Reversed-Phase High-Performance Liquid Chromatographic Separation of Mono- and Divinyl Chlorophyll Forms Using Pyridine-Containing Mobile Phases and a Polymeric Octadecylsilica Column

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Key Words

Column liquid chromatography
Mono- and divinyl chlorophylls
Polymeric ODS phases
Pyridine containing mobile phases

Summary

The separation of chlorophyll forms was studied employing a wide bore polymeric octadecylsilica column and pyridine containing mobile phases, giving consideration to considering the influence of mobile phase composition and column temperature on the resolution of monovinyl forms from their divinyl analogues. A method involving gradient elution and operating at 15 °C is proposed for the separation of several polar and non-polar mono- and divinyl chlorophylls from etiolated tissues of higher plants and from marine phytoplankton. The advantages of pyridine as a mobile phase additive in the reversed-phase liquid chromatography of chlorophylls are discussed.

Introduction

Available experimental evidence indicates that chlorophylls (chls) are formed via multiple biosynthetic routes. Although additional biosynthetic details need to be established, most chlorophyll *a* appears to be formed via two routes populated by divinyl (DV) and monovinyl (MV) tetrapyrroles [1]. This biosynthetic heterogeneity explains the occurrence of various mono- or divinyl components, either porphin -protochlorophyllide (Pchlide)- or chlorin -chlorophyllide (chlide), chlorophyll- derivatives, during the greening of etiolated tissues in different higher plants [1–3]. It also underlies the variety of chlorophyll *c* forms described in chromophyte algae [4–6] and explains the prevalence of DV-chl *a* (chl a_2) and DV-chl *b* (chl b_2) in the euphotic zone of tropical oceans [7, 8].

Analysis of photosynthetic pigments is important in the study of photosynthetic mechanisms, especially chloroplast biogenesis in both plants and algae [9]. In the marine context, the study of these compounds is interesting not only from the physiological point of view: many taxonomic considerations are based on the distribution of certain chl forms within different algal groups [10, 11] and in marine ecology studies they have been considered to be indicators of the abundance, diversity, productivity and degradation of phytoplankton in seas and oceans [12, 13].

Chloropigments exhibit a wide range of polarities, from the acidic chlides, Pchlides and chl *c* forms, to the non-polar phytol esterified chls *a* and *b*. Many of them are structurally very similar, in certain cases differing from one to another in no more than the position of a double bond (Figure 1). Because of the complexity of pigment extracts and the minor differences among chlorophyll forms, separation of individual species is difficult.

The separation of chlorophyll mixtures has been the subject of considerable research effort spanning several decades. Most HPLC separations of monovinyl chlorophyll forms from their respective divinyl analogues have been achieved by employing polyethylene columns [3, 4, 14, 15]. These methods are lengthy and do not allow the simultaneous separation of polar and non polar pigments in the same chromatographic run. On the other hand, most of the methodologies achieving the global separation of free and esterified pigments occurring in algae or etiolated tissues from higher plants, show several multicomponent peaks corresponding to the

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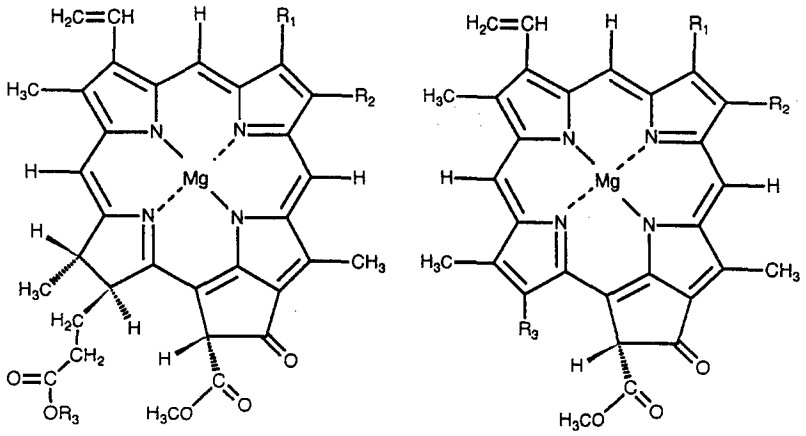

