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An optimized method for automated analysis of algal pigments by HPLC

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Abstract

A recent development in algal pigment analysis by high-performance liquid chromatography (HPLC) is the application of automation. An optimization of a complete sampling and analysis protocol applied specifically in automation has not yet been performed. In this paper we show that automation can only be successful if the various methodological aspects of the sampling and analysis protocol are considered in coherence. We introduce an optimized protocol that involves freeze-drying of the sample, subsequent extraction in 90% acetone and the application of water-packing during analysis. The method was evaluated on both natural plankton populations and a broad spectrum of microalgal cultures: *Thalassiosira weisflogii* (Bacillariophyceae), *Emiliania huxleyi* (Prymnesiophyceae), *Phaeocystis globosa* and *Phaeocystis antarctica* (Prymnesiophyceae) and *Pyramimonas* sp. (Prasinophyceae). Whereas pigment extracts were unstable in methanol, with recorded chlorophyll *a* losses from 10% to 60% per day, pigment degradation rates in acetone were generally less than 1% over 18 h storage in the autosampler (4 °C). In addition, it was found that the extraction efficiency of acetone significantly increased upon freeze-drying prior to extraction. Increases as high as 50–60% were measured in *P. antarctica*. The application of water-packing of the sample during injection resulted in improved peak shape and peak separation, without diluting the pigment concentrations. Automation is especially beneficial for application in the field, when mixed algal assemblages and low biomass put a high demand on the sensitivity as well as reproducibility of the method.

Keywords: Automation; Freeze-drying; HPLC pigment analyses; Microalgae; Water-packing

1. Introduction

Pigment analysis by liquid chromatography has become a favourite tool for marine researchers. Pigments can be used to determine the biomass and composition of the algal community (e.g. van Leeuwe et al., 1998; Wright and Jeffrey, 1987) and provide insight in the physiological condition of the phytoplankton (Geider et al., 1993; van Leeuwe and Stefels, 1998). With the development of CHEMTAX, a program to estimate the biodiversity of phytoplankton based on pigment fingerprints (Wright et al., 1996), the routine analysis of pigments in field studies has strongly increased.

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There are a multitude of high-performance liquid chromatography (HPLC) methods described in the literature for measuring chlorophylls and carotenoid pigments (see Jeffrey et al., 1997 and references therein). With the increased need for large databases of chlorophylls as well as accessory pigments in biological oceanography, a method is required with a high throughput, which is sensitive for as many pigments as possible and provides reproducible data. Reproducibility is not only of importance within laboratories but also between laboratories. The latter is becoming of ever-greater relevance now that the increasing variety of applications in itself is a source of error that troubles the intercomparison of data (Lasata et al., 1996; Claustre et al., 2004). A recent development in HPLC analysis that has the potential to address the necessary demands is automation.

The requirements for sample handling during automation are different from individual sample analysis. So far, an optimization of the complete sampling and analysis protocol has not yet been performed, with incomparable data as a result (e.g. Claustre et al., 2004, references therein; Gibb et al., 2001). In order to successfully use automated HPLC, all aspects of sample handling, from filtration to chromatography, need to be considered in coherence. In this paper we present a complete and optimized method that is based on the following priorities:

1. Stability of the pigment extract.
2. Optimization of the extraction efficiency of pigments.
3. Good resolution of peak shape and separation.

Stability of the pigment extract during storage in the autosampler is of prime importance and therefore the selection of the extraction solvent requires special consideration. The application of various extraction solvents has been thoroughly discussed by Wright et al. (1997), but so far not been discussed in the context of automation. In this paper we will reconsider the use of the two most used extraction solvents, methanol and acetone. We will present here a highly reproducible method based on the extraction in 90% acetone, in combination with freeze-drying and water-packing.

