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HPLC-analysis of algal pigments: comparison of columns, column properties and eluents

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Abstract

The effects of columns (Nucleosil C_{18}ODS, MZ-PAH, YMC-PACK C_{30}), column properties (inner diameters of 4 mm, 3 mm and 2 mm, pore-width 10 nm and 30 nm) and eluents (methanol, acetonitrile, acetone, water) were tested on the separation of algal pigments. The length of columns was 250 mm and particle size was 5 μm. Flow rates and gradients were adjusted to optimize peak separation; remaining chromatographic conditions were kept constant. The resolution of chromatographic systems was tested with pigment standards and various algal cultures. Total flow rate and retention times decreased with decreasing inner diameter, whereas pressure, sensitivity and peak-width increased. Pore width had negligible effects on the chromatographic separation of pigments under the test conditions. Only with acetonitrile as eluent were all the taxonomically important pigments resolved adequately: zeaxanthin (Cyanophyceae), lutein (Chlorophyceae), fucoxanthin (Bacillariopyceae), alloxanthin (Cryptophyceae), peridinin (Dinophyceae).

Introduction

The separation of algal pigments by high pressure liquid chromatography has become a commonly used method which lacks the deficiencies of spectrophotometrical methods (Bidigare *et al.*, 1985; Jacobson & Rai, 1990; Neveux *et al.*, 1990). By the simultaneous separation of additional pigments further information is obtained on the community composition of the phytoplankton assemblage (Wilhelm *et al.*, 1991; Yacobi *et al.*, 1991; Lami *et al.*, 1992), its biomass (Wilhelm & Manns, 1991) and its degradation by sedimentation (Hurley & Armstrong, 1990; Leavitt & Carpenter, 1990; Yacobi *et al.*, 1991) or alteration of pigments by grazing (e.g. Gieskes & Kray, 1986b; Leavitt *et al.*, 1989). This additional information on the state of phytoplankton represents the actual benefits of high pressure liquid chromatography.

Our comparison of methods revealed wide variations of e.g. filtration volume (Gieskes & Kraay, 1986b), handling of filtered material (Gieskes & Kraay, 1983, 1986a), reagents used for extraction (Bowles *et al.*, 1985; Webb *et al.*, 1992) and extraction procedures (Vernet & Lorenzen, 1987; Hurley & Armstrong, 1990; Wilhelm *et al.*, 1991; Downes *et al.*, 1993). Utilized mobile phases were: methanol-water, acetone-methanol (Mantoura & Llewellyn, 1983); methanol-water, ethylacetate-methanol (Gieskes & Kraay, 1986a, 1986b; Klein *et al.*, 1986, Klein & Sournia, 1987); methanol-water, acetonitrile-ethylacetate (Wilhelm *et al.*, 1991; Wright *et al.*, 1991); acetonitrile-water, ethylacetate (Wright & Shearer, 1984); methanol, water (Bidigare *et al.*, 1985).

Usually a silica-based matrix was employed as the filling material for columns; column dimensions ranged from 8.0 mm × 1000 mm (Bidigare *et al.*, 1985) to 4.0 mm × 250 mm (e.g. Wilhelm *et al.*, 1991) and flow rates from 9.9 ml min^{-1} (Bidigare *et al.*, 1985) to 0.8 ml min^{-1} (Gieskes & Kraay, 1986b).

Apparently no ideal method exists for the separation of algal pigments by HPLC. However, the method proposed by Mantoura and Llewellyn (1983) is often employed, which includes the use of an ion-pairing reagent (ipr). According to the authors, dissociation

of compounds is suppressed by the ipr and peak separation is thereby enhanced. They recommended the presence of ipr in both sample and mobile phase. Other authors, however, such as Gieskes and Kraay (1986b) and Wright *et al.* (1991) do not use ipr in either sample or mobile phase.