Compound (abbreviation)	Main structure	R_1	R_2	R_3
Monovinyl Chlorophyllide *a* (MV-chlide *a*)	A	CH_3	CH_2–CH_3	H
Divinyl Chlorophyllide *a* (DV-chlide *a*)	A	CH_3	CH=CH_2	H
Monovinyl Protochlorophyllide *a* (MVPchlide *a*)	B	CH_3	CH_2–CH_3	CH_2–CH_2–COOH
Divinyl Protochlorophyllide *a* (DVPchlide *a* or Mg DVP)	B	CH_3	CH=CH_2	CH_2–CH_2–COOH
Monovinyl chlorophyll *a* (chl *a* or chl a_1 or MV-chl *a*)	A	CH_3	CH_2–CH_3	phytyl
Divinyl chlorophyll *a* (chl a_2 or DV-chl *a*)	A	CH_3	CH=CH_2	phytyl
Monovinyl chlorophyll *b* (chl *b* or chl b_1 or MV-chl *b*)	A	CHO	CH_2–CH_3	phytyl
Divinyl chlorophyll *b* (chl b_2 or DV-chl *b*)	A	CHO	CH=CH_2	phytyl
Chlorophyll c_1(chl c_1)	B	CH_3	CH_2–CH_3	CH=CH–COOH
Chlorophyll c_2(chl c_2)	B	CH_3	CH=CH_2	CH=CH–COOH
Monovinyl chlorophyll c_3 (MV-chl c_3)	B	$COOCH_3$	CH_2–CH_3	CH=CH–COOH
Divinyl chlorophyll c_3(DV-chl c_3)	B	$COOCH_3$	CH=CH_2	CH=CH–COOH

Figure 1
Structures of the chlorophyll forms studied in this work. The abbreviations commonly found in the literature for these pigments are also indicated.

coelution of the mono- and divinyl analogues of a particular chlorophyll form [16–22].

We introduced the use of polymeric octadecyl silica columns for the analysis of chloropigments [23, 24]. The enhanced shape selectivity exhibited by these stationary phases allowed the simultaneous separation of various polar chlorophyll *c* pigments (including that of the monovinylic chl c_1 from its divinyl analogue, chl c_2), and chls *a*, *b* and the phytol-substituted chlorophyll *c*-like pigment from marine phytoplankton. The use of a similar column allowed the detection and isolation of several new chlorophylls [6]. These methods, however, failed in the separation of monovinyl chlorophyll *a* (chl *a*) from its divinyl analogue (chl a_2) and of chl *b* from the divinylic chl b_2.

A similar performance is shown by octadecyl polyvinyl alcohol polymer columns, on which Shioi et al. [25] separated various mono and divinyl acidic chlorophyll species involved in chloroplast biogenesis together with their corresponding apolar derivatives esterified with different alcohols.

Taking advantage of the increase in shape selectivity that polymeric ODS phases exhibit under reduced temperatures [26], a method was proposed that achieved a partial separation of chl a_1 from chl a_2 at 10 °C while the pair chl b_1-chl b_2 remained unresolved [27]. The first separation of the complete array of algal chlorophylls, including the mono- and divinyl analogues of polar and non polar forms, was achieved by Van Lenning et al. [28] using either isothermal elution or a temperature gradient (and thus a dynamic modification of the selectivity) during the analysis.

All the above cited methods using polymeric ODS or ODP materials as stationary phases employ ammonium acetate buffered eluents, based on that first developed by Zapata et al. [29]. In a recent paper we have studied the use of different organic amines as counterions in the ion-pair separation of algal chlorophylls on either

monomeric or polymeric ODS stationary phases [30]. In this investigation we studied the separation of algal and higher plant chlorophyll forms when pyridine containing mobile phases are used with a polymeric ODS column.

