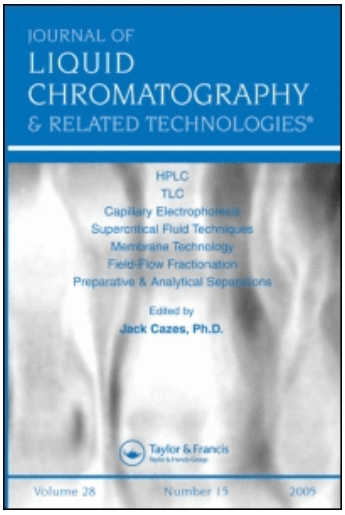

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Comparison of Extraction and HPLC Methods for Marine Sedimentary Chloropigment Determinations

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Abstract: This study compared different extraction and high performance liquid chromatography (HPLC) combinations for the analysis of chloropigments in sediments and microalgae (phytoplankton). A significant literature review is also included. Extractants in the present study included 100% acetone, tetrahydrofuran, and an aqueous mixture of methanol, acetone, and dimethylformamide. HPLC methods involved gradient elution methods with or without ion pairing reagents over C_{18} silica based columns. Though slight differences were found, most notably with the extraction of steryl chlorin esters, this intercomparison reveals that both extraction and HPLC protocols investigated, in any combination, perform well for the analysis of chlorophylls and their derivatives in sediment and phytoplankton samples.

Keywords: Chloropigments *a*, Sediments, Analysis, HPLC, Extraction

INTRODUCTION

Chlorophyll *a* and other pigments in aquatic/marine systems are of significant interest to limnologists, oceanographers, and organic geochemists.[1–7] Analysis of these compounds is not always an easy task, as they often occur in low concentrations and have many isomeric forms. There are many different parent chloropigments and even more carotenoids, which often

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differ only slightly in their chemical structure, generating coeluting or partially coeluting peaks, and standards for direct comparisons are not always available. All of these compounds are unstable and are affected by numerous factors (light, heat, oxygen, acids, bases, etc.) which transform the native biopigments into a wide variety of derivatives. These transformations not only occur in senescing/dead cells[3,4] or in the natural geochemical environment,[8–10] but also during analysis.[1,11] Aggressive extraction conditions[12] or analytical methods (derivatizations, GC, HPLC/MS)[7] can result in satisfactory precision but can also form artifacts.

Numerous extraction methods have been applied to sediments and it is not the intent of this short treatise to review all solvent combinations. When dealing with surface to 'near surface' (e.g., <1 m depth below surface {dbs}) sediments, one of the main obstacles to the extraction of lipophilic substances, pigments in the present case, is water. That is, a dehydration step(s) is required in order to effectively extract lipophilic compounds from an aqueous milieu. This is quite commonly performed by transitioning from aqueous miscible solvents (methanol {MeOH}, acetone {ACE}) to more lipophilic solvent systems or by lyophilization (freeze drying) sediments prior to extraction. For example, ACE/MeOH extraction with ultrasound has been reported for the extraction of lake[13,14] or marine sediments.[15] Another system, used for the extraction of deep sea diatomaceous oozes, entailed MeOH, MeOH/ACE, ACE, ACE/Benzene, and Benzene extractions, in that order, with ball milling under nitrogen to affect the dehydration and then complete extraction of pigments.[16] Lyophilization, for the dehydration step, has often been reported,[14] though a recent study reveals lower yields from freeze dried sediments versus native wet sediments.[17] The use of buffers, such as bicarbonate[5] or ammonium acetate[18] has also been reported for pigment extraction from sediments, though this practice is not as widespread as adding magnesium carbonate for plant extractions[19] used to be.

Determination of pigments in seawater has become quite well known, due to development of reversed phase HPLC techniques[20–25] and is detailed in a UNESCO monograph.[1] There are different methods used, but often the method worked out by Mantoura and Llewellyn[26] with ion pairing and buffers or slight modifications[7,27,28] are applied.

HPLC methods applied to microalgal pigments range from simple isocratic and step isocratic methods[29] to a variety of elaborate gradient elution systems.[30–33] The highly polar chlorophylls-c and congeners have been separated over polyethylene based HPLC columns.[34] The monovinyl and divinyl chlorophylls are found to be best resolved using C_8 (octyl) rather than C_{18} (octadecyl) columns.[29,35] Certain highly unstable chlorophyll derivatives of the cyclopheophorbide series are susceptible to significant alteration over silica based reversed phase (rp) columns, but can be isolated in high purity using polymeric supported C_{18} stationary phases.[36] Applications of RP-HPLC to sediment extracts have involved techniques both with[4,37,38] and without[39–41] ion pairing reagents. To date, a comparison

of rpHPLC methods with and without ion pairing on the identical sediment extracts has yet to appear.

