

Comparison of Diode Array and Electrochemical Detection in the C₃₀ Reverse Phase HPLC Analysis of Algae Carotenoids

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A determinação qualitativa e quantitativa de carotenóides pode fornecer diferentes e importantes informações sobre os organismos que os contêm. Na análise de pigmentos por HPLC diversos detectores podem ser utilizados, como *diode array* (DAD) e eletroquímico (ED). O presente trabalho tem como objetivo desenvolver um método por HPLC utilizando uma coluna C₃₀ para a identificação e quantificação de dezesseis pigmentos em diferentes classes de algas, além de comparar as respostas obtidas nos detectores DAD e ED por meio da análise dos resultados de precisão e exatidão. Apesar do ED ser geralmente um detector mais sensível que o DAD, os resultados de precisão e exatidão foram mais satisfatórios para o DAD. O método desenvolvido foi eficiente para a análise quantitativa dos pigmentos de cianobactérias e diferentes classes de algas, sendo que o padrão cromatográfico encontrado em cada classe foi discutido.

Qualitative and quantitative determination of carotenoids pigments can provide valuable information about the organisms in which this important class of compounds is found. In the HPLC analysis of pigments, diode array (DAD), electrochemical (ED) and other kinds of detector may be used. The aim of this work is to develop an HPLC method using a C₃₀ column to identify and quantify sixteen different pigments from algae. A further aim is to compare precision and accuracy obtained by DAD and ED. ED is normally more sensible than DAD. On the other hand, the highest precision and accuracy was obtained with DAD. In conclusion, the method was efficient for quantitative and qualitative analyses of pigments from cyanobacteria and different microalgae classes. Their pigment patterns for several organisms are also discussed.

Keywords: carotenoids, chlorophylls, HPLC-DAD, HPLC-ED, algae

Introduction

Carotenoids are pigments found mostly in plants and algae. Their biological function in these organisms is to act as antioxidants,^{1,2} membrane stabilizers^{3,4} and light harvesters in photosynthetic organisms.⁵ More than 700 natural carotenoids from different sources have been isolated and chemically characterized. They are derived from the same basic C₄₀ isoprenoid skeleton.^{6,7} Algae, in particular, have several structurally different pigments in their photosynthetic apparatus (Figure 1) making them an important and sometimes unique commercial source

of carotenoids.¹ Moreover, the carotenoid profile in algae may provide information about their photoacclimation, photoprotection responses,^{8,9} phylogenetic distribution¹⁰ and serve as important biomarkers of environmental impacts.^{11,12} Alternative methods to detect and quantify these pigments in other different matrixes are also valuable tools for investigating their biological activity and bioavailability.¹³

Due to improved separation efficiency, carotenoid analyses are routinely performed by reversed-phase HPLC. Many methods for the separation of pigments from algae have been developed.^{14,15} However, the complexity of their composition makes the resolution of all pigments from different algae species difficult in a single run method.¹⁶ Another challenge is the identification and the guarantee

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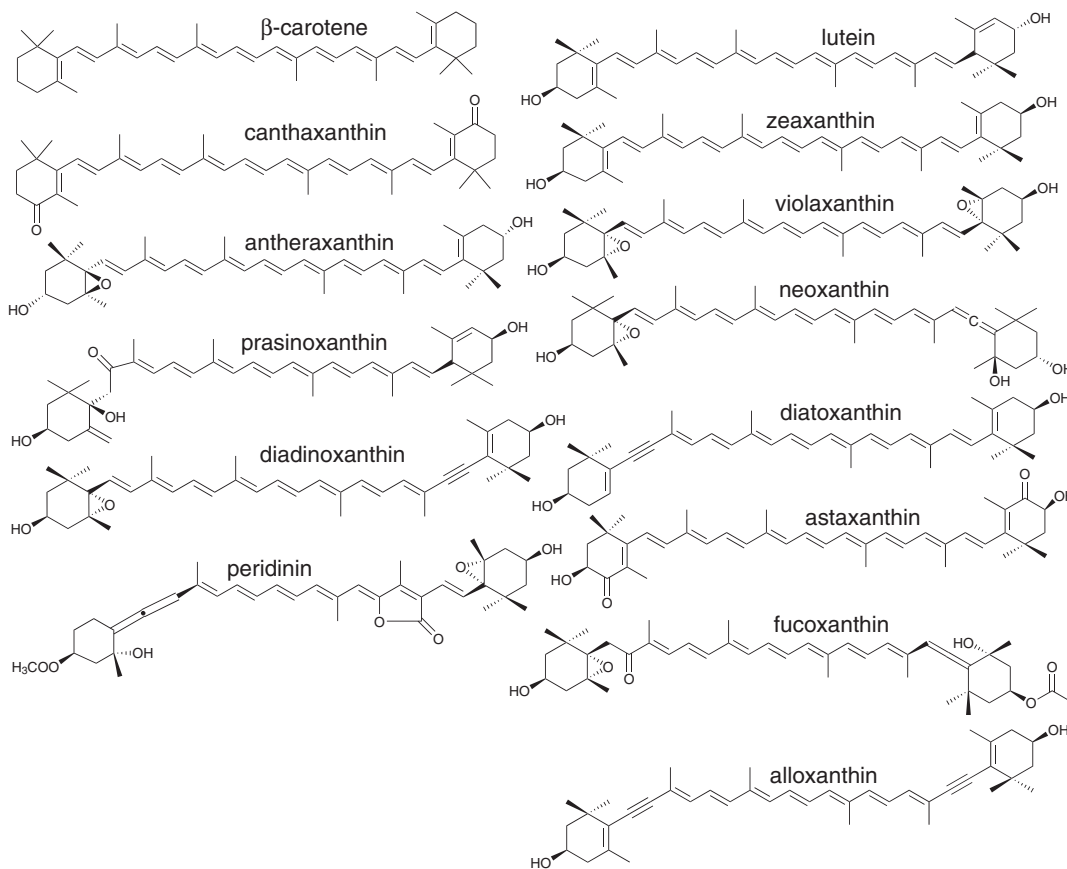


