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ORIGINAL ARTICLE

# HPLC separation of cyanobacterial and algal photosynthetic pigments

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## Abstract

Photosynthetic pigments analysis has become a standard tool in ecological and physiological studies of photosynthetic organisms. With numerous methods previously published, there is no one ideal protocol that could satisfy all the research needs. Therefore, regarding the purpose of HPLC pigment analyses, a suitable method should be chosen. In this study, two $C_{18}$ columns (i.e., LiChrospher 100 RP18e and Spherisorb ODS2) and three sets of eluents (based on acetone, acetonitrile, methanol, and water) were used to develop three separation protocols. They were then examined with respect to their resolution and sensitivity, by analyzing pigment extracts obtained from 10 cyanobacterial and algal cultures and two types of environmental samples, i.e. phytoplankton and microphytobenthos. All the protocols provided highly repeatable results and allowed for the separation of all taxonomically most relevant chlorophylls and carotenoids. They had similar resolution and sensitivity. The Syst1 method was the shortest, while Syst2 had better resolution of pigments in the middle part of the protocol when most of the diagnostic pigments are separated (i.e., alloxanthin, diatoxanthin, lutein, chlorophyll *b*). Syst3, on the other hand, enabled distinguishing the highest number of pigments, including their derivatives and degradation products. The results showed that all protocols may be used for routine analysis of cyanobacterial and algal pigments in various sample types.

**Keywords** Carotenoids · Chlorophylls · HPLC · Marker pigments · Photosynthetic pigments

## Introduction

Photosynthetic pigments analysis has become an integral component in ecological and physiological studies of photosynthetic organisms. Since the introduction of high-performance liquid chromatography in the 1980s, it has been widely applied for separation and identification of chlorophylls and carotenoids, their derivatives as well as degradation products (e.g. Van Heukelem et al. 1994, Zapata et al. 2002, Mendes et al. 2007).

The composition of photosynthetic pigments may be used for the chemotaxonomic assessment of algal communities. The chlorophyll *a* concentration has been and still is readily used to estimate the biomass of algal communities. However, the subsequent development of the marker (or diagnostic) pigments concept allowed for the detection and biomass estimation of particular taxonomic groups or species (e.g., Stoń and Kosakowska 2000, 2002; Schlüter et al. 2000; Schlüter et al. 2004; 2014; Stoń-Egiert et al. 2010). Chlorophylls and carotenoids are widely distributed in the environment. Some of them, like chlorophyll *a*, $c_2$ or β-carotene, are quite ubiquitous among algae, whereas others, mainly xanthophylls, are characteristic for particular algal classes or divisions, e.g. alloxanthin and α-carotene for *Cryptophyceae*, fucoxanthin for *Bacillariophyceae*, *Chrysophyceae*, *Prymnesiophyceae*, lutein for *Chlorophyceae*, *Prasinophyceae* and it is also present in higher plants, and peridinin for *Dinophyceae* (e.g. Brotas and Plante Cuny 1998, 2003). Phycobilinproteins, an important light harvesting complexes found in various taxonomic groups such as cryptophytes and rodophytes, also can be used as a biomass proxy of those groups as well as valuable markers in their taxonomy (e.g., Lawrenz et al. 2011; Asencio and Hoffmann 2013). However, phycobiliproteins being water-soluble complexes require phosphate buffers for extraction, while chlorophylls and carotenoids are extracted using organic solvents; thus phycobiliprotenis cannot be analyzed together with them using HPLC technique (Lawrenz et al. 2011 and references therein). Regarding the fact that pigments in environmental samples are usually investigated

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with the purpose of the whole community description, the characteristics of phycobiliproteins would require separate procedures (i.e. extraction and quantification) to be performed, and that could be seen as of little use (considering the aim of a study). Especially that numerous carotenoids, which can be simultaneously detected and quantified with HPLC technique, can be applied as indicators of the presence of cyanobacteria and phycobiliproteins-containing algae (e.g. alloxanthin, canthaxanthin, echinenon, myxoxanthophyll, zeaxanthin).

Characteristics of photosynthetic pigments also enable the evaluation of an algal physiological state. Pigments concentrations and ratios change due to light and nutrient conditions. Chlorophyll *a* is the primary photosynthetic pigment. Other chlorophylls support its photosynthetic functions, changing correspondingly with varying light conditions. Carotenoids, on the other hand, giving their physiological roles can be separated into two groups, i.e. photosynthetic (i.e. fucoxanthin, fucoxanthin-like pigments, peridinin, prasinoxanthin and α-carotene) (PSC) and photoprotective (i.e. alloxanthin, diadinoxanthin, diatoxanthin, lutein, neoxanthin, violaxanthin, zeaxanthin, β-carotene) (PPC) ones (e.g. Stoń and Kosakowska 2000; Eisner et al. 2003). Photosynthetic xanthophylls enable better utilization of low light intensities, increasing its efficiency. In contrast, the concentration of photoprotective ones increases at higher light intensities to protect photosystem against excessive irradiance and photoinhibition (e.g. MacIntyre et al. 2002; Goss and Jakob 2010; Jahns and Holzwarth 2012).

The HPLC-based separation of photosynthetic pigments in natural samples of phytoplankton and microphytobenthos communities has been extremely challenging (e.g. Brotas and Plante Cuny 1998, Goela et al. 2014, Tamm et al. 2015, Chai et al. 2016, Pniewski and Sylwestrzak 2018). Complex mixtures of algal species contain pigments of various molecular structures and properties (from the polar chlorophylls to non-polar carotenes), which may be difficult to separate as some of the pigment pairs differ only in the presence or position of double bonds (Zapata et al. 2000). Currently used methods differ in sensitivity, resolution and separation time (Mendes et al. 2007). Various dual or ternary solvent systems are employed using columns containing most commonly, but not restricted to, octadecysilica (ODS) $C_{18}$ or octylsylica (OS) $C_8$ stationary phases, while mobile phases usually consist of methanol, acetonitrile, ethyl acetate or acetone. Additionally, ion-pairing reagents such as ammonium acetate or tetraethylammonium acetate are also used (e.g., Kraay et al. 1992, Shmid &Stich 1995, Zapata et al. 2000, Van Heukelem and Thomas 2001, Jayaraman et al. 2011).

Although many various separation protocols and their modifications have been published, there is no ideal method providing satisfactory results in all research situations. Therefore, this paper aimed to present three protocols differing in the resolution and sensitivity of photosynthetic pigment separation and quantification. In this paper, two different columns and three different sets of eluents were tested. The methods were evaluated using a variety of algal cultures and mixtures as well as natural algal community samples, including phytoplankton and microphytobenthos.

## Materials & methods

### Reagents

Solvents for extraction and chromatography, including acetone, methanol, acetonitrile, were HPLC-grade (T.J.Baker). For the preparation of the ion-pairing reagent, ammonium acetate (Sigma-Aldrich) was used.