Analysis of fresh algal cultures or phytoplankton (bioseston) is inherently more facile than dealing with sedimentary bitumen, wherein a multitude of biopigments and their derivatives co-occur in more complicated mixtures than from either pure algal cells or seston. The present study compares reversed phase HPLC analyses using (a) repetitive acetone extractions and a C_{18} endcapped stationary phase with an ACE/water gradient for HPLC separations[2,42–45] with that using tetrahydrofuran[4] or methanol (MeOH)/acetone (ACE)/dimethylformamide (DMF)/water[46] for the extractions of sediments[4] or algae/phytoplankton,[46] respectively, and an ion pairing multisolvent HPLC system and C_{18} stationary phase.[3–5] The two stages of these procedures, extraction and HPLC analysis, are then compared.

While we have looked into certain extraction differences, the main point of this work is the comparison of HPLC separations of marine sedimentary chloropigments in the '*a*' series using systems with or without ion pairing methodology.

EXPERIMENTAL

Samples

Two sediment types from the Gulf of Gdańsk (Baltic Sea) were used for these intercomparisons. These were a sandy sediment, characterized by low organic carbon, collected at the Wisła mouth and clay rich sediment of high organic carbon content collected from Gdańsk Deep (water depth ~100 m).

In addition to sediment samples, unialgal cultures and phytoplankton were investigated. These were (a) a diatom species also abundant in the Baltic (*Cyclotella meneghiniana*), (b) a dinoflagellate (*Amphidinium carterae*), and (c) a green alga (*Stephanoptera* sp.). All cultures were obtained from Carolina Biological Supply Company (Burlington, N. Carolina, USA).

Samples of mixed marine phytoplankton were collected from the near bottom surficial floc (water depth ~2 m) in a marine lagoon (Boca Raton, Florida, U.S.A.: 26° 21′0.99′× 80° 04′33.03″).

Pigment Extraction

Frozen sediment (0.2–15 g) was placed into the glass centrifuge tube and left to thaw. Next, the sample was centrifuged to remove excess water, decanted, and the pellet was then extracted. Extractions of sediments were compared using both the method of Marine Pollution Laboratory, Institute of Oceanology, Polish Academy of Sciences (MPL IO PAS)[2,43] – extraction method I and that of the Organic Geochemistry Group at the Florida Atlantic

University (OGG-FAU)-extraction method IIa. The later method was originally developed for use with carbonate marl sediments.[4]

Following extraction, sediments were dried at 60°C and weighed.

For extraction of phytoplankton and algae from the cultures the method of MPL IO-PAS[2,45] – extraction method I, and a different method used by OGG-FAU[3,5,46] – extraction method IIb, were compared.

Extraction efficiencies were compared using the FAU-OGG-HPLC methods IIa-b, as described below.[3–5,46] Pigment concentrations were normalized to dry weight (g) of sediment or to volume (L) of water in the case of algal cultures and phytoplankton.

Extraction of Pigments from Sediments

Solvents

All solvents were purchased in Optima grade purity (Fisher Scientific Inc., Pittsburg, Pennsylvania, USA). Tetrahydrofuran contained butylated hydroxytoluene (BHT) as a peroxide inhibitor.

Extraction Method I

After removing excess water by centrifugation, the sediment sample was covered with 15 mL of acetone, mixed and sonicated 2–3 min, centrifuged again (10 min, 2500 rpm), and the extract decanted. This extraction procedure was repeated until subsequent supernatants were colorless (usually no more than three times). Sequential acetone extracts were pooled and transferred to a separate funnel for liquid-liquid partitioning. Here, the acetone extract was diluted with water and benzene added in proportion: acetone extract/benzene/water (15/1/10, v/v/v). The benzene layer was then transferred to a glass vial and evaporated to dryness in a stream of argon and kept at −20°C until analyzed by HPLC.[2,43]

Extraction Method IIa

A sediment sample was thawed, mixed by hand, centrifuged, decanted to remove excess water, mixed with 5 mL of tetrahydrofuran (THF), sonicated for *ca.* 2 min, and placed in a refrigerator(∼4°C) to steep for 1 hour. The sample was centrifuged, decanted, the supernatant was passed through a 0.45 μm filter, and the solvent was removed in a stream of dry nitrogen.[4]

Extraction of Pigments from Phytoplankton and Algal Cultures

Extraction Method I

The procedure was similar to that one used for sediments. Microalgae filtered onto glass fiber filters (Whatman GF/F) were frozen. The folded filter was next placed in the glass centrifuge tube covered with 15 mL of acetone, ground with a glass bar, and sonicated for 2–3 min, centrifuged (10 min, 2,500 rpm) and the supernatant decanted. The extraction was

repeated until subsequent supernatants were colorless.[2,45] Additional workup followed that given above for sediment extract method I.