We wanted to develop a simple HPLC-method permitting the determination of all taxonomically relevant pigments and thus the seasonal and spatial changes of the major phytoplankton groups in lakes of differing trophic status. 'Simple' refers mainly to the treatment of samples and to the necessary technical HPLC equipment. We used the method of Mantoura and Llewellyn (1983) initially, but failed to separate lutein and zeaxanthin. As both carotenoids are used as 'markers' to distinguish between Chlorophyceae and Cyanophyceae (cf. Wilhelm *et al.*, 1991) we tried to improve the method.

Method

Reagents

The solvents for extraction (acetone) and chromatography (acetone, acetonitrile, methanol, water) were HPLC-grade (Merck). The ion-pairing reagent (ipr) consisted of 2 g tetraethylammonium acetate (Aldrich, 99%) and 5 g ammonium acetate (Merck, p.a.) dissolved in 300 mL water.

Extraction of samples

Water samples were filtered on glass fibre filters (Whatman GF/F, ⌀ 27 mm). Filtration volume ranged between 300 mL (small eutrophic lakes) and 1000 mL (Lake Constance). Subsequently filters were placed in dark coloured glass vials, covered with 3.8 mL 90% acetone and placed in a water bath for 5 min at 55 °C; the 'hot' extraction prevented the formation of chlorphyllide by the enzyme chlorophyllase (Barret & Jeffrey 1971). After cooling to room temperature, 213 μL ipr were added, resulting in a final sample volume of 4 mL. Concentration of ipr in the extraction sample and in the chromatographic eluent was identical. Finally the extract was sonicated for 3 min to disperse ipr and purged through a disposable filter unit (Schleicher & Schüll Spartan 30B) into a dark-coloured sample vial. If the subsequent HPLC-estimation was not possible, samples were stored at -15 °C. We tested pigment composition of the following algae: *Pavlova lutheri* (strain B926-1) *Stephanodiscus hantzschii* (strain B66.81), *Cyclotella meneghiniana* (strain 1020-1a), *Aphanizomenon gracile* (strain B32.79), *Synechococcus rubescens* (strain B3.81), *Euglena gracilis* (strain 1224-5/3), *Chlorella mirabilis* (strain 211-30), *Oocystis lacustris* (strain B81.80), *Monoraphidium contortum* (strain B47.80), *Scenedesmus acuminatus* (strain B38.81), *Chlamydomonas reinhardtii* (strain 11-32a) and *Cryptomonas ovata* (strain B979-3). All algal strains were obtained from the algal collection of the Institut für Pflanzenphysiologie, Universität Göttingen. Identification of violaxanthin, neoxanthin, diadinoxanthin, diatoxanthin and α-carotene was obtained by diode-array detection from these cultures (cf. Leavitt & Carpenter, 1990; Wright *et al.*, 1991; Wilhelm *et al.*, 1991; Van Heukelem *et al.*, 1992). Identification of carotenoids was verified by e.g. the reaction of the epoxy-group after acidification (Britton, 1988; Sykes, 1988). Tested pigment standards were chlorophyll *a* and *b*, α- and β-carotene (Sigma), phaeophorbid (Wako) and canthaxanthin (Fluka). Astaxanthin, β-cryptoxanthin, echinenone, canthaxanthin (trans), fucoxanthin, lutein and zeaxanthin were donated by Dr H. E. Keller, Hoffman La Roche AG, Basel, Switzerland. Peridinin was obtained by extracting a culture of Peridinium gatunense, donated by Prof. W. Ullrich, Botanisches Institut der Universität Darmstadt. The algal samples were extracted as described above, standards were dissolved directly in acetone.

HPLC-system

The Kontron HPLC-System consisted of two-piston pumps 422 (flow range of pump heads: 0.01 mL min^{-1} to 2 mL min^{-1}), an auto-sampler with cooled sample storage (AS 360), an eluent mixing chamber (M 800), a valco injection valve with a 50 μL sample-loop, a diode array detector with 8 μL cell volume and 10 mm light path and a fluorometrical detector with 15 μL cell-volume. Operation of the chromatographic system and analysis of chromatograms were achieved with the data system 450 MT2. During chromatographic separation eluents were degassed constantly.