### Algal cultures and natural algal community samples.

The cultures used in the experiment were obtained from the Culture Collection of Baltic Algae (University of Gdańsk, Poland), including three cyanobacterial strains, i.e. *Aphanizomenon* sp. BA-69, *Geitlerinema amphibium* BA-13, *Nodularia spumigena* BA-15, and four algal species, i.e. two benthic diatoms: *Bacillaria* cf. *paxillifera* BA-14, *Nitzschia aurariae* BA-158 and two green algae: *Chlorella vulgaris* BA-02 and *Monoraphidium contortum* BA-05; Kalmar Algae Collection (Linnaeus University, Sweden) – the dinoflagellate *Amphidinium carterae* KAC 28 and diatom *Thalassiosira weissflogii* KAC 24; and the CAUP Culture Collection (Charles University, Czech Republic) – cryptophyte *Cryptomonas* sp. CAUP F 105. All cultures were grown in f/2 enriched artificial seawater (Guillard 1975) with the salinity of 8, prepared using marine salt (TropicMarine®), except for *Cryptomonas* sp. which was grown in freshwater. The cultures were kept at a constant temperature of 17 °C and two different light intensities, i.e. 30 μmol photons $^{-2}$ $s^{-1}$ (low light, LL) and 150 μmol photons $^{-2}$ $s^{-1}$ (high light, HL) in a 16 h:8 h light-dark cycle for 10 days. After this time, cells were filtered onto the Whatman GF/C filters and immediately frozen at −60 °C until further processing. Seawater and sediment samples were obtained at the station near Władysławowo located in the coastal shallows of the Puck Lagoon (54°43' N, 18°34′ E). Water samples (1 L), similarly to algal cultures, were filtered through the Whatman GF/C filters and kept frozen at −60 °C. Sediment samples were collected using corer, and the top 1 cm of the sediment was cut off, frozen, and then freeze-dried (as described in Pniewski et al. 2015).

### Extraction procedures

Photosynthetic pigments from algal cultures and phytoplankton samples were extracted in a similar manner; pigments were extracted with 4 ml of cold 90 % acetone for 4 h at −20 °C (Strickland & Parsons, 1972), in the dark.

Subsequently, samples were centrifuged (7000 rpm, 10 min) and filtered through the PTFE filter (Pniewski et al. 2015). Pigments from the sediment samples (1 g) were extracted using the same procedure as described above, but using 2 ml of cold acetone solution instead of 4 ml.

### HPLC pigment analysis

Prepared extracts were analyzed with Waters liquid chromatograph system comprising dual system pump Waters 515, Diode Array Detector 440 set at 440 nm. Pigment separation was performed using three protocols based on the reverse phase chromatography (RP-HPLC) technique, employing two types of $C_{18}$ columns and three sets of eluents, i.e. (1) the method using LiChrospher 100 RP18e (Merck) column and methanol-water eluent A and methanol-acetone eluent B; (2) the method using the same column as previously but with acetonitrile-water eluent A and methanol-acetone eluent B; and (3) the method employing Spherisorb ODS2 (Waters) column and again methanol-water and methanol-acetone based eluents. The methods examined herein will be referred to as Syst1, Syst2, and Syst3, respectively. All details regarding eluents and analytical gradients are presented in Table. 1. An injection volume was 40 μl. In Syst3, samples were first diluted with distilled water in the volume ratio of 3:1 to allow better early peaks separation. Pigments were identified from their absorbance spectra and retention times. The resolution between critical pigment pairs was evaluated using the following formula $R_s = 2(Rt_2\text{-}Rt_1)/W$, where $Rt_1$ and $Rt_2$ are the retention times of given peaks, and W is the sum of peaks' width at the baseline (Mendes et al. 2007). The HPLC system was calibrated using high purity pigment standards purchased from DHI (Institute for Water and Environment, Denmark), i.e., chlorophyll *a*, *b*, $c_2$, anteraxanthin, cantaxanthin, diadinoxanthin, diatoxanthin, echinenone, fucoxanthin, lutein, myxoxanthophyll, neoxanthin, peridinin, violaxanthin, zeaxanthin, α-carotene and β-carotene. Pigments were quantified following the procedure described by Mantoura and Repeta (1997). Calibration curves were used to calculate the response factor ($f_p$) for all pigments using the equation $f_p = W_p/A_p$, where $W_p$ is the weight of the pigment, while $A_p$ is its area (Stoń-Egiert and Kosakowska 2005; Mendes et al. 2007). Limits of detection (LOD) and quantification (LOQ) were calculated as described by Hooker et al. (2005), assuming that the signal-to-noise ratios (SNR) for LOD and LOQ equal 3 and 10, respectively.

**Table 1** Characteristics of the chromatographic columns and analytical gradients used in tested separation procedures

| | | Solvent (%) | |
|---|---|---|---|
| Time (min) | Flow (ml $min^{-1}$) | A | B |
| Column: LiChrospher 100 18RPe (endcapped), dimensions: 250 mm × 4 mm, octadecylsilica bonded phase, particle size: 5 μm, carbon load 21.6 % | | | |
| Analytical protocol 1 (Syst1) | | | |
| | | Methanol:water* (80:20 v:v) | Methanol:acetone (70:30 v:v) |
| 0 | 0.8 | 70 | 30 |
| 10 | 0.8 | 20 | 80 |
| 15 | 0.8 | 0 | 100 |
| 30 | 0.8 | 0 | 100 |
| 35 | 0.8 | 70 | 30 |
| 40 | 0.8 | 70 | 30 |
| Analytical protocol 2 (Syst2) | | | |
| | | Acetonitrile:water‡ (50:50 v:v) | Methanol:acetone (60:40 v:v) |
| 0 | 0.8 | 60 | 40 |
| 5 | 0.8 | 20 | 80 |
| 17 | 0.8 | 20 | 80 |
| 20 | 0.8 | 5 | 95 |
| 27 | 0.8 | 5 | 95 |
| 30 | 0.8 | 0 | 100 |
| 42 | 0.8 | 0 | 100 |
| 45 | 0.8 | 60 | 40 |
| 50 | 0.8 | 60 | 40 |
| Column: Spherisorb (Spher), dimension: 150 mm × 4.6 mm, octadecylsilica bonded phase (ODS2), particle size: 3 μm, carbon load 11.5% | | | |
| Analytical protocol 3 (Syst3) | | | |
| | | Methanol:water* (80:20 v:v) | Methanol:acetone (80:20 v:v) |
| 0 | 0.7 | 55 | 45 |
| 2.5 | 0.7 | 55 | 45 |
| 25 | 0.7 | 10 | 90 |
| 27 | 0.7 | 0 | 100 |
| 44 | 0.7 | 0 | 100 |
| 46 | 0.7 | 55 | 45 |
| 50 | 0.7 | 55 | 45 |

*buffered with 200 mM ammonium acetate (final concentration)

‡buffered with 10 mM ammonium acetate (final concentration)

## Results and discussion

Methods were tested using a variety of samples, including cyanobacterial and algal strains, mixed extracts as well as environmental samples. The separation of photosynthetic pigments within one run was satisfactory with all three methods used. Overall, a total number of 37 pigments were detected, including 10 chloropigments and 27 carotenoids and their derivatives. Table 2 compiles pigments and their derivatives detected in extracts obtained for each analyzed algal strain and environmental sample in order to show and compare their elution order and retention times. The extract in which a particular peak was observed was specified in the Source column. The absorption maxima recorded for each peak (as measured in the eluents mixture by DAD detector) during separation procedure were also included, as they may

