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RESEARCH ARTICLE

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RP-HPLC determination of phytoplankton pigments—comparison of calibration results for two columns

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Abstract In the experiment reported the results obtained with two different columns for the RP-HPLC analysis of phytoplankton pigment components were compared. LiChroCART Hypersil ODS and LiChroCART LiChrospher 100 RP18e columns were used exchangeably. Two groups of high-purity pigment standards of reference phytoplankton strains (23 chlorophylls and carotenoids in the first case and 25 in the second case) were subjected to chromatographic analysis. All calibration parameters (retention times, calibration curves, spectrum shapes, detection thresholds) for qualitative and quantitative identification of pigments were determined. The results indicate that both columns used in the analysis ensure sufficient resolution and can be used interchangeably for the analysis of pigments present in naturally existing phytoplankton, even when present in only trace amounts.

Introduction

Since the 1980s, reverse-phase high performance liquid chromatography (RP-HPLC) has been commonly used for the separation, identification and quantification of large groups of polar and non-polar pigments. Normal-phase HPLC systems are best suited for separating only a few groups of pigments with similar polarities and structures (Gieskes and Kraay 1983). The idea to employ the RP-HPLC in environmental studies was introduced by Mantoura (Mantoura and Llewellyn 1983) and has been developed by many researchers (Bidigare et al. 1985; Wright et al. 1991; Wright and Jeffrey 1997; Heukelem and Thomas 2001; Marty et al. 2002; Rodriguez et al. 2002; Brotas and Plante-Cuny 2003). The resolution of compounds is based on adsorption and desorption processes on the coating of chromatographic columns (stationary phase) in the presence of the suitable solvents' composition (mobile phase).

There are many disciplines of environmental studies in which qualitative and quantitative characteristics of pigments obtained by the use of chromatographic techniques have found applications and are indispensable. The use of taxonomically important chlorophylls and carotenoids in the direct identification of phytoplankton classes, their biomass, condition and stage of development in seawater samples has increased due to advancements in these techniques (Wright and Enden 2000; Mackey et al. 2002). The knowledge of photosynthetic and photoprotectant pigment concentrations facilitates the determination of the absorption properties of phytoplankton (Woźniak et al. 2000) and of the rate of photosynthesis in natural waters (MacIntyre et al. 2000) approximated from satellites (Woźniak et al. 1997; Claustre et al. 2004). Additionally, the radioactively labeled taxon-specific pigments can be used to distinguish primary producers in the field (Gieskes and Kraay 1989) and for the mathematical analysis of pigment fingerprints (Gieskes et al. 1988; Rodriguez et al. 2002).

Solvents and their time-variable composition, temperature-controlled systems (ensuring stable retention times) and different columns were tested during years of investigations. Octadecylo silane (ODS) C_{18}, C_8 and even C_{30} chain columns with different diameters, lengths and particle sizes have, with different degrees of effectiveness, found application in the analysis of pigments (Schmid and Stich 1995; Zapata et al. 2000; Barlow et al. 2002; Rodriguez et al. 2002). Octadecyl silica C_{18} columns (with polymeric, as well as monomeric coating)

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have been commonly used for the determination of algal pigments. At present, over 50 phytoplankton pigments and their degradation products can be separated in a single analysis (Wright et al. 1991; Wright and Jeffrey 1997). Polymeric C_{18} columns were used to separate carotenoid pairs, such as lutein and zeaxanthin, 19′hexanoyloxyfucoxanthin and 9′cis-neoxanthin, and α- and β-carotene (Heukelem et al. 1992). The employment of C_8 columns allowed separation of divinyl and monovinyl chlorophyll *a* and *b* (Goericke and Repeta 1992). In addition, the incorporation of a pyridine-containing mobile phase to the HPLC pigment analysis, either in combination with polymeric C_{18} columns or monomeric C_8 columns, allows separation of chlorophyll *c* pigments (Zapata et al. 2000, 2001). The pigment resolution may also be enhanced by adjusting the temperature of the column (Heukelem et al. 1994). Also the solvent composition can be modified, for example, by the use of highly ionic solvents with chlorophylls *c1*, *c2* and *c3* separated on the C_{18} column (Kraay et al. 1992).

The RP-HPLC technique is highly accurate (±6%, Latasa et al. 1996; Claustre et al. 2004) in routine pigment measurements, even for trace amounts of <0.1 μg dm^{-3} (Roy et al. 1996; Stoń et al. 2002). The qualification and quantification of individual chlorophylls and carotenoids is mainly based on their properties in the visible light range. The identification relies on comparisons of the observed diode array spectroscopy during elution, or by transferring HPLC fractions to standard solvents and comparing their visible absorption spectra with reference standards or literature values, or by co-chromatography with authentic standards (Wright et al. 1991). Quantitative characterization of eluted pigments is based on peak area and excitation coefficients published in the literature (Mantoura and Llewellyn 1983; Latasa et al. 1996; Jeffrey 1997a; Bjørland et al. 2003) and on Lambert–Beer's law. Concentrations of pigments can also be calculated using internal (e.g. canthaxanthin) and external standards (Andersen et al. 1996; Mantoura and Repeta 1997; Obayashi and Tanoue 2002; Qian et al. 2003).

