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Research Article

SIMULTANEOUS SEPARATION OF CHLOROPHYLLS AND CAROTENOIDS BY RP-HPLC IN SOME ALGAE AND CYANOBACTERIA FROM THE SOUTHERN BALTIC

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Abstract

RP-HPLC (reversed-phase high-performance liquid chromatography) was used to analyse chlorophyll and carotenoid pigments in cyanobacteria and algae from the Baltic Sea, belonging to different taxonomic groups. The following species were used: *Cyclotella meneghiniana* - diatom, *Oocystis submarina* - green alga and *Phormidium amphibium* - cyanobacterium. Investigations on a favourable method of chlorophyll and carotenoid pigment separation have been carried out. This method allowed to separate the following pigments: lutein and zeaxanthin; diadinoxanthin and fucoxanthin; chlorophyll c_1 and c_2, into sharp peaks. It could be presumed that the method would be useful and universal in the identification of pigments in other algal and cyanobacteria species and natural phytoplankton assemblages.

INTRODUCTION

At present, high-performance liquid chromatography (HPLC) is commonly applied in chlorophyll and carotenoid analysis. It has the advantage of speed and sensitivity, in addition to protecting pigments from degradation by oxygen (Rowan 1989). HPLC now seems to be the most commonly used method for ecological work, *e.g.* for examining algal blooms or detecting the types of

symbionts present in marine organisms. Algal pigments can play an important role as biomarkers in identifying individual algae classes in naturally existing phytoplankton. For example, chlorophyll *b* and lutein are considered to be markers for green algae, fucoxanthin for diatoms, myxoxanthophyll for cyanobacteria, alloxanthin for cryptophytes and peridinin for dinoflagellates (Wright *et al*. 1991, Kirk 1994).

The most common method of pigment separation is the reversed-phase system with isocratic or gradient flow of eluents. Isocratic elution is a fast and simple method in the separation of pigments characterized by low polarity. When pigments of different polarity are separated, gradient elution is recommended (Roy 1987). Although reversed-phase high-performance liquid chromatography (RP-HPLC) can separate carotenoid and chlorophyll pigments into sharp peaks, certain separations, in particular: lutein and zeaxanthin; diadinoxanthin, dinoxanthin and fucoxanthin; neoxanthin and 19'hexanyloxyfucoxanthin; and chlorophyll c_1 and c_2, are not always possible (Rowan 1989). The aim of the present work was to elaborate a method providing reliable and simultaneous separation of chlorophyll and carotenoid pigments in cyanobacteria and algae from the Baltic Sea, belonging to different taxonomic groups. To verify the method, three strains of photoautotrophic organisms (diatom, green alga and cyanobacteria) from Baltic phytoplankton were used.

MATERIAL AND METHODS

The material used in chromatographic analyses was isolated from the Baltic coastal waters and maintained as monocultures in the Culture Collection of Baltic Algae (CCBA) in the Institute of Oceanography, Gdynia (Latała & Misiewicz 2002). The following strains were used: *Cyclotella meneghiniana* Kützing (Bacillariophyceae) - AL-10/586, *Oocystis submarina* Lagerheim (Chlorophyta) - AL-2/682 and *Phormidium amphibium* (Ag. ex Gom.) Anagnostidis & Komárek (Cyanobacteria) - AL-13/994. The batch cultures were carried out on sterile media, cyanobacteria - BG-11 (Stanier et al. 1971) and algae f/2 (Guillard 1975). The media were prepared from Baltic water, 8 PSU salinity. The water was filtered through GF/C Whatman glass filters and kept in darkness in plastic containers. The following incubation conditions were applied: irradiance (10 µmol photons$\cdot m^{-2} \cdot s^{-1}$ PAR), photoperiod - L:D 16:8 and temperature - 18 ºC.

At the exponential growth phase the cultures were filtered through GF/C Whatman glass filters (19 mm diameter). To improve the extraction yield (Rowan 1989) the filters were kept at -20 ºC for at least 30 min prior to

extraction. The extraction of chlorophyll and carotenoid pigments was carried out in cold 90% acetone (HPLC grade), in darkness, at -20 ºC for 4 hours (Strikland & Parsons 1972). To improve extraction, the cells were disintegrated for 2 min. in an ultrasonic bath. To remove cell emulsion and filter particles the pigment extract was centrifuged for 5-10 min. (5000 RPM). Then the sample was filtered through the PTFE filter (0.45 µm pore diameter).