Extraction Method IIb

The filter with the algae was folded several times and inserted into the pre-chilled glass mortar of a 15 mL tissue homogenizer immersed in crushed ice. Next, 3 mL of methanol/acetone/DMF/water – (30/30/30/10 v/v/v/v) containing an internal standard (copper mesoporphyrin-IX dimethyl ester) were added and ground in several *ca.* 30 sec spurts with a pre-chilled PFTE coated stainless steel pestle. The homogenate was then sonicated in several *ca.* 10–20 sec spurts, and allowed to steep in a refrigerator ($\sim 4^{\circ}$C) for 1 hr. The sample was then centrifuged, decanted, and filtered through a 0.45 μm syringe filter.[3,5,46]

HPLC Analyses

HPLC analyses were carried out using two methods: method I used in MPL IO PAS, described earlier[2,43] and method II used in OGG FAU.[3–5] For comparison of HPLC methodologies, the extracts of green algae (*Stephanoptera* sp.) and the clay sediments from the Baltic Sea were utilized.

Solvents, Chemicals

Solvents were purchased in Optima grade purity (Fisher Scientific Inc., Pittsburg, Pennsylvania, USA). Ammonium acetate (Fisher Scientific Inc., Pittsburg, Pennsylvania, USA) and tetrabutylammonium acetate (Sigma-Aldrich, St. Louis, Missouri, USA) were purchased in HPLC grade.

HPLC Method I

A sample was dissolved in acetone and 20 μL injected through a Merck (Darmstadt, Germany) Model 100RP18 end capped guard column (4 mm × 4 μm) onto a Merck (Darmstadt, Germany) Lichrospher 100RP18 end capped column (250 × 4 mm, 5 μm). The HPLC system consisted of two Type 64 Knauer (Berlin, Germany) pumps equipped with a photodiode array type detector (Chrom-a-Scope). Separations were affected at room temperature with an acetone-water gradient system (Table 1) at the flow rate 1.00 mL/min. Absorption spectra were collected over the range of 360–700 nm. Solvents used were pre-filtered and sparged with helium.

Individual pigments were identified based on retention time for the gradient system used and spectral characteristics for each compound (Table 2).

An aliquot of the extract to be analyzed by HPLC was diluted with acetone and its absorption was measured at 660 nm using a

Table 1. The gradient system used in HPLC method I (A–acetone; B–water)

Time (min)	Solvent A (%)	Solvent B (%)
0	80	20
10	85	15
20	95	5
40	100	0
50	100	0
60	80	20

spectrophotometer. Pigment concentration in the sample was calculated according to the following equation:[2,44]

$$c_1 = E_{\lambda\,max} \cdot \%A_{\lambda\,max} \cdot v \cdot D \cdot 1000/(\varepsilon \cdot l \cdot w \text{ or } L)$$
$$c_2 = c_1 \cdot 1000/M_{cz}$$

where: c = pigment concentration in 1 g of the dried sediment or 1 L of water (μg or nmol), $E_{\lambda max}$ = extinction measured spectrophotometrically, at the maximum, at the longer wavelength (~660 nm), $\%A_{\lambda max}$ = the % of the pigment peak area in the total area of all the integrated peaks of the $HPLC_{DAD}$ chromatogram registered at the maximum wavelength (~660 nm), v = the volume of acetone solution prepared for HPLC analysis (mL), D = the dilution factor (dilution of the solution prepared for HPLC, for spectrophotometric measurement), ε = the extinction coefficient for the pigment at 660 nm (mL $mg^{-1} \cdot cm^{-1}$), l = the optical path length (cm), w = weight of dried sediment (g) or the volume of seawater or culture medium (L), and M_{cz} = molecular mass.

Extinction coefficients (Table 2) for chlorophyll *a* and pheophytin *a* were taken from literature.[20,47] It was assumed that the molar extinction coefficients for all pheophorbides *a*, pyropheophytin *a* and steryl chlorins *a* were the same as for pheophytin *a*[48] and mass differences (M_{cz}) were taken into consideration.