2. Material and methods

2.1. Cultures

Pigments were extracted from exponentially growing cultures of a variety of marine microalgal species that were selected for the diversity in their pigment composition as well as their differences in global distribution. *Pyramimonas* sp. (Prasinophyceae; isolated from the Weddell Scotia Confluence, RUG culture collection) and *Phaeocystis antarctica* (Prymnesiophyceae; colonies, CCMP 1871) were grown at 4 °C in F/10 medium (based on Admiraal and Werner, 1983); *Phaeocystis globosa* (Prymnesiophyceae; single cells, isolated from the Marsdiep, RUG culture collection), *Emiliania huxleyi* (Prymnesiophyceae, isolated from the Oslo fjord, RUG culture collection) and *Thalassiosira weisflogii* (Bacillariophyceae, CCMP 1049) were grown at 12 °C in F/2 medium. All algae were cultured in 1-l Fernbach flasks exposed to ca. 20 μmol photons m^{-2} s^{-1}. Cells were harvested by filtering measured volumes of culture through Whatman GF/F glass-fibre filters under gentle vacuum (<0.3 kPa) and dim light. After filtration, filters were immediately snap-frozen in liquid nitrogen (Mantoura et al., 1997), wrapped in aluminium foil and stored at −80 °C until analyses.

2.2. Analytical methods

2.2.1. Freeze-drying

Filters in aluminium foil were placed in open beakers in a Labconco freeze-drier (FreeZone) and dried for 48 h at −50 °C; pressure <1 Pa. The beakers held paper tissues to which liquid nitrogen was added, in order to ensure that the filters were kept frozen while placing them in the freeze dryer.

2.2.2. Extraction

The extraction procedure was carried out under dim light conditions to prevent photooxidation of pigments (Kowalewska and Szymczak, 2001; Nelson, 1993). Filters were extracted with 5 ml of extraction solvents in ground-glass stoppered dark-brown centrifuge tubes, either direct or after freeze-drying for 48 h. Cold acetone (90% aqueous) or cold, buffered methanol (98%, 2% ammonium acetate, 0.5 M, pH 7.1) were used for extraction. For simplicity, the extraction solvents will be referred to simply as *acetone* and *methanol*.

Filters that were extracted in acetone were left in the dark for 48 h (vortexed after 24 h) either at 4 °C or at −20 °C. Whereas at −20 °C the pigment extract may be more stable, reproducibility may benefit from an enhanced temperature as equilibrium processes involved in the extraction are temperature related.

Filters that were extracted in methanol were placed in centrifuge tubes. After addition of 5 ml ice-cold methanol the filters were disrupted by sonication (Sonics, Vibra Cell; 2 times 30 s at 10 W) while kept

on ice. The extract was then filtered over a 0.2 μm cellulose acetate filter (Schleiger and Schuell).

Approximately 1.8 ml of the pure pigment extract was transferred into 2 ml glass sample vials and sealed with teflon lined crimp caps.

2.2.3. HPLC

The HPLC system used was a Waters liquid chromatography system (Model 2690), a cooled autosampler (4 °C) and a Waters 996 diode-array detector. The chromatography method is based on the method by Kraay et al. (1992). The reversed phase column was a 150×3.9 mm Waters DeltaPak C18 column (17% carbon load, fully endcapped, particle size 5 μm) protected by a guard column (Waters Nova-Pak, C18, particle size 5 μm). The column was maintained at a constant temperature of 28 °C using a column thermostat (Waters Column heater Model 2690).

The gradient was based upon a ternary solvent system (Table 1). All solvents were degassed nanograde HPLC solvents (Lab-Scan). Solvent A consisted of 85% methanol/water (v/v), buffered with 0.5 M ammonium acetate (final concentration), solvent B consisted of 90% acetonitrile/water (v/v) and solvent C of ethyl acetate. The flow rate was kept at 0.8 ml min^{-1}.

The column was equilibrated prior to use by flushing with 60% mobile phase A and 40% mobile phase B (v/v) for 5 min. Sample vials were maintained at 4 °C in the autosampler. Sample volumes were injected under the control of an external personal computer that supported Millenium software. Pigments were detected by diode-array spectroscopy (wavelength range: 400 to 750 nm, 1.2 nm spectral resolution). Data were recorded and processed by Millenium software (peak identification on the basis of retention time in combination with comparison with a spectral library). The detection limit of the system is 0.01 mg/l. The coefficient of variation of replicate injections was typically <1%. A suit of standards was always processed with every batch of samples. Standards were obtained from DHI Water Quality Institute (Horsholm, Denmark).

