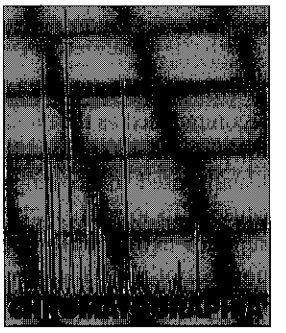
Losses of Chlorophylls and Carotenoids in Aqueous Acetone and Methanol Extracts Prepared for RPHPLC Analysis of Pigments



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Key Words

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Phase changes
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Summary

RPHPLC methods for analysis of photosynthetic pigments (chlorophylls and carotenoids) usually require addition of water to methanol or acetone extracts to prevent distortion of early-eluting peaks corresponding to the more polar compounds. In this work we have investigated the short-(< 2 min) and long-term (up to 48 h) effect of adding water to acetone and methanol extracts from two marine phytoplankton species, *Emiliania huxleyi* and *Dunaliella tertiolecta*. Solvent extracts were prepared and separated into fractions that were subsequently diluted with water to 90%, 80%, 70%, 60%, 50%, and 40% for methanol, and the same range extended to 30% and 20% for acetone. Changes in pigment concentration with time were followed spectrophotometrically and chromatographically. Losses of pigments as a result of precipitation were clearly observed immediately after dilution of acetone extracts to 60% or less and methanol extracts to 80% or less. For chlorophyll *a* the most substantial losses were recorded for 50% acetone (up to 27% decrease) and for 70% methanol (31% decrease). This effect increased considerably with time. Only for 90% and 80% acetone were the initial concentrations of all the pigments unchanged after 24 h, and even up to 48 h. In contrast, more than 60% and 57% of the initial amounts of chlorophyll *a* were lost after 24 h in 50% acetone and 70% methanol extracts, respectively. These losses increased to 83% and 60% after 48 h. There was a clear correlation between the polarity of a pigment and the polarity of the solvent at which maximum precipitation occurred. Losses of pigment from pure acetone and methanol extracts with time were also observed, although we attribute these to pigment degradation rather than precipitation. Some of the losses occurring with time can be avoided by use of autosamplers in which the sample can be mixed with water immediately before injection.

Introduction

A wide range of chlorophylls and carotenoids can be used to estimate the abundance and taxonomic composition of phytoplankton populations [1–4]. Reversed-phase high-performance liquid chromatography (RPHPLC) is the analytical tool of choice for the analysis of pigments from field and cultured species of marine phytoplankton; this has been reviewed by Jeffrey and Vesk [5]).

Measurement of phytoplankton pigments starts with a concentration step in which cells are harvested, by filtration, on to glass fiber filters, usually 25 or 47 mm in diameter. The material retained is subsequently extracted with an appropriate organic solvent and the extract injected on to the chromatographic column.

In the field the amount of sample collected is often limited by the water volume available or the filtration time; this complicates the detection and quantification of important pigments present in trace amounts (ng L^{-1} seawater). For HPLC analysis, the detection limit can be reduced by using a minimum volume of extraction solvent (down to 1.5 mL [6]) and by increasing the volume of extract injected into the chromatograph (up to 500 µL [7]). Use of a very low extracting volume, however, causes problems, because of the handling of a relatively large filter and the considerable errors that can arise as a result of even slight evaporation of the very volatile solvents used. Increasing the injection volume can lead to chromatographic problems, the worst of which is distortion of early eluting peaks.

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Although methanol has recently been recommended as the most suitable solvent for pigment extraction [8], 90% acetone is usually used in oceanographic work because of its lower toxicity and because the superior extraction properties of methanol are not always apparent when natural samples are extracted [9]. When 90% acetone is used as injection solvent in HPLC with methanol as mobile phase, early-eluting peaks, corresponding to the more polar pigments, are distorted [10]. This effect is worse the larger the amount of sample injected. We have, in fact, observed that even methanol extracts, if large volumes are injected, can furnish distorted early-eluting peaks.

Peak distortion was originally attributed to differences between the solvent strengths of the mobile phase and the injection solvent. This explanation was questioned by Zapata and Garrido [10]. They observed peak distortion and splitting of early-eluting pigment peaks when employing sample solvents the strengths of which exactly matched those of other injection solvents affording correct peak shapes. These authors demonstrated that peak distortion could be avoided by adding an appropriate amount of water to the injection solvent. According to Castells and Castells [11] and Castells et al. [12], the phenomenon of initial peak distortion seems to be more a consequence of differences between the viscosities of the sample solvent and mobile phase. Increasing the viscosity of the sample solvent by addition of water clearly mitigated this problem, although it did not solve it for high-volume injections.