**Table 2** Pigments detected by each separation protocol (as described in Table 1), their corresponding retention times (Rt), absorption maxima measured during the elution and sources

| | Pigment | Retention time (Rt) (min) | | | Absorption maxima ($\lambda_{max}$) (nm) | | | Source* |
|---|---|---|---|---|---|---|---|---|
| | | Syst1 | Syst2 | Syst3 | Syst1 | Syst2 | Syst3 | |
| 1 | Chlorophyllide *a* | 6.33 | 6.07 | 3.92 | 433, 667 | 432, 618, 665 | 430, 618, 667 | 1–9 |
| 2 | Chlorophyll $c_1$ + $c_2$ | 7.71 | 6.88 | 5.52 | 447, 584, 633 | 445, 581, 630 | 447, 584, 633 | 1,3,8,10,S,W |
| 3 | Peridinin | 8.58 | 9.58 | 6.04 | 474 | 474 | 474 | 1 |
| 4 | Peridinin-derivative | 9.25 | 7.30 | 6.73 | 472 | 475 | 472 | 1 |
| 5 | Peridinin-derivative | 10.01 | 10.04 | 7.46 | 464 | 469 | 467 | 1 |
| 6 | Fucoxanthin | 10.33 | 10.77 | 8.455 | 450 | 450, 467 | 452 | 3,8,10,S,W |
| 7 | Oscillatoxanthin | 10.60 | | | 496, 530 | | | 2 |
| 8 | Neoxanthin | 11.09 | 11.39 | 9.613 | 413, 438, 464 | 413, 438, 467 | 411, 435, 464 | 4,7 |
| 9 | Pheophorbide *b* | | | 9.98 | | | 437, 598, 652 | S,W |
| 10 | 4-ketomyxoxanthophyll-like pigment | 11.58 | 11.84 | 11.00 | 481, 508 | 484, 511 | 484, 508 | 9 |
| 11 | Violaxanthin | 12.04 | 12.70 | 12.73 | 440, 469 | 416, 440, 469 | 440, 469 | 4,7,W |
| 12 | Fucoxanthin-derivative | 12.22 | 13.19 | 11.80 | 445 | 445 | 450 | 3,8 |
| 13 | Dinoxanthin | 12.66 | 13.95 | 12.73 | 442, 469 | 440, 469 | 440, 469 | 1 |
| 14 | Fucoxanthin-derivative | 12.68 | 13.97 | | 442 | 440 | | 3,8 |
| 15 | Pheophorbide *a* | | | 13.33 | | | 411, 608, 667 | S,W |
| 16 | Aphanizophyll | 12.68 | 13.57 | 13.51 | 474, 506 | 476, 508 | 474, 506 | 2 |
| 17 | Astaxanthin | 12.77 | 14.11 | | 479 | 479 | | 7 |
| 18 | Diadinoxanthin | 13.14 | 14.65 | 13.80 | 447, 476 | 447, 476 | 445, 476 | 3,8,S,W |
| 19 | Antheraxanthin | 13.67 | 15.36 | 14.85 | 447, 474 | 447, 476 | 445, 474 | 4,7,W |
| 20 | Myxoxanthophyll | 13.90 | 15.74 | 15.58 | 474, 506 | 476, 508 | 474, 506 | 6 |
| 21 | Alloxanthin | 14.17 | 17.20 | 16.23 | 452, 481 | 452, 481 | 450, 476 | 5,S,W |
| 22 | Diatoxanthin | 14.66 | 18.81 | 17.31 | 452, 479 | 455, 481 | 452, 479 | 3,8,S,W |
| 23 | Lutein | 15.01 | 20.34 | 18.10 | 445, 474 | 447, 474 | 445, 474 | 4,7,W |
| 24 | Zeaxanthin | 15.04 | 20.06 | 18.43 | 452, 479 | 452, 479 | 450, 479 | 4,6,7,S |
| 25 | Monadoxanthin | 15.47 | 21.10 | 18.41 | 447, 476 | | 447, 476 | 5 |
| 26 | Canthaxanthin | 17.19 | 23.82 | 23.00 | 476 | 476 | 476 | 2,9 |
| 27 | Chlorophyll *b* | 18.96 | 27.47 | 26.26 | 467, 601, 650 | 467, 599, 650 | 467, 601, 650 | 4,7,S,W |
| 28 | Crocoxanthin | 20.24 | 28.61 | 28.85 | 445, 476 | 447, 476 | 447, 474 | 5 |
| 29 | Chlorophyll *a* allomer | 20.02 | 29.21 | 28.41 | 430, 618, 665 | 432, 613, 665 | 430, 616, 665 | 1–9,S,W |
| 30 | Chlorophyll *a* | 21.36 | 31.26 | 30.40 | 430, 618, 665 | 430, 618, 665 | 430, 618, 665 | 1–9,S,W |
| 31 | Chlorophyll *a* epimer | 22.24 | 32.78 | 31.42 | 430, 623, 665 | 430, 623, 665 | 430, 616, 665 | 1–9,S,W |
| 32 | Echinenone | 22.84 | 32.23 | 32.12 | 462 | 462 | 462 | 9 |
| 33 | Pheophytin *b* | | | 38.50 | | | 435, 535, 655 | W |
| 34 | Pheophytin *a* | | | 43.30 | | | | S,W |
| 35 | $\alpha$-carotene | 32.67 | 40.63 | 42.03 | 445, 474 | 447, 474 | 445, 474 | 5 |
| 36 | $\beta$-carotene | 34.02 | 41.54 | 43.30 | 452, 479 | 452, 479 | 452, 479 | 2–9,S,W |
| 37 | cis-$\beta$-carotene | | | 44.08 | | | 445, 472 | 6 |

*The Source column specifies the origin of the extract (i.e. cyanobacterial/algal culture and/or environmental sample) in which particular pigment was detected; 1. *Amphidinium carterae* KAC 28, 2. *Aphanizomenon* sp. BA-69, 3. *Bacillaria* cf. *paxillifera* BA-14, 4. *Chlorella vulgaris* BA-02, 5. *Cryptomonas* sp. CAUP F 105, 6. *Geitlerinema amphibium* BA-13, 7. *Monoraphidium contortum* BA-05, 8. *Nitzschia aurariae* BA-158, 9. *Nodularia spumigena* BA-15, 10. *Thalassiosira weissflogii* KAC 24, W – water (phytoplankton) sample, S – sediment (microphytobenthos) sample

vary from values provided for pigment standards (which are usually measured in specific extraction solvent). The highest number of pigments (32) was identified with the systems Syst3. Comparing all three protocols, the elution orders of the main pigments generally were the same; however, some inversions mainly regarding pigment derivatives or degradation products were also observed. Pigment retention times varied. The shortest ones were observed in the system Syst1.

In the systems Syst2 and 3 retention times were comparable, except for the pigments eluting as last ones, namely α-carotene and β-carotene (Table. 2).

## Pigments composition and resolution

Regarding the analysis of 10 cyanobacterial and algal monocultures belonging to 5 taxonomic groups, all separation protocols were successful and provided sufficient resolution of almost all main pigments. Two pigments, i.e. chlorophyll *a* and β-carotene were common to all studied strains. The only exception was *Cryptomonas* sp. CAUP F 105 which contained α-carotene instead of β-carotene (Fig. 1, Table. 2). In all tested strains a range of xanthophylls was identified. Cyanobacteria were the most diverse in this respect; in *Aphanizomenon* sp. BA-69 oscillatoxanthin and aphanizophyll were found, in *N. spumigena* BA-15 extract canthaxanthin and 4-ketomyxoxanthophyll-like pigment as well as aphanizophyll were identified, whereas in *G. amphibium* BA-13 myxoxanthophyll and zeaxanthin were present. In diatoms (*B.* cf. *paxillifera* BA-14, *N. aurariae* BA-158, *T. weissflogii* KAC 24) chlorophylls $c_1$ and $c_2$ co-eluted, while other pigments (fucoxanthin, diadinoxanthin, diatoxanthin) posed no problems in separating them. Regarding other tested chlorophyll *c*-containing algae, i.e. the dinophyte and cryptophyte, they possessed chl $c_2$ only. In *A. carterae* KAC 28 extracts peridin, diadinoxanthin and diatoxanthin as well as small amounts of dinoxanthin were identified, whereas in *Cryptomonas* sp. CAUP F 105 alloxanthin, monadoxanthin and crocoxanthin were found. For most of the pigments observed in the tested strains of green algae (*C. vulgaris* BA-02 and *M. contortum* BA-05), i.e. neoxanthin, violxanthin, antheraxanthin and chlorophyll *b*, their full resolution was obtained, except for the pigment pair lutein / zeaxanthin.