To analyse pigment composition a Waters liquid chromatograph equipped with a diode array and fluorescent detectors was used. The absorption spectrum from 350 to 700 nm for each of the separated pigments was measured, detection at 440 nm absorbance. The separation was carried out at the ambient temperature, on the Vydac 201TP (C18) analytical column (dimensions 250 x 4.6 mm). Pigments were analysed by reversed-phase HPLC using the method of Mantoura & Llewellyn (1983). The column was protected by a guard column. The separation method was modified and adapted for the examined material. The changes concerned the eluent composition as well as the separation conditions and they are described in detail in the next chapter. The eluents used for chromatography were filtered and degassed with helium before the analysis. The flow rate was 1 $cm^3 \cdot min^{-1}$ throughout the separation. Pigments were identified by comparison of both retention times (elution sequence) and absorption spectra with those of pigment standards and pigments obtained from algal reference cultures. High purity pigment standards (myxoxanthophyll, zeaxanthin, β–carotene and chlorophyll *a*) were purchased from the International Agency for ^{14}C Determination VKI in Denmark. *Dunaliella tertiolecta* (SAG 13.86), *Synechococcus rubescens* (SAG B3.81), *Chroomonas salina* (SAG B980-1) and *Euglena gracilis* (Univ. NSW, strain Z) were used as reference cultures (Wright et al. 1991). As a result of the separation of reference and investigated cultures the following pigments were identified: neoxanthin, violaxanthin, lutein and chlorophyll b (from *D. tertiolecta),* cis-β-carotene (from *S. rubescens),* chlorophyll c (from *C. salina*) and diadinoxanthin (from *E. gracilis*). On the other hand, fucoxanthin (a common pigment in diatoms and present in great amounts in the culture examined) was identified by comparison of its absorption spectrum, elution sequence and retention time with literature data (Jeffrey *et al*. 1997).

RESULTS

To elaborate the most effective method of chromatographic analysis, pigments were separated in different gradient mixtures of eluents and different times of gradient program were applied. Investigations for a favourable method of chlorophyll and carotenoid pigment separation have been carried out. 0.5 M

(7.7 g in 200 cm^3) of ammonium acetate : methanol (20:80 v/v) and acetone : methanol (20:80 v/v) were used in mobile phases as eluents A and B respectively. Before the injection of pigment extract (20μl) the column was conditioned by an isocratic flow of eluents (40% of A and 60% of B) for ca. 15 min. Pigment separation was performed according to the gradient system (0-10 min. 20% A and 80% B - gradient, 10-20 min 20% A and 80% B - isocratic, 20-22 min 0% A and 100% B - gradient, 22-32 min 0% A and 100% B - isocratic, 32-40 min 40% A and 60% B - gradient).

Fig. 1 illustrates chromatograms of the extracts obtained from *C. meneghiniana* (diatom), *O. submarina* (green alga) and *P. amphibium* (cyanobacterium). The separation of the pigments proved to be very satisfactory. The following pigments were detected: fucoxanthin, diadinoxanthin, β–carotene, cis-β–carotene, chlorophyll c_1, chlorophyll c_2 and chlorophyll *a* in *C. meneghiniana*; neoxanthin, violaxanthin, lutein, zeaxanthin, β–carotene, chlorophyll *b* and chlorophyll *a* in *O. submarina*; myxoxanthophyll, zeaxanthin, β–carotene, cis-β–carotene and chlorophyll a in *P. amphibium*. In Table 1 spectral properties of pigments and their retention times are presented. Since the reversed-phase liquid chromatographic method was applied, the more polar pigments (fucoxanthin, neoxanthin, violaxanthin, chlorophyll c_1 and c_2) were eluted at the beginning and the nonpolar ones (carotenes) - at the end.

Table 1

Spectral properties of chlorophyll and carotenoid pigments.