Figure 1. Structure of the carotenoids studied.

of purity of all peaks, since carotenoids are structurally similar. Consequently, in order to obtain a wide range of information, several methods with different detectors are available in the literature. The diode array detector (DAD) is by far the most common detector used in carotenoids analyses, as it makes use of the absorption properties of these compounds in the visible region. DAD can also furnish some structural information for analyte identification. This characteristic makes DAD an important tool for characterization, since the spectrum associated with a given retention time can be compared with known analytical standard profiles.¹⁷ However, spectra of carotenoids cannot be used as conclusive evidence of structure, since many carotenoids present very similar absorption profiles. Moreover, the detection limit of DAD can be a major disadvantage.¹⁷ Accordingly, other detectors, notably the electrochemical detector (ED) have been used to counter the disadvantages of the DAD. ED is based on the oxidation and reduction properties of analytes. Since carotenoids are electroactive, the HPLC-ED system can be another option for their analysis.¹⁸⁻²⁰

Since carotenoids can be analyzed by either an electrochemical or a diode array detector, the aim of this work was to develop an HPLC method to identify and

quantify sixteen carotenoids and chlorophylls from algae using a C₃₀ column and to compare the precision and accuracy values and overall performance obtained with DAD and ED.

Experimental

Materials

All solvents were HPLC or reagent grade, from Sigma Chemical Company (St. Louis, USA), Tedia (São Paulo, Brazil) and Mallinckrodt (Paris, USA). Deionized water was obtained using a Milli-Q water purification system (Millipore, Bedford, USA). Stock solutions of alloxanthin (1.04 mg L⁻¹), antheraxanthin (0.625 mg L⁻¹), canthaxanthin (0.559 mg L⁻¹), diadinoxanthin (1.197 mg L⁻¹), diatoxanthin (0.935 mg L⁻¹), fucoxanthin (1.438 mg L⁻¹), lutein (1.296 mg L⁻¹), neoxanthin (1.137 mg L⁻¹), peridinin (0.984 mg L⁻¹), prasinoxanthin (1.281 mg L⁻¹), violaxanthin (0.814 mg L⁻¹), and zeaxanthin (0.591 mg L⁻¹) in ethanol were purchased from DHI Water & Environment (Copenhagen, Denmark) and stored at -80 °C. β -carotene, astaxanthin and chlorophyll *a* and *b* were purchased from Sigma (St. Louis, USA).

Instrumentation and chromatography

The HPLC instrument consisted of a SCL-10AVP Shimadzu system equipped with two LC-10AD pumps, a SIL-10ADVP automatic sample injector, a DGU-14A degasser, and a SPD-M10AVP photodiode-array detector. The Coulochem III ED (ESA Inc., Chelmsford, MA) was coupled in series with the photodiode-array detector and consisted of a guard cell (Model 5021) and an analytical cell (Model 5010) with two porous graphite working electrodes, palladium reference electrodes and platinum counter electrodes.

Chromatographic separations were carried out on a C₃₀ column (Ultrasorb, 250 x 4.6 mm, 5 µm, Phenomenex) at 1.0 mL min⁻¹ and room temperature, using as mobile phase: (A) MeOH:H₂O:NH₄Ac buffer 1 mol L⁻¹ (pH 4.6) (90:8:2) and (B) MeOH:MTBE:NH₄Ac buffer 1 mol L⁻¹ (pH 4.6) (30:68:2). The gradient elution was performed as follows: a linear increase from 5 to 10% of solvent B (0-15 min); maintaining 10% B for 10 min; a linear gradient (10 min) to 15% B followed by another linear gradient (5 min) to 40% B and then an increase to 45% of solvent B in 2 min, an isocratic elution for 20 min and an increase to 100% B in 1 min and maintaining 100% B for 5 min, for a total run time of 68 min.