Without knowing the final mechanism, researchers quickly noticed the benefits of adding water in preventing the distortion of early-eluting peaks from the more polar pigments. Extract/water ratios varying from 1:0.3 to 1:1 (*v/v*) are usually used [6, 13–16].

A problem that has not yet been addressed systematically is the effect of addition of water on the stability of the whole array of pigments present in marine and freshwater samples. Photosynthetic pigments are lipidic molecules highly soluble in organic solvents. If water is added to these solutions, the increase in polarity might induce grouping of the initially dissolved lipidic compounds, especially the less polar, and promote the formation of micelles, eventually leading to their precipitation as aggregates [17, 18]. When, furthermore, refrigerated autosamplers are used, pigment samples can spend several hours in a solvent which is very polar relative to some of the pigments. These conditions clearly favor precipitation of the most non-polar pigments.

In this work we have investigated the short- and long-term effects of addition of water to acetone and methanol extracts of pigments. We found that water added within the range frequently reported in the literature can lead to considerable losses of pigment. We show the upper limit of water that can be added to methanol and acetone extracts without causing significant losses of pigment; this thus implies an upper limit for injection volumes. Finally, we make some recommendations related to the problem studied here.

Experimental

Pigments Sources and Sample Preparation

Pigments were extracted from two marine phytoplankton species, *Emiliania huxleyi* and *Dunaliella tertiolecta*, species selected for the experiments because they are commonly available in culture collections and because their main pigments cover the whole array from the most polar compounds to the most non-polar. *E. huxleyi* contains, as major pigments in decreasing order of polarity, chlorophyll c_2, chlorophylls c_3 (monovinyl and divinyl forms), fucoxanthin, 19′-hexanoyloxyfucoxanthin, diadinoxanthin, diatoxanthin, a non-polar chlorophyll *c*-like compound, monovinyl-chlorophyll *a* (hereinafter chlorophyll *a*), and β,ε- and β,β-carotene. *D. tertiolecta* contains neoxanthin, violaxanthin, lutein, zeaxanthin, monovinyl-chlorophyll *b* (hereinafter chlorophyll *b*), chlorophyll *a*, and β,ε- and β,β-carotene.

Immediately before analysis cultured cells were harvested on to 25-mm Whatman GF/F glass fiber filters (0.7 µm nominal pore) which were then folded and repeatedly blotted with absorbent paper until no humidity was observed in the later. We estimated by weight that after this procedure the amount of water remaining in the filter was always less than 0.1 mL. Pigments were subsequently extracted by placing the filter in a polypropylene tube filled with a large volume (30 mL) of methanol or acetone (Prolabo, HPLC grade). This tube was shaken by hand. After 5 min extracts were filtered twice through Whatman GF/F filters to eliminate cell and filter debris. Series of injections of the extracts were then started immediately.

Apparatus

Spectrophotometric analysis of the extracts was performed with a Uvikon 923 (Bio-Tek Kontron) spectrophotometer, operating between 400 and 750 nm. HPLC analysis of the extracts was performed with a ThermoQuest chromatograph, including a P2000 solvent module, an A/S 3000 autosampler, a UV-3000 absorbance detector (λ = 440 nm), a FL2000 fluorescence detector (λ_{ex} = 430 ± 40 nm, λ_{em} = 662 nm), and an SN 4000 controller. HPLC pigment analysis was performed by means of the method proposed by Zapata [19], with minor modifications of the solvent gradient as specified below. The column used was a 150 mm × 4.6 mm, 3.5 µm particle, Waters Symmetry C_8 protected with a 3 mm × 4.6 mm guard column containing the same stationary phase. The column was thermostatted at 25 °C by means of a water jacket (Alltech) connected to a recirculating water bath. The mobile phase mixtures employed were methanol-acetonitrile-0.25 M aqueous pyridine solution, adjusted at pH 5 with acetic acid, 50:25:25 (*v/v*), mobile phase A, and acetonitrile-acetone, 80:20 (*v/v*), mobile phase B. The mobile phase gradient (flow rate 1.0 mL min^{-1}) was (time, %A, %B): (0, 100, 0), (10, 77, 23), (23, 77, 23), (25, 65, 35), (35, 60, 40), (38, 25, 75), (46, 15, 85), (48, 0, 100), (57, 0, 100). HPLC-grade solvents and analytical-grade pyridine and acetic acid were from SDS.