Table 1
The solvent gradient that was applied for chromatography (after Kraay et al., 1992; solvent A: 85% methanol/water (v/v), buffered with 0.5 M ammonium acetate (final concentration), solvent B: 90% acetonitrile/water (v/v), solvent C: ethyl acetate)

Time (min)	% A	% B	% C
0	60	40	0
2	0	100	0
7	0	80	20
17	0	50	50
21	0	30	70
28.5	0	30	70
29.5	0	100	0
30	60	40	0
35	60	0	0

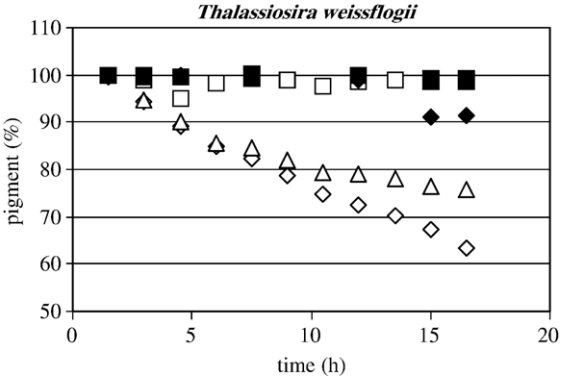

Fig. 1. Pigment concentrations followed over 18 h, in extracts from *Thalassiosira weissflogii*. Pigments are expressed as a percentage of the concentration of the first injection ($t=0$). Open symbols: methanol extraction, closed symbols: acetone extraction. Diamonds: chlorophyll c_2, triangles: chlorophyll *a*, squares: fucoxanthin.

2.3. Experiments

2.3.1. Stability of the pigment extract

To test the relative stability of acetone and methanol pigment extracts in the autosampler, duplicate volumes of a culture were harvested on filters. One filter was extracted with 90% acetone in combination with freeze-drying, the other filter was extracted with methanol. From each of these crude extracts, aliquots of 1.8 ml were dispensed into autosampler vials. These samples were then analysed with 1.5 h intervals, for a period of 18 h. Replicate cultures were tested for each extraction solvent (acetone: $n=3$; MeOH: $n=2$).

2.3.2. Extraction efficiency

The extraction efficiency was tested for acetone versus methanol for all species except *E. huxleyi*. Four constant volume subsamples of a culture were harvested on filters. One filter was extracted directly in acetone; a second filter was extracted in methanol. The samples extracted in methanol were analysed immediately after extraction. Two additional filters were extracted in acetone after freeze-drying over 48 h, one of which was extracted at 4 °C and the other at −20 °C. Since it was the aim of the study to optimize an automated procedure, freeze-drying only was applied to filters that were acetone extracted. The efficiency was tested on duplicate cultures.

2.3.3. Water-packing

To test the extent to which water-packing improved the analyses of the more polar components, the same acetone extract was injected twice according to one of two alternative methods. In the first method, the autosampler injected 60 µl of the pigment extract directly onto the column. In the second ("water-packing") mode, the syringe was filled with 20 µl of water (Milli-Q), then 60 µl of the pigment extract and another 20 µl of water (Milli-Q), respectively. This "water-packed" sample was then injected onto the column.

This experiment was only carried out for samples extracted in acetone. The problems with peak resolution that are of concern here do not occur with methanol extracts (Zapata and Garrido, 1991).

2.4. Statistical analysis and method validation

Effects of the various treatments were tested for significance by ANOVA and multiple regression analyses. Effects were considered significant at $p<0.05$.

To test the validity of the method for application on natural algal populations, field samples that were collected in the Westerscheld (The Netherlands) were extracted both in 90% acetone and buffered MeOH. Stability of the pigment extracts was recorded over 18 h in the autosampler. In addition, field samples collected in the Weddell Sea (Antarctica) were analysed to confirm the sensitivity of the method under conditions of very low biomass (chlorophyll *a* concentrations <0.2 µg/l).