A limited number of high-purity pigment standards exists for the calibration of spectrometers and HPLC detectors. Due to difficulties resulting from a very labile structure of pigment molecules, susceptible to degradation by light, heat, oxygen, acids and bases, the preparation of pigment standards is very complicated. Some synthetic chlorophyll and carotenoid standards are available from commercial sources, e.g. chlorophyll *a* and *b*, canthaxanthin, lutein, lycopene, zeaxanthin, α-carotene and β-carotene. In most cases, laboratories prepare and purify their own standards from reference phytoplankton monocultures with well-known pigment composition, or from other sources, such as tomatoes, leaves of maize, or other higher plants. Detailed information, full description and guidelines for the preparation of 17 chlorophyll and 29 carotenoid standards, chemotaxonomically significant in phytoplankton ecology, from reference strains of monocultures were given by Jeffrey (1997b) and Repeta and Bjørland (1997). In general, the approach is the same: pigments are extracted from suitable biological sources by organic solvents, purified by thin-layer or semi-preparative liquid chromatography, crystallized and transferred to an appropriate solvent (90% acetone in the case of chlorophylls; ethanol, diethyl ether, hexane, or petroleum ether for carotenoids) and characterized by spectrophotometry.

The aim of this paper is to present the results of comparative analysis of chromatographic resolution by the use of two types of columns: LiChroCART Hypersil ODS and LiChroCART LiChrospher 100 RP18e. Two groups of high-purity pigment standards from The International Agency for ^{14}C Determination, DHI Institute for Water and Environment, Denmark, were subjected to chromatographic analysis in order to calibrate these two HPLC systems. The external calibration results obtained (such as spectrum shapes in appropriate solvent compositions, retention times, slopes of calibration curves, sensitivity, or detection thresholds) provide possibilities to use interchangeable analytical columns for separation, isolation and quantification of the pigments present in environmental samples.

Materials and methods

Pigment standards

Two groups of high-purity certified chlorophyll and carotenoid standards, isolated from reference strains of phytoplankton monocultures, were obtained from The International Agency for ^{14}C Determination, DHI Institute for Water and Environment, Denmark. The first group of 23 pigments consisted of chlorophylls (*a*, *b*, *c*, *c2*, *c3*) and carotenoids (alloxanthin, antheraxanthin, aphanizophyll, 19′butanoyloxyfucoxanthin, canthaxanthin, α- and β-carotenes, diadinoxanthin, echinenone, fucoxanthin, 19′haxanoyloxyfucoxanthin, lutein, myxoxanthophyll, neoxanthin, peridinin, prasinoxanthin, violaxanthin, zeaxanthin). The second group consisted of 25 pigments, chlorophylls *a*, *b*, *c2* and *c3*, chlorophyllide *a*, divinyl chlorophyll *a*, pheophythin *a,* alloxanthin, antheraxanthin, 19′butanoyloxyfucoxanthin, canthaxanthin, α- and β-carotenes, diadinoxanthin, diatoxanthin, echinenone, fucoxanthin, 19′haxanoyloxyfucoxanthin, lutein, myxoxanthophyll, neoxanthin, peridinin, prasinoxanthin, violaxanthin and zeaxanthin. Pigments were supplied in 2.5 cm^3 sealed vials: the extracts were in 90% acetone for chlorophylls and in 100% ethanol for carotenoids. The parameters of reference pigments are given in Table 1.

These compounds were subjected to chromatographic analysis. Pigment standards were injected onto a chromatographic system, calibration curves were prepared and many factors (such as the response factor f_p and detection thresholds) were established.

Table 1 Parameters [symbol, solvent, extinction coefficient ($dm^3\ g^{-1}\ cm^{-1}$), concentration ($mg\ dm^{-3}$) and source of origin] of reference pigment standards obtained from The International Agency for ^{14}C Determination, DHI Institute for Water and Environment, Denmark (*I,II* the first and second group of chlorophylls and carotenoids, respectively)

Pigment	Pigment symbol		Solvent	Extinction coefficient ($dm^3\ g^{-1}\ cm^{-1}$)		Concentration ($mg\ dm^{-3}$)		Source of origin	
	I	II		I	II	I	II	I	II
Chlorophyll *a*	Chla-102	Chla-106	90% acetone	87.67 at 664.3 nm		2.213	1.287	Cyanophyceae	
Chlorophyll *b*	Chlb-101	Chlb-104	90% acetone	51.36 at 646.8 nm		1.451	1.253	Chlorophyceae	
Chlorophyll *c*	Chlc-101		90% acetone	42.6 at 631 nm		0.791		Bacillariophyceae	
Chlorophyll *c2*	Chlc2-101	Chlc2-105	90% acetone	40.4 at 630.9 nm		1.098	0.760	Cryptophyceae	
Chlorophyll *c3*	Chlc3-101	Chlc3-105	90% acetone	346 at 452.9 nm		1.493	0.507	Prymnesiophyceae	Pelagophyceae
Chlorophyllide *a*		Chlia-101	90% acetone		127 at 664 nm		0.757		Cyanophyceae
Divinyl chlorophyll *a*		Dva-103	90% acetone		87.67 at 664.3 nm		0.859		*Prochlorococcus* sp.
Pheophythin *a*		Phta-101	90% acetone		51.2 at 667 nm		1.813		Prymnesiophyceae
Alloxanthin	Allo-102	Allo-104	100% ethanol	262 at 453 nm		1.063	1.032	Cryptophyceae	
Antheraxanthin	Ant-101	Ant-107	100% ethanol	235 at 446 nm		0.630	0.466	Chlorophyceae	
Aphanizophyll	Apha-101		100% ethanol	125.3 at 475 nm		1.135		Cyanobacteria	
19′Butanoyloxy fucoxanthin	But-103	But-109	100% ethanol	160 at 446 nm		0.484	0.566	Pelagophyceae	
Canthaxanthin	Cant-101	Cant-111	100% ethanol	200 at 440 nm	207.5 at 476 nm	1.333	0.685	Roth Chemicals	Cyanophyceae
α-Carotene	Acar-104	Acar-110	100% ethanol	288 at 444 nm		0.445	0.710	Cryptophyceae	
β-Carotene	Acar-101	Beta-106	100% ethanol	262 at 453 nm		0.660	0.670	Cyanophyceae	
Diadinoxanthin	Ddx-103	Ddx-107	100% ethanol	262 at 446 nm		0.633	1.313	Bacillariophyceae	
Diatoxanthin		Dtx-106	100% ethanol		262 at 449 nm		0.638		Bacillariophyceae
Echinenone	Echi-102	Echi-107	100% ethanol	188.1 at 440 nm		1.254	0.913	Cyanophyceae	
Fucoxanthin	Fuco-101	Fuco-104	100% ethanol	160 at 449 nm		1.379	1.596	Bacillariophyceae	
19′Hexanoyloxy fucoxanthin	Hex-102	Hex-107	100% ethanol	160 at 447 nm		0.556	1.368	Prymnesiophyceae	
Lutein	Lut-104	Lut-108	100% ethanol	255 at 445 nm		0.659	1.314	Chlorophyceae	
Myxoxanthophyll	Myxo-101	Myxo-102	100% ethanol	125.3 at 440 nm		1.219	1.186	Cyanophyceae	
Neoxanthin	Neox-102	Neox-104	100% ethanol	244.3 at 439 nm		0.986	1.137	Chlorophyceae	
Peridinin	Peri-102	Peri-105	100% ethanol	132.5 at 472 nm		1.428	1.101	Dinophyceae	
Prasinoxanthin	Pras-102	Pras-104	100% ethanol	160 at 454 nm		1.226	1.282	Prasinophyceae	
Violaxanthin	Viol-103	Viol-109	100% ethanol	255 at 443 nm		0.625	1.103	Chlorophyceae	
Zeaxanthin	Zea-101	Zea-112	100% ethanol	254 at 450 nm		0.843	0.729	Cyanophyceae	