Experimental

Methanol, acetonitrile, acetone (HPLC grade), pyridine and acetic acid (analytical grade) were from Merck (Darmstadt, Germany).

200 µL aliquots of sample solution were injected into an HPLC system consisting of a Beckman (Fullerton, California, USA) System Gold chromatograph including a Model 126 programmable solvent module, a Model 210 injection valve and a Model 167 diode array detector together with a Hitachi (Tokyo, Japan) F1050 spectrofluorimeter (excitation wavelength 440 nm, emission wavelength 660 nm) interfaced with a Beckman 406 analog interface module. Columns were thermostatted with a water bath controlled at different set temperatures by means of the simultaneous use of a refrigerated chiller with immersion probe and a temperature controlled immersion heater-circulator (both from Selecta, Barcelona, Spain).

The column was a Vydac 201 TP (250 mm × 4,6 mm I.D., polymeric octadecylsilica, 5 µm particle size, 300 Å pore size) (The Separation Group, Hesperia, California, USA).

Eluent A consisted in 8 parts of organic modifier (a methanol/acetonitrile mixture in different proportions) and 2 parts of aqueous pyridine (0.25 M pyridine, pH adjusted to 5.0 with acetic acid) while eluent B was always acetone. The flow rate was 1.2 mL min^{-1}.

Pigment standards were obtained from 4 days old etiolated and phototransformed cucumber (*Cucumis sativus*) cotyledons [3, 31], from unialgal cultures of several species of known pigment composition (the prymnesiophytes *Emiliania huxleyi*, *Isochrysis galbana* and *Pavlova gyrans* and the prasinophyte *Micromonas pusilla*) and from oceanic samples taken south of Gran Canaria (Canary Islands, Spain). Peak identity was confirmed as previously described [6, 23, 24, 28].

Natural seawater samples (1,5 L) were filtered and extracted as described in [28].

Results and Discussion

In a previous paper [30] we showed that the use of pyridinium ion as the counterion in ion pair reversed phase HPLC of chlorophylls gave the highest retention of acidic chlorophylls together with an enhanced selectivity, which reached the best overall results when employed together with polymeric ODS column stationary phases. However, two important pigments, DV-chl c_3 and DV-Pchlide *a* (the latter compound is also known as Mg-divinylprotoporphyrin a_5 monomethyl ester, MgDVP) coeluted. In an attempt to separate these components, we tested the effect of a change in mobile phase composition. When the organic modifier (80 % of the eluent) was changed from methanol to acetonitrile, while keeping constant the content of aqueous pyridine (20 % of the eluent) and the temperature (27 °C), a general decrease in retention and a dramatic change in selectivity of acidic algal chlorophylls was observed (Figure 2), resulting in a higher relative retention of the more oxygenated DV-chl c_3. The esterified forms, eluting at the end of the chromatogram are not affected except by a slight decrease in retention. The four acidic chlorophylls tested eluted, well separated, in eluents containing between 25 and 55 % acetonitrile. An eluent consisting of methanol 45 %, acetonitrile 35 % and 0.25 M pyridine (pH 5) 20 % was finally selected, as it achieves a good separation of polar chlorophylls and of the carotenoids that usually accompany them in chromophyte algae. An example is shown in Figure 3, in which all the pigments from the prymnesiophyte *Isochrysis galbana* are well separated, including the phytol substituted chlorophyll *c*-like pigment, recently described in this species [32]. The most striking feature of this system is the enhanced retention of chls c_1 and c_2 that elute in the chromatogram *after* the fucoxanthin peak and are completely resolved.