Chromatographic Procedures

Fresh acetone and methanol extracts of the pigments, 20 and 75 µL, respectively, were injected directly into the chromatographic system without causing peak distortion. Peak areas from these initial extracts were used as the references for subsequent analyses. Solvent extracts were then diluted with water to 90%, 80%, 70%, 60%, 50%, 40% and, for acetone extracts only, the same range extended to 30% and 20%. Dilution was always performed immediately before sample injection – the time between addition of water and effective sample injection on to the column (including autosampler cycle) was always

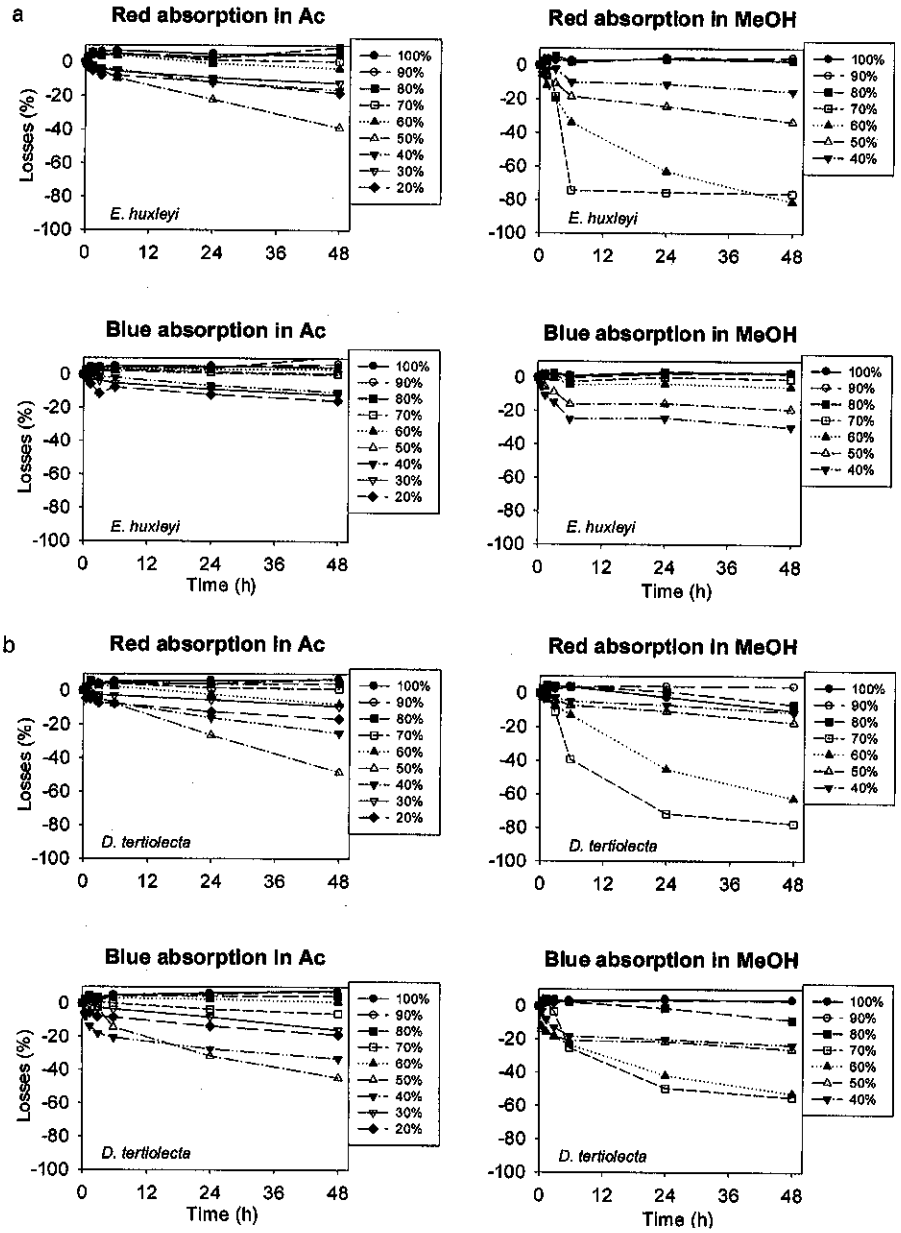

Figure 2. Variation with time of the red and blue absorption maxima of extracts of (**a**) *Emiliania huxleyi*, and (**b**) *Dunaliella tertiolecta* in 100%, 90%, 80%, 70%, 60%, 50%, and 40% methanol, and the same range extended to 30% and 20% for acetone. 'MeOH' and 'Ac' denote methanol and acetone. Losses were calculated as for Table I.