Columns

After preliminary tests of eight columns, we kept columns of Macherey-Nagel (Nucleosil C_{18}ODS, particle size 5 μm, 10 nm and 30 nm pore width), Mz-Analysentechnik (MZ-PAH C_{18}, particle size 5 μm, 10

nm pore width) and YMC (YMC-Pack C_{30}, particle size 5 μm, 10 nm pore width) for further tests. The length of all columns was 250 mm and the inner diameters were 4 mm, 3 mm or 2 mm. All columns were maintained at 35 °C.

Chromatographic eluents and separation

Eluent A1 was made from 700 mL MeOH and 300 mL H_2O in which 2 g TEAA and 5 g AA were separately dissolved and united by filtration through Schleicher and Schüll RC55-filters (pore width 0.45 μm). Eluent A2 was made from 50% acetonitrile and 50% water. Prior to use, all eluents were degassed by sonification for 10 min. Eluent B always consisted of 600 mL MeOH and 400 mL acetone (by volume). When using eluent A1 chromatographic separations started with an initial mixing ratio of 50% A:50% B. The mixing ratio changed to 98% B within 10 to 14 min and was kept constant for 8 to 14 min. Thereafter, the mixing ratio returned to the initial ratio within 6 to 12 min. Specific flow rates and gradients were adjusted to column properties to optimize peak separation. When using eluent A2, the mixing ratio declined from 60% A2 to 20% A2 within 5 min. After an isocratic hold for 12 min, the mixing ratio was again reduced to 5% A2 within 3 min. After a second isocratic hold for 20 min, the original mixing ratio was achieved after 5 min and kept there for 15 min before the next separation was started. No ipr was added to the eluent A2. The use of ipr was restricted to the extraction of samples.

Results

Eluent A1, Nucleosil C_{18} ODS

The effects of column diameter were tested with Nucleosil C_{18} ODS columns. With decreasing inner diameter the total flow rate decreased, whereas retention times, sensitivity (area μg^{-1}) and the pressure of columns increased (Table 1). Pressure increments did not exceed tolerable limits of the HPLC-pumps.

The high sensitivity of the 2 mm column was counteracted by the increase in base-width of peaks, which affected the ratio of height/base-width. This increase was especially observed with the carotenoids and rendered an adequate resolution of succeeding carotenoid peaks impossible. The phenomenon in base width was not observed in "later" peaks e.g. chlorophylls and phaeophytins. The 3-mm column with 30 nm pore-width had a higher column pressure than the same column with 10 nm pore-width. Valuation of additional criteria did not show a general difference between both 3 mm columns (Table 1).

Table 1. Effect of inner diameter and pore width on pressure (MPa), flow-rate (ml min^{-1}), retention time (min), base-width (min) and height (mV), area (mV min) of peak for the three pigments fucoxanthin, chlorophyll *a* and β-carotene.

Column	4 mm id, 10 nm	3 mm id, 10 nm	2 mm id, 10 nm	3 mm id, 30 nm
Pressure	7	9	13	14
Flow rate	1.4	0.5	0.2	0.5
Fucoxanthin				
Retention time	4.5	7.3	11.0	6.0
Base width	0.6	0.7	1.4	0.8
Height	669.2	1384.9	677.1	722.1
Area	86.3	241.9	328.0	237.5
Area μg^{-1}	4.2	11.6	31.5	11.4
Chlorophyll a				
Retention time	11.1	15.7	24.5	15.2
Base width	0.3	0.5	0.7	0.3
Height	346.5	693.9	699.0	1075.0
Area	41.4	108.3	152.8	124.4
Area μg^{-1}	1.4	4.2	12.2	4.3
β-carotene				
Retention time	13.4	21.4	33.4	18.3
Base width	0.4	0.7	0.8	0.3
Height	195.0	385.4	480.0	390.1
Area	19.7	104.8	157.9	56.4
Area μg^{-1}	3.4	17.9	27.0	9.7

The 4-mm column had the shortest retention times, but the highest flow rate and a poor peak resolution of the closely grouped pigments chlorophyll *b* – chlorophyll *b'* – cryptoxanthin – chlorophyll *a* – chlorophyll *a'* – echinenone.