3. Results

3.1. Stability of pigments in the autosampler

In general, pigments extracted in 90% acetone were stable over the 18-h storage period in the autosampler. Significant degradation was observed in methanol extracts (Fig. 1). A comparison of the degradation

Table 2
Degradation over 18 h of the principle pigments in 90% acetone and methanol extracts from *Pyramimonas* sp., *P. antarctica* and *P. globosa*, *T. weisflogii* and *E. huxleyi* (acetone: $n=3$, MeOH: $n=2$) and natural samples ($n=4$)

	Pigment	90% acetone			MeOH		
		Slope (% ml^{-1} h^{-1})	r^2	p	Slope (% ml^{-1} h^{-1})	r^2	p
Pyramimonas sp.	Lutein	0.08	0.13	ns	−0.53	0.48	<0.001
	Chlorophyll *b*	0.04	0.05	ns	−1.20	0.94	<0.05
	Chlorophyll *a*	0.05	0.18	ns	−1.08	0.91	<0.001
	β-carotene	0.00	0.28	ns	−0.70	0.61	<0.05
P. antarctica	Chlorophyll c_3	−0.51	0.56	<0.05	−1.08	0.74	<0.001
	Chlorophyll $c_{1,2}$	−0.23	0.34	ns	−0.40	0.39	<0.05
	Fucoxanthin	0.00	0.00	ns	−0.37	0.43	<0.05
	Hexfuco	−0.01	0.01	ns	−0.30	0.33	<0.05
	Diadinoxanthin	0.16	0.26	ns	−0.15	0.04	ns
	Chlorophyll *a*	−0.03	0.24	ns	−0.79	0.72	<0.001
P. globosa	Chlorophyll c_3	−0.06	0.04	ns	−0.07	0.73	<0.05
	Chlorophyll $c_{1,2}$	−0.03	0.02	ns	−0.09	0.67	<0.05
	Fucoxanthin	0.06	0.57	<0.05	0.00	0.00	ns
	Diadinoxanthin	0.07	0.23	ns	−0.22	0.75	<0.05
	Chlorophyll *a*	−0.04	0.04	ns	−0.54	0.93	<0.001
T. weisflogii	Chlorophyll $c_{1,2}$	−0.06	0.40	ns	−2.30	0.98	<0.001
	Fucoxanthin	−0.04	0.01	ns	0.004	0.0003	ns
	Diadinoxanthin	−0.07	0.06	ns	−0.31	0.83	<0.001
	Chlorophyll *a*	−0.07	0.20	ns	−1.50	0.91	<0.001
E. huxleyi	Chlorophyll $c_{1,2}$	−0.54	0.92	<0.001	−1.49	0.73	<0.001
	Fucoxanthin	−0.24	0.85	<0.05	−0.54	0.50	<0.05
	Diadinoxanthin	−0.54	0.96	<0.001	−0.59	0.66	<0.001
	Chlorophyll *a*	−0.15	0.87	<0.05	−2.53	0.95	<0.001
	β-carotene	−1.10	0.92	<0.001	−0.69	0.69	<0.001
Natural populations	Chlorophyll $c_{1,2}$	0.32	0.85	ns	−0.24	0.53	<0.05
	Fucoxanthin	0.07	0.31	ns	−0.03	0.38	ns
	Chlorophyll *a*	0.03	0.08	ns	−0.40	0.99	<0.05

Slope as derived from time-course analyses (see e.g. Fig. 1); ns: not significant.

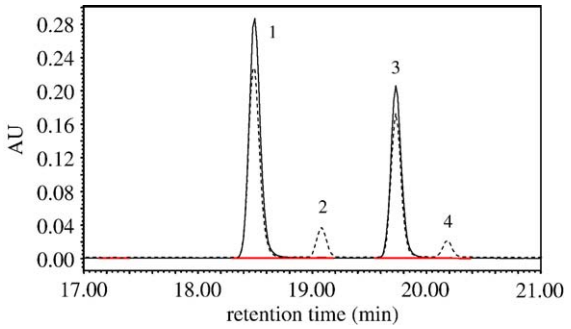

Fig. 2. Section of chromatograms of *Pyramimonas* sp. extracted in methanol at $t=0$ (solid line) and $t=18$ h (dotted line). Absorbance at 450 nm. Peak identification: (1) chlorophyll *b*; (2) chlorophyll *b* epimer; (3) chlorophyll *a*; (4) chlorophyll *a* allomer.