Apparatus

Pigments were isolated using the RP-HPLC technique. The chromatographic system was equipped with a Hewlett-Packard HP 1050 pump, diode array detector (model HP 1100), an HP 1046 fluorescence detector and a Rheodyne injector with a 100 µl sample loop. Two types of C_{18} analytical columns: LiChroCART Hypersil ODS (dimension: 250×4 mm, particle size: 5 µm; Merck) and LiChroCART LiChrospher 100 RP18e (dimension: 250×4 mm, particle size: 5 µm; Merck) were used for comparative analysis of analytical column responses and efficiency.

The diode array absorbance detector "dad" was set at $\lambda = 440$ nm. During a single analysis, the full spectral range of 350–700 nm, with steps of 1 nm and a 0.4 s time resolution, was recorded. The fluorescence detector with the excitation wavelength $\lambda_{ex} = 431$ nm and emission $\lambda_{em} = 660$ nm was only used to confirm the presence of chloropigments in the extract.

Solvents and chromatographic analysis

The solvents used for chromatography were filtered and degassed with helium before use. Mobile phases used in the gradient elution were composed of the primary eluant (A), consisting of methanol and 1 M ammonium acetate (80:20 v/v), and the secondary eluant (B), prepared from methanol and acetone (60:40 v/v). In addition, 1 M ammonium acetate was used as the ion-pairing reagent, and it is recommended that it be present in both the sample and the mobile phase, to improve pigment separation and suppress the dissociation of the isolated compounds (Mantoura and Llewellyn 1983). The separation was achieved by changing the solvent mixture composition (Mantoura and Llewellyn 1983; Barlow et al. 1993; Stoń and Kosakowska 2002). The solvent composition was changed linearly from 100% of solvent A to 100% of solvent B in 10 min after injection, and was isocratically held at a constant flow rate of 0.8 ml min^{-1} till the end of the analysis (25 and 32 min for first and second type of column, respectively). The equilibrium state was attained after 10 min, when the solvent composition returned to the initial conditions.

Statistical analysis

The repeatability of the chromatograms and the method's precision were also checked. The analyses were based on monocultures grown in laboratory conditions and using pigment standards. The statistical analysis was based on 36 repetitions of the chromatographic resolution of the extracts. The margin of error of the adopted method ranged from 1.28% to 5.55%, with a mean value of ±2.9%.

Results

Chromatographic resolution

The separation of chlorophylls and carotenoid pigment standards by the use of two types of columns yielded satisfactory results. Sufficient resolution of all reference pigments in a single run was achieved. Representative absorbance chromatograms of the pigment standard mixture are shown in Fig. 1. The results obtained indicate that chlorophylls *c1* and *c2* elute as a single peak. Likewise, lutein and zeaxanthin were not completely resolved in either type of analytical column. A comparison of the results obtained with pigments separated on different columns showed some differences in the elution times of individual components of the mixtures. Although the sequence of