Next, a mixture of pigment extracts obtained from cyanobacterial and algal cultures was analyzed using the three methods tested. The mixture consisted of extracts obtained from *A. carterae* KAC 28 HL, *Cryptomonas* sp. CAUP F 105 LL, *N. spumigena* BA-15 HL, *C. vulgaris* BA-02 HL and *T. weissflogii* KAC 24 HL. Most of the tested diagnostic pigments (i.e. alloxanthin, fucoxanthin, peridinin, chlorophyll *b*) were fully separated with all tested protocols, while other pigments co-eluted (Fig. 2, Tables. 2, 3). As mentioned above, none of the methods discriminated chlorophyll $c_1$ and $c_2$. Generally, the resolution of polar chlorophylls c (i.e. chl $c_1$, $c_2$, $c_3$, MgDVP) is important from the taxonomical point of view when analyzing phytoplankton samples as they may be indicative of certain algal classes (e.g. chl $c_3$ is present in species belonging to *Prymnesiophyceae*, *Chrysophyceae*, and MgDVP is typical of *Prasinophyceae*) (Jeffrey and Vesk 1997). Full separation of polar chlorophylls requires polymeric-packed $C_{18}$ or monomeric $C_8$ columns (e.g. Rodríguez et al. 1998; Zapata et al. 2000; Mendes et al. 2007). In this study, Syst3 clearly discriminated chl $c_3$ from chl $c_1 + c_2$ (an analysis of a commercial mixture of pigments from DHI, data not shown). Some of the cyanobacterial pigments co-eluted with algal ones. Aphanizophyll and diadinoxanthin were completely separated with this system only. Myxoxanthophyll was not separated from alloxanthin in Syst3 and only Syst1 allowed for their full resolution. The main problem was a separation of lutein and zeaxanthin. These xanthophylls were not separated with the LiChrospher column (Syst1 and 2), while with Spherisorb (Syst3), partial resolution was obtained (Rs = 0.91) (Table. 3.) (Tamm et al. (2015) were able to fully separate them using two combined Spherisorb columns). The separation of both pigments is crucial from both physiological as well as chemotaxonomical point of view. Lutein and zeaxanthin are present in green algae cells, and their separation is important to properly evaluate their photoacclimation and photoprotection mechanisms (Masjídek et al. 2004; Jahns and Holzwarth 2012). Furthermore, both pigments are considered to be diagnostic ones; lutein indicates the presence of chlorophytes (although one must be aware of the fact that it also occurs due to the presence of higher plant debris), while zeaxanthin is the marker pigment for cyanobacteria (Brotas and Plante Cuny 1998). In some cases, chlorophyll derivatives prevented the full resolution of some xanthophylls. The Syst1 method did not allow separating crocoxanthin from chl *a* allomer, in the Syst2 method echinenone co-eluted with chl *a* epimer, while none of the methods enabled separation of *β*-carotene and phaeophytine *a* (Tables. 2, 3).

Environmental samples (i.e. phytoplankton and microphytobenthos) were analyzed with Syst3 as it was shown to distinguish the highest number of peaks. Pigment composition in both environmental samples was quite similar; main carotenoids included fucoxanthin as well as diadinoxanthin and diatoxanthin, zeaxanthin, alloxanthin and lutein (Fig. 3). However, both samples differed with respect to the proportion of particular pigments in the carotenoid pool. Among chloropigments chlorophylls *a*, *b* and $c_{1+2}$ were found. Additionally, several chlorophylls degradation products were identified, i.e., chlorophillde *a*, pheophorbides *a* and *b*, as well as pheophytin *a* and *b*. There were also some other peaks of unidentified pigments. Their quantities were minute, and thus the absorption spectra were unclear. In the analyzed samples pheopigments did not interfere with the identification and quantification of carotenoids, except for pheophytin *a* which was not separated from β-carotene.

## Duration of the procedures

The duration of the separation may vary greatly from 30 even up to 60 min (e.g. Schmidt & Stich 1994, Pinckney et al. 2001, Zapata et al. 2000, Jodłowska and Latała 2003).

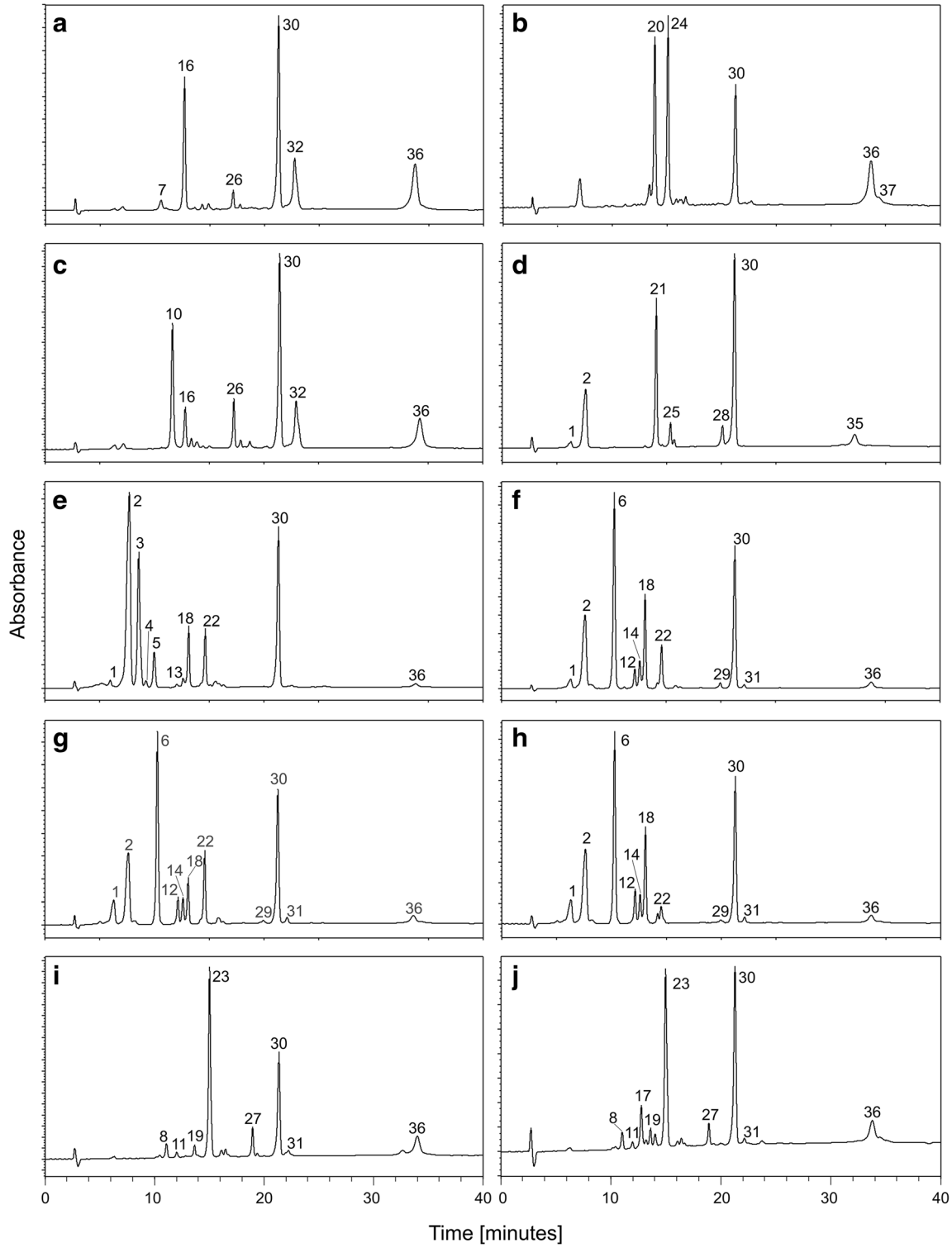


**Fig. 1** Photosynthetic pigment composition from the cyanobacterial and algal cultures obtained using the Syst1 procedure; (A) *Aphanizomenon* sp. BA-69, (B) *Geitlerinema amphibium* BA-13, (C) *Nodularia spumigena* BA-15, (D) *Cryptomonas* sp. CAUP F 105, (E) *Amphidinium carterae* KAC 28, (F) *Bacillaria* cf. *paxillifera* BA-14, (G) *Nitzschia aurariae* BA-158, (H) *Thalassiosira weissflogii* KAC 24, (I) *Chlorella vulgaris* BA-02 and (J) *Monoraphidium contortum* BA-05. Pigments are labeled according to the Table 2

