determined.

Assessment

Chromatographic resolution—The separation of chlorophylls and carotenoid pigments by the two studied methods yielded satisfactory results in phytoplankton, microphytobenthic, and culture samples. A total of 37 pigments were detected in the samples analyzed: 25 carotenoids and 12 chloropigments (Table 3). A comparison of the results obtained with the two methods shows differences in the elution times of individual pigments (Table 3) and several inversions in elution order (Figs. 1, 2, 3), as mentioned in Zapata et al. (2000). This is the case for the pigment pairs lutein (peak 26)/zeaxanthin (peak 27), neofucoxanthin (peak 18)/diadinoxanthin (peak 20), and Chl b (peak 29)/crocoxanthin (peak 30). In the C₈ method, 19'-hexanoyloxyfucoxanthin (peak 12) elutes after neoxanthin (peak 13), prasinoxanthin (peak 15), and violaxanthin (peak 16), whereas it elutes before this set of pigments with the C₁₈ column.

For the C₈ method, in phytoplankton and/or culture samples, violaxanthin (peak 16) co-eluted or partially co-eluted with 19'-hexanoyloxyfucoxanthin (peak 12; Figs. 1B, 3B; $R_s < 0.8$), and prasinoxanthin (peak 15) with an unknown carotenoid (peak 17; Fig. 1B; $R_s = 0.85$); in microphytoben-

Table 3. List of pigments obtained for the C₁₈ and C₈ columns, with the corresponding retention times and absorption maxima.

Number	Pigment	Retention time (min)		λ_{\max} (nm)	
		Column C ₁₈	Column C ₈	Column C ₁₈	Column C ₈
1	Peridininol	7.23	6.19	474	475
2	Chlorophyll c ₃	7.58	7.30	454, 586	458, 591
3	Chlorophyllide a	7.91	9.88	431, 665	429, 664
4	MgDVP	–	10.43	–	438, 576, 627
5	Chlorophyll c ₂	8.69	10.68	445, 581, 630	452, 584, 633
6	Chlorophyll c ₁	–	11.70	–	447, 581, 632
7	Fucoxanthinol	9.90	–	446, 470	–
8	Unknown peridinin derivative	10.67	14.82	474	474
9	Peridinin	10.88	13.77	475	475
10	19'-Butanoyloxyfucoxanthin	11.26	17.31	445, 470	447, 469
11	Fucoxanthin	12.38	18.18	448, 465	450, 464
12	19'-Hexanoyloxyfucoxanthin	12.76	20.91	445, 470	447, 469
13	Neoxanthin	12.91	19.10	414, 438, 466	414, 437, 466
14	Unknown fucoxanthin derivative	13.05	17.09	449, 469	449, 472
15	Prasincoxanthin	13.51	19.67	454	455
16	Violaxanthin	13.81	20.54	417, 441, 471	417, 440, 470
17	Unknown carotenoid $\lambda_{444\text{ nm}}$	14.21	19.56	424, 444, 467	424, 444, 468
18	Neofucoxanthin	14.60	23.90	442, 462	443, 458
19	Unknown violaxanthin derivative	15.15	23.51	414, 441, 471	414, 441, 469
20	Diadinoxanthin	15.45	23.06	424, 448, 477	424, 446, 476
21	Diadinochrome	16.53	24.77	408, 432, 458	408, 430, 458
22	Antheraxanthin	16.60	24.13	424, 448, 476	423, 446, 475
23	Alloxanthin	16.84	24.85	429, 454, 483	429, 453, 482
24	Unknown carotenoid $\lambda_{443\text{ nm}}$	17.41	25.71	422, 443, 472	419, 443, 471
25	Diatoxanthin	17.77	25.51	430, 454, 482	428, 453, 481
26	Lutein	18.24	26.21	425, 447, 475	425, 447, 475
27	Zeaxanthin	18.71	26.04	430, 454, 481	428, 453, 479
28	Pheophorbide a*	19.86	15.93	410, 506, 536, 609, 666	409, 507, 536, 608, 665
29	Chlorophyll b	22.97	30.20	457, 596, 646	462, 599, 648
30	Crocoxanthin	24.80	29.92	426, 446, 475	429, 447, 477
31	Chlorophyll a allomer	25.30	31.36	428, 613, 662	428, 619, 663
32	Chlorophyll a	25.82	31.72	430, 617, 663	431, 617, 663
33	Chlorophyll a epimer	26.21	32.21	430, 615, 663	429, 617, 663
34	Pheophytin a ₁	28.20	34.00	409, 504, 535, 609, 666	409, 506, 535, 608, 665
35	Pheophytin a ₂	29.70	35.60	410, 507, 536, 609, 667	409, 506, 535, 608, 665
36	β , ϵ - Carotene	29.71	35.48	426, 449, 476	427, 447, 475
37	β , β - Carotene	30.00	35.77	430, 454, 481	429, 454, 480