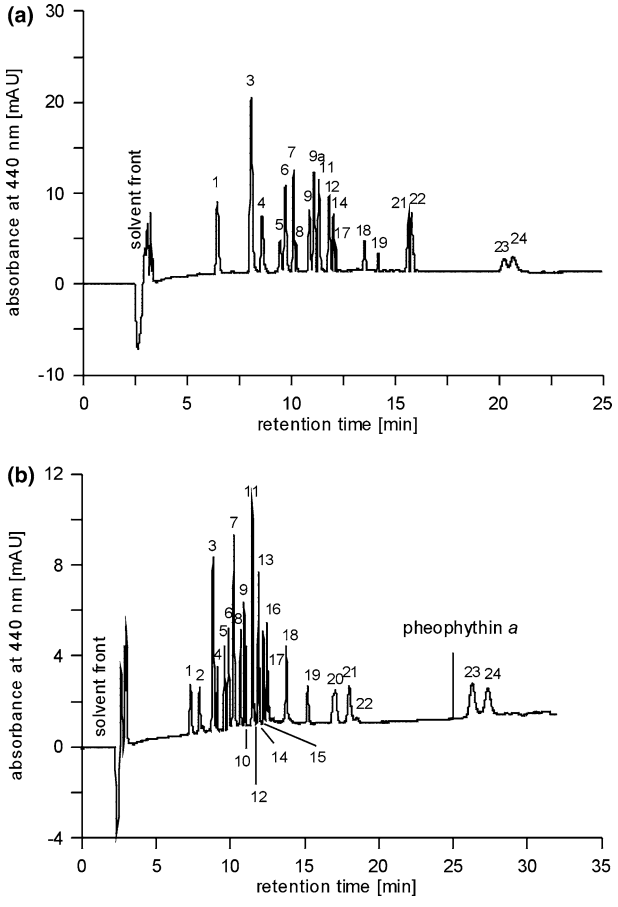

Fig. 1 Chromatograms of resolved mixtures of phytoplankton pigment standards obtained using an RP-HPLC system equipped with two types of analytical columns: **a** LiChroCART Hypersil ODS and **b** LiChroCART LiChrospher 100 RP18e (*1* chlorophyll *c3*; *2* chlorophyllide *a*; *3* chlorophyll *c1* + *c2*; *4* peridinin; *5* 19′butanoyloxyfucoxanthin; *6* fucoxanthin; *7* 19′haxanoyloxyfucoxanthin; *8* neoxanthin; *9* prasinoxanthin; *9a* aphanizophyll; *10* violaxanthin; *11* diadinoxanthin; *12* antheraxanthin; *13* alloxanthin; *14* myxoxanthophyll; *15* diatoxanthin; *16* lutein; *17* zeaxanthin; *18* canthaxanthin; *19* chlorophyll *b*; *20* divinyl chlorophyll *a*; *21* chlorophyll *a*; *22* echinenone; *23* α-carotene; *24* β-carotene)

retained and eluted pigments remained mostly the same in the two systems, in the case of LiChroCART Hypersil ODS, the retention times of pigments were shorter than those found with LiChroCART LiChrospher 100 RP18e. The greatest differences in elution times were observed for α-carotene and β-carotene (~7 to 8 min). This delay may have resulted from the molecular structure of these two non-oxygenated compounds. As a consequence, the time of a single analysis was prolonged from 25 min for LiChroCART Hypersil ODS to 32 min for LiChroCART LiChrospher 100 RP18e. The values of the chromatographic parameters obtained for each pigment in the two columns are given in Table 2.

Purity of pigments

The purity of chlorophyll and carotenoid standards (>95%) was confirmed by an analytical chem-station procedure. This procedure allows assessment of whether the eluting peak is spectrally pure, or contains impurities due to simultaneous retention of several compounds, or incomplete separation of peaks, and/or background absorption The peak shape and purity curves are shown in Fig. 2 for fucoxanthin.

Spectral properties of chlorophylls and carotenoids

Identification and quantification of pigments by HPLC is based on the characteristic absorption properties of these compounds in the visible spectral range. Pigments differ from each other in the shape and position of their absorption maxima. Carotenoids absorb light in the range 400–500 nm, displaying two or three distinct absorption maxima in this region. Chlorophylls additionally absorb in the red part of the light spectrum. The ability of carotenoids to absorb light is due to their system of regularly alternating single and double bonds (a chromophore). The chromophore structure in algal carotenoids consists of 9–11 aliphatic double bonds in conjunction with no, one, or two endocyclic double bonds. Spectral properties of carotenoids are not influenced by functional groups. Under given conditions (solvent, temperature) the spectrum is a characteristic feature of the absorbing molecules, and is therefore used not only in quantitative measurements but also to confirm the identity of the pigment.

Results of the comparative analysis of the spectra of two groups of reference pigments are presented in Fig. 3. The wavelengths of the absorption maxima differed between pigments and were generally shifted by about 1–5 nm relative to the same pigments separated on the other type of column. The spectra overlap only in the cases of neoxanthin and alloxanthin (Fig. 3, 9 and 15). Regarding other pigments, the shifts in the absorption maxima result from different retention times and, hence, different compositions of solvents during elution. The respective values of λ_{max} of individual pigments are listed in Table 2.

Calibration parameters

Calibration curves were prepared based on a series of injections of known concentrations of pigment standards on the chromatographic system. Six to ten different concentrations were injected for each pigment. Such a method of external standard calibration allows establishment of the relationships between the weight of the pigment injected on the chromatographic column and its peak area.

The resultant chromatographic peak areas are related to pigment masses by the response factor (or calibration curve). Calibration factors for each pigment, designated "response factors" (f_p, ng mAU^{-1} s^{-1}) are obtained. The value of parameter f_p is representative of an individual pigment isolated from the pigment mixture. The f_p values and correlation coefficients obtained by the use of two types of chromatographic columns are given in Table 2. The calibration curves for two groups of standards are shown in Fig. 4.