methanol (Figure 1). The characteristics of both phenomena clearly indicate, however, that losses occurring under each different conditions arise from different processes. Thus, the losses in pure solvent affect only some pigments i. e. acetone was very reactive toward chlorophyll *b* (as already reported by Wright et al. [8]) and methanol toward chlorophylls c_3 and, to a lesser extent, chlorophyll *a*. We attribute these losses to pigment degradation because of the sensitivity of a pigment to a specific solvent.

We attributed the pattern of pigment losses observed in extracts after addition of water to precipitation. The increase in the polarity of the solvent mixture probably induced grouping of the initially dissolved pigments and promoted the formation of micelles, eventually leading to precipitation of the aggregates. In a preliminary experiment employing extracts from grass we had previously observed the formation of such pigment aggregates visible to the naked eye in 60 and 70% methanol. These aggregates were highly enriched in chlorophylls *a* and *b* (data not shown). Although aggregates of this size were not observed in this work, the results clearly imply that the same phenomenon occurred in the experiment with the two phytoplankton species.

It is well known [18, 20] that in a wide range of water-miscible organic solvents (e. g. alcohols, acetone, tetrahydrofuran) chlorophylls are present in solution as solvated monomers. When water is added, more or less stable dispersions are formed. These dispersions were very soon recognized as chlorophyll aggregates, because they were non-fluorescent and because precipitates were invariably formed on standing [20]. The formation of these aggregates was explained in terms of solvation (coordination) of the Mg atom of the chlorophylls by nucleophilic solvents. When water is added it replaces the solvent ligands [21, 22]. Being a bifunctional ligand, water both coordinates the Mg atom and at the same time forms hydrogen-bonds with the oxygen functions of another chlorophyll-water adduct, thereby providing an easy route to self-assembly [20–22]. It is known that the formation of the chlorophyll aggregates is a gradual process depending on time, the nature of the organic solvent, the water content, the temperature, and the bulk properties of the solvent mixture [21, 22]. Increased losses with time, as a result of precipitation, might be described in the same way.

An initially counter-intuitive result was that the greatest losses of pigments by precipitation did not occur at the most polar dilutions. As is apparent from Table I, there was a clear correlation between the polarity of the pigment and the polarity of the solvent mixture at which its precipitation was maximum, i. e. there was a critical dilution for the greatest precipitation of each pigment. This phenomenon can be explained in terms of the formation of lipid colloids that can contribute to keeping the pigments in suspension. Polar lipids, from membrane structures, are major components of pigment extracts in organic solvents. These lipids remain dissolved when the proportion of organic solvent in the mixture is sufficiently high. When water content reaches a critical value, lipid micellar associations or lipid bilayer vesicles can be formed in which the polar ends of the molecules are distributed in the outer part of the micelle [23]. Pigments can partially dissolve in the mono- or bilayer vesicles and such systems can provide clear and relatively stable dispersions in an aqueous environment [24]. It has been demonstrated that chloroplast glycolipids, which always accompany pigments in methanol or acetone extracts, can form such colloidal structures [25, 26].

Table I. continued

Dunaliella tertiolecta Acetone	100%	90%	80%	70%	60%	50%	40%	30%	20%
Time = 24 h									
Neoxanthin	− 4	− 2	0	+ 2	− 2	− 7	−18	**−41**	−37
Violaxanthin	− 4	+ 2	+ 2	+ 3	− 4	−31	−23	**−45**	−38
Zeaxanthin	− 7	0	5	3	− 5	**−58**	−17	−30	−27
Lutein	− 6	0	3	3	1	**−52**	−12	−25	−14
Chlorophyll *b*	−10	+ 2	+ 3	+ 1	− 6	**−59**	−24	−19	−18
Chlorophyll *a*	− 3	+ 3	+ 3	+ 2	− 7	**−61**	−20	−53	−33
Carotenes	− 3	+ 4	+ 4	**−19**	−13	− 6	− 2	− 7	− 8
Time = 48 h									
Neoxanthin	− 5	− 5	+ 1	0	− 1	− 4	−27	**−46**	−45
Violaxanthin	0	+ 1	+ 1	0	−12	−46	−32	**−52**	−52
Zeaxanthin	− 6	0	1	3	− 1	**−84**	−15	−33	−26
Lutein	− 4	0	2	2	1	**−77**	−15	−32	−14
Chlorophyll *b*	−16	+ 2	0	0	−10	**−82**	−37	−25	−25
Chlorophyll *a*	− 3	+ 3	+ 4	+ 2	−12	**−89**	−35	−29	−40
Carotenes	− 1	+ 4	+ 4	**−31**	−19	−10	− 5	− 8	−11