It is dependent on system applied, flow rate, separation temperature, solvents used and column characteristics (diameter, particle size etc.) (e.g. Van Heukelem et al. 1994; Van Heukelem and Thomas 2001; Stoń-Egiert and Kosakowska 2005). In this study, the duration of the pigment separation differed among methods. The longest was with Syst3, which

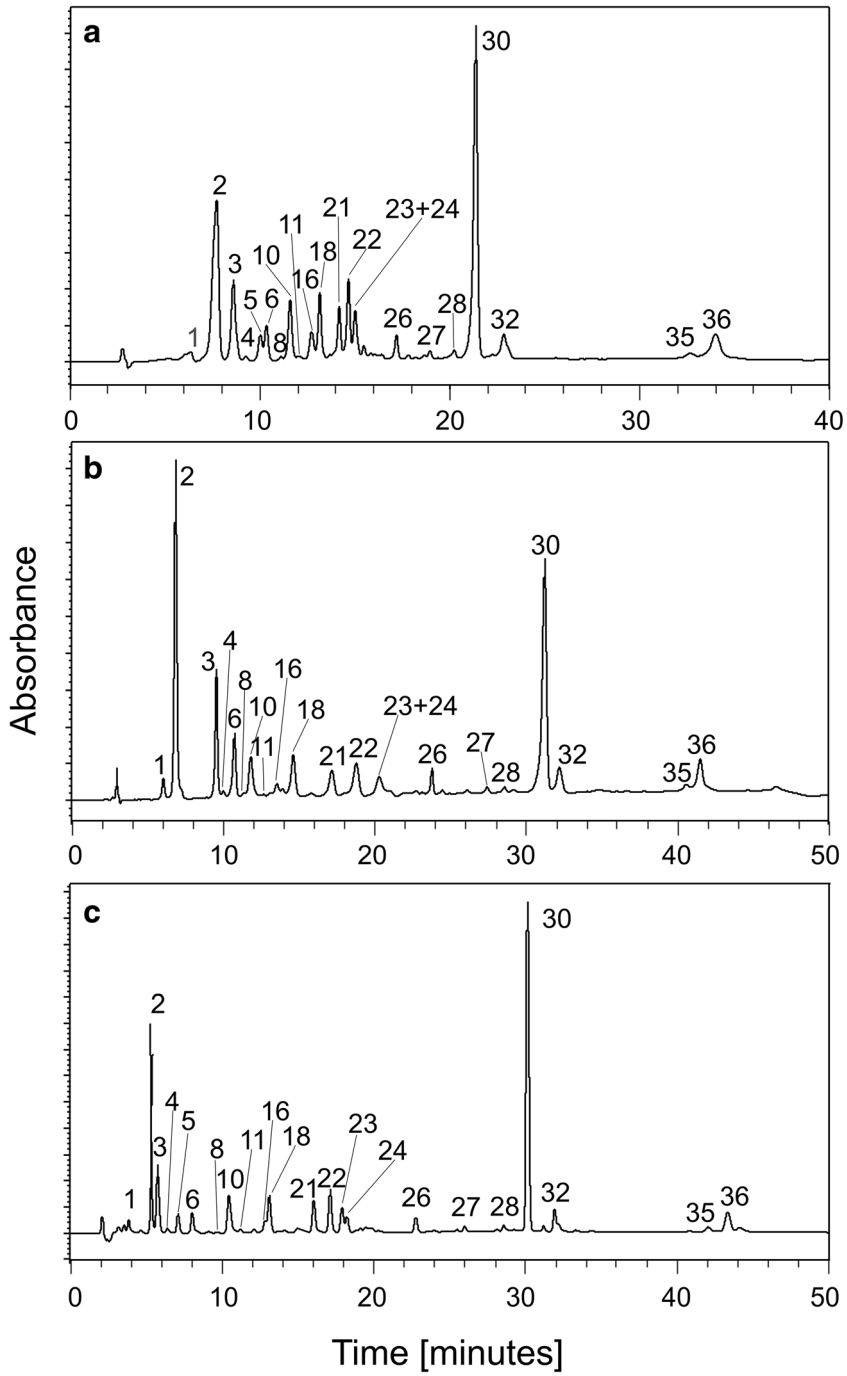


**Fig. 2** Chromatograms obtained for the mixture of pigment extracts from *Amphidinium carterae* KAC 28, *Chlorella vulgaris* BA-02, *Cryptomonas* sp. CAUP F 105, *Nodularia spumigena* BA-15 and *Thalassiosira weissflogii* KAC 24 with the (A) Syst1, (B) Syst2 and (C) Syst3 separation procedures. Pigments are labeled according to the Table 2

may be attributed to the lowest flow rate of 0.7 ml/min as well as the low elution strength of the solvent B consisting of the lowest proportion of acetone (20 %) compared to B solvents in other methods tested herein. In Syst1 and 2 the same column (LiChrospher) and flow rate (0.8 ml/min) were employed. Thus it may be tentatively assumed that the difference in the duration of the procedure resulted from the solvents used and gradients applied.

## The use of water and the addition of an ion-pairing agent for better pigments separation

Dilution of the extracts with water is sometimes recommended to obtain higher and sharper peaks for polar pigments (Wright et al. 1991). Van Leeuwe et al. (2006) suggested water packing, which prevents sample dilution and thus changes in pigments' concentrations as well as broadening of the peaks.

**Table 3** Resolution ($R_s$) of various pigment pairs estimated based on the results obtained from tested separation procedures described in Table 1

| | Pigment pair | Separation procedure | | |
|---|---|---|---|---|
| | | Syst1 | Syst2 | Syst3 |
| 1 | chl$c_1$ / chl$c_2$ | – | – | – |
| 2 | chlc / peridinin | +++ | +++ | ++ |
| 3 | peridinin / fucoxanthin | +++ | +++ | +++ |
| 4 | fucoxanthin / neoxanthin | +++ | + | +++ |
| 5 | aphanizophyll / diadinoxanthin | + | +++ | + |
| 6 | antheraxanthin / alloxanthin | +++ | +++ | +++ |
| 7 | myxoxanthophyll / alloxanthin | + | +++ | +++ |
| 8 | alloxanthin / diatoxanthin | +++ | +++ | +++ |
| 9 | diatoxanthin / lutein | + | +++ | +++ |
| 10 | lutein / zeaxanthin | – | – | ++ |
| 11 | zeaxanthin / monadoxanthin | +++ | +++ | – |
| 12 | chl*a* allomer / crocoxanthin | – | +++ | +++ |
| 13 | chl*a* epimer / echinenon | +++ | – | +++ |
| 14 | $\beta$-carotene / phaeophytin *a* | – | – | – |
| 15 | $\alpha$-carotene / $\beta$-carotene | +++ | ++ | +++ |

+++ – Rs > 1, ++ – Rs = 1–0.85, + – Rs = 0.85–0.5, – - Rs < 0.5

However, such water packing is not always possible, and the addition of water may be the only option to obtain better resolution of polar pigments that elute at similar times. Unfortunately, the dilution of a sample with water leads to a lower concentration of a given pigment, and thus a smaller peak area is observed, which subsequently causes lower sensitivity of a method. In this study, a water dilution procedure was only used with Syst3 to improve the separation of two pigments i.e., chl *c* and peridinin, which had very similar retention times resulting in their poor separation. Regarding that, the peridinin is a pigment marker such modification was necessary to facilitate its full separation.