Discussion

Within the framework of the experiment presented, the separation ability of two C_{18} chromatographic columns (LiChroCART Hypersil ODS and LiChroCART LiChrospher 100 RP18e) was compared. Different pigment standards were subjected to chromatography in order to obtain calibration parameters. The standard group encompassed 8 chlorophylls and 19 different carotenoids, with different concentrations (Table 1). For example, the concentration of lutein was equal to 0.659 mg dm^{-3} in the first group, but in the second group was almost twice as high (1.314 mg dm^{-3}). Pigments originated from reference phytoplankton classes. The role of marker pigments in identifying and quantifying individual species has been well recognized. For example, fucoxanthin is considered to be a marker of diatoms; zeaxanthin-cyanobacteria; 19′-hexanoyloxyfucoxanthin-prymnesiophytes; 19′butanoyloxyfucoxanthin-pelagophytes; alloxanthin-cryptophytes; prasinoxanthin-prasinophytes; peridinin-dinoflagellates; and chlorophyll *b* and lutein-chlorophytes (Andersen et al. 1996; Jeffrey and Vesk 1997).

The chromatographic system and procedure presented herein can be used to separate chlorophylls and their derivatives, as well as carotenes and their oxygenated forms, called xanthophylls. Only in the case of zeaxanthin and lutein were the resolution results unsatisfactory. Complete resolution of the above pigment pair was accomplished only by the use of a polymeric-packed chromatographic column (Heukelem et al. 1992; Schmid and Stick 1995). A single analysis allowed isolation and

Table 2 Combination of calibration parameters: retention time (min), detection thresholds (ng per 100 µl), response factors f_p (ng mAU^{-1} s^{-1}), correlation coefficients and wavelength of maximum absorption λ_{max} (nm)—of individual standard pigments obtained by RP-HPLC equipped with different analytical columns (*I* LiChroCART Hypersil ODS; *II* LiChroCART LiChrospher 100 RP18e)

Pigment	Retention time (min)		Detection limit (ng per 100 µl)		Response factor f_p (ng mAU^{-1} s^{-1})		Correlation coefficient		λ_{max} (nm)	
	I	II	I	II	I	II	I	II	I	II
Chlorophyll *c3*	6.44	7.26	1.493	0.507	0.1228	0.1259	0.9968	0.9965	458, 592	458, 590
Chlorophyllide *a*		7.86		0.757		0.1751		0.9811		418, 434, 581, 622, 667
Chlorophyll *c1*	8.28		0.791		0.0663		0.9293		449, 585, 635	
Chlorophyll *c2*	8.28	8.99	0.549	0.380	0.0588	0.0626	0.9919	0.9737	449, 583, 634	447, 581, 632
Peridinin	8.69	9.74	1.428	1.101	0.1471	0.1777	0.9961	0.9789	475	477
19′Butanoyloxy fucoxanthin	9.67	9.97	0.968	0.566	0.1190	0.1033	0.9746	0.9864	449, 457, 468	448, 470
Fucoxanthin	9.78	10.16	0.689	0.319	0.0967	0.1164	0.9964	0.9852	450, 466	449, 470
19′Hexanoyloxy fucoxanthin	10.35	10.38	1.112	0.274	0.1386	0.1076	0.9916	0.9828	445, 470	446, 472
Neoxanthin	10.07	10.75	0.986	0.227	0.0765	0.0957	0.9849	0.9755	414, 438, 466	414, 438, 466
Prasinoxanthin	10.13	11.05	0.613	0.641	0.1067	0.1566	0.9774	0.9973	457	455, 473
Violaxanthin	10.42	11.42	0.625	0.221	0.0965	0.0655	0.9896	0.9199	417, 440, 469	418, 441, 470
Aphanizophyll	10.64		2.270		0.2540		0.9832		461, 475, 508	
Diadinoxanthin	11.81	11.56	0.633	0.263	0.0638	0.0843	0.9981	0.999	428, 447, 476	425, 447, 477
Antheraxanthin	10.91	11.74	0.315	0.466	0.0910	0.0903	0.9975	0.9728	432, 447, 475	423, 448, 475
Alloxanthin	11.25	11.99	0.531	0.206	0.0785	0.081	0.9758	0.9972	426, 454, 483	426, 453, 483
Myxoxanthophyll	11.38	11.94	0.609	0.593	0.1520	0.1865	0.9976	0.9973	452, 477, 507	453, 477, 507
Diatoxanthin		12.34		0.319		0.0948		0.9965		426, 452, 482
Lutein	11.87	12.53	0.329	0.263	0.0988	0.1158	0.9877	0.97	423, 446, 475	422, 447, 474
Zeaxanthin	11.96	12.81	0.421	0.365	0.0824	0.1048	0.9777	0.9967	425, 450, 479	425, 453, 481
Canthaxanthin	13.63	13.74	1.333	0.685	0.0163	0.115	0.9932	0.9133	371, 471	478
Chlorophyll *b*	14.15	15.57	7.255	3.759	0.4275	0.6009	0.9545	0.9351	468, 605, 651	468, 600, 650
Divinyl Chlorophyll *a*		17.00		4.295		0.3263		0.9458		386, 441, 616, 667
Chlorophyll *a*	15.54	17.75	2.213	2.574	0.4458	0.4147	0.9567	0.8649	432, 619, 665	433, 616, 665
Echinenone	15.70	17.97	1.254	0.913	0.1477	0.1493	0.9960	0.9974	472	463, 480
Pheophythin *a*		24.99		36.26		2.6865		0.5964		410, 505, 535, 609, 666
α-Carotene	20.37	27.06	2.275	0.710	0.0844	0.0809	0.9836	0.9869	425, 446, 475	427, 448, 475
β-Carotene	20.58	28.56	3.300	3.350	0.0957	0.113	0.9639	0.9951	430, 453, 481	426, 453, 480

resolution of a mixture of 25 pigments present in the extract. Only the duration of the analysis depends on the type of column used (25 min for LiChroCART Hypersil ODS and 32 min for LiChroCART LiChrospher 100 RP18e); the sequence of eluted pigments remains unchanged.