With the 3 mm column with 10 nm pore-width, which had the best test result (Table 1), we obtained a general improvement, but did not separate zeaxanthin and lutein. Using the eluent system of Mantoura and Llewellyn (1983), Van Heukelem *et al.* (1992) separated both pigments by using a polymeric reversed-phase C_{18} column (Vydac 201 TP). This column, however, was not available with an inner diameter of 3 mm. As we wanted to maintain the aforementioned advantages of the reduced inner diameter, especially the low flow rate, we tested a column with similar separation char-

acteristics, the MZ-PAH C_{18}, which was available with an inner diameter of 3 mm.

Eluent A1, MZ-PAH C_{18}

With the MZ-PAH C_{18} as column, we immediately obtained the separation of zeaxanthin and lutein (Table 2), but fucoxanthin was superposed on chlorophyll *c* (diode array detector) and chlorophyll *c* overlapped phaeophorbid *a* (fluorometrical detector). As the MZ-PAH C_{18} column was sensitive to temperature changes, we tested the temperatures at 20 °C, 35 °C and 40 °C to enhance peak resolution. Decreasing the temperature from 35 °C to 20 °C reversed the elution sequence of fucoxanthin and chlorophyll *c*. Increasing the temperature to 40 °C affected neither elution sequence nor peak resolution. As the boiling point of acetone is 56.2 °C, we did not test temperatures >40 °C because we wanted to ensure unattended operation of the HPLC system. The MZ-PAH C_{18} column always separated zeaxanthin and lutein, but we could not overcome the disadvantages mentioned above.

Eluent A2, MZ-PAH C_{18}

In view of the results achieved so far concerning adequate peak resolution, we started to test acetonitrile as eluent. The use of acetonitrile was promising from the start. After optimizing the chromatographic conditions, including the introduction of a second gradient (cf. methods), we finally achieved the separation of fucoxanthin, neoxanthin, violaxanthin, flucoxanthin-like, astaxanthin, diadinoxanthin, lutein and zeaxanthin. Cryptoxanthin – chlorophyll *a*, diatoxanthin – lutein (diode array detector) and phaeophorbide – chlorophyll *c* (fluorometric detector) remained unresolved (Table 2).

Eluent A2, Nucleosil C_{18} ODS

Testing the Nucleosil C_{18} ODS column with eluent A2 did not show obvious improvements. Chromatographic results remained almost unaffected and no obvious improvement in peak resolution was apparent (Table 2).

Eluent A2, YMC-Pack C_{30}

Sander *et al.* (1994) applied the YMC-Pack C_{30} (= YMC SIL200, A. Kuppka, YMC, pers. comm. 1994) for the separation of carotenoids, a column which is especially suited for the separation of isomeric substances. Zeaxanthin and lutein separated well (Table 2), but astaxanthin – diadinoxanthin and canthaxanthin – chlorophyll *b* remained unresolved. While optimizing peak resolution, we also tested the effects of ipr. When extracting *Cyclotella meneghiniana*, without the addition of ipr, chlorophyll *c*1 and chlorophyll *c*2 eluted as two well resolved peaks (Fig. 1). Both peaks were identified by the dad estimation of their spectral absorbance data. When adding ipr, only one peak 'chlorophyll *c*' occurred (Fig. 1). Thus, the original purpose of ipr, 'enhancing peak separation by suppressing dissociation of compounds' (Mantoura & Llewellyn, 1983) is reversed. This phenomenon was only observed with the YMC Pack C_{30}. The column had good peak resolution, but retention times were not stable. Within two successive runs observed retention times differed up to 5 min. Reducing the share of water in the eluents (A. Kuppka, YMC, pers. comm. 1994) lowered the variations of retention times but also lowered the sep-

Table 2. Retention times (min) of pigments for the three columns Nucleosil C_{18} ODS (MN), MZ-PAH (MZ) and YMC-Pack (YMC) and for the combined columns MN-MZ.