The chromatographic parameters, capacity factor (*k*) and separation factor (*α*) were calculated to evaluate the separation efficiency. The injection volume of standards and samples was 50 µL and all ultraviolet-visible spectra were recorded from 200 to 800 nm. For quantitative analyses chromatograms were integrated at 445 nm. The ED was operated in the DC-mode and the settings were as follows: +850 mV at the guard channel (to improve the baseline signal by oxidizing interfering compounds possibly present in the mobile phase) and +600 mV at the first cell (channel 1). For confection of hydrodynamic voltammograms, data were acquired in channel 1, which was set at different potentials in each run (+100 to +900 mV, in 50 mV increments).

Sample preparation

Non-axenic cultures of *Prorocentrum minimum* (Pavillard) (Proro.min.-C2) (Dinophyceae), *Minutocellus polymorphus* (Hargraves and Guillard) Hasle, von Stosch and Syvertsen (Min.poly.-CF1) (Bacillariophyceae), *Isochrysis galbana* Parke (Iso.g.-USA1) (Prymnesiophyceae), *Tetraselmis gracilis* (Kyllin) Butcher (Tetra.g.-C1) (Prasinophyceae), *Hillea* sp. (Hil.-PB1) (Cryptophyceae), and *Synechococcus lividus* Copeland (Syn.liv.-C1) (Cyanophyceae) were obtained from the Culture Collection

of the Oceanographic Institute of the University of São Paulo (Brazil). The cultures were grown in Guillard f/2 medium²¹ at 20 ± 1 °C on a 12 h light / 12 h dark cycle with cool-white fluorescent light at an irradiance of 120 µmol photons m⁻² s⁻². Light intensity was measured by a Biospherical Instrument QSL 100 photometer with a 4p scalar sensor. Samples were collected in the exponential phase at midday, ground with liquid nitrogen and dried in a speed-vacuum (SpeedVac®, Savant, City, USA). The homogeneous, freeze-dried samples were weighed, dissolved in methanol:acetone (1:1, v/v) and sonicated for 15 min. The extracts were then centrifuged and filtered through a 0.45 µm membrane (Millex HN nylon, 13 mm, Millipore). Aliquots (50 µL) of each extract (3 mg mL⁻¹) were injected into the HPLC system. The pigment content was expressed as µg per mg of dried weight (µg mg⁻¹ DW).

Peak identification

Most peaks were identified according to the characteristics of their electronic absorption spectra and chromatographic retention times by comparing retention times with those of the corresponding standards.

Quantitative analysis

Calibration was performed using dilutions of stock standard solutions. The respective peak areas obtained in the DAD (445 nm) and in the first channel of the ED (+ 600 mV) were plotted vs. the nominal concentrations. For precision assays, samples with the same concentration were analyzed five times and the intra-assay relative standard deviation (RSD) was calculated. The inter-assay RSD was determined by analyzing the samples on five different days. The intra- and inter-assay accuracy was evaluated by assessing the agreement between the measured and nominal concentrations of the analytes.

Results and Discussion

Many methods are available in the literature for analysis and quantification of carotenoids and chlorophylls in different matrices due to the importance and applicability of these compounds.^{14-16,22-24} The C₃₀ stationary phase has been widely used for carotenoid separations since, in general, it provides significant resolution of complex mixtures.^{22,23} In addition to the column selection, mobile phase choice is extremely important. In the case of ED, the presence of an electrolyte is required. Hence, researchers have employed mobile phases containing salt or buffer solutions such as MeOH:MTBE:acetate buffer 1 mol L⁻¹

(pH 4.6) (95:3:2) and MeOH:MTBE:acetate buffer 1 mol L⁻¹ (pH 4.6) (25:73:2)²⁴ or 13.44 mmol L⁻¹ lithium perchlorate in MeOH:EtOH:2-propanol (88:24:10).¹⁹ In the present work, we chose to use ammonium acetate (NH₄Ac) buffer, which reduces losses or degradation by minimizing the acidity effects of the free silanol groups present in the derivative silica stationary-phase.¹⁷ After testing different mobile phases and elution gradients, the most suitable was the method described in the experimental section. This method was able to resolve a total of sixteen pigments. Retention times, separation (α) and capacity (k) factors are presented in Table 1. A good selectivity of mobile phase for all the peaks was attained as indicated by the separation values higher than 1. Low capacity factor values indicate low retention in the stationary phase, while the longer the analyte is retained, the greater capacity factor and consequently, the longer total run time. The capacity factors between 0.5 and 20 indicate that a proper solvent strength was maintained throughout the analysis.^{25, 26} Capacity factor values higher than 20, which were observed for some analytes, are consistent with the long total run time.