*Retention time of pheophorbide a correspond to the pigment standard.

thos, diatoxanthin (peak 25) partially co-eluted with an unknown carotenoid (peak 24; Figure 2B; $R_s = 0.91$). This introduces an error on pigment quantification, although in cases of partial co-elution modern software generally allows satisfactory resolution through peak analysis tools. Pigment pairs lutein (peak 26)/zeaxanthin (peak 27) and β , ϵ -carotene (peak 36)/ β , β -carotene (peak 37) were completely resolved with both studied methods ($R_s > 1$). In general, carotenoid resolution was higher with the C₁₈ method as some pigments partially co-eluted with the C₈ method.

One of the major differences between the two methods was the capacity of the C₈ column to discriminate chlorophylls c₁, c₂ and MgDVP (peaks 6, 5, and 4, respectively; $R_s > 1.1$), whereas these chloropigments eluted as a single peak with the C₁₈ column (Figs. 1, 2, 3). Nevertheless, in microphytobenthic samples, fucoxanthinol (peak 7), a degradation product of fucoxanthin, partially co-eluted with Chl c₁ in the C₈ method ($R_s = 0.64$; Fig. 2B); with the C₁₈ method, the fucoxanthinol peak was clearly separated from the chlorophylls c₁ + c₂ + MgDVP peak ($R_s = 2.17$; Fig. 2A). Although not

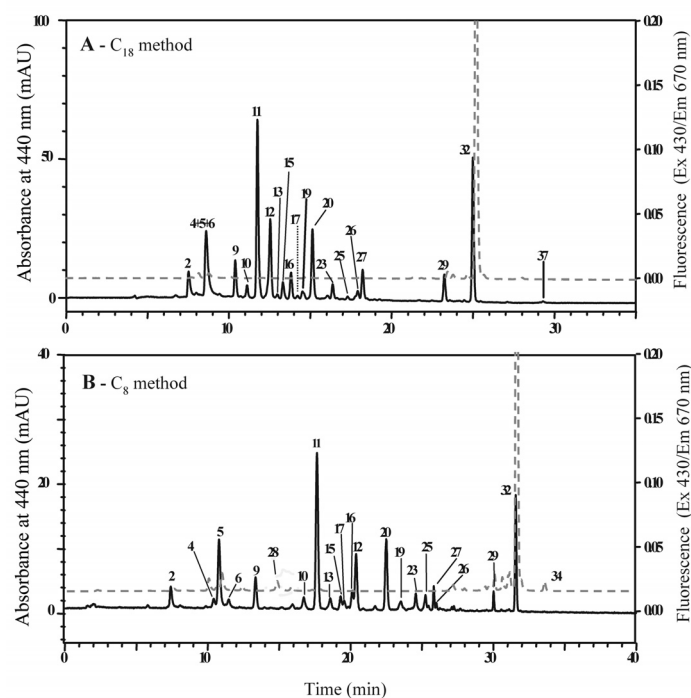


Fig. 1. Example of HPLC chromatogram of absorbance (continuous line; 440 nm) and fluorescence (broken line; Ex. 430 nm; Em. 670 nm) obtained for a phytoplankton sample with the C_{18} (A) and C_8 (B) HPLC method. Pigments are labeled according to Table 3.