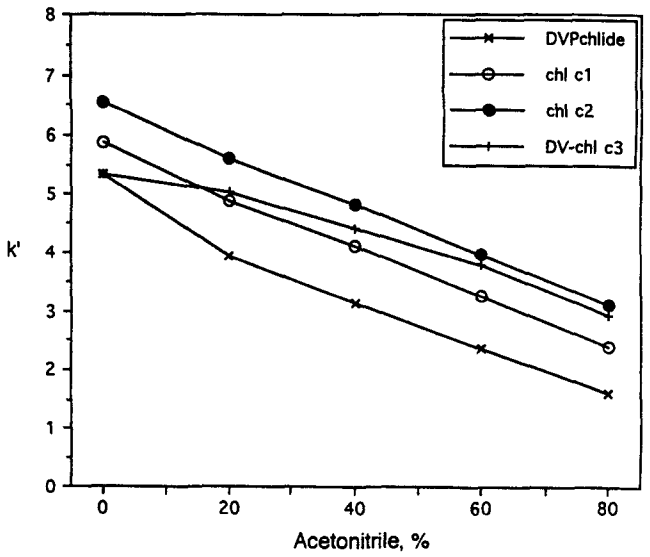

Figure 2
Variation of polar chlorophyll forms capacity factors with percent acetonitrile in mobile phase A. Gradient: linear from 5 to 60 % B in 28 min., then 60 to 100 % B in a further 4 min and then kept at 100 % B during 6 min. Temperature: 27 °C. For other operating conditions, see text.

Employing this mobile phase, the chromatogram of a natural seawater sample extract from the Canary Islands showed no separation of the mono- and divinyl forms of chls *a* and *b* at 27 °C (Figure 4 A). As it has been previously demonstrated that acetonitrile containing mobile phases buffered with ammonium acetate are able to achieve baseline resolution of both pairs of pigments when employed at subambient temperatures [28],

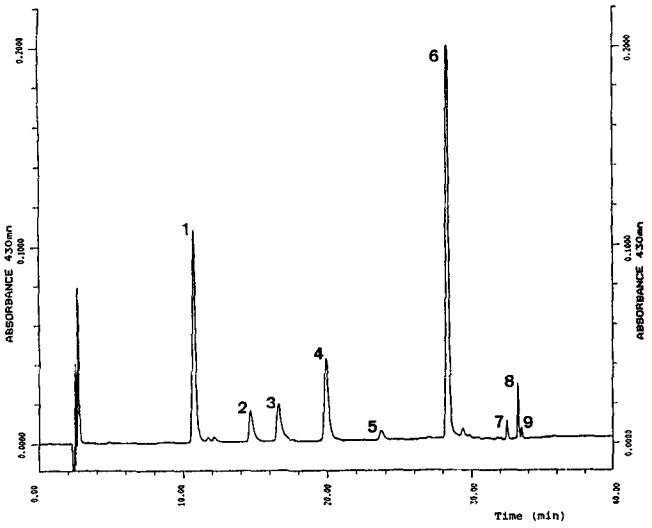

Figure 3

Chromatogram of a pigment extract of the microalga *Isochrysis galbana*. Eluents: A, methanol / acetonitrile / 0.25 M aqueous pyridine (pH 5), 45/35/20 (v/v/v); B, acetone. Gradient and temperature as in Figure 2. Detection by absorbance at 430 nm. For other conditions, see experimental. Peak identification: **1** = Fucoxanthin; **2** = chl c_1; **3** = chl c_2; **4** = Diadinoxanthin; **5** = Diatoxanthin; **6** = chl a; **7** and **8** = phytol-substituted chl *c*-like pigments; **9** = β,β-carotene.