Table 1. Peak number (P_n), retention time (Rt), maximum wavelength (λ_{\max}), capacity factor (k) and separation factor (α) between peaks (indicated in parenthesis) of standard pigments analysis obtained by HPLC-DAD (445 nm)

Pigments	P _n	Rt/min	λ_{\max} / nm	k	α
Peridinin	1	7.3	475	2.5	1.7 (1/2)
Fucoxanthin	2	10.9	450	4.2	1.2 (2/3)
Neoxanthin	3	12.8	435	5.1	1.2 (3/4)
Prasinixanthin	4	15.4	459	6.3	1.1 (4/5)
Violaxanthin	5	17.1	438	7.1	1.3 (5/6)
Astaxanthin	6	21.2	476	9.1	1.1 (6/7)
Diadinoxanthin	7	22.9	445	9.9	1.2 (7/8)
Antheraxanthin	8	26.5	445	11.6	1.2 (8/9)
Alloxanthin	9	30.5	451	13.5	1.2 (9/10)
Diatoxanthin	10	36.2	450	16.2	1.1 (10/11)
Lutein	11	38.3	445	17.2	1.0 (11/12)
Zeaxanthin	12	40.1	450	18.1	1.1 (12/13)
Canthaxanthin	13	44.8	474	20.3	1.1 (13/14)
Chlorophyll <i>b</i>	14	48.9	459	22.3	1.2 (14/15)
Chlorophyll <i>a</i>	15	56.5	420	25.9	1.2 (15/16)
β -carotene	16	66.8	451	30.8	-

In addition to DAD, another possible technique for carotenoid analysis is ED, which detects the electroactivity of a compound.²⁷ In order to determine the potential of maximum response and thus improve the selectivity and sensitivity, each standard was injected into the HPLC system

and analyzed by varying the applied potential in each run.²⁸ With these data, it was possible to obtain hydrodynamic voltammograms, as shown in Figure 2 for β -carotene, lutein and chlorophyll *a*. In a previous study, Ferruzzi *et al.*²⁴ determined the maximum response for several carotenoids and obtained somewhat different results using a system with an array of electrochemical cells (8, 12 or 16 channels) connected in series. This multi-channel system offers the possibility of obtaining the hydrodynamic voltammograms in a single run. However, the maximum response can be underestimated if not submitted to mathematical corrections. Oxidations and reductions in the electrochemical cells are not reversible processes and each species has its own intrinsic redox potential.²⁹⁻³⁰ When using electrochemical cells in series, increasing potentials are applied. If the first cell detects the analyte, the next cell will detect a smaller quantity of sample. Consequently, the resultant signal will

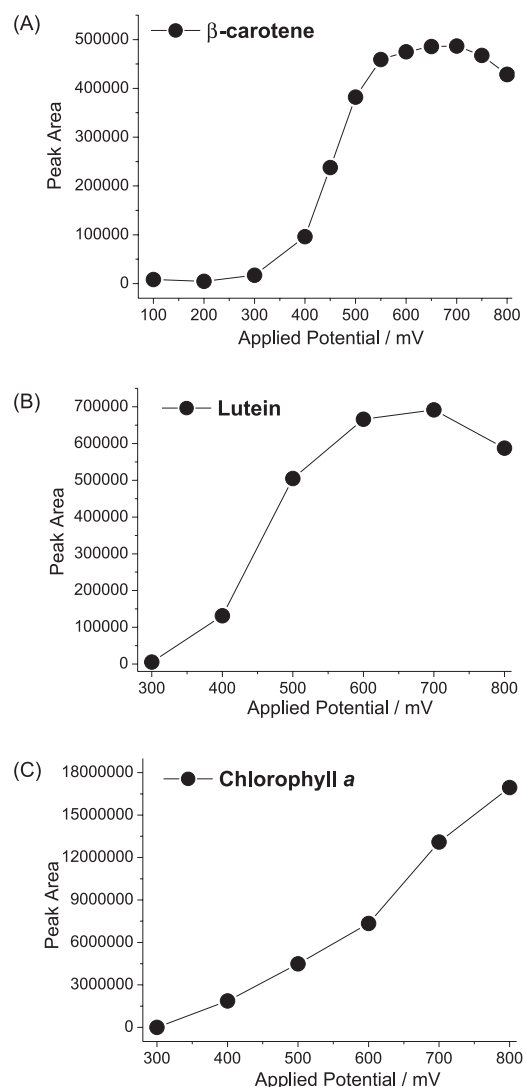


Figure 2. Hydrodynamic voltammograms of (A) β -carotene, (B) lutein and (C) chlorophyll *a*.

be the real response minus the response in the previous cell and so on for the other cells. Thus, the maximum response is not exactly what would be obtained if only one electrode, operating at the exact redox potential of the analyte, were employed. For example, the potential in which β -carotene presented maximum response in a multi-channel system was approximately 340 mV and in a one channel system the maximum response was at 600 mV. Furthermore, the oxidation potential of lutein maximum response was 400 and 650 mV, respectively.²⁴ Although the use of electrochemical cells connected in series is very useful for a wide dynamic range of analyses,³¹ including those with completely different redox potentials, an undesirable decrease in the sensitivity of the method can occur when the redox potentials cover only a narrow range of potentials.