rates, as calculated from the time course of pigment concentrations, showed that in acetone extracts of *Pyramimonas* sp., *P. antarctica*, *P. globosa* and *T. weisflogii* and natural algal populations no significant degradation was observed for all pigments identified, except for chlorophyll c_3 (*P. antarctica*) and fucoxanthin (*P. globosa*) (Table 2). However, the pigment extract derived from *E. huxleyi* was unstable in acetone. The stability of pigments in methanol was always lower than in acetone, for all species studied and for field samples, although the degradation rate of β-carotene in *E. huxleyi* was faster in acetone (Table 2). In general, the chlorophylls were more negatively affected than the xanthophyll pigments. Degradation rates in methanol varied with species. Highest rates were again recorded in *E. huxleyi* extracts, with a rate of 2.5% h^{-1} for chlorophyll *a*. In contrast, moderate degradation rates were recorded for *P. globosa*, in which chlorophyll *a* degradation measured only 0.5% h^{-1}.

The decrease in chlorophyll *a* in methanol extracts was accompanied by an increase in the number of derivatives identified as allomers and epimers of chlorophyll *a* (Fig. 2). The increased concentrations of these chlorophyll derivates, however, did not compensate for the overall loss of chlorophyll *a*. The same was observed for chlorophyll *b*, which underwent similar allomerization and epimerization reactions. No increase in the Mg-free derivates, phaeophytin *a* and *b* and phaeophorbide *a* and *b*, nor in chlorophyllides *a* or *b* was observed.

3.2. Extraction efficiency

Freeze-drying (FD) significantly and equally improved the extraction efficiency of all pigments (Table 3). Pigment concentrations in samples that had not been freeze-dried prior to extraction were 20–50% lower

Table 3
Extraction efficiency of various treatments as a percentage of acetone extraction in combination with FD at 4 °C over 48 h

		Ac/4 [a]	Ac/−FD [a]	Ac/−20 [a]	MeOH [a]
Pyramimonas sp.	Lutein	100 (3.7)	77 (1.9)	94 (6.7)	129 (24.6)
	Chlorophyll *b*	100 (0.3)	79 (1.9)	96 (9.3)	111 (20.1)
	Chlorophyll *a*	100 (1.9)	80 (1.4)	96 (8.6)	110 (21.0)
	β-carotene	100 (1.5)	83 (5.0)	96 (7.9)	73 (19.3)
P. antarctica	Chlorophyll c_3	100 (1.4)	54 (9.0)	113 (19.3)	118 (1.6)
	Chlorophyll $c_{1,2}$	100 (7.9)	54 (8.5)	107 (13.8)	105 (1.6)
	Fucoxanthin	100 (3.0)	46 (5.1)	110 (4.7)	123 (9.8)
	Hexfuco	100 (1.0)	48 (4.6)	112 (4.9)	116 (5.4)
	Diadinoxanthin	100 (4.9)	42 (6.3)	119 (11.3)	130 (4.1)
	Chlorophyll *a*	100 (6.2)	64 (3.3)	115 (2.4)	107 (2.1)
P. globosa	Chlorophyll c_3	100 (2.3)	84 (5.3)	87 (2.4)	152 (20.9)
	Chlorophyll $c_{1,2}$	100 (2.2)	85 (4.9)	91 (0.2)	154 (19.6)
	Fucoxanthin	100 (3.9)	82 (2.0)	89 (2.9)	150 (22.9)
	Diadinoxanthin	100 (4.7)	73 (9.7)	90 (3.6)	152 (23.3)
	Chlorophyll *a*	100 (8.5)	89 (1.3)	93 (3.0)	151 (18.2)
T. weisflogii	Chlorophyll $c_{1,2}$	100 (0.9)	82 (9.0)	94 (7.4)	81 (6.1)
	Fucoxanthin	100 (1.9)	85 (6.1)	98 (2.1)	81 (5.0)
	Diadinoxanthin	100 (8.1)	84 (4.4)	101 (0.7)	73 (7.1)
	Chlorophyll *a*	100 (1.1)	84 (6.7)	100 (2.4)	80 (8.2)
	β-carotene	100 (2.9)	97 (8.0)	99 (2.7)	101 (1.8)

Data in parentheses indicate the standard deviation ($n=2$–3; multiple injections were not considered in the variance analyses: given the high degradation rates observed in methanol extraction, only the first injection was considered relevant for this test).