HPLC Method II

Separations were carried out using ThermoSeparation Products (Waltham, Massacusetts, USA) Model 4100 HPLC quaternary pump, a Rheodyne (Rohnert Park, California, USA) model 7120 injector (100 μL loop), a Waters (Billerica, Massachusetts, USA) NovaPack C18 column (3.9 × 150, 4 μm particle size), and a Waters (Billerica, Massachusetts, USA) 990 photodiode array (PDA) detector. Electronic absorption spectra were recorded through the range of 330–800 nm. Gradient elution involved Solvent A = 0.5 M solution of ammonium acetate in methanol/water (85/15 v/v), Solvent B = acetonitrile/water (90/10 v/v),

Table 2. HPLC Method I–The pigments determined, their retention times, absorption maxima, extinction coefficient values and molecular mass

No.	Pigment	Abbreviation[a]	Retention time (min)	Absorption maxima (nm)	Extinction coefficient[b] ($ml \cdot mg^{-1} \cdot cm^{-1}$)	Molecular mass ($g \cdot mol^{-1}$)
1	Chlorophyllide *a*	Chlide *a*	1.80	430, 662	88	614
2	Pheophorbides I[c]	Phides I	3.20–4.00	408, 666	52	600
3	Pheophorbides II[c]	Phides II	4.30–5.50	408, 666	52	600
4	Chlorophyll *a* allomers	Chl *a* allom	19.00–20.10	428, 662	88	908
5	Chlorophyll *a*	Chl *a*	20.40	430, 662	88	892
6	Chlorophyll *a* epimer	Chl *a*′	21.20	430, 662	88	892
7	Pheophytin *a*	Phytin *a*	25.40	408, 664	52	870
8	Pheophytin *a* epimer	Phytin *a*′	26.10	408, 664	52	870
9	Pyropheophytin *a*	Pyrophytin *a*	28.30	410, 664	52	812
10	Steryl chlorin esters	SCEs	32.00–40.00	408, 664	52	850

[a]Ref. #1.
[b]Extinction coefficients, see references in text.
[c]– Groups of compounds with spectra very similar to pheophorbides.

Table 3. The gradient system used in HPLC method II (A–0.5 M solution of ammonium acetate in methanol:water (85:15 v/v); B–acetonitrile:water (90:10 v/v); C–ethyl acetate)

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	60	40	0
5	60	40	0
10	0	100	0
40	0	30	70
45	0	30	70
46	0	0	100
47	0	100	0
48	60	40	0

and Solvent C = ethyl acetate as detailed in Table 3.[3–5,46] The pigments were identified based on retention time and spectral properties of particular compound. Over 75 known compounds formed the basis for QA/QC during these comparisons.[3–5] Quantitative analysis was carried out based on the in-house Excel™ spreadsheet ('PIGCALC') of OGG FAU that includes molar coefficients taken from the literature[3–5, cf.1] for each pigment. The peaks were integrated at 440 nm (chlorophylls, carotenoids) and 410 nm (for pheopigments). The method used by the OGG-FAU lab is a modification of that by Mantoura and Llewellyn,[19] which was used by many other authors for the analysis of algal cultures and marine phytoplankton.[1,18,49,50]

RESULTS AND DISCUSSION

Results for sediment extracts prepared by methods I (100% acetone) and IIa (tetrahydrofuran), and analyzed using HPLC method II, are presented in Table 4. The differences between the mean values for each extraction method were analyzed mathematically using Students t-test. There was a higher extraction efficiency of chlorophyll *a*, pheophytin *a*, and pyropaeophytin *a*, while using the extraction method I. The differences in the relevant means for pheophytin *a* were 9.7% for the sandy sediment and 12.8% for the clay sediment, while for pyropheophytin *a*, these differences were 21.4% and 9.8%, respectively. However, these values were not statistically significant. For chlorophyll *a* the mean differences were 28.5% for the sandy sediment and 18.4% for the clay sediment and were statistically significant ($p < 0.05$). When the extraction method IIa was used, there were higher mean values for pheophorbide II (= pyropheophorbide-*a*). Extraction method

Table 4. Chloropigment *a* content (nmol · g^{-1} d.w.) determined using two different extraction methods (I and IIa) for sandy and clay sediment from the Baltic Sea (mean ± standard deviation, n = 3)

	Phides I	Phides II	Chl *a* allo	Chl *a*	Chl *a*′	Phytin *a*	Phytin *a*′	Pyrophytin *a*
Sandy sediment								
Method I	<d.l.[a]	2.1 ± 0.08	0.4 ± 0.09	1.3 ± 0.11	0.3 ± 0.05	1.9 ± 0.14	0.2 ± 0.06	1.4 ± 0.26
Method IIa	<d.l.	3.0 ± 0.11	0.2 ± 0.02	0.9 ± 0.06	0.1 ± 0.07	1.8 ± 0.07	0.2 ± 0.05	1.1 ± 0.13
p (*t*-test)	—	0.01	n.s.[b]	0.04	n.s.	n.s.	n.s.	n.s.
Clay sediment								
Method I	45.6 ± 3.21	51.4 ± 1.21	1.6 ± 0.14	102.2 ± 3.67	17.0 ± 2.55	61.6 ± 3.46	9.9 ± 0.95	53.2 ± 4.72
Method IIa	51.9 ± 1.38	91.4 ± 8.44	1.4 ± 0.14	83.4 ± 4.37	11.4 ± 0.41	53.7 ± 5.55	7.5 ± 0.45	48.0 ± 0.40
p (*t*-test)	n.s.	0.02	n.s.	0.04	n.s.	n.s.	n.s.	n.s.