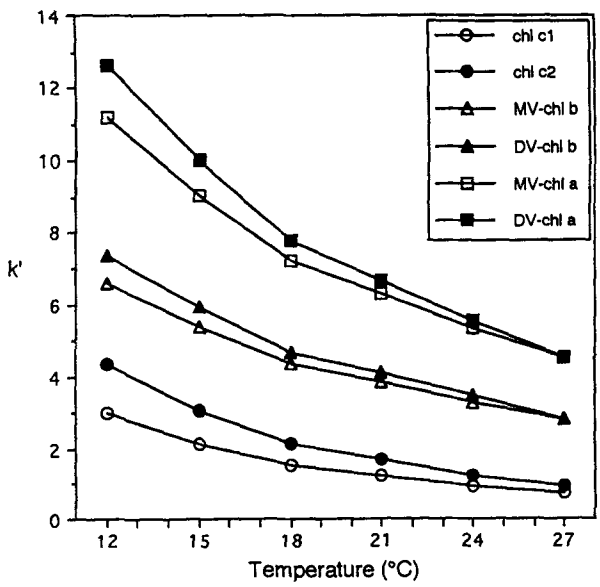

Figure 5

Variation of capacity factors of selected mono- and divinyl chlorophyll forms with column temperature. Isocratic elution with 55 % A and 45 % B. Eluents as in Figure 3; other conditions as described in Experimental.

a mixture containing polar (chl c_1, chl c_2) and non polar (MV-chl *b*, DV-chl *b* and MV-chl *a*, DV-chl *a*) pigments was chromatographed at different temperatures under isocratic conditions (Figure 5). The acidic chls c_1 and c_2 separated well even at temperatures above 25 °C. The mono- and divinyl chls *a* and *b* showed increased separation as the temperature was decreased, reaching

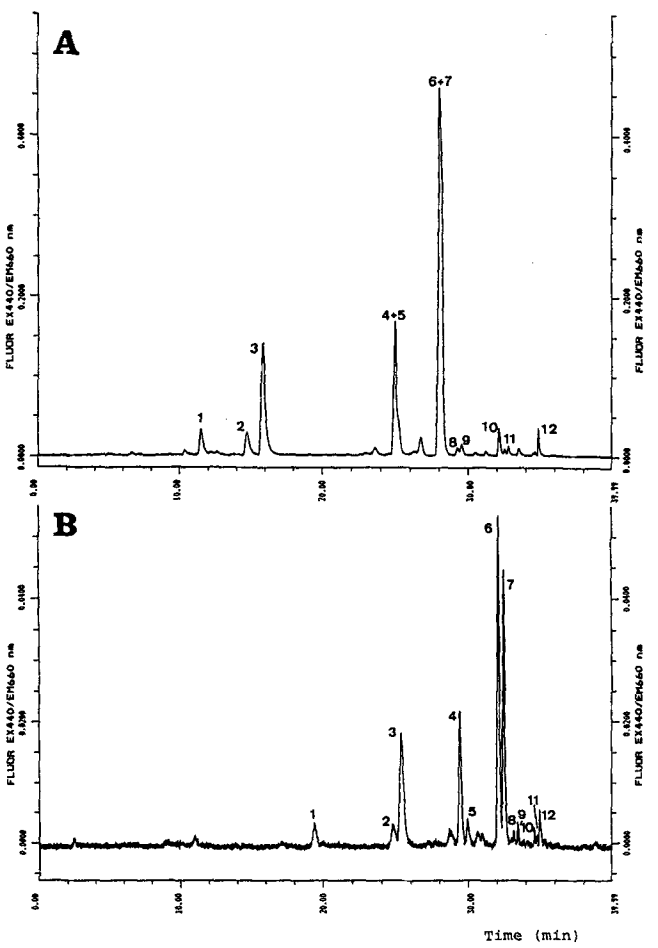

Figure 4

Chromatograms of a chlorophyll extract of a natural seawater sample from the Canary Islands. A) column temperature, 27 °C; B) column temperature, 15 °C. Eluents and gradient as in Figure 3, detection by fluorescence (for other conditions, see Experimental). Peak identification: **1** =, DVPchlide *a*; **2** = DV-chl c_3; **3** = chl c_2; **4** = chl *b*; **5** = chl b_2; **6** = chl *a*; **7** = chl a_2; **8** = chl *a* '; **9** = chla_2 '; **10** = phytol-sustituted chl *c*-like pigment; **11** = phytol-sustituted chl *c*-like pigment; **12** = pheophytin *a*.