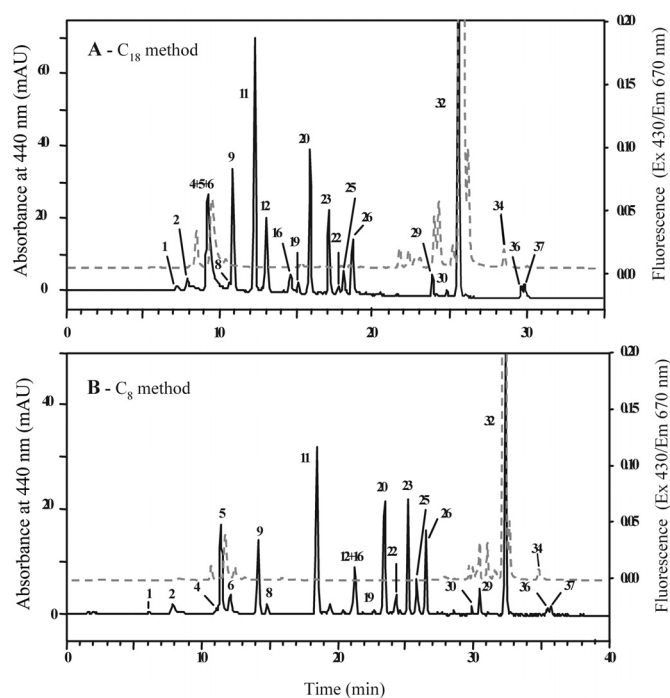


Fig. 3. HPLC chromatograms of absorbance (continuous line; 440 nm) and fluorescence (broken line; Ex. 430 nm; Em. 670 nm) obtained for a mixture of *Scrippsiella trochoidea*, *Rhodomonas* sp., *Cylindrotheca closterium*, *Coelastrum reticulatum* and *Emiliana huxleyi* with the C_{18} (A) and C_8 (B) HPLC method. Pigments are labeled according to Table 3.

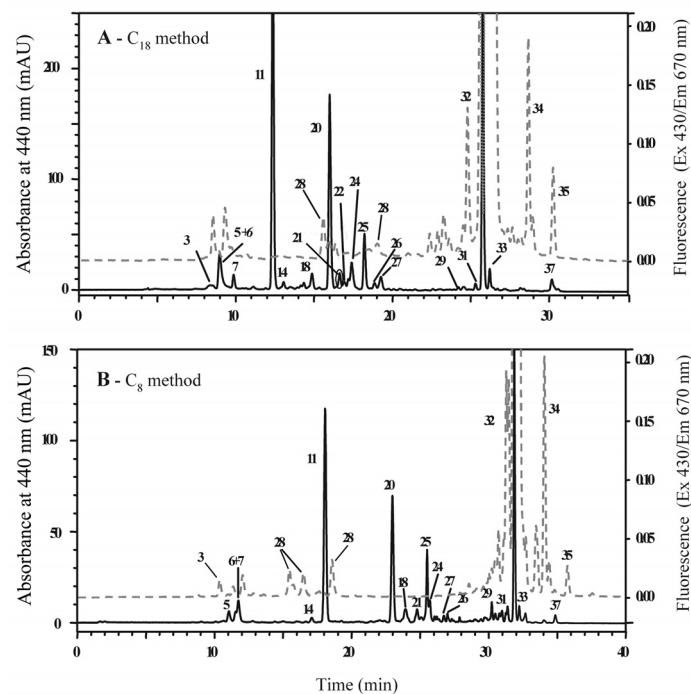


Fig. 2. Example of HPLC chromatogram of absorbance (continuous line; 440 nm) and fluorescence (broken line; Ex. 430 nm; Em. 670 nm) obtained for an intertidal muddy sediment sample (microphytobenthos) with the C_{18} (A) and C_8 (B) HPLC method. Pigments are labeled according to Table 3.

present in the samples analyzed, divinyl Chl *a* standard injected in some of the runs was not separated from Chl *a* with the C_{18} method, whereas the C_8 method clearly separated both pigments ($R_s = 1.22$). Therefore, the C_8 method is essential for the separation of the latter pigments as well as of polar chlorophylls *c*.