Methanol	100%	90%	80%	70%	60%	50%	40%
Initial time							
Neoxanthin	–	− 1	+ 1	+ 2	− 4	−22	**−27**
Violaxanthin	–	0	+ 1	− 1	− 7	−16	**−16**
Zeaxanthin	1	0	3	−14	**−17**	− 8	
Lutein	–	1	1	− 3	**−12**	−10	− 6
Chlorophyll b	–	+ 1	+ 1	**−20**	−14	− 6	− 3
Chlorophyll a	–	+ 1	− 1	**−24**	−11	− 4	− 3
Carotenes	–	− 2	**− 9**	− 8	− 5	− 3	− 3
Time = 24 h							
Neoxanthin	− 5	− 3	− 2	− 4	−19	**−34**	−30
Violaxanthin	− 7	− 9	−19	−44	**−61**	−30	−26
Zeaxanthin	− 5	− 3	− 4	**−70**	−62	−31	−11
Lutein	− 4	− 1	− 2	**−67**	**−60**	−27	−11
Chlorophyll b	− 8	− 7	**−65**	**−63**	−60	−41	−18
Chlorophyll a	−69	− 3	**−73**	**−62**	−59	−22	−11
Carotenes	−12	−18	−54	**−63**	−57	−16	−11
Time = 48 h							
Neoxanthin	−10	− 5	− 3	− 4	−27	**−41**	−32
Violaxanthin	−13	−18	−31	**−63**	**−89**	**−50**	**−34**
Zeaxanthin	−11	−11	0	**−94**	**−95**	**−54**	**−10**
Lutein	− 9	− 5	− 4	**−87**	**−94**	**−48**	**−17**
Chlorophyll b	−10	− 8	−72	**−87**	**−93**	**−64**	**−33**
Chlorophyll a	−84	− 5	−85	**−87**	**−93**	**−43**	**−20**
Carotenes	−20	−29	−77	**−87**	**−91**	**−30**	**−17**

trix and the solvent. Thus, chlorophyll *a* was more degraded by methanol in extracts of *E. huxleyi* than in those of *D. tertiolecta*, whereas chlorophylls c_3 and chlorophyll *b* seemed to be especially sensitive to methanol and acetone, respectively.

Chromatographic analysis revealed that only for 90% and 80% acetone extracts were no losses of pigments observed after 24 h and 48 h at 4 °C. We assume that losses after 24 h were more influenced by precipitation whereas losses after 48 h were a mixed response to pigment degradation (Figure 1) and precipitation.

Spectrophotometry

Results from spectrophotometric analysis were very similar to those from chromatography, implying that the losses measured by HPLC were not related to problems with the chromatography. The main difference from the chromatographic results was the smaller extent of the decreases in absorption than the decreases in pigment concentration measured by HPLC. The pattern of the losses was identical, absorption decreases being largest at intermediate dilutions (Figure 2). After only 6 h, absorption in the red maximum region (contributed mostly by chlorophyll *a* and *b*) of extracts in 70% methanol decreased by more than 70% and 40% for extracts from *E. huxleyi* and *D. tertiolecta*, respectively. The decrease was also substantial for 60% methanol, especially after 24 h, and became as large as that for 70% methanol after 48 h (Figure 2). For the other methanolic dilutions the decrease in absorption was smaller. Only for extracts in 90% methanol was absorption constant for 48 h.

Decreasing absorption of the acetone extracts was also important, although less dramatic than for methanol extracts. Red absorption of 100%, 90%, 80%, and 70% acetone extracts kept at 4°C remained constant throughout the experiment. A decrease of ca 10% was observed for 60%, 30% and 20% acetone extracts over 24 h. Again, the largest decreases in absorption occurred for the 50% and 40% extracts (more than 22% and 12%, respectively).