[a] Ac/4: acetone extraction at 4 °C, after freeze-drying; Ac/-FD: acetone extraction without freeze-drying prior to extraction; Ac-20: acetone extraction at −20 °C, after freeze-drying; MeOH: methanol extraction.

compared to treated samples. Especially for *P. antarctica* an important increase (ca. 50%) in efficiency upon FD was observed.

Temperature did not significantly affect the extraction efficiency (Table 3). The only exception was found for *P. globosa*, where the extraction was more efficient at 4 °C than at −20 °C.

The difference in extraction efficiency between methanol and acetone was strongly species dependent. Whereas in *P. globosa* methanol extraction was 50% more efficient, in *T. weisflogii* acetone was more efficient (Table 3; $p<0.001$). In general, methanol extracts were more variable.

3.3. Signal improvement using water-packing

Water-packing strongly improved the peak shape and thereby peak separation, especially for the chlorophyll *c*'s and the fucoxanthins (Fig. 3A, B, peak nos. 1–6). The effect diminishes with decreasing peak polarity. Improved peak resolution is reflected in changes in peak area and height: water-packing resulted in a more than three-fold improvement of the peak height of polar pigments, whereas peak areas improved almost two-fold. Good peak separation was also achieved with natural algal populations, even at very low concentrations of algal biomass (Fig. 4).

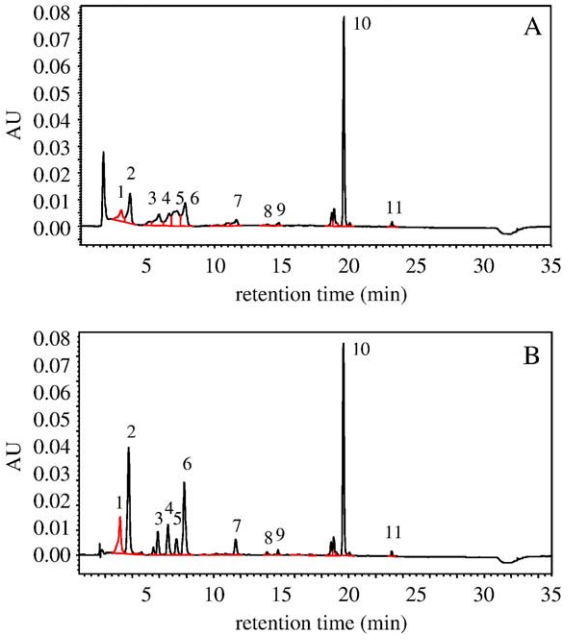

Fig. 3. Chromatograms of *Phaeocystis antarctica* extracted in 90% acetone, (A) without water-packing and (B) with water-packing. Absorbance at 450 nm. Peak identification: (1) chlorophyll c_3; (2) chlorophyll $c_{1,\ 2}$; (3) 19′-butanoyl-oxyfucoxanthin; (4) fucoxanthin; (5) 19′-hexanoyloxyfucoxanthin-like; (6) 19′-hexanoyloxyfucoxanthin; (7) diadinoxanthin; (8) diatoxanthin; (9) lutein; (10) chlorophyll *a*; (11) β-carotene.