Peak number	Pigment	Retention time [min]	Absorption maximum [nm]
1	Fucoxanthin	4,960	449,6
2	Chlorophyll c_1	6,127	442,4 632,0
3	Neoxanthin	6,392	(411,0) 435,1 464,1
4	Chlorophyll c_2	7,093	449,6 635,7
5	Violaxanthin	7,775	(417,7) 438,8 471,4
6	Diadinoxanthin	10,443	446,0 475,0
7	Myxoxanthophyll	10,462	475,0 504,1
8	Chlorophyll *b*	12,075	467,8 650,4
9	Lutein	12,542	446,0 475,0
10	Zeaxanthin	13,775	453,3 478,7
11	Chlorophyll *a*	15,892	431,5 665,0
12	β-Carotene	26,612	453,3 478,7
13	Cis-β-Carotene	27,512	447,2 471,4

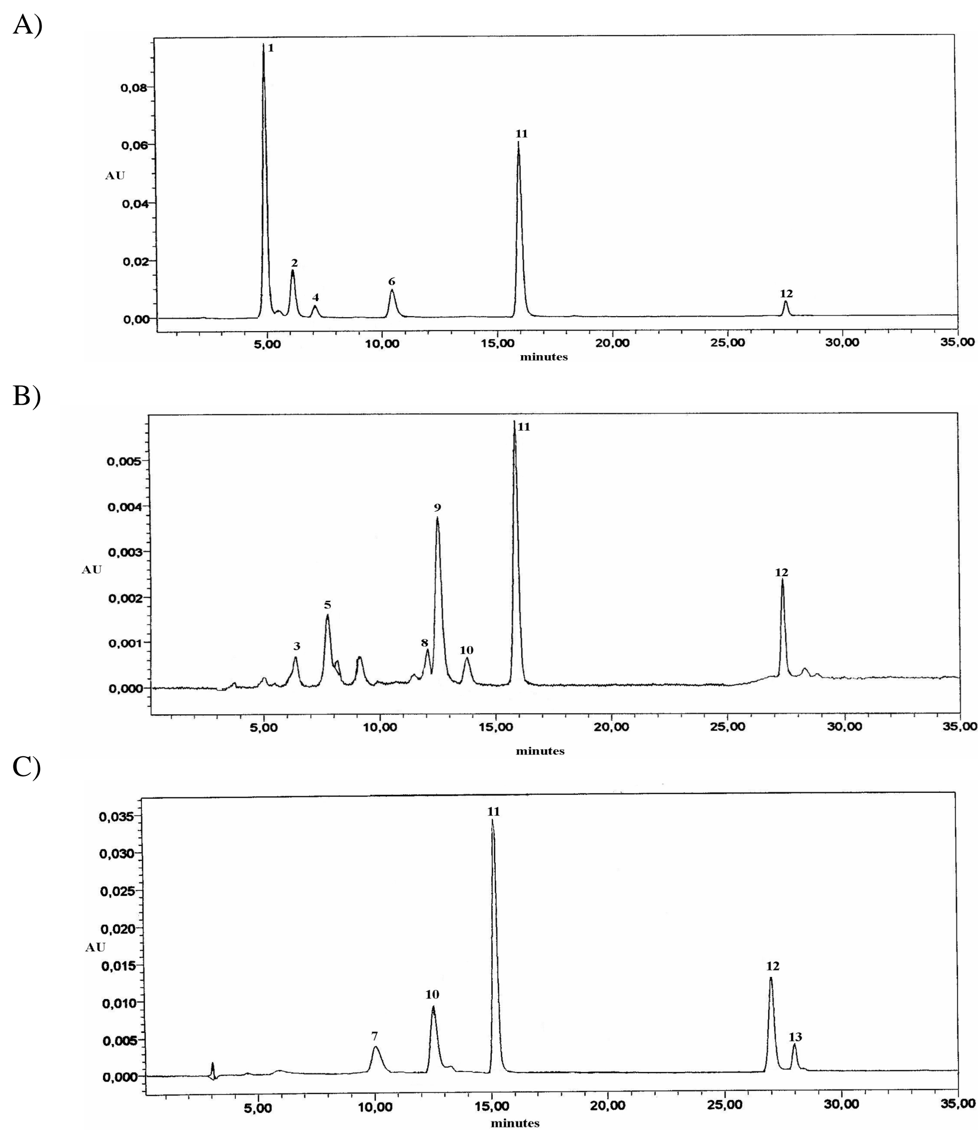

Fig. 1. Chromatographic separation of pigments extracted from *Cyclotella meneghiniana* (A), *Oocystis submarina* (B) and *Phormidium amphibium* (C). AU - absorbance at 440 nm, peak numbers are denoted like in Tab. 1.