[a]<d.l.–Below detection limit.
[b]n.s.–Difference not statistically significant ($p > 0.05$).

Table 5. Chloropigment *a* content (nmol · L^{-1}) determined using two different extraction methods (I and IIb) in algae cultures and seawater (mean ± standard deviation, n = 3)

	Chlide *a*	Phides II	Chl *a* allo	Chl *a*	Chl *a*′	Phytin *a*	Phytin *a*′	Pyrophytin *a*	SCEs
Cyclotella meneghiniana									
Method I	<d.l.[a]	<d.l.	<d.l.	928.8 ± 12.24	26.5 ± 3.38	30.8 ± 1.17	<d.l.	<d.l.	<d.l.
Method IIb	<d.l.	<d.l.	<d.l.	865.4 ± 30.87	36.8 ± 2.35	19.3 ± 1.71	<d.l.	<d.l.	<d.l.
p (*t*-test)	—	—	—	n.s.[b]	n.s.	0.01	—	—	—
Amphidinium carterae									
Method I	<d.l.	<d.l.	<d.l.	269.4 ± 2.53	7.0 ± 0.2	<d.l.	<d.l.	<d.l.	<d.l.
Method IIb	<d.l.	<d.l.	<d.l.	243.1 ± 16.59	9.8 ± 1.18	<d.l.	<d.l.	<d.l.	<d.l.
p (*t*-test)	—	—	—	n.s.	n.s.	—	—	—	—
Stephanoptera sp.									
Method I	<d.l.	<d.l.	<d.l.	621.6 ± 40.49	15.6 ± 1.76	19.2 ± 0.79	<d.l.	<d.l.	<d.l.
Method IIb	<d.l.	<d.l.	<d.l.	667.1 ± 13.93	18.7 ± 0.19	12.8 ± 1.33	<d.l.	<d.l.	<d.l.
p (*t*-test)	—	—	—	n.s.	n.s.	0.02	—	—	—
Surface seawater									
Method I	<d.l.	0.6 ± 0.04	<d.l.	1.8 ± 0.03	<d.l.	0.5 ± 0.05	<d.l.	<d.l.	<d.l.
Method IIb	<d.l.	0.7 ± 0.04	<d.l.	1.9 ± 0.06	<d.l.	0.4 ± 0.01	<d.l.	<d.l.	<d.l.
p (*t*-test)	—	n.s.	—	n.s.	—	n.s.	—	—	—
Near-bottom seawater									
Method I	<d.l.	4.2 ± 0.07	0.5 ± 0.7	5.5 ± 0.46	1.2 ± 0.23	4.1 ± 0.16	0.6 ± 0.10	4.9 ± 0.51	1.8 ± 0.11
Method IIb	<d.l.	4.1 ± 0.12	0.07 ± 0.19	5.0 ± 0.14	1.2 ± 0.07	2.8 ± 0.15	0.3 ± 0.09	2.5 ± 0.19	0.2 ± 0.09
p (*t*-test)	—	n.s.	n.s.	n.s.	n.s.	0.01	n.s.	0.02	0.01

[a]<d.l.–Below detection limit.

[b]n.s.–Difference not statistically significant ($p > 0.05$).