	MN-MZ	YMC	MZ	MN
Chlorophyll *c*1 + *c*2	9.49	7.47	5.26	4.23
Peridinin	14.10	--	--	--
Fucoxanthin	15.86	11.64	9.24	9.91
Neoxanthin	16.40	13.33	9.77	--
Violaxanthin	18.07	13.95	10.59	10.60
Fucoxanthin-like	18.50	14.44	10.40	10.91
Astaxanthin	--	--	11.46	11.20
Diadinoxanthin	--	--	11.78	--
Astaxanthin-diadinoxanthin	20.46	18.98	--	--
Alloxanthin	23.11	--	--	--
Diatoxanthin	24.56	27.08	--	12.16
Lutein-diatoxanthin	--	--	13.39	--
Lutein	25.07	23.53	--	12.49
Zeaxanthin	25.53	26.32	13.80	--
Canthaxanthin	27.88	--	15.78	14.19
Chlorophyll *b*	30.20	--	16.86	16.33
Canthaxanthin-chlorophyll *b*	--	28.77	--	--
Chlorophyll *b'*	30.64	30.10	17.59	16.96
β-cryptoxanthin	32.02	34.30	--	17.95
β-cryptoxanthin-chlorophyll *a*	--	--	18.99	--
Chlorophyll *a*	32.40	32.37	--	18.50
Echinenone-chlorophyll *a'*	33.43	--	--	--
Chlorophyll *a'*	--	32.37	19.57	--
Echinenone	--	34.89	19.77	19.08

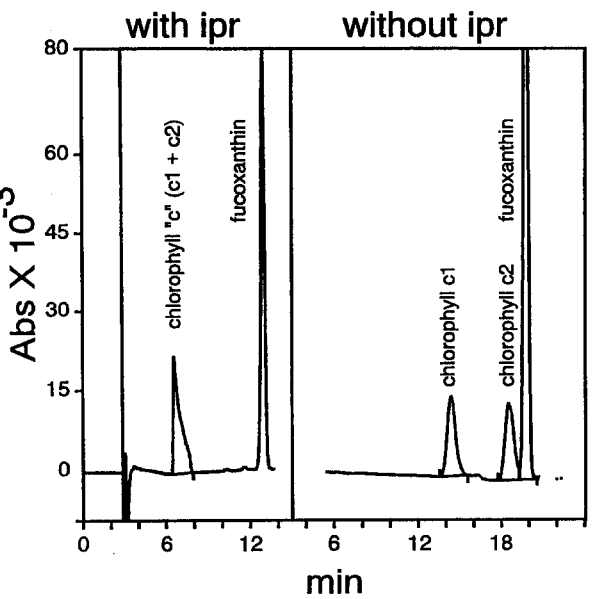

Fig. 1. Effect of ipr on the separation of chlorophyll *c*1 and *c*2, using the YMC-PACK C_{30} column.

aration capabilities of the column. We did not want to change eluents, trying so might have solved this problem. Sander *et al.*, 1994, used methanol and methyl-t-butyl ether as eluents.