Another method for improvement of polar pigments separation is the addition of an ion-pairing agent, e.g., ammonium acetate or pyridine (e.g. Schmid and Stich 1995; Zapata et al. 2000). Methods Syst1 and 3 originally included an ion-pairing agent, namely ammonium acetate, although in different concentrations (Table. 1). In the original protocol by Schmid and Stich (1995), which was used to develop Syst2, an ion-pairing agent was not included in the eluents (its use was restricted to the pigment extractions). When the Schmid and Stich (1995) analytical gradient was applied to the LiChrosphere column, polar pigments had shortened retention times and eluted right

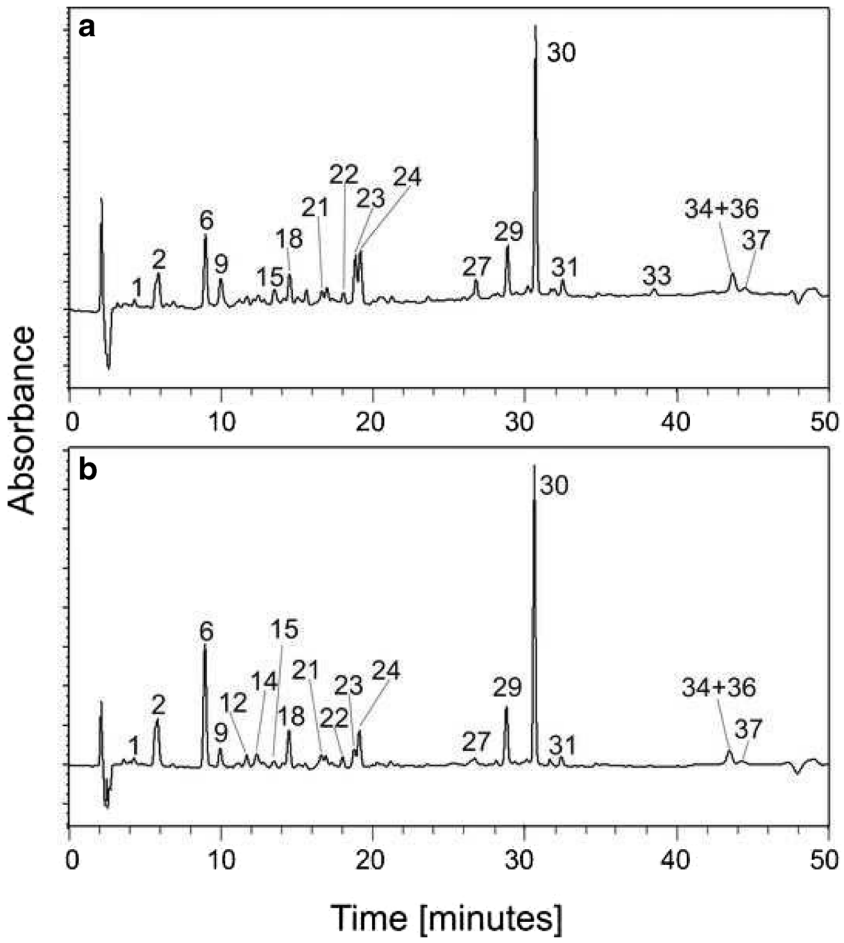


**Fig. 3** Chromatograms obtained for the environmental samples, i.e. (A) phytoplankton and (B) microphytobenthos with the Syst3 separation procedure. Pigments are labeled according to the Table 2

after the solvent front (data not shown). The addition of ammonium acetate in a low concentration (10 mM) as well as gradient adjustments extended pigments' retention enabling their proper separation.

## Calibration

For each analyzed pigment standard a calibration curve was obtained by plotting a series of peak surface areas against their corresponding concentrations. The correlation coefficients were satisfactory and all of them exceeded the value of 0.95 (Table. 4). The slopes of calibration curves differed among pigments and methods used. For most of the pigments, their response factor ($f_p$) value was the highest in the Syst3 method. In two other methods, Syst1 and 2, response factors calculated for most pigments were very similar, including chlorophyll $c_1 + c_2$, fucoxanthin, neoxanthin, violaxanthin, diadinoxanthin, canthaxanthin, chlorophyll *a* and *b*, echinenone, β-carotene (Table. 4). For the rest of them, i.e., peridinin, myxoxanthophyl, alloxanthin, diatoxanthin, lutein, zeaxanthin and α-carotene, the $f_p$ values differed, with those obtained for Syst2 being steeper (except for peridinin and α-carotene).

## Limit of detection

Detection limits (LOD) for analyzed pigments were low with values usually below 1 ng 100 $\mu l^{-1}$, and they were no different or in some cases better than those reported in other studies (e.g. Stoń-Egiert and Kosakowska 2005). The lowest detection thresholds varied among pigments and the methods used (Table. 4). The comparison of LODs showed that neither of the methods could be considered distinctively more sensitive than the other. The sensitivity of chromatographic methods depends on both mobile and stationary phases' characteristics (e.g. Stoń-Egiert and Kosakowska 2005; Mendes et al. 2007). Furthermore, Mendes et al. (2007) pointed out that the sensitivity of the system may also depend on a flow rate applied. They argued that in their study, the composition of the eluents could not explain the sensitivity of the tested systems as pigments' extinction coefficients were comparable at the time of their elution irrespective of the column type and solvents applied. They explained that at slower flow rates pigments are eluted in smaller amounts, which increases absorbance and in turn, affects peak area. In this study, different columns, solvents (eluents A were based on methanol or acetonitrile and eluents B contained different proportions of acetone, Table. 1) and flow rates (0.8 for Syst1 and 2 and 0.7 for Syst3) were employed, therefore they all contributed to the differences observed in the systems' sensitivity.

## Spectral characteristics

Spectral characteristics that are the basis for the pigment identification may also vary depending on separation conditions (Stoń-Egiert and Kosakowska 2005 and references therein). In this study, for several pigments, including peridinin, violaxanthin, zeaxanthin, cantaxanthin, echinenone, β-carotene as well as chlorophyll *a* and *b*, their absorption maxima were identical in all separation systems. For the rest of them, a shift within the range of 1 to 5 nm in their absorption