Several pheophorbide and pheophytin *a* pigments were observed. Some of these Chl breakdown products, particularly abundant in microphytobenthic samples, co-eluted with carotenoids. In the C_{18} method, different pheophorbide *a* peaks in natural samples co-eluted with diadinoxanthin (peak 20) and lutein (peak 26), and pheophytin *a*₂ (peak 35) with β , β -carotene (peak 37; Fig. 2A). In the C_8 method, pheophorbide *a* (peak 28) co-eluted with fucoxanthin (peak 11; Fig. 2B). As pheopigments *a* do not absorb at the wavelength range where carotenoids are usually detected, they do not interfere in their quantification. However, the absorbance of carotenoids around 410 nm interferes with the quantification of pheopigments *a* with the diode array detector. Absorbance of pheopigments *a* at 666 nm was usually too small for identification and quantification. For this reason, the double detection with the diode array and fluorescence detector (approximately one order of magnitude more sensitive) is essential for the quantification of pheophorbide and pheophytin *a* pigments.

Chromatographic sensitivity—The C_{18} method had significantly lower limits of detection (LOD) and quantification (LOQ) than the C_8 method. For the C_{18} method, LODs for zeaxanthin, prasinoxanthin and diadinoxanthin were 0.08, 0.16, and 0.08 ng of pigment, respectively. The corresponding values for the C_8 method were significantly higher: 0.15, 0.21, 0.17 ng of pigment, respectively. Low concentrations of zeaxanthin (0.45 ng 100 μL^{-1}), prasinoxanthin (0.57 ng 100 μL^{-1}), and diadinoxanthin (0.54 ng 100 μL^{-1}) standards were detected with both methods (above LOD), but were not quantifiable with the C_8 method (below LOQ values of 0.50, 0.70 and 0.55 ng of pigment, respectively; Fig. 4). In natural samples, for example, β , β -carotene (peak 37), detected in trace amounts with the C_{18} method in phytoplankton samples was not detected with the C_8 method (below LOD, Fig. 1). Pigment response factors (f_p , $\text{ng} \cdot (\text{mAU} \cdot \text{s})^{-1}$) were lower for the C_{18} method for all pigments analyzed, as shown by the steeper slopes of the calibration curves obtained with commercially available standards of zeaxanthin, prasinoxanthin, and diadinoxanthin (Fig. 5).

Differences in the sensitivity of the two studied methods could be related to the characteristics of both the stationary and mobile phases. Therefore, the four combinations of column type and elution gradient were tested for pigment sensitivity. Pigment areas were significantly lower when the C_8 elution gradient was used and were independent of the type of column, as shown for fucoxanthin, alloxanthin, and zeaxanthin (Fig. 6). Hence, we can conclude that differences in sensitivity are due to the characteristics of the mobile phase. These could be either the extinction coefficients of the pigments in the mobile phase solvents or in the flow rate used (0.6 mL min^{-1} and 1.0 mL min^{-1} for the C_{18} and C_8 methods, respectively). Extinction coefficients of the pigments in the solvent mixture at the time of elution were similar for both the C_{18} and C_8 methods, and could not explain the differences in method sensitivity. For example, Chl *a* extinction coefficient

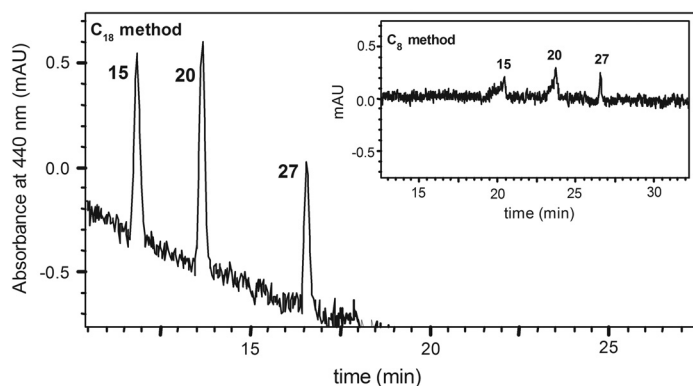


Fig. 4. HPLC chromatogram of absorbance at 440 nm obtained for a mixture of pigment standards (zeaxanthin, prasinoxanthin, and diadinoxanthin) in trace amounts for the C_{18} method. Inset: Corresponding chromatogram with the C_8 method. [Zeaxanthin; peak 27] = 0.45 ng 100 μL^{-1} ; [Prasinoxanthin; peak 15] = 0.57 ng 100 μL^{-1} ; [Diadinoxanthin; peak 20] = 0.54 ng 100 μL^{-1} . Pigments are labeled according to Table 3.