The duration of analysis depends on the isolation system applied (e.g. solvents, appropriate temperature) and on the type of column, its diameter, particle size, pore size and number of plates. The analysis may last as long as 60 min (Heukelem and Thomas 2001); 40 min when a C_8 column (150×4.6 mm, 3.5 μm particle size) thermostated at 25°C is employed (Zapata et al. 2000), or 20 min when using 3 μm Hypersil MOS (100×4.6 mm, C_8; Shandon) for separation of chlorophyll *a* from divinyl chlorophyll *a* and zeaxanthin from lutein (Vidussi et al. 1996).

The calibration parameters obtained during the analysis were different for two kinds of stationary phases. Spectral characteristics allowing qualitative identification of pigments were very close only in the case of alloxanthin (λ_{max} = 426, 454, 483 nm) and neoxanthin (λ_{max} = 414, 438, 466 nm). In other pigments, the absorption spectrum was shifted by 1–5 nm (all details are presented in Table 2). Spectral characteristics vary not only between individual pigments but also with temperature and solvent within the pigment itself. The data published by Jeffrey and Humphrey (1975) and Bjørland (1997) present data on how solvent effects the

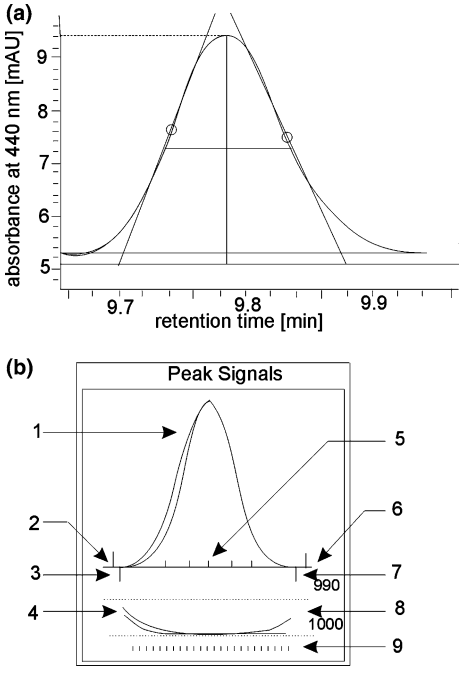

Fig. 2 Confirmation of the peak purity on the example of fucoxanthin: **a** peak shape and **b** peak signals window showing similarity and threshold curves (*1* peak signal plots; *2* peak start; *3* initial reference spectrum; *4* similarity curve; *5* position of selected peak spectra; *6* peak end; *7* final reference spectrum; *8* threshold curve; *9* spectral tick marks)

algal carotenoid chromofores. The different λ_{max} values and different shapes of spectra presented in Fig. 3 may have resulted from not the same solvent composition during retention.

The slopes of the calibration curves differed not only between different pigments but also for the same pigment when different columns were used. Different response factors (f_p, ng mAU^{-1} s^{-1}) were observed in most cases. The values of factor f_p are representative of individual pigments isolated from the pigment mixture. The linear approximation of f_p, which resulted from a sequence of injections of fixed pigment amounts onto chromatographic systems, yielded satisfactory correlation (correlation coefficients ranged from 0.92 to 0.99 in the case of the first group and from 0.6 to 0.99 for the second group of pigments). Poor correlation (0.59, wide scatter of points) was noted for pheophythin *a*. This pigment also has a higher detection threshold (36.3 ng per 100 μl), which means that pheophythin *a* can only be determined in samples with a high content of degradation products. Other chlorophyll *a* derivatives, chlorophyllide *a* and divinyl chlorophyll *a*, revealed little scatter of points, and, thus, the values of f_p were close to 1 (0.98 and 0.94, respectively).

Slopes of the calibration curves of chlorophyll *c3*, antheraxanthin, echinenone and chlorophyll *a* were similar for both column types. Slopes of the calibration curves of chlorophyll *c2*, peridinin, fucoxanthin, neoxanthin, prasinoxanthin, diadinoxanthin, alloxanthin, myxoxanthophyll, lutein, zeaxanthin, chlorophyll *b* and *β*-carotene were steeper for the second group of pigments, separated by LiChroCART LiChrospher 100 RP18e. In the other cases the situation was reversed.

The detection thresholds of isolated pigments were very low and in most of the cases did not exceed 1 ng per 100 μl. The comparison of the detection thresholds of chlorophylls and carotenoids in both columns indicated that the second type of columns was more sensitive: from 1.02 times for myxoxanthophyll up to 3.2, 4.06 and 4.34 times for 19′hexanoyloxyfucoxanthin, α-carotene and neoxanthin, respectively. Only prasinoxanthin, antheraxanthin, chlorophyll *a* and *β*-carotene could be detected at lower concentrations by the use of the LiChroCART Hypersil ODS analytical column. The minimum delectable concentration of *β*-carotene was similar in both systems ~3.3 and 3.35 ng per 100 μl. The results obtained also indicate that pheophythin *a* is difficult to detect because of its very high threshold concentration, 36.7 ng per 100 μl.