DISCUSSION

To separate carotenoid and chlorophyll pigments the reversed-phase (RP) method was used. The method is generally accepted since it provides the best separation of pigments of different polarity (Mantoura & Llewellyn 1983, Roy 1987). Moreover, it gives good results only in combination with gradient elution. On the other hand, the RP method with isocratic elution could not be applied to the separation of polar pigments (*e.g*. chlorophylls *c*, chlorophyllide, xanthophylls). It could be used only for separating pigments of low polarity *e.g*. chlorophyll *a* and phaeophytin *a*. The employed RP-HPLC method provided a separation of pigments of taxonomic importance commonly occurring in Chlorophyta: neoxanthin and violaxanthin, lutein and zeaxanthin. Separate peaks of diadinoxanthin, fucoxanthin, chlorophyll c_1 and chlorophyll c_2 in the analysis of pigments extracted from *C. meneghiniana* were obtained. It is an undoubted achievement since Mantoura & Llewellyn (1983) could not separate those pigments in *Phaeodactylum* sp. In *P. amphibium* a taxonomically important pigment (myxoxanthophyll), commonly occurring in cyanobacteria, was found.

In the separation of carotenoid and chlorophyll pigments an ion-pair reagent (ipr) method (Mantoura & Llewellyn 1983, Schmid & Stich 1995) is often used. The method provides very good results of the main pigment separation in a much shorter time, less than 30 min. As ipr (P solution) Mantoura & Llewellyn (1983) used tetrabutylammonium acetate (1.5 g) and ammonium acetate (7.7 g). In eluent A it was used in proportion 20 (P solution) : 80 (methanol). In our method the buffer reagent of the tetrabutylammonium acetate type was not applied because it did not contribute to the separation improvement of the investigated pigments.

Vydac 201TP (C18) column was chosen on the basis of literature data (Roy 1987, Epler & Sander 1992, Van Heukelem *et al*. 1992, Sander & Wise 1993, Schmid & Stich 1995, Garrido & Zapata 1998). Polymeric C18 columns are the only columns enabling the separation of lutein and zeaxanthin in solvents containing methanol (Epler & Sander 1992). Schmid & Stich (1995) could not separate those pigments by the method of Mantoura & Llewellyn (1983). However, when they used the Vydac 201TP (C18) column, the intended effect was achieved. Using this column and the eluents - A: ammonium acetate - methanol (20 : 80) and B: acetate - acetonitryle (40 : 60) Garrido & Zapata (1997, 1998) obtained satisfactory results of pigment separation. They detected chlorophyll c_1, c_2 and c_3 in *Emiliania huxleyi* and *Isochrysis galbana* (Prymnesiophyceae). However, Sander *et al*. (1994) failed to separate chlorophyll c_1 and c_2 in *C. meneghiniana* when ipr was not used.

Table 2

Methods of pigment detection by HPLC with respect to the separation of pigment pairs.

Pigment pair	Mantoura & Llewellyn 1983	Wright et al. 1991	Van Heukelem et al. 1992	Present work
Chlorophyll c_1 and Chlorophyll c_2	-	-	+	+
Lutein and Zeaxanthin	-	+	+	+
Neoxanthin and 19'-hexanyloxyfucoxanthin	-	-	+	not tested
Diadinoxanthin and Fucoxanthin	+	+	+	+
Separation method	LiChrospher 100 RP-18 **Eluent A:** 80:20 methanol:P (7.7 g ammonium acetate and 1.5 g tetrabutylammonium acetate in 200 ml) **Eluent B:** 80:20 methanol:aceton	Spherisorb ODS2 **Eluent A:** 80:20 methanol:0.5 M ammonium acetate **Eluent B:** 90:10 acetonitrile:water **Eluent C:** ethyl acetate	Vydac 201TP (C18) **Eluent A:** 80:20 methanol:0.5 M ammonium acetate **Eluent B:** 70:30 methanol:aceton	Vydac 201TP (C18) **Eluent A:** 80:20 methanol:0.5 M ammonium acetate **Eluent B:** 80:20 methanol:aceton

In Table 2 four methods of pigment separation by HPLC are compared. Owing to the modification of the method of pigment identification by HPLC it was possible to obtain separated peaks of such pigments as chlorophyll c_1 and c_2, lutein, zeaxanthin, diadinoxanthin and fucoxanthin. It could be presumed that the modified method of pigment separation may be especially useful in the identification of pigments in extracts obtained from taxonomically different algal monocultures.

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