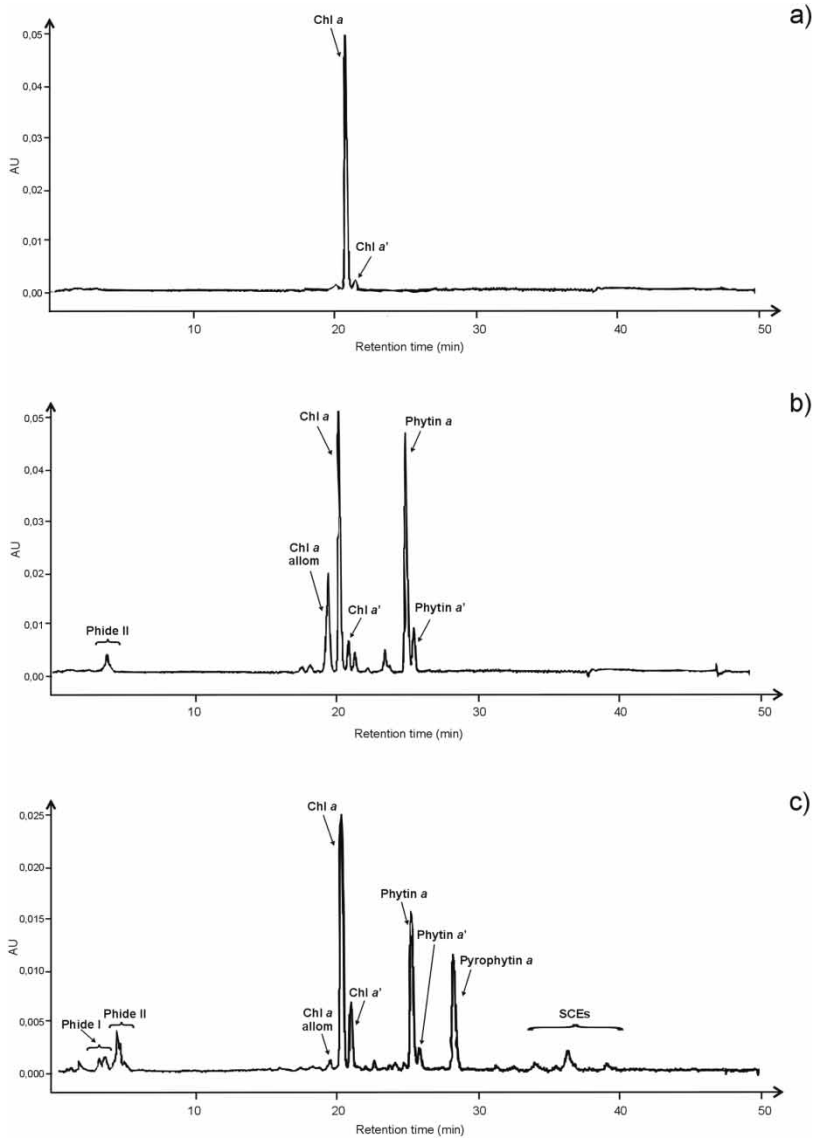

Figure 1. Examples of chloropigments a chromatogram ($\lambda = 660$ nm) obtained using extraction method I and HPLC method I, extract of: a) diatom culture (*Cyclotella meneghiniana*), b) surface (1 m) seawater from Baltic Sea, c) clay sediment from Baltic Sea.

I is concluded as being a bit better than method IIa for extraction of the early diagenetic products of chlorophyll *a* from sandy and clay based sediments. It is noted here, that extraction method IIa was originally developed for sulfidic carbonate marls.[4]