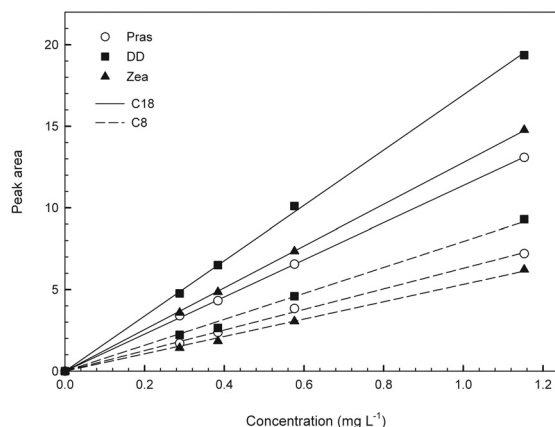


Fig. 5. Comparison of calibration curves of zeaxanthin (Zea), prasinoxanthin (Pras), and diadinoxanthin (DD) obtained with the C_{18} and C_8 HPLC method.

at 664.3 nm were 85.9 and 82.4 $\text{L g}^{-1} \text{cm}^{-1}$ for the C_{18} and C_8 solvent mixture, respectively, at the time of elution.

Applying flow rates of 0.6 and 1 mL min^{-1} to both C_{18} and C_8 methods clearly demonstrates that this parameter affects peak area and hence sensitivity with significantly higher peak areas when a slower flow rate was used, as shown for fucoxanthin (Fig. 7). Lower flow rates elute pigments in smaller volumes of solvent, and absorbance therefore increases. When flow rates slower than 1.0 mL min^{-1} are applied to the C_8 method, non-polar pigments such as β -carotene do not elute within the 40 min run. This is a significant disadvantage of C_8 method.

Discussion

An HPLC method can be evaluated on the basis of several criteria, such as resolution, sensitivity, and rapidity. Pig-

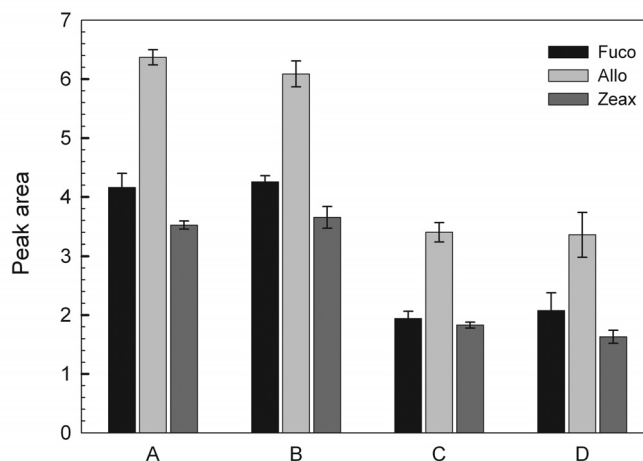


Fig. 6. Peak areas (mean \pm standard deviation, $n = 3$) of fucoxanthin (0.342 mg L^{-1}), alloxanthin (0.312 mg L^{-1}), and zeaxanthin (0.281 mg L^{-1}) standards for A. C_{18} column + C_{18} elution gradient; B. C_8 column + C_{18} elution gradient; C. C_{18} column + C_8 elution gradient; and D. C_8 column + C_8 elution gradient.