Eluent A2, Nucleosil C_{18} ODS – MZ-PAH C_{18}

Discouraged by our unsuccessful efforts to combine stable retention times and high peak resolution of in the YMC-Pack C_{30} column, we coupled the Nucleosil C_{18} ODS and the MZ-PAH C_{18} columns. We were fortunate, since the separation properties of columns added to and increased peak resolution. Unresolved peaks were reduced to astaxanthin-diadinoxanthin, which can be identified by their differing spectral absorbance characteristics and to echinenone-chlorophyll a', which may be distinguished by separate readings in the photometrical (dad) and fluorometrical detector. Although total column length was now 500 mm, retention times were remarkably constant. In five successive runs, the observed variation of retention times for the last eluting β-carotine was $\leq 0.5\%$ (= 0.19 min). By coupling the two columns we achieved a chromatographic separation of peaks, which included all taxonomically important carotenoids, with a resolution of ≥ 1 (Fig. 2).

Discussion

Reducing the inner diameter of the column had positive effects (lower flow rate, higher sensitivity) and negative effects (higher pressure and retention time). The described characteristics followed from our chosen chromatographic conditions.

The 2 mm column had the lowest flow rate and the highest sensitivity. Both 'positive' effects were counteracted by the 'negative' effects, especially the high retention times. Furthermore increased base-width of peaks (Table 1) rendered an adequate resolution of succeeding carotenoid peaks impossible. As, in addition, the general handling of this column was difficult (e.g. equilibration), it was discarded.

Although the 4 mm column had the shortest retention times, it was dismissed because of the high flow rate, especially as the peak resolution and the sensitivity were not as good as in both 3 mm columns.

The 3-mm column with 30 nm pore-width had a higher column pressure than the same column with 10 nm pore-width. The evaluation of additonal criteria (cf. Table 2) did not show a general difference between both 3 mm columns (Table 1). In the 3 mm column with 10 nm pore-width the 'positive' effects were prevalent (low flow rate, good resolution of peaks, intermediate retention times, ease of handling).

Using this column and the solvent system of Mantoura and Llewellyn (1983), we were able to improve the resolution of peaks. However we were unable to separate lutein and zeaxanthin with any of the chromatographic conditions. We hesitated to use acetonitrile as mobile phase, not only because it is hazardous, but also because the spectrophotometric data on carotenoids are only available for acetone (cf. Mantoura & Llewellyn, 1983). However, replacing methanol (eluent A1) with acetonitrile (eluent A2) improved the separation and resolution of peaks, except when applied with the Nucleosil C_{18} ODS column. The best results were obtained by coupling the Nucleosil C_{18} ODS and the MZ-PAH C_{18} column. The number of superposing peaks (astaxanthin-diadinoxanthin and echinenone-chlorophyll a') was reduced to a minimum. All other tested substances were well separated. Coupling both columns increased the total column length to 500 mm. We could not detect any disadvantages caused by this high column height, except that it is unusual. The high total column length increased the duration of our chromatographic pigment separation, which is about 1.2- to 3-fold longer than that of previously reported methods (Van Heukelem *et*