Since the carotenoids and chlorophylls tested herein exhibited maximum responses at similar potentials, a potential of + 600 mV was used in the first cell to obtain analytical curves, precision and accuracy parameters. For DAD analysis, the wavelength selected was 445 nm. Chromatograms of β -carotene, lutein and chlorophyll *a* obtained at the same run in both detectors are presented in Figure 3. Peak Area Ratio (P.A.R.) between DAD and ED responses showed a higher peak for all carotenoids

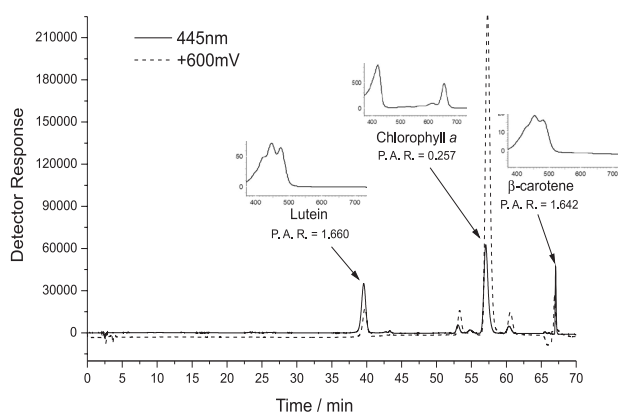


Figure 3. HPLC-DAD (445 nm), HPLC-ED (+ 600mV) chromatograms and UV spectra of standard β -carotene, lutein and chlorophyll *a*. Peak Area Ratio (P.A.R.) between DAD and ED responses are presented. Chromatographic conditions are described in the text.

by DAD than by ED, as shown for β -carotene and lutein in Figure 3. On the other hand, chlorophylls were more responsive to ED than to DAD. Although chlorophylls absorb at 445 nm, the highest absorption wavelength of chlorophyll *a* is at approximately 420 nm (spectra shown in Figure 3), resulting in a more intense signal at ED and a much lower absorption at 445 nm.

In the quantification of algal carotenoids, analytical curves were constructed by injecting different concentrations of each pigment. Curves were constructed using both DAD (445 nm) and ED (+ 600 mV) data. Intra- and inter-day precision and accuracy were also calculated for all pigments using the results obtained with both detectors (Table 2).

Comparing the results obtained, it is possible to conclude that, in this case, the DAD exhibited higher precision and accuracy than ED (Table 2). ED is normally more sensitive than DAD. However, for analytes exhibiting high molar extinction coefficients, such as the carotenoids, DAD may be a more sensitive detector and may improve the precision and accuracy of the detection. Indeed, the results obtained by both HPLC coupled DAD and ED showed that the light absorbing properties of the carotenoids provide a better detection performance than their electroanalytical properties. Although carotenoids are easily oxidized at + 600 mV, our data suggest that their electroanalytical properties are less optimal for detection.

The HPLC-DAD chromatograms of pigment extracts from the six species of marine phytoplankton are shown in Figure 4. The pigments β -carotene and chlorophyll *a* are present in all algae studied (Figure 4 and Table 3). It is known that extraction conditions can interfere in the chromatogram profile due to differences in the polarity of carotenoids.³² Using our extraction protocol, peridinin and diadinoxanthin were the main carotenoids identified in the dinoflagellate *Prorocentrum minimum* (Table 3). These pigments are typically found in this class of marine microalgae.^{33,34} The chromatogram obtained for the diatom *Minutocellus polymorphus* showed one major peak, which was further identified as fucoxanthin (Figure 4) and two lower intensity peaks, identified as diadinoxanthin and diatoxanthin. Likewise, Sigaud-Kutner *et al.*³⁵ found the

Table 2. Intra- and inter-day precision and accuracy (n = 5) assays of representative pigments. Data obtained from DAD (445 nm) and ECD (+ 600 mV)

	445 nm				+ 600 mV			
	Precision (%)		Accuracy (%)		Precision (%)		Accuracy (%)	
	Intra-	Inter-	Intra-	Inter-	Intra-	Inter-	Intra-	Inter-
Prasinoxanthin	3.1	2.5	1.3	1.1	9.8	10.4	1.5	0.9
Antheraxanthin	3.2	4.5	0.4	0.4	8.4	7.1	0.4	0.4
Diatoxanthin	1.4	0.9	1.1	0.8	9.7	2.1	0.71	0.8
Zeaxanthin	5.1	1.8	1.3	1.2	16.6	1.8	5.8	1.2