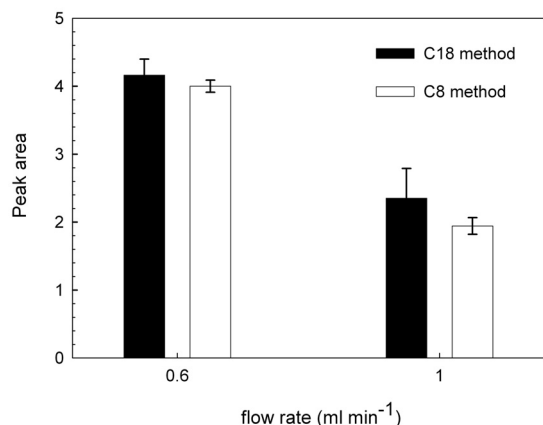


Fig. 7. Influence of flow rate on fucoxanthin standard (0.342 mg L^{-1}) peak area for the C_{18} and C_8 method (mean \pm standard deviation, $n = 3$).

ments should be resolved in a single run, detected in trace amounts, and the analysis time should be the shortest possible to allow numerous samples to be processed. Although a high number of HPLC methods for pigment analysis have been published over the past years, no method is ideal for all pigments and all sample types (phytoplankton and microphytobenthos).

The C_8 method allowed the discrimination of Chl a and divinyl Chl a , as well as chlorophylls c_1 , c_2 and MgDVP, pigments that co-eluted in the C_{18} method. This result can be explained in terms of column polarity, considering that its increased value in the C_8 column allows the discrimination of compounds with small differences in molecular polarity (Rodríguez et al. 1998). Furthermore, the pyridinium ion, as mobile phase additive, is a more hydrophobic ion pair reagent than ammonium acetate, increasing the retention of acidic chlorophylls (Zapata et al. 2000). The advantage of the C_8 method to resolve chlorophylls c_1 and c_2 is not so effective in microphytobenthic samples, because fucoxanthinol, a common degradation product of fucoxanthin, interferes with the quantification of Chl c_1 . The C_8 method should be chosen if a separation between chlorophylls c_1 , c_2 , and MgDVP or Chl a and divinyl Chl a is necessary.

Kraay et al. (1992) reported a modification of the Wright et al. (1991) method using a high carbon load C_{18} column and a high ion strength solvent capable of separating chlorophylls c_1 and c_2 . In our study, using a C_{18} monomeric column and the same solvent gradient, separation of chlorophylls c_1 and c_2 was not obtained. Rodríguez et al. (1998) reported the use of four different monomeric C_{18} columns, one of them with a higher carbon load than the one originally used by Kraay et al. (1992) and did not achieve the separation of the chlorophylls c_1 and c_2 . Therefore, separation of both chlorophylls c in Kraay et al. (1992) is probably not related to the high carbon load of the C_{18} column as the authors state, but instead is related to the fact that the RSil column originally employed was in fact a polymeric or partially polymeric (intermediate) column.

The C_{18} method has a shorter elution program and a lower solvent flow rate, making it cheaper and faster than the C_8 method. Furthermore, the C_{18} method had higher carotenoid resolution and significantly lower detection limits than the C_8 method. The C_{18} method is, therefore, more suitable for the detection of pigments present in trace amounts in both phytoplankton and microphytobenthic samples. Van Leeuwe et al. (2006) stresses the importance of optimized extraction and automation on the reproducibility and sensitivity of the HPLC method, particularly when low microalgae biomass is present. Our results prove that method flow rate is crucial also.

Comments and recommendations

Each method must be chosen to meet the objectives of each kind of study, taking into consideration the advantages and disadvantages of the methods, as well as the type of sample (phytoplankton or microphytobenthos). The C_{18} method is cheaper, has faster sample throughput and higher sensitivity. Hence, the C_{18} method should be used in the absence of polar chlorophylls c and divinyl Chl a or, in case the separation of these pigments is not essential, as is the case with microphytobenthos samples. On the other hand, if your target is to characterize phytoplankton assemblages in natural samples with unknown pigment composition, the C_8 method would be preferred thanks to its good overall resolution and capacity to resolve polar chlorophylls c and the pair Chl a /divinyl Chl a , not separated by the C_{18} method. The lower sensitivity of the C_8 method may constitute a major disadvantage in oceanic samples with low pigment concentrations and might require different sample preparation, including larger filtration volumes, smaller filters, and smaller extraction volumes. Future work regarding the C_8 method might include adjustment of the elution conditions (gradient and elution time) to allow the use of a lower solvent flow rate.

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