Discussion

Important losses of most photosynthetic pigments were observed after addition of water to acetone and methanol extracts of *E. huxleyi* and *D. tertiolecta* (Table I). Losses of pigment with time were also observed for extracts in pure acetone and

Table I. Losses of pigment (%), relative to the respective 100% solvent extract of *Emiliania huxleyi* and *Dunaliella tertiolecta* at time 0 (<2 min after extract preparation), after 24 h, and after 48 h. Values are calculated by use of the formula $(A_{dil} \times A_{ref}^{-1} \times f_{dil}^{-1} - 1) \times 100$, where A_{dil} is the area of the peak in the sample diluted with water, A_{ref} the area with the 100% solvent, and f_{dil} the water dilution factor. Values in italics are those which deviate by more than 5% from the reference. Values in bold indicate maximum degradation for each pigment. Pigments are ordered from highest to lowest polarity.

Emiliania huxleyi Acetone	100%	90%	80%	70%	60%	50%	40%	30%	20%
Initial time									
Total chlorophylls c_3	–	+ 2	0	+ 2	– 1	– 5	*–10*	*–28*	***–57***
Chlorophyll c_2	–	+ 2	– 3	– 2	– 3	– *8*	*–16*	*–35*	***–60***
19′–hex-fucoxanthin	–	+ 2	+ 4	+ 4	+ 3	– 1	*–18*	***–41***	*–27*
Diadinoxanthin	–	+ 2	+ 4	+ 4	+ 3	– 1	*–27*	*–26*	*–20*
Non-polar chlorophyll *c*	–	+ 4	+ 3	+ 4	– 2	***–33***	*–11*	+ 1	0
Chlorophyll *a*	–	+ 3	+ 4	+ 4	– 3	*–23*	*–12*	– 4	*–10*
Carotenes	–	+ 4	+ 2	– 1	***–12***	+ 1	+ 2	+ 3	0
Time = 24 h									
Total chlorophylls c_3	– 4	0	– 3	– 1	– 4	– *7*	*–10*	*–37*	***–58***
Chlorophyll c_2	– 3	– 3	– 3	– 4	– 4	– *7*	*–16*	*–40*	***–68***
19′-hex-fucoxanthin	– 3	0	0	+ 1	+ 1	– 2	*–23*	***–46***	*–28*
Diadinoxanthin	– 4	+ 2	+ 2	+ 1	– 3	*–17*	***–49***	*–44*	*–44*
Non-polar chlorophyll *c*	– 2	+ 1	0	– 1	*–16*	***–34***	– 4	– 3	+ 4
Chlorophyll *a*	– 1	+ 3	+ 3	+ 1	*–17*	***–64***	*–23*	*–34*	*–55*
Carotenes	– 5	+ 1	– 1	*–16*	***–71***	*–24*	– 4	– 3	– *8*
Time = 48 h									
Total chlorophylls c_3	– 5	+ 2	+ 3	0	0	– 3	– *7*	*–33*	***–65***
Chlorophyll c_2	0	– 2	– 2	– 1	– 5	– *9*	*–19*	*–45*	***–65***
19′-hex-fucoxanthin	– 3	0	0	0	+ 1	– 3	*–22*	***–43***	*–31*
Diadinoxanthin	– 3	0	– 1	0	– 5	*–26*	*–55*	*–51*	*–48*
Non-polar chlorophyll *c*	+ 3	+ 3	+ 3	+ 2	*–15*	***–42***	+ 4	1	– 3
Chlorophyll *a*	0	+ 4	+ 4	– 1	*–26*	***–83***	*–34*	*–47*	*–69*
Carotenes	– 1	+ 2	+ 2	*–15*	***–90***	*–31*	– 3	– 2	– *7*