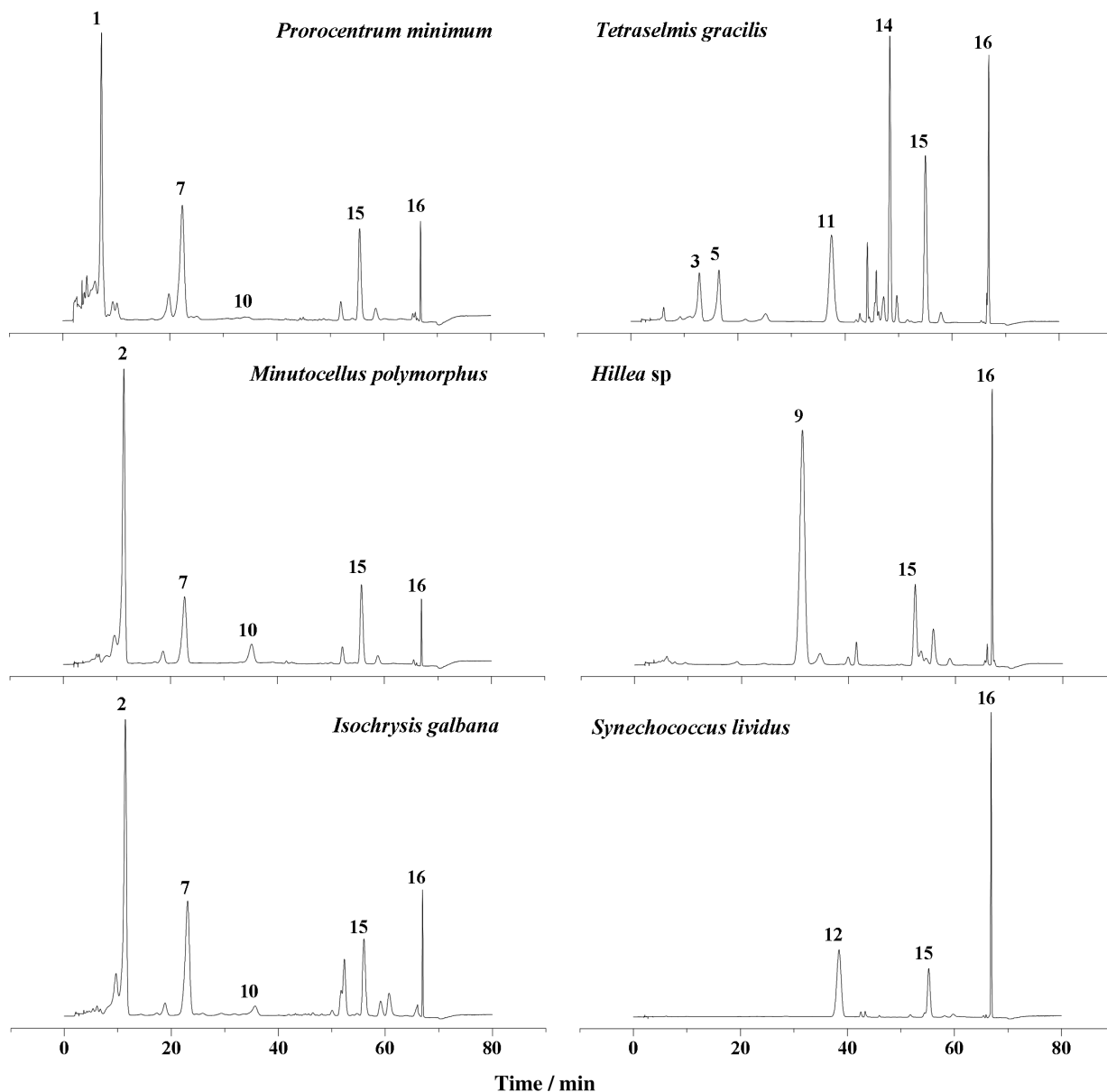


Figure 4. HPLC-DAD (445 nm) chromatograms of pigments from cyanobacteria and different microalgae. Chromatographic conditions are described in the text and peaks are identified in Tables 1 and 3.

same pattern of pigments in this species. Additionally, the xanthophylls fucoxanthin, diadinoxanthin and diatoxanthin were also present in the prymnesiophyceae *Isochrysis galbana*. Previous studies showed that this species contains other pigments such as chlorophyllide *a*, chlorophyll *c*₁ and *c*₂, β -carotene.³⁶

According to Egeland *et al.*³⁷ prasinophytes may contain either pigments similar to those produced by common green algae (β -carotene, lutein, zeaxanthin and the epoxides violaxanthin and neoxanthin) or carotenoids unique to this algal class (prasinoxanthin, anhydroprasinoxanthin, uriolide, anhydrouriolide, micromonal, anhydromicromonal, micromonol, anhydromicromonol and dihydrolutein),

where prasinoxanthin is the most abundant carotenoid. In our *Tetraselmis gracilis* (Prasinophyceae) strain, the carotenoids neoxanthin, violaxanthin and lutein were detected and the concentrations estimated. Chlorophyll *b* was also identified in *T. gracilis*.