**Table 4** Limits of detection (LOD), response factors ($f_p$) and correlation coefficients (*r*) estimated for pigment standards based on the results obtained from tested separation procedures described in Table 1

| | Pigment | LOD (ng 100 $\mu l^{-1}$) | | | $f_p$ (ng 100 $\mu l^{-1}$) | | | *r* | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Syst1 | Syst2 | Syst3 | Syst1 | Syst2 | Syst3 | Syst1 | Syst2 | Syst3 |
| 1 | Chlorophyll $c_2$ | 0.32 | 0.61 | 0.23 | 0.049 | 0.044 | 0.053 | 1.000 | 0.999 | 0.973 |
| 2 | Peridinin | 0.23 | 0.34 | 0.65 | 0.153 | 0.122 | 0.122 | 0.995 | 1.000 | 0.999 |
| 3 | Fucoxanthin | 0.41 | 0.34 | 0.46 | 0.089 | 0.087 | 0.071 | 1.000 | 0.998 | 0.999 |
| 4 | Neoxanthin | 0.47 | 0.20 | 0.34 | 0.062 | 0.059 | 0.059 | 1.000 | 0.999 | 0.999 |
| 5 | Violaxanthin | 0.96 | 0.26 | 0.16 | 0.055 | 0.054 | 0.047 | 1.000 | 0.996 | 0.999 |
| 6 | Diadinoxanthin | 0.56 | 0.24 | 0.29 | 0.059 | 0.059 | 0.071 | 1.000 | 1.000 | 0.999 |
| 7 | Myxoxanthophyll | 0.18 | 1.01 | 0.78 | 0.131 | 0.162 | 0.112 | 0.996 | 1.000 | 0.997 |
| 8 | Alloxanthin | 0.34 | 0.48 | 0.37 | 0.075 | 0.089 | 0.057 | 1.000 | 0.998 | 0.995 |
| 9 | Diatoxanthin | 0.46 | 0.35 | 0.17 | 0.059 | 0.070 | 0.069 | 1.000 | 1.000 | 0.998 |
| 10 | Lutein | 0.60 | 0.51 | 0.46 | 0.060 | 0.075 | 0.055 | 0.999 | 1.000 | 0.997 |
| 11 | Zeaxanthin | 0.32 | 0.69 | 0.43 | 0.080 | 0.108 | 0.064 | 0.999 | 0.997 | 0.998 |
| 12 | Canthaxanthin | 0.67 | 0.50 | 0.93 | 0.117 | 0.107 | 0.096 | 0.999 | 0.983 | 0.999 |
| 13 | Chlorophyll *b* | 0.40 | 1.69 | 0.67 | 0.312 | 0.335 | 0.263 | 0.998 | 1.000 | 0.997 |
| 14 | Chlorophyll *a* | 0.37 | 1.63 | 1.16 | 0.274 | 0.295 | 0.275 | 0.999 | 0.999 | 0.987 |
| 15 | Echinenone | 0.30 | 1.42 | 0.54 | 0.097 | 0.105 | 0.069 | 0.998 | 0.998 | 0.993 |
| 16 | α-carotene | 1.74 | 0.41 | 0.54 | 0.099 | 0.082 | 0.065 | 0.988 | 0.996 | 0.999 |
| 17 | β-carotene | 1.82 | 0.47 | 0.90 | 0.105 | 0.110 | 0.088 | 0.955 | 0.998 | 0.999 |

**Table 5** Coefficient of variations (C.V., %) for retention times and concentrations estimated for pigment standards based on the results obtained from tested separation procedures described in Table 1

| | Pigment | Retention times | | | Concentration | | |
|---|---|---|---|---|---|---|---|
| | | Syst1 | Syst2 | Syst3 | Syst1 | Syst2 | Syst3 |
| 1 | Chlorophyll $c_2$ | 0.33 | 0.88 | 0.93 | 2.25 | 1.51 | 4.71 |
| 2 | Peridinin | 0.42 | 0.15 | 0.62 | 1.18 | 4.21 | 4.09 |
| 3 | Fucoxanthin | 0.22 | 0.61 | 0.62 | 2.22 | 4.85 | 4.10 |
| 4 | Neoxanthin | 0.24 | 0.32 | 0.79 | 2.61 | 3.42 | 2.01 |
| 5 | Violaxanthin | 0.16 | 1.00 | 0.75 | 5.15 | 0.47 | 1.17 |
| 6 | Diadinoxanthin | 0.15 | 0.59 | 0.73 | 0.10 | 4.78 | 2.13 |
| 7 | Myxoxanthophyll | 0.11 | 1.49 | 0.72 | 0.56 | 6.95 | 3.70 |
| 8 | Alloxanthin | 0.30 | 1.38 | 0.93 | 3.25 | 1.46 | 1.80 |
| 9 | Diatoxanthin | 0.13 | 0.90 | 0.67 | 2.95 | 8.03 | 5.32 |
| 10 | Lutein | 0.15 | 1.81 | 0.63 | 4.64 | 1.23 | 0.62 |
| 11 | Zeaxanthin | 0.30 | 1.64 | 0.79 | 6.88 | 0.55 | 1.74 |
| 12 | Canthaxanthin | 0.16 | 0.72 | 0.49 | 7.44 | 0.81 | 3.79 |
| 13 | Chlorophyll *b* | 0.18 | 0.49 | 0.38 | 0.11 | 2.26 | 7.33 |
| 14 | Chlorophyll *a* | 0.16 | 0.70 | 0.24 | 1.96 | 1.43 | 0.12 |
| 15 | Echinenone | 0.33 | 0.76 | 0.34 | 2.04 | 1.37 | 4.98 |
| 16 | $\alpha$-carotene | 0.40 | 0.37 | 0.37 | 5.91 | 6.64 | 0.80 |
| 17 | $\beta$-carotene | 0.34 | 0.39 | 0.41 | 3.81 | 4.32 | 1.44 |

maxima was observed (Table. 2). Regarding the fact that pigments are characterized by different absorption maxima in different solvents, it can be assumed that differences observed here resulted from the different solvent composition at the moment of pigment elution.

### System repeatability

The results obtained with each method were highly reproducible. Their repeatability was evaluated based on the coefficient of variation (C.V.) calculated for retention times and concentrations of each standard pigment (shown in Table. 4) used for the calibration of separation systems. The C.V. values obtained for each pigment were summarized in Table 5. Overall, retention times varied by less than 1 %. Whereas the range of C.V. variations calculated for pigment concentrations was similar to those reported in other studies (e.g. Mateos and García-Mesa 2006), and on average, it was ca. 3 % for each separation method.

## Conclusions

HPLC methods allow precise determination and quantification of photosynthetic pigments in extracts of various origins, from algal cultures to environmental samples, in order to establish contribution of various taxonomic groups into the biomass of the community, describe its seasonal changes, conclude on the presence and intensity of various physiological mechanisms as well as to describe certain ecological aspects of water habitats (e.g. the origin of organic matter) (e.g. Brotas and Plante Cuny 2003; Cartaxana et al. 2003; Goela et al. 2014; Pniewski et al. 2015; Tamm et al. 2015; Pniewski and Sylwestrzak 2018; Stoń et al. 2002). Despite the fact that numerous methods have been published previously (suffice to mention Wright et al. 1991, Zapata et al. 2000), there is no one ideal protocol that could satisfy all the research needs. Therefore, regarding the purpose of such analyses, most suitable separation system should be employed. In this study, two columns and three sets of eluents were used to develop three separation systems, and then their resolution and sensitivity were examined. The methods are fairly easy; they use simple eluents based on methanol and ammonium acetate as an ion-pairing agent and utilize a linear gradient in analytical protocols. All of them allow for separation of all taxonomically most relevant chlorophylls and carotenoids. They had similar resolution and sensitivity which with respect to particular pigments was better than reported in other studies. The Syst1 method was the shortest, while Syst2 had better resolution of pigments in the middle part of the protocol when most of the diagnostic pigments are separated (i.e., alloxanthin, diatoxanthin, lutein, chlorophyll *b*). In Syst3 the problematic pair zeaxanthin / lutein was partially separated. The lack of full resolution may be managed with modern software implemented with peak analysis tools. The methods described herein have been proven to be efficient in the analysis of unialgal cultures as well as various environmental samples such as phytoplankton and microphytobenthos.