In conclusion, employment of the chromatographic systems discussed here allows precise determination of the pigments present in natural samples, even if only present in low amounts (< 0.1 μg dm^{-3}). Our experiments have provided unique calibration parameters for RP-HPLC systems equipped with two types of columns: LiChroCART Hypersil ODS and LiChroCART LiChrospher 100 RP18e. Sufficient resolution of all pigments present in the mixture was achieved. Both columns used provided similar results in the isolation of

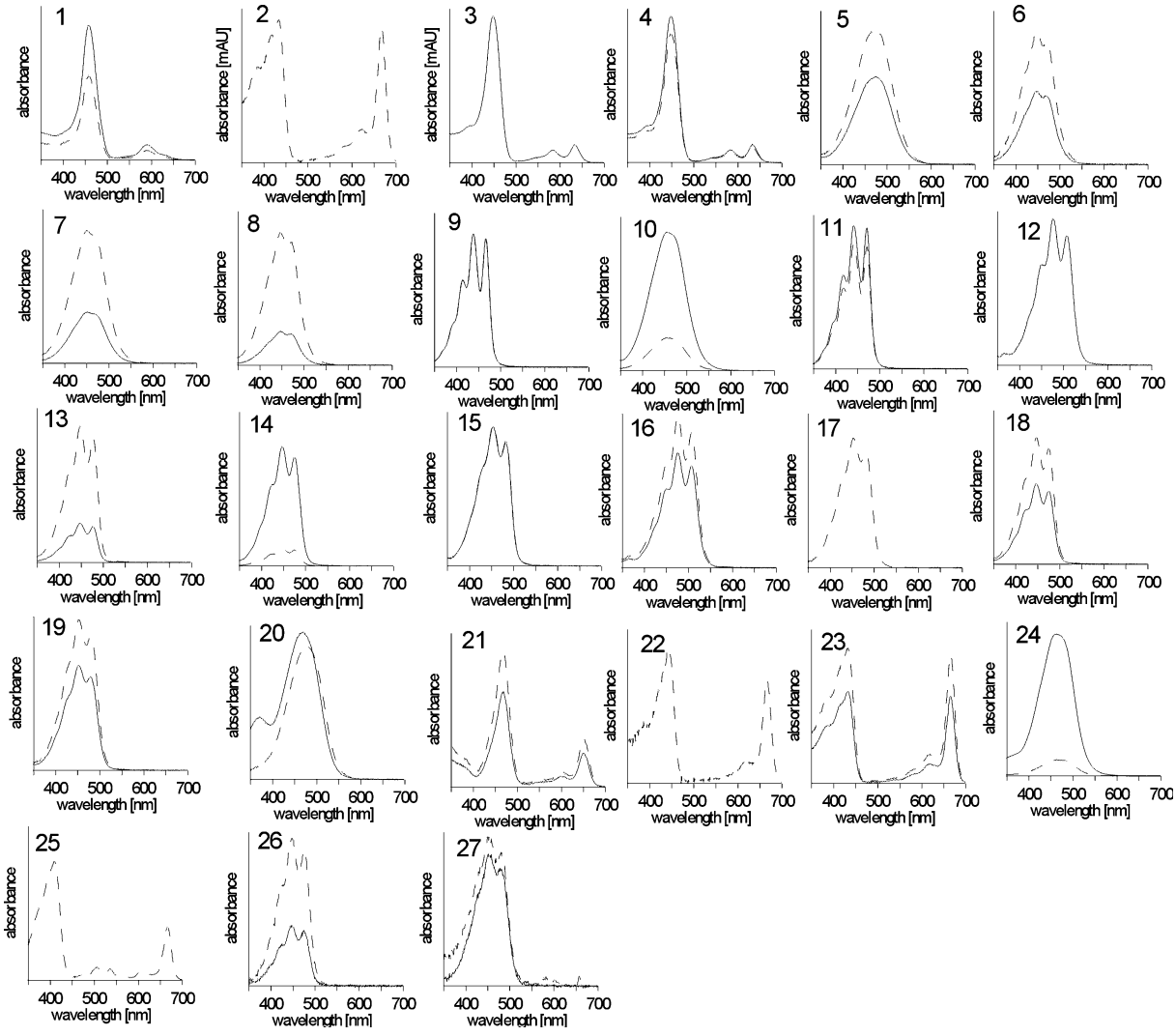

Fig. 3 Comparison of on-line spectrum shapes of pigments obtained during elution by diode-array detector. *Continuous lines* represent the first group of reference pigments isolated by LiChroCART Hypersil ODS; *broken lines* represent the second group of standards isolated by the use of the LiChroCART LiChrospher 100 RP18e column (*1* chlorophyll *c3*; *2* chlorophyllide *a*; *3* chlorophyll *c1*; *4* chlorophyll *c2*; *5* peridinin; *6* 19′but-fucoxanthin; *7* fucoxanthin; *8* 19′hex-fucoxanthin; *9* neoxanthin; *10* prasinoxanthin; *11* violaxanthin; *12* aphanizophyll; *13* diadinoxanthin; *14* antheraxanthin; *15* alloxanthin; *16* myxoxanthophyll; *17* diatoxanthin; *18* lutein; *19* zeaxanthin; *20* canthaxanthin; *21* chlorophyll *b*; *22* divinyl chlorophyll *a*; *23* chlorophyll *a*; *24* echinenone; *25* pheophythin *a*; *26* α-carotene; *27* β-carotene)

pigments. Except for two pigment pairs, the chlorophylls *c1* and *c2* and zeaxanthin and lutein, all other chlorophylls and carotenoids injected onto the chromatographic columns were separated to the baseline. Even though zeaxanthin and lutein are hard to separate, the absorption spectrum of combined peaks could allow a statement on which one dominated. Although both pigment groups analyzed differed in amount and type, it was possible to establish calibration parameters for the two HPLC systems. Unfortunately, only the group of compounds tested on LiChroCART LiChrospher 100 RP18e possessed chlorophyll *a* derivatives (chlorophyllide *a* and pheophythin *a*), which may exclude the use of the LiChroCART Hypersil ODS analytical column in certain environmental analyses, for example, of sediment samples. Pigment concentrations in samples of naturally living phytoplankton can be determined by the use of both types of columns. The pigments analyzed are characteristic biomarkers of different phytoplankton groups. The LiChroCART LiChrospher 100 RP18e column was tested on a wide group of pigments and in most chlorophylls and carotenoids yielded better detection results. However, in the case of prasinoxanthin, antheraxanthin, chlorophyll *a* and β-carotene, the use of LiChroCART Hypersil ODS allows detection of these pigments at lower concentrations. In conclusion, the columns used in the analysis are interchangeable.