The genus *Cryptophyta* displays two acetylenic class-specific marker compounds alloxanthin and monadoxanthin, plus crocoxanthin, chlorophyll *a* and *c*₂, zeaxanthin, and α - and β -carotene. Alloxanthin is very often the most abundant carotenoid.³⁸ Our strain of *Hillea sp* (Cryptophyceae) contained alloxanthin as the main carotenoid. Due to the lack of further available standards, it was not possible to characterize the other pigments (Figure 4).

Table 3. Quantification of the pigments present in algae measured by comparison to external standards using HPLC-DAD (445 nm)

Pigments	Pigments concentration ($\mu\text{g mg}^{-1}$ DW) measured in different species					
	<i>P. minimum</i>	<i>M. polymorphus</i>	<i>I. galbana</i>	<i>T. gracilis</i>	<i>Hillea</i> sp	<i>S. lividus</i>
Alloxanthin	ND	ND	ND	ND	2.34	ND
Antheraxanthin	ND	ND	ND	0.11	ND	ND
Astaxanthin	ND	ND	ND	ND	ND	ND
Canthaxanthin	ND	ND	ND	ND	ND	ND
β -Carotene	0.31	0.25	0.84	2.64	1.51	2.85
Chlorophyll <i>a</i>	8.02	8.46	14.84	33.32	6.31	12.58
Chlorophyll <i>b</i>	ND	ND	ND	16.53	ND	ND
Diadinoxanthin	0.62	0.42	1.31	ND	ND	ND
Diatoxanthin	0.02	0.19	0.17	ND	ND	ND
Fucoxanthin	ND	1.76	3.18	ND	ND	ND
Lutein	ND	ND	ND	1.74	ND	ND
Neoxanthin	ND	ND	ND	1.17	ND	ND
Peridinin	2.22	ND	ND	ND	ND	ND
Prasinolaxanthin	ND	ND	ND	ND	ND	ND
Violaxanthin	ND	ND	ND	0.72	ND	ND
Zeaxanthin	ND	ND	ND	ND	ND	1.44

* The absence of a pigment was indicated as not detected (ND).

A peculiarity of freshwater cyanobacteria is the presence of the primary ketocarotenoids canthaxanthin and echinenone and the monocyclic carotenoid-glycoside myxoxanthophyll. The later is typical of many freshwater cyanoprokaryota and apparently is a class-specific pigment since it has not been detected in eukaryotic algae up to date.³⁸ However, under conditions used by us, these pigments were not detected. It is also known that cyanobacteria are not capable of ϵ -ring biosynthesis;³⁹ therefore, α -carotene and lutein are not found. Instead, zeaxanthin and β -carotene are always present in this group. Indeed, in our cultures of *Synechococcus lividus* (Cianophyceae), zeaxanthin was detected as the major carotenoid (Figure 4 and Table 3).

Conclusion

This manuscript reports a new method for the HPLC analysis of carotenoids and chlorophylls. Results obtained using two different detectors were compared and better accuracy and precision was observed for HPLC-DAD. The method was efficiently applied to quantitative and qualitative analyses of different algal species and should prove a powerful tool for monitoring of pigments in complex matrices.

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References

- Cardozo, K. H. M.; Guaratini, T.; Barros, M. P.; Falcão, V. R.; Tonon, A. P.; Lopes, N. P.; Campos, S.; Torres, M. A.; Souza, A. O.; Colepicolo, P.; Pinto, E.; *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2007**, *146*, 60.
- Guaratini, T.; Medeiros, M. H. G.; Colepicolo, P.; *Quim. Nova* **2007**, *30*, 206.
- Havaux, M.; *Trends Plant Sci.* **1996**, *3*, 147.
- Barros, M. P.; Pinto, E.; Colepicolo, P.; Pedersen, M.; *Biochem. Biophys. Res. Commun.* **2001**, *288*, 225.
- Holt, N. E.; Zigmantas, D.; Valkunas, L.; Li, X. P.; Niyogi, K. K.; Fleming, G. R.; *Science* **2005**, *307*, 433.
- Fraser, P. D.; Bramley, P. M.; *Prog. Lipid Res.* **2004**, *43*, 228.
- van den Berg, H.; Faulks, R.; Granada, H. F.; Hirschberg, J.; Olmedilla, B.; Sandmann, G.; Southon, S.; Stahl, W.; *J. Sci. Food Agric.* **2000**, *80*, 880.
- Rodriguez, F.; Chauton, M.; Johnsen, G.; Andresen, K.; Olsen, L. M.; Zapata, M.; *Mar. Biol.* **2006**, *148*, 963.
- Schubert, N.; Garcia-Mendonza, E.; *J. Phycol.* **2008**, *44*, 1437.
- Tran, D.; Haven, J.; Qiu, W. G.; Polle, J. E. W.; *Planta* **2009**, *229*, 723.