baseline resolution at 15 °C. In consequence, this temperature was selected. Figure 4 B shows the chromatogram obtained when the same natural sample extract was analyzed under identical chromatographic conditions except that the column temperature was lowered. All pigments were, as expected, more retained. The higher relative retention of the divinyl forms can be explained by the increased shape selectivity (especially with regard to differences in planarity) of bonded phases (mainly those of a polymeric nature), with decreased temperatures [33]. This increased selectivity allows the separation of 8-ethyl- and 8-vinyl-substituted chlorophylls based on the differences in their molecular shape: in 8-vinyl derivatives (i.e. divinyl forms), the substituent and the tetrapyrrolic macrocycle are located in the same plane (as conjugated systems are greatly stabilized when the double bonds are coplanar) whereas 8-ethyl derivatives (monovinyl forms) adopt a non planar conformation, so that the steric hindrance is the smal-

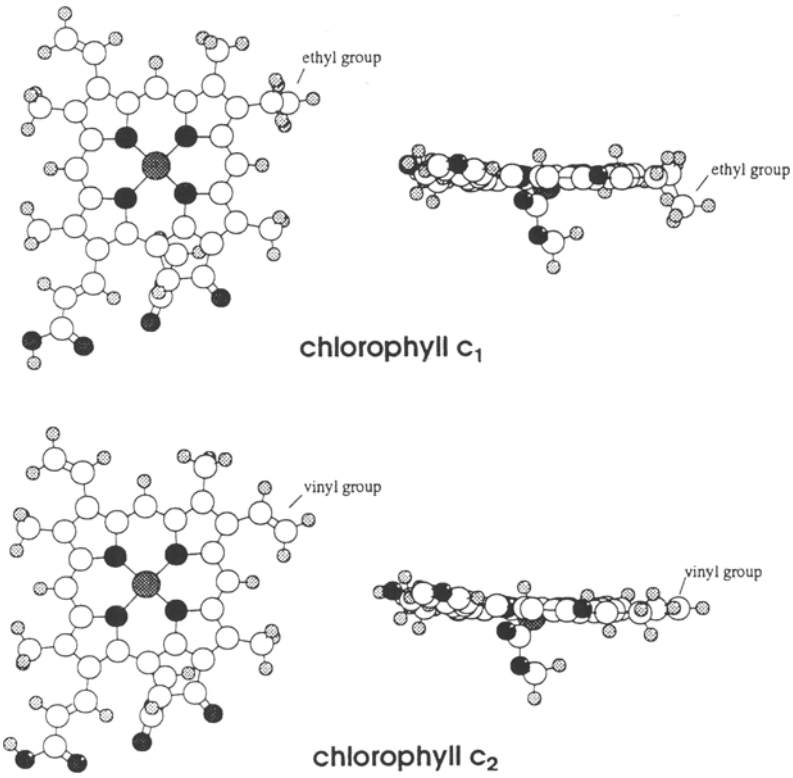

Figure 6
Frontal (macrocycle parallel to the paper, -left-) and lateral (macrocycle perpendicular to the paper, -right-) views of the molecular models of chlorophylls c_1 and c_2.

lest. An example of this is shown in Figure 6 for the pair chl c_1-chl c_2 (This property was first applied by Hanamoto and Castelfranco [31] who employed different monomeric ODS columns operated at 0 °C and ion-pair elution to separate the pairs of acidic pigments MV-chlide *a*, DV-chlide *a* and MV-Pchlide *a*, DV-chlide *a*. This method, however, was extremely lengthy, requiring at least 75 min for the complete separation of just four pigments and did not allow the simultaneous analysis of esterified compounds). The difference in molecular shape induced by the presence of an ethyl or a vinyl substituent in position 8 is somewhat reduced if a voluminous group is present at position 7 (see Figure 1, substituent R_1) of the macrocycle (i.e. -CHO in chl *b* or -$COOCH_3$ in chl c_3). In these cases, the vinyl group appears slightly twisted from the macrocycle plane to relieve steric tension. These facts could explain the difficulties in separating chl *b* from chl b_2 in certain methods that successfully resolve chl *a* and chl a_2 [8, 27].