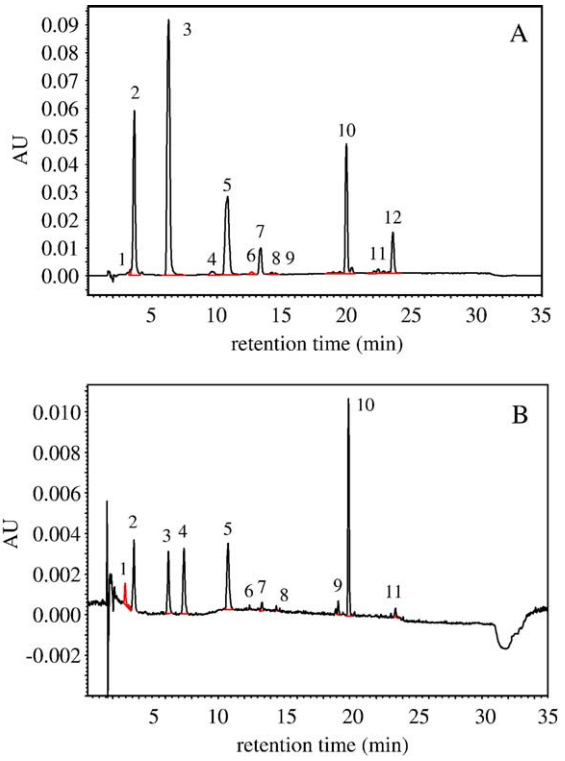

Fig. 4. Chromatograms of field samples. (A) Westerscheldt. Peak identification: (1) chlorophyllide *a*; (2) chlorophyll $c_{1,\ 2}$; (3) fucoxanthin; (4) pheophorbide *a*; (5) diadinoxanthin; (6) antheraxanthin; (7) diatoxanthin; (8) lutein; (9) chlorophyll *b*; (10) chlorophyll *a*; (11) pheophytin *a*; (12) β-carotene. (B) Weddell Sea. Peak identification: (1) chlorophyll c_3; (2) chlorophyll $c_{1,\ 2}$; (3) fucoxanthin; (4) 19′-hexanoyloxyfucoxanthin; (5) diadinoxanthin; (6) alloxanthin; (7) diatoxanthin; (8) unknown; (9) chlorophyll *b*; (10) chlorophyll *a*; (11) β-carotene.

4. Discussion

In this paper, we show that the successful application of automation in the analysis of algal pigments depends on the selection of an appropriate extraction procedure. A serious pitfall involved in automation is the use of methanol in combination with prolonged sample storage in the autosampler (e.g. Claustre et al., 2004). Our study clearly shows that this combination can result in rapid degradation of algal pigments thus invalidating the analysis. Pigments extracted in 90% acetone appeared to be stable for at least 18 h in an autosampler kept in darkness and cooled at 4 °C. The application of freeze-drying prior to the extraction procedure resulted in a significant increase in extraction efficiency and thus

proved a valuable improvement in the process of pigment analysis. In addition, automation allows the routine practice of water-packing, which results in enhanced peak resolution of the more polar peaks.

Both acetone and methanol are widely applied in the extraction of algal pigments. The highest extraction efficiency generally is achieved by using methanol as an extraction solvent in combination with mechanical disruption (Wright et al., 1997). The prime criterion for selection of the most appropriate solvent for a method based on automation, however, has to be based on solvent stability. The stability of pigments is low in methanol. Several papers already have described how methanol promotes the formation of allomers of chlorophyll (Bowles et al., 1985; Brereton et al., 1994). Acetone, on the other hand, provides a stable environment. This was indeed shown in our study. Although pigment extracts can safely be stored for up to 18 h, a limit of 12 h (see Fig. 1) may be pursued. From theory, acetone is known to have a lower extractability of chlorophylls from the protein matrix (Nakamura and Watanabe, 2001). However, this study showed that the extraction efficiency of acetone relative to methanol was species dependent; e.g. in the diatom *T. weisflogii* extraction was 50% more efficient in acetone than in methanol. Further support for acetone is found in the basic chemical properties of both solvents. While acetone and methanol have the same polarity index, acetone has greater eluotrophic strength than methanol for carbon-rich substrates, which results in sharper peaks (Stock and Rice, 1967).