11. Hollnagel, H. C.; Di Mascio, P.; Asano, C. S.; Okamoto, O. K.; Stringher, C. G.; Oliveira, M. C.; Colepicolo, P.; *Braz. J. Med. Biol. Res.* **1996**, *29*, 105.
12. Bjørnland, T.; Haxo, F. T.; Liaaen-Jensen, S.; *Biochem. Syst. Ecol.* **2003**, *31*, 1147.
13. Nakagawa, K.; Kiko, T.; Hatade, K.; Asai, A.; Kimura, F.; Sookwong, P.; Tsuduki, T.; Arai, H.; Miyazawa, T.; *Anal. Biochem.* **2008**, *381*, 129.
14. Cardozo, K. H. M.; Oliveira, M. A. L.; Tavares, M. F. M.; Colepicolo, P.; Pinto, E.; *Biol. Rhythm Res.* **2002**, *33*, 371.
15. Di Mascio, P.; Hollnagel, H. C.; Sperança, M.; Colepicolo, P.; *Biol. Chem.* **1995**, *376*, 297.
16. Yuan, J. P.; Gong, X. D.; Chen, F.; *J. Agric. Food Chem.* **1997**, *45*, 1952.
17. Feltl, L.; Pacáková, V.; Štulík, K.; Volka, K.; *Curr. Anal. Chem.* **2005**, *1*, 93.
18. Liu, D.; Gao, Y.; Kispert, L. D.; *J. Electroanal. Chem.* **2000**, *488*, 140.
19. Finckh, B.; Kontush, A.; Commentz, J.; Hübner, C.; Burdelski, M.; Kohlschütter, A.; *Anal. Biochem.* **1995**, *232*, 210.
20. Hermans, N.; Cos, P.; Berghe, D. V.; Vlietinck, A. J.; Bruyne, T. *J. Chromatogr., B* **2005**, *822*, 33.
21. Guillard, R. R. L. In *Culture of Marine Invertebrate Animals*; Smith, W. L.; Chanley, M. H., eds.; Plenum Publishing Corporation: New York, 1975.
22. Sander, L. C.; Sharpless, K. E.; Pursch, M.; *J. Chromatogr., A* **2000**, *880*, 189.
23. Schlatterer, J.; Breithaupt, D. E.; *J. Agric. Food Chem.* **2006**, *54*, 2267.
24. Ferruzzi, M. G.; Sander, L. C.; Rock, C. L.; Schwartz, S. J.; *Anal. Biochem.* **1998**, *256*, 74.
25. Dolan, J. W.; *LCGC* **1990**, *5*, 1030.
26. Inbaraj, B. S.; Chien, J. T.; Chen, B. H.; *J. Chromatogr., A* **2006**, *1102*, 193.
27. Frank, H. A.; Brudvig, G. W.; *Biochemistry* **2004**, *43*, 8607.
28. Andreoli, R.; Careri, M.; Manini, P.; Mori, G.; Musci, M.; *Chromatographia* **1997**, *44*, 605.
29. Guaratini, T.; Vessecchi, R. L.; Lavarda, F. C.; Campos, P. M. B. G. M.; Naal, Z.; Gates, P. J.; Lopes, N. P.; *Analyst* **2004**, *129*, 1223.
30. Guaratini, T.; Vessecchi, R.; Pinto, E.; Colepicolo, P.; Lopes, N. P.; *J. Mass Spectrom.* **2005**, *40*, 963.
31. Castro-Gamboa, I.; Cavalheiro, A. J.; Cardoso, C. L.; Silva, D. H. S.; Furlan, M.; Bolzani, V. S.; *J. Braz. Chem. Soc.* **2003**, *14*, 771.
32. Szymczak-Zyła, M.; Louda, J. W.; Kowalewska, G.; *J. Liq. Chromatogr. Relat. Technol.* **2008**, *31*, 1162.
33. Wright, S. W.; Jeffrey, S. W.; Mantoura, R. F. C.; Llewellyn, C. A.; Bjørnland, T.; Repeta, D.; Welschmeyer, N.; *Mar. Ecol. Prog. Ser.* **1991**, *77*, 183.
34. Jeffrey, S. W. In *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*; Jeffrey, S. W.; Mantoura, R. F. C.; Wright, S. W.; eds.; Unesco Publishing: Paris, 1997.
35. Sigaud-Kutner, T. C. S.; Pinto, E.; Okamoto, O. K.; Latorre, L. R.; Colepicolo, P.; *Physiol. Plant.* **2002**, *114*, 566.
36. Zapata, M.; Garrido, J. L.; *J. Phycol.* **1997**, *33*, 209.
37. Egeland, E. S.; Eikrem, W.; Thronsen, J.; Wilhelm, C.; Zapata, M.; Liaaen-Jensen, S.; *Biochem. Syst. Ecol.* **1995**, *23*, 747.
38. Schagerl, M.; Donabaum, K.; *Int. J. Limnol.* **2003**, *39*, 35.
39. Armstrong, G. A.; Hearst, J. E.; *FASEB J.* **1996**, *10*, 228.

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