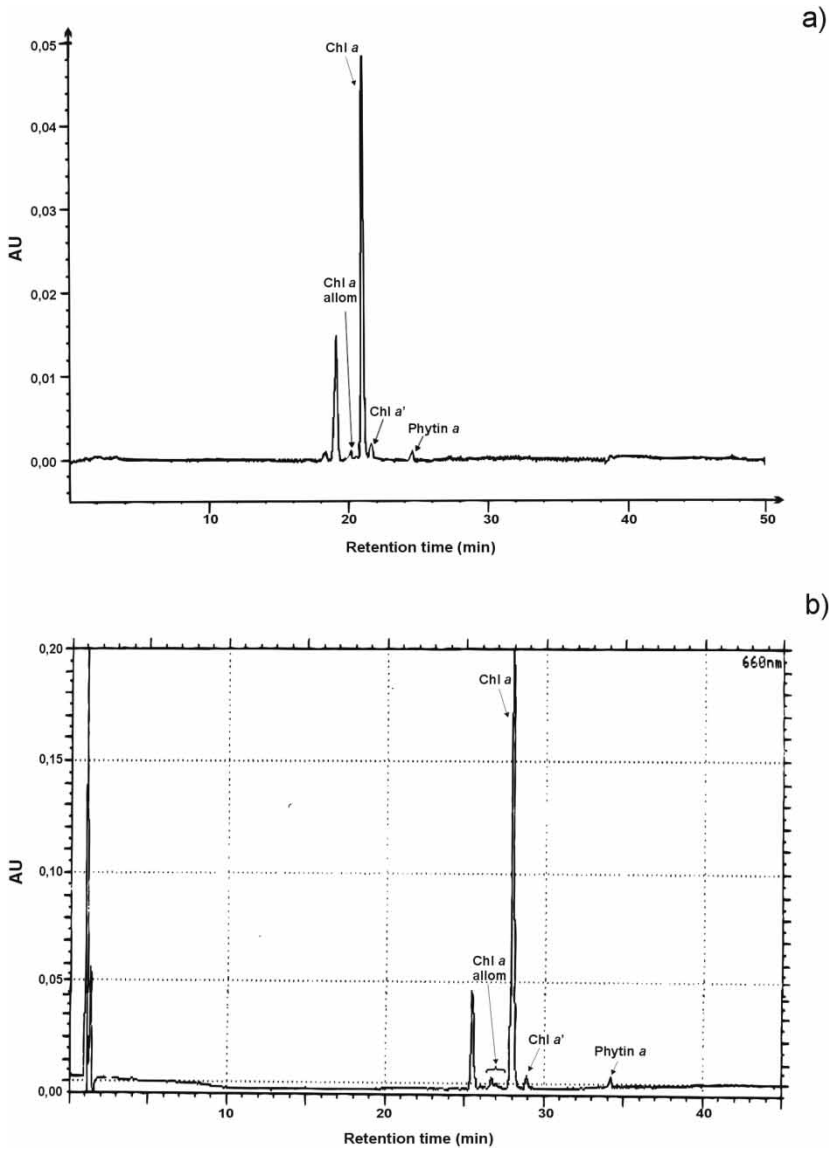

Figure 2. Examples of chloropigments a chromatogram ($\lambda = 660$ nm) from extract of the green algae culture (*Stephanoptera* sp.) (extraction method I) obtained using two different HPLC methods: a) method I, b) method II.

The results for samples of algae and marine phytoplankton extracted using extraction methods I (100% acetone) and IIb (methanol/acetone/DMF/water) are presented in Table 5. The amount of chlorophyll *a* extracted was slightly higher when the method I was used, both in the case

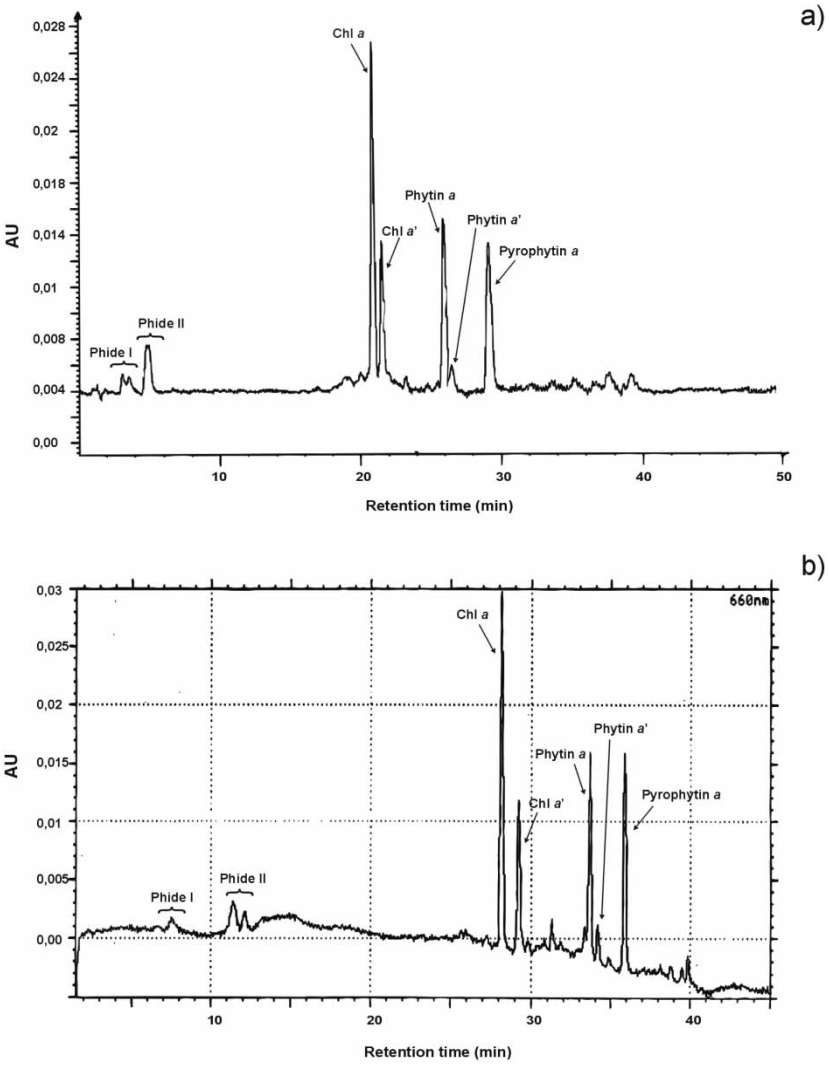

Figure 3. Examples of chloropigments a chromatogram ($\lambda = 660$ nm) from extract of sediment from Baltic Sea (extraction method I) obtained using two different HPLC methods: a) method I, b) method II.