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## References

Asencio AD, Hoffmann L (2013) Chemosystematic evaluation of the genus Scytonema (cyanobacteria) based on occurrence of phycobiliproteins, scytonemin, carotenoids and mycosporine-like amino acid compounds. Eur J Phycol 48:331–344

Brotas V, Plante Cuny MR (1998) Spatial and temporal patterns of microphytobenthic taxa of estuarine tidal flats in the Tagus estuary (Portugal) using pigment analysis by HPLC. Mar Ecol Prog Ser 171: 43–57

Brotas V, Plante Cuny MR (2003) The use of HPLC pigment analysis to study microphytobenthos communities. Acta Oecolog. https://doi.org/10.1016/S1146-609X(03)00013-4

Cartaxana P, Jesus B, Brotas V (2003) Pheophorbide and pheophytin a-like pigments as useful markers for intertidal microphytobenthos grazing by *Hydrobia ulvae*. Estuar Coast Shelf Sci 58:293–297

Chai C, Jiang T, Cen J, Ge W, Lu S (2016) Phytoplankton pigments and functional community structure in relation to environmental factors in the Pearl River estuary. Oceanologia 58:201–211

Eisner LB, Twardowski MS, Cowles TJ, Perry MJ (2003) Resolving phytoplankton photoprotective: photosynthetic carotenoid ratios on fine scales using in situ spectral absorption measurements. Limnol Oceanogr 48:632–646

Goela PC, Danchenko S, Icely JD, Lubian LM, Cristina S, Newton A (2014) Using CHEMTAX to evaluate seasonal and interannual dynamics of the phytoplankton community off the south-west coast of Portugal. Estuar Coast Shelf Sci 151:112–123

Goss R, Jakob T (2010) Regulation and function of xanthophyll cycle-dependent photoprotection in algae. Photosynth Res 106:103–122

Hooker SB, Van Heukelem L, Thomas CS, Claustre H, Ras J, Barlow R, Sessions H, Schlüter L, Perl J, Trees C, Stuart V, Head E, Clementson L, Fishwick J, Llewellyn C, Aiken J (2005) The second SeaWiFS HPLC analysis round-Robin experiment (SeaHARRE-2). NASA TM-2005-212785, Washington

Jahns P, Holzwarth AR (2012) The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. Biochim Biophys Acta 1817:182–193

Jayaraman S, Knuth ML, Cantwell M, Santos A (2011) High performance liquid chromatography analysis of phytoplankton pigments using a $C_{16}$-amide column. J Chromatogr A 1218:3432–3438

Jeffrey SW, Vesk M (1997) Introduction to marine phytoplankton and their pigment signatures. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Publishing, Paris, pp 37–84

Jodłowska S, Latała A (2003) Simultaneous separation of chlorophylls and carotenoids by RP-HPLC in some algae and cyanobacteria from the southern Baltic. Oceanol Hydrobiol Stud 2:81–89

Kraay GW, Zapata M, Veldhuis MJW (1992) Separation of chlorophylls $c_1$, $c_2$, $c_3$ of marine phytoplankton by reversed-phase-$C_{18}$-high-performance liquid chromatography. J Phycol 28:708–712

Lawrenz E, Fedewa EJ, Richardson TL (2011) Extraction protocols for the quantification of phycobilins in aqueous phytoplankton extracts. J Appl Phycol 23:865–871

MacIntyre HL, Kana TM, Anning T, Geider RJ (2002) Photoacclimation of photosynthesis irradiance response curves and photosynthetic pigments in microalgae and cyanobacteria. J Phycol 38:17–38

Mantoura RFC, Repeta DJ (1997) Calibration methods for HPLC. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Publishing, Paris, pp 407–428

Masjídek J, Kopecký J, Koblížek M, Torzillo G (2004) The xanthophyll cycle in green algae (Chlorophyta): its role in the photosynthetic apparatus. Plant Biol 6:342–349

Mateos R, García-Mesa JA (2006) Rapid and quantitative extraction method for the determination of chlorophylls and carotenoids in olive oil by high-performance liquid chromatography. Anal Bioanal Chem 385:1247–1254

Mendes CR, Cartaxana P, Brotas V (2007) HPLC determination of phytoplankton and microphytobenthos pigments: comparing resolution and sensitivity of a $C_{18}$ and $C_8$ method. Limnol Oceanogr Methods 5:363–370

Pinckney JL, Richardson TL, Millie DF, Paerl HW (2001) Application of photopigment biomarkers for quantifying microalgal community composition and in situ growth rates. Org Geochem 32:585–595

Pniewski F, Sylwestrzak Z (2018) Influence of short periods of increased water temperature on species composition and photosynthetic activity in the Baltic periphyton communities. Biologia. https://doi.org/10.2478/s11756-018-0122-6

Pniewski FF, Biskup P, Bubak I, Richard P, Latała A, Blanchard G (2015) Photo-regulation in microphytobenthos from intertidal mudflats and non-tidal coastal shallows. Estuar Coast Shelf Sci 152:152–161

Rodríguez F, Zapata M, Garrido JL (1998) High performance liquid chromatographic separation of chlorophyll *c* forms from marine phytoplankton on octylsilica bonded phases. Chromatographia 48: 677–680

Schlüter L, Garde K, Kaas H (2004) Detection of the toxic cyanobacteria *Nodularia spumigena* by means of a 4-keto-myxoxanthophyll-like pigment in the Baltic Sea. Mar Ecol Prog Ser 275:69–78

Schlüter L, Mohlenberg F, Havskum H, Larsen S (2000) The use of phytoplankton pigments for identifying and quantifying phytoplankton groups in coastal areas: testing the influence of light and nutrients on pigment/chlorophyll *a* ratios. Mar Ecol Prog Ser 192: 49–63

Schlüter L, Mohlenberg F, Kaas H (2014) Temporal and spatial variability of phytoplankton monitored by a combination of monitoring buoys, pigment analysis and fast screening microscopy in the Fehmarn Belt estuary. Environ Monit Assess 186:5167–5184

Schmid H, Stich HB (1995) HPLC-analysis of algal pigments: comparison of columns, column properties and eluents. J Appl Phycol 7: 487–494

Stoń J, Kosakowska A (2000) Qualitative and quantitative analysis of Baltic phytoplankton pigments. Oceanologia 42:449–471

Stoń J, Kosakowska A (2002) Phytoplankton pigments designation – an application of RP-HPLC in qualitative and quantitative analysis. J Appl Phycol 14:205–210

Stoń J, Kosakowska A, Łotocka M, Łysiak-Pastuszak E (2002) Pigment composition in relation to phytoplankton community structure and nutrient content in the Baltic Sea. Oceanologia 44:419–437

Stoń-Egiert J, Kosakowska A (2005) RP-HPLC determination of phytoplankton pigments – comparison of calibration results for two columns. Mar Biol 147:251–260

Stoń-Egiert J, Łotocka M, Ostrowska M, Kosakowska A (2010) The influence of biotic factors on phytoplankton pigment composition and resources in Baltic ecosystems: new analytical results. Oceanologia 52:101–125

Tamm M, Freiberg R, Tõnno I, Nõges P, Nõges T (2015) Pigment-based chemotaxonomy – a quick alternative to determine algal assemblages in large shallow eutrophic lake. PLoS One. https://doi.org/10.1371/journal.pone.0122526

Van Heukelem L, Lewitus AJ, Kana TM, Craft NE (1994) Improved separations of phytoplankton pigments using temperature-controlled high performance liquid chromatography. Mar Ecol Prog Ser 114:303–313

Van Heukelem L, Thomas CS (2001) Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. J Chromatogr A 910:31–49

Van Leeuwe MA, Villerius LA, Roggeveld J, Visser RJW, Stefels J (2006) An optimized method for automated analysis of algal pigments by HPLC. Mar Chem 102:267–275

Wright SW, Jeffrey SW, Mantoura RFC, Llewellyn BT, Repeta D, Welschmeyer N (1991) Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. Mar Ecol Prog Ser 77:183–196

Zapata M, Rodríguez F, Garrido JL (2000) Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C8 column and pyridine-containing mobile phases. Mar Ecol Prog Ser 195:29–45