The extraction efficiency of acetone increases considerably when freeze-drying is applied prior to extraction. Freeze-drying may break up the protein matrix of membranes, creating accessibility for the extraction solvent even with more recalcitrant algae (Buffan-Dubau and Carman, 2000; Chen et al., 2003). The effect of freeze-drying on extraction efficiency was again found to be species specific (Table 2). This was well illustrated by the experiments with the two strains of *Phaeocystis*. Whereas both species had a similar pigment composition, freeze-drying had a greater effect on *P. antarctica* than on *P. globosa*. This difference may be ascribed to the fact that cells in *P. antarctica* were embedded in a colonial matrix that possibly decreased the extraction efficiency when not freeze-dried, whereas *P. globosa* was in the form of single cells.

Another important advantage of the application of freeze-drying is that it inhibits the activity of chlorophyllase. One of the difficulties in pigment extraction involves the release of chlorophyllase, an enzyme found in many marine taxa (Jeffrey, 1974; Jeffrey and Hallegraeff, 1987). The enzyme catalyses the hydrolysis of the bond linking the phytol chain to the propionic acid residue of chlorophylls *a* and *b*, thereby converting chlorophyll to its corresponding chlorophyllide (Barrett and Jeffrey, 1964; Draziewicz, 1994). Chlorophyllase is, however, mainly active in an aqueous environment (Barrett and Jeffrey, 1964). It appears that dehydration by freeze-drying creates conditions that inhibit enzyme activity. In our method, pigments were extracted in 90% acetone rather the 100% acetone recommended by Jeffrey and Hallegraeff (1987). In theory some remaining chlorophyllase activity could not be excluded in our pigment extract, yet no indication for such activity was observed. Negative reports on the combination of freeze-drying and storage of filter material can most likely be ascribed to high storage temperatures (e.g. Mantoura et al., 1997), and subsequent sorption of water from ambient (and humid) air.

Direct injection of acetone extracts onto a chromatographic column using the gradients currently employed for HPLC pigment analysis produces broadly resolved peaks for the more polar compounds (this study; Zapata and Garrido, 1991; Wright et al., 1991). Water-packing was applied to improve peak resolution of the more polar peaks. The leading and trailing water packets overcome the peak broadening normally associated with injecting acetone onto methanol-based gradients. At the same time, a water-packed sample retains both the pigment mass and concentration of the original extract, allowing the maximum mass of pigment to be loaded onto the column in a minimum loading volume. Comparison of the chromatograms obtained with the water-packing protocol with that of direct injection shows a substantial improvement. A strong increase in peak height, up to a factor of almost 4 for the most polar components, the chlorophyll *c*'s, is achieved, besides a better peak separation. This development is an important benefit especially when high concentrations of breakdown products are present as such peaks often form shoulders on peaks of interest. Furthermore, an increasing amount of unknown analogue products of chlorophyll *c*'s and fucoxanthins are being described (see Zapata et al., 2004 and references therein) and most of them elute at very similar retention times. Under such conditions an optimal peak resolution is a prerequisite for adequate pigment analyses. When applying water-packing, a total amount of water should be chosen as to arrive at a final acetone concentration of 69% or less (Zapata and Garrido, 1991).

Sample dilution with Milli-Q water prior to injection has been suggested as an alternative means to improve chromatography of the more polar compounds (Wright et al., 1991). While dilution does improve the sharpness of the polar peaks, dilution also reduces the peak areas of all other peaks if the injection volume is held constant. This leads to an undesirable decrease in sensitivity. Increasing the injection volume to compensate for the dilution only broadens the loading band, which results again in broadened peaks and reduced peak separation. Clearly, the water-packing method made possible by automation forms a refined alternative to improve peak resolution.

This study illustrated that not only the extraction efficiency but also the stability of the extract varies with species. The difference in extract stability between species is an important finding that needs to be taken into consideration for laboratory experiments. When working with mixed algal populations, this impediment introduces an error that cannot be avoided. The high analytical reproducibility that is introduced with automation minimizes this error. In addition, the enhanced sensitivity that is provided with the method can be advantageous when encountering low algal biomass (Fig. 4B). It is therefore that the implementation of automation may be beneficial for application in the field, where conditions often put a high demand on the sensitivity as well as reproducibility of the method.

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