of diatom *Cyclotella meneghiniana* and dinoflagellate *Amphidium carterae*, while in the case of green alga *Stephanoptera* sp. method IIb was more efficient. The mean amounts of chlorophyll *a* obtained by the two methods differed in *ca* 9.5% for diatom, 9.8% for dinoflagellate, and 6.8% for green alga. These differences are not statistically significant. While using extraction method I, slightly higher amounts of pheophytin *a* were obtained during

Table 6. Chloropigment *a* content determined using two different HPLC methods (I and II) in extracts of green algae (*Stephanoptera* sp.) (nmol · L^{-1}) and sediment from the Baltic Sea (nmol · g^{-1} d.w.) (mean ± standard deviation, n = 4)

	Phides I	Phides II	Chl *a* allo	Chl *a*	Chl *a*′	Phytin *a*	Phytin *a*′	Pyrophytin *a*
Stephanoptera sp.								
Method I	<d.l.[a]	<d.l.	0.1 ± 0.01	53.8 ± 1.14	0.4 ± 0.04	2.4 ± 0.11	<d.l.	<d.l.
Method II	<d.l.	<d.l.	0.3 ± 0.07	58.7 ± 1.32	0.6 ± 0.05	3.2 ± 0.12	<d.l.	<d.l.
p (*t*-test)	—	—	n.s.[b]	n.s	n.s.	n.s.	—	—
Sediment								
Method I	29.5 ± 1.45	42.8 ± 0.68	<d.l.	100.7 ± 3.17	25.5 ± 1.31	50.9 ± 1.78	5.3 ± 0.48	35.8 ± 0.30
Method II	36.5 ± 1.17	57.2 ± 2.98	<d.l.	109.3 ± 3.65	30.3 ± 2.35	64.8 ± 1.83	7.2 ± 0.40	47.0 ± 0.42
p (*t*-test)	n.s.	0.02	—	n.s.	n.s.	0.02	n.s.	0.01

[a]<d.l.–below detection limit.

[b]n.s.–difference not statistically significant ($p > 0.05$).

extraction. For the surface seawater samples, where the pigment content was low, the results of the two methods were very similar. In the case of the near bottom water, the amounts of chlorophyll *a* were also similar but the absolute amounts of pheophytin *a*, pyropheophytin *a*, and steryl chlorins of chlorophyll *a* were much higher for the (repetitive) extraction method I. Statistical analysis indicated, that these differences (27.8% for pheophytin *a*, 49.5% for pyropheophytin *a*, and 88.9% for steryl derivatives) were significant ($p < 0.05$).

Extraction methods I (100% acetone) and IIb (methanol/acetone/DMF/water) were found to give similar results for the extraction of the fresh algae. It is noted that the mixture of methanol/acetone/DMF/water (30:30:30:10, v/v/v/v) has proven to give excellent results for the extraction of all lipophilic pigments from fresh microalgal, including recalcitrant filamentous cyanobacteria and chlorophytes of periphyton.[46] In the case of the more degraded algal material, containing the less polar chlorophyll *a* derivatives (pheophytin *a*, pyropheophytin *a* and steryl chlorins) extraction method I (100% acetone) was found to be slightly more efficient than method IIb. This is likely caused by usage of an overall less polar solvent in extraction method I.

The two HPLC methods tested gave similar results. Examples of chromatograms of the extracts from algae culture, phytoplankton, and sediment obtained using HPLC method I are presented here as Figure 1. No artifacts could be shown to be formed during these analyses (Figure 1a). Comparisons of chromatograms of the same extract, from an algae culture or sediment, and determined by both HPLC methods (I and II) are presented in Figures 2 and 3, respectively. As found, the same chloropigment-*a* peaks are visible in chromatograms obtained by either HPLC method and peak resolution in each is more than adequate for valid peak integration.

HPLC method II gives slightly higher values than method I (Table 6). The mean differences in the case of chlorophyll-*a* in the green alga extract were 8.3%, and in case of the sediment extract 7.8%, both not being statistically significant. Somewhat higher and more statistically significant differences were obtained for the quantitation of certain chlorophyll-*a* derivatives. Re-evaluation of integration wavelengths and extinction coefficients may be required in order to sort out these differences.

Though slight differences were found, most notably with the extraction/HPLC protocols tested do perform well for the isolation, identification, and quantification of chlorophyll-*a* and its derivatives in sediment samples.

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