Methanol	100%	90%	80%	70%	60%	50%	40%
Initial time							
Total chlorophylls c_3	–	+ 1	+ 2	– 1	– *6*	*–13*	***–29***
Chlorophyll c_2	–	+ *6*	+ 5	+ 3	– 3	*–13*	***–28***
19′-hex-fucoxanthin	–	+ 2	+ 2	0	– *6*	*–33*	*–32*
Diadinoxanthin	–	+ 2	+ 1	0	*–17*	***–27***	*–19*
Non-polar chlorophyll *c*	–	+ 1	– 2	***–32***	*–12*	– 4	– 3
Chlorophyll *a*	–	0	– 3	***–31***	*–24*	*–12*	*–12*
Carotenes	–	– 1	***–22***	*–13*	– *8*	– 4	*–8*
Time = 24 h							
Total chlorophylls c_3	*–64*	– 1	– 1	– 2	– *8*	*–18*	*–37*
Chlorophyll c_2	*–36*	+ 3	+ *6*	+ 1	– *9*	*–21*	*–38*
19′-hex-fucoxanthin	– 1	0	0	0	*–11*	*–43*	*–42*
Diadinoxanthin	– 3	– 2	– *6*	*–16*	*–50*	*–48*	*–38*
Non-polar chlorophyll *c*	+ 1	0	*–12*	*–69*	*–32*	*11*	+ 5
Chlorophyll *a*	*–32*	– 3	– *8*	*–57*	*–45*	*–37*	*–28*
Carotenes	– 1	– 4	*–72*	*–63*	*–51*	*–27*	*–18*
Time = 48 h							
Total chlorophylls c_3	*–65*	*–12*	– 2	– 3	*–10*	*–23*	*–40*
Chlorophyll c_2	*–79*	– 3	+ 4	– 1	*–14*	*–58*	*–49*
19′-hex-fucoxanthin	– 4	– 1	0	– 1	*–14*	*–49*	*–47*
Diadinoxanthin	– *7*	– *6*	*–12*	*–28*	*–67*	*–58*	*–46*
Non-polar chlorophyll *c*	– 4	+ 2	*–15*	*–72*	*–60*	*–61*	*14*
Chlorophyll *a*	*–60*	– *7*	*–11*	*–60*	*–67*	*–82*	*–42*
Carotenes	– *9*	– *9*	*–69*	*–71*	*–77*	*–61*	*–34*

Dunaliella tertiolecta Acetone	100%	90%	80%	70%	60%	50%	40%	30%	20%
Initial time									
Neoxanthin	–	+ 1	+ 4	+ 2	+ 4	– 2	*–11*	*–37*	*–28*
Violaxanthin	–	+ 2	+ 5	+ 5	+ 3	– 1	*–10*	*–25*	*–17*
Zeaxanthin	–	– 1	1	2	– 3	– 4	*–20*	*–16*	*–13*
Lutein	–	2	4	4	3	– 5	*–18*	*–14*	*–12*
Chlorophyll *b*	–	+ 2	+ 3	+ 3	0	*–15*	*–12*	– *7*	– *7*
Chlorophyll *a*	–	+ 2	+ 4	+ 4	– 1	*–14*	– *7*	– 5	– 7
Carotenes	–	+ 3	+ 4	0	– *5*	+ 1	+ 3	– 2	– 4

less than 2 min. Peak areas obtained during this initial series of injections were compared with those from the reference samples to enable estimation of possible short-term losses of pigment. The reference samples and diluted extracts were maintained at 4 °C in the autosampler, and re-injected during two further series performed after 24 and 48 h to enable estimation of long-term variation in pigment concentrations.

Because the duration of each chromatographic analysis was ca 1 h, the procedures used for the initial series of injections implied that the pure methanol and acetone extracts had been standing for ca 7 h before the last dilution occurred. We evaluated possible variations in pigment concentrations during this period by means of an additional HPLC experiment in which undiluted extracts were injected successively for 24 h.