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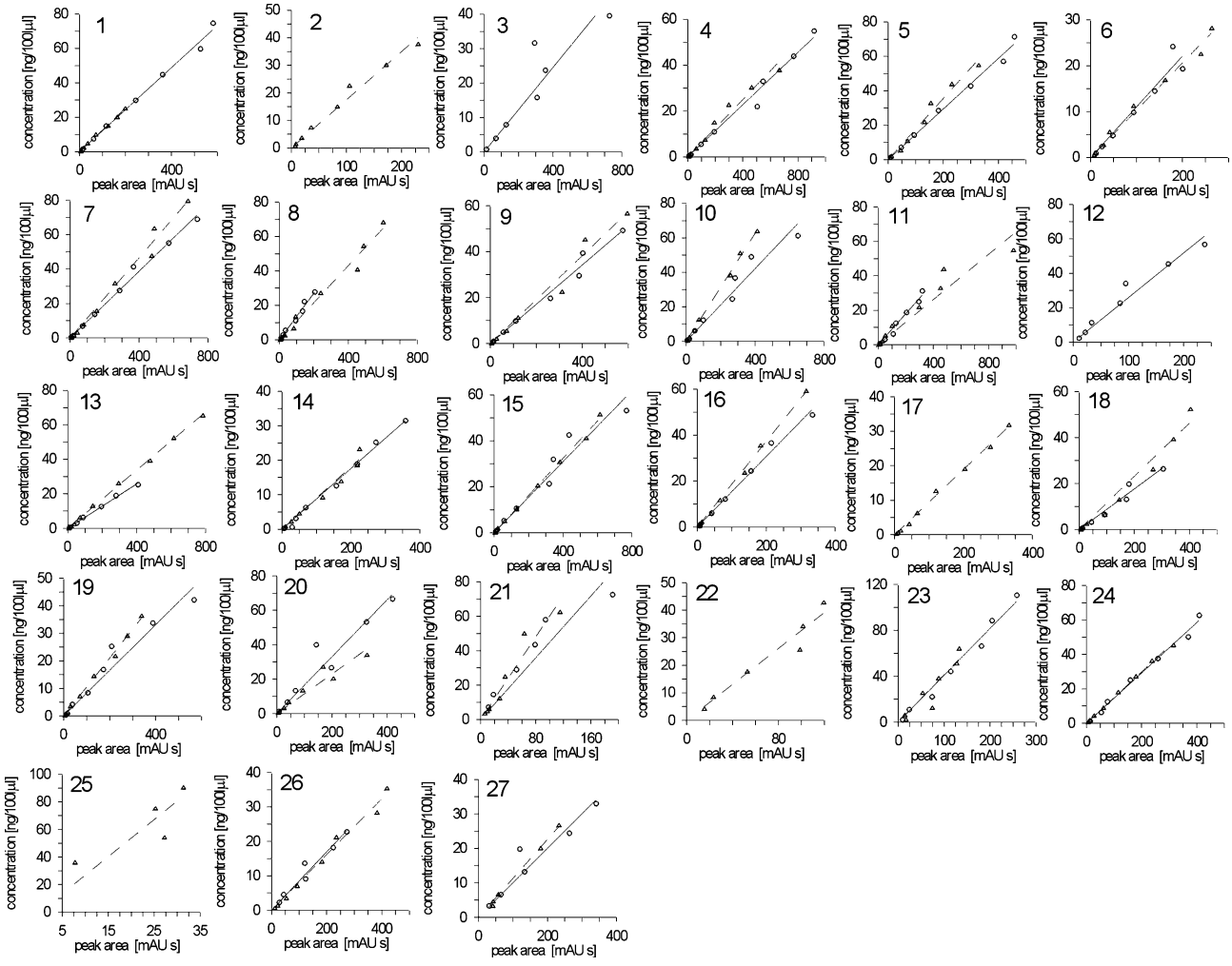

Fig. 4 Comparison of calibration curves of individual pigments obtained by resolution on two different columns: LiChroCART Hypersil ODS (*continuous lines*) and LiChroCART LiChrospher 100 RP18e (*broken lines*) (*1* chlorophyll *c3*; *2* chlorophyllide *a*; *3* chlorophyll *c1*; *4* chlorophyll *c2*; *5* peridinin; *6* 19′but-fucoxanthin; *7* fucoxanthin; *8* 19′hex-fucoxanthin; *9* neoxanthin; *10* prasinoxanthin; *11* violaxanthin; *12* aphanizophyll; *13* diadinoxanthin; *14* antheraxanthin; *15* alloxanthin; *16* myxoxanthophyll; *17* diatoxanthin; *18* lutein; *19* zeaxanthin; *20* canthaxanthin; *21* chlorophyll *b*; *22* divinyl chlorophyll *a*; *23* chlorophyll *a*; *24* echinenone; *25* pheophythin *a*; *26* α-carotene; *27* β-carotene)

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