The method can be modified by changing the gradient profile to fulfill specific analytical situations. For example, a gradient was developed for monitoring the greening process of etiolated tissues of higher plants. A typical situation is depicted in Figure 7, in which the chlorophyll forms occurring in etiolated cucumber cotiledons after flash irradiation are separated in less than 25 min. In this chromatogram the peaks eluting after min 20 correspond to protochlorophyllide esters [25]. If these apolar components are absent from the sample (they can be easily eliminated by passing the sample through a solid-phase extraction cartridge, as suggested in [15]), the complete separation of mono- and divinyl chlides and Pchlides can be completed in *less than 10 min, even under isocratic elution* (results not shown).

The behaviour of other pigments was studied employing a mixture of chlorophylls of marine origin (Figure 8). The method allows the rapid separation of most of the pigments described in marine phytoplankton, including the monovinyl-divinyl pairs chl *a* - chl a_2, chl *b* - chl b_2, chl c_1 - chl c_2, and the newly characterized MV chl c_3 - DV chl c_3 [34]. Interestingly, the peak corresponding to the polar chlorophyll c-like pigment from *Pavlova gyrans* [35] appeared as a doublet, suggesting it can actually be a mixture of two substances. Work directed at the characterization of these pigments is currently in progress.

Pyridine shows several properties which make it a preferred additive in the liquid chromatography of acidic chlorophylls:

- It is miscible with water and most organic solvents [36] and shows adequate vicosity (0.88 cP at 25 °C) and boiling point (115 °C) values [37].
- Although it is strongly absorbing in the ultraviolet, it is transparent in the visible region of the spectrum, thus not interfering with pigment detection.
- Being a tertiary amine, it does not react with acetone (frequently employed as eluent in RP-

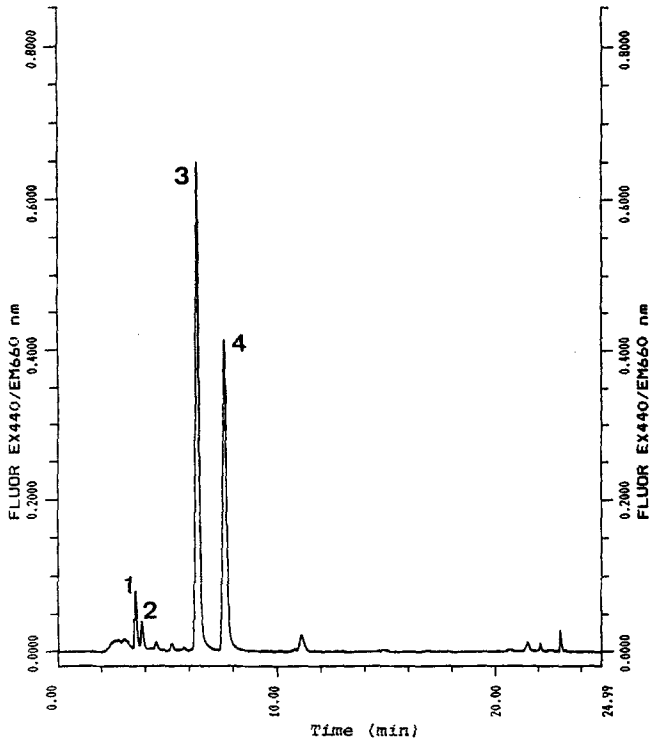

Figure 7

Separation of pigments in a extract of etiolated cucumber cotyledons after flash irradiation. Eluents as in Figure 3. Gradient: linear from 35 % B to 55 % B in 8 min, isocratic hold at 55 % B from min 8 to min 16, then from 55 % B to 100 % B in 4 min and then kept at 100 % B for 4 min more. Peaks: **1** = MV-chlide *a*; **2** = DV-chlide *a*; **3** = MVPchlide *a*; **4** = DVPchlide *a*.