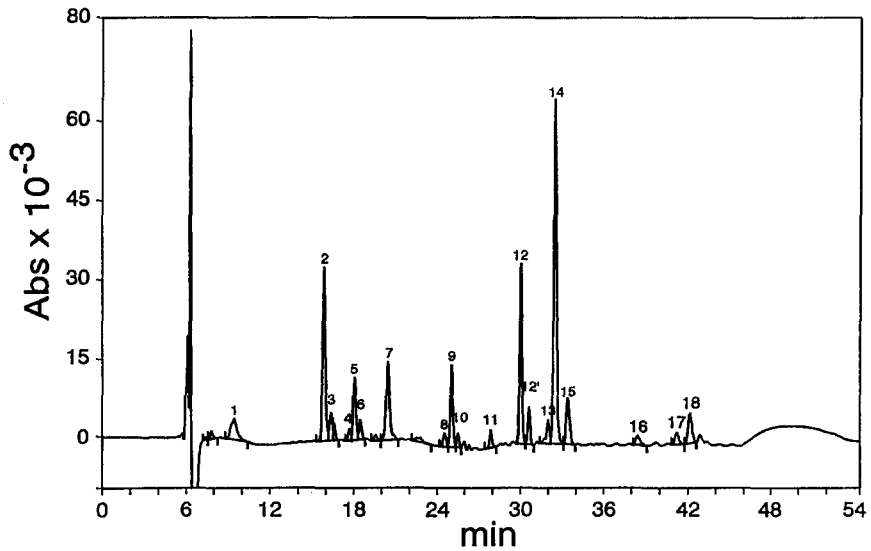

Fig. 2. Dad peak separation using the coupled columns Nucleosil C_{18} ODS and MZ-PAH (1 = chlorophyll *c*, 2 = fucoxanthin, 3 = neoxanthin, 4 = fucoxanthin-like, 5 = violaxanthin, 6 = fucoxanthin-like, 7 = astaxanthin-diadinoxanthin, 8 = diatoxanthin, 9 = lutein, 10 = zeaxanthin, 11 = canthaxanthin, 12 = chlorophyll *b*, 12′ = chlorophyll *b*′, 13 = cryptoxanthin, 14 = chlorophyll *a*, 15 = echinenone, 16 = phaeophytin *b*, 17 = α-carotene, 18 = β-carotene).

al., 1992: 19.5 min; Wilhelm *et al.*, 1991: 45 min), but thereby we gained a peak resolution ≥1 and avoided 'rider peaks' (cf. Yacobi *et al.*, 1991). Due to our low flow rate of 0.5 mL min^{-1}, the total consumption of eluent during this time was 30 mL, including 15 min for the equilibration of the column (Van Heukelem *et al.*, 1992: 29.3 mL; Wright *et al.*, 1991: 30 mL; Wilhelm *et al.*, 1991: 45 mL). The retention times were remarkably constant and we presumed that they were obtained by omitting the use of ipr during the chromatographic separation.

We finally achieved our aim, at least partially. The sample preparation is restricted to a sufficient minimum. The small filtration volume (300–1000 mL) reduced vacuum 'stress' on phytoplankton cells during the filtration process. Tests of extraction efficiency proved a final sample volume of 4 mL as sufficient. Reducing the inner diameter of the column to 3 mm increased the sensitiveness of the chromatographic method and enabled the low flow rate. The HPLC equipment was based on two pumps only, pump heads, however, were specially designed for low flow range (0.01 mL min^{-1} to 2 mL min^{-1}). The small sample loop (50 μL) prevented the column from overloading. The use of a diode array detector (dad) is, as we know by now, indispensable to scrutinize peak purity. With the developed HPLC-protocol, all major taxonomically relevant carotenoids, chlorophylls and their degradation products (cf. Wright *et al.*, 1991, Table 5) are distinguished. Re-running samples at differing temperatures (Van Heukelem *et al.*, 1994) is not necessary. For surveying habitat and seasonally specific changes in the phytoplankton communities of mesotrophic Lake Constance and neighbouring small eutrophic lakes our method is adequate and permits unattended automated sample treatment.

Appendix

HPLC-protocol for the analysis of algal pigments

Column 1: Nucleosil C_{18} (Macherey and Nagal, Düren, Germany), 5 μm particle size, 10 nm pore-width, length 250 mm, inner diameter 3 mm

Column 2: MZ-PAH C_{18} (MZ-Analysentechnik, Mainz, Germany), 5 μm particle size, 10 nm pore-width, length 250 mm, inner diameter 3 mm

Oven:	35 °C	
Eluent A:	water : acetonitrile	50 : 50
Eluent B:	methanol : acetone	60 : 40
Gradient:	start	60% A
	0–5 min	20% A
	17–20 min	5% A
	40–45 min	60% A
	re-equilibration	15 min

HPLC: Kontron-Instruments, Neufahrn, Germany,

Wavelength settings of detectors:

Diode array detector

channel 1	444 nm
channel 2	515 nm (distinction of diadinoxanthin astaxanthin)

Fluorometrical detector:	Excitation	Emission
0–25 min	409	670
23–33 min	465	656
33–45 min	409	670

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