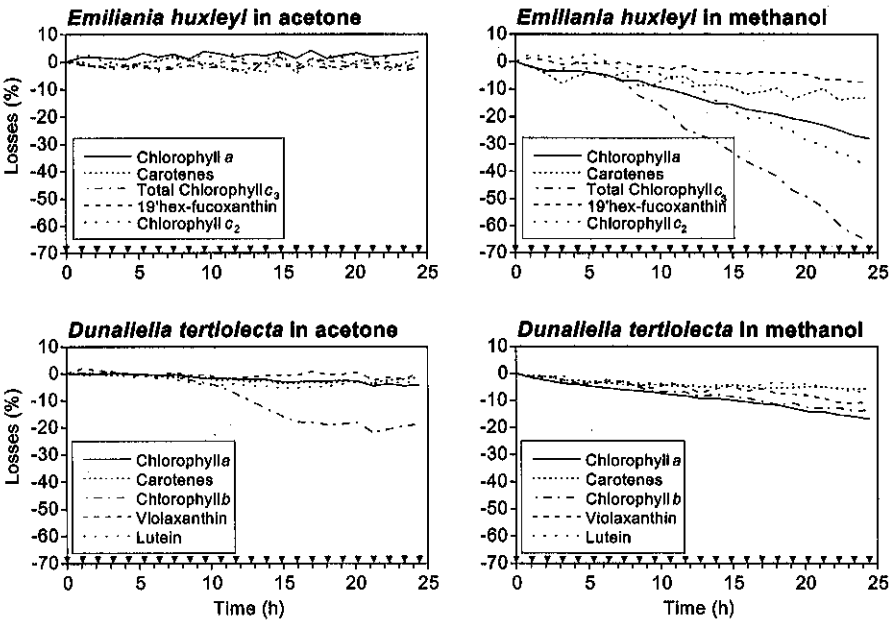

Figure 1. Variation with time of losses of pigment (%) in acetone and methanol extracts of *Emiliania huxleyi* and *Dunaliella tertiolecta*. Losses were calculated as for Table I. Triangles over the *x*-axis mark the times of successive injections. Losses were calculated as for Table I.

Spectrophotometric Procedures

Variations in pigment concentrations were also evaluated by spectrophotometry, mainly to check whether losses of pigment observed in HPLC analysis were related to chromatographic problems. Acetone and methanol extracts were diluted with water, to the same range of concentrations as employed for the HPLC experiment, immediately before the first readings. Because of solvent and pigment-composition differences, the wavelengths for the blue and red maxima were 443–453 nm and 662–669 nm for *E. huxleyi* and 430–438 nm and 662–669 nm for *D. tertiolecta*. The first readings provided the reference data for all further readings, which were performed after 30, 75, 180, 360 min (6 h), 24 h and 48 h. Between these intervals, the samples were stored at 4 °C (i. e. the same temperature as the HPLC samples in the autosampler).

Ninety five percent methanol was used as a blank in the reference cell for all spectrophotometric readings. The use of different solvents in the reference and the sample cells did not affect our results because the absorption measurements were compared with the initial reading from the same dilution. Spectrophotometric measurements could not, however, be used to compare pigment concentrations in the different dilutions because of the lack of extinction coefficients for all the solvent-water mixtures studied.

Results

Chromatography

Results from the time-series experiments for pure acetone and methanol revealed only small losses of some of the pigments during the first 7 h after preparation of the extracts (Figure 1). This period was the time before dilution of the extracts to 20% acetone, all other dilutions were processed more quickly. We used ±5% deviation from the initial value (5.2% for chlorophylls c_3 in methanol, the maximum loss observed in this 7-h period) to distinguish differences arising from the treatment from errors arising as a result of experimental design and methodology.

With this criterion in mind the chromatographic results presented in Table I are clearly indicative of losses of pigment less than 2 min after dilution of extracts to 60% acetone or less or to 80% methanol or less. The most substantial losses of chlorophyll *a* were recorded in 50% acetone (up to 23%) and in 70% methanol (up to 31%); behavior of chlorophyll *b* and the non-polar chlorophyll *c* was similar. For the carotenes, the most hydrophobic pigments analyzed in this study, losses were highest for 70% acetone (up to 12%) and 80% methanol (up to 22%) in contrast with chlorophylls c_3 and c_2, the most polar pigments, for which losses were highest for 20% acetone (up to 60%) and 40% methanol (up to 29%).

The losses increased substantially with increasing time, restricting even more the 'safe' dilutions to 80–90% acetone (Table I). The pattern of losses being greatest for intermediate dilution was maintained. Thus for acetone losses of carotenes were highest for 70% and 60%, losses of chlorophylls *a* and *b*, the non-polar chlorophyll *c*, zeaxanthin, and lutein were highest for 50%, losses of neoxanthin, violaxanthin and 19′-hexanoyloxyfucoxanthin were highest for 30%, and losses of chlorophylls c_3 and c_2 were highest for 20%. The pattern was similar for methanol extracts but with the highest losses of pigment occurring at lower dilutions; thus most lutein losses occurred for 60–70% methanol compared with 50% acetone (Table I).

It was evident that there was a critical dilution at which losses of each pigment were the highest. The polarity of the 'critical dilution' for each pigment varied with the polarity of the pigment, i. e. the more polar the pigment the more polar the dilution at which losses of that pigment were the greatest. It thus seems that the polarities of both pigment and solvent play an important role in losses attributable to precipitation (see below).

In the time-series performed with pure solvent extracts, significant losses of some pigments, unrelated to polarity, occurred after several hours (Figure 1). It seems that these losses were a consequence of degradation, because of the susceptibility of some molecules to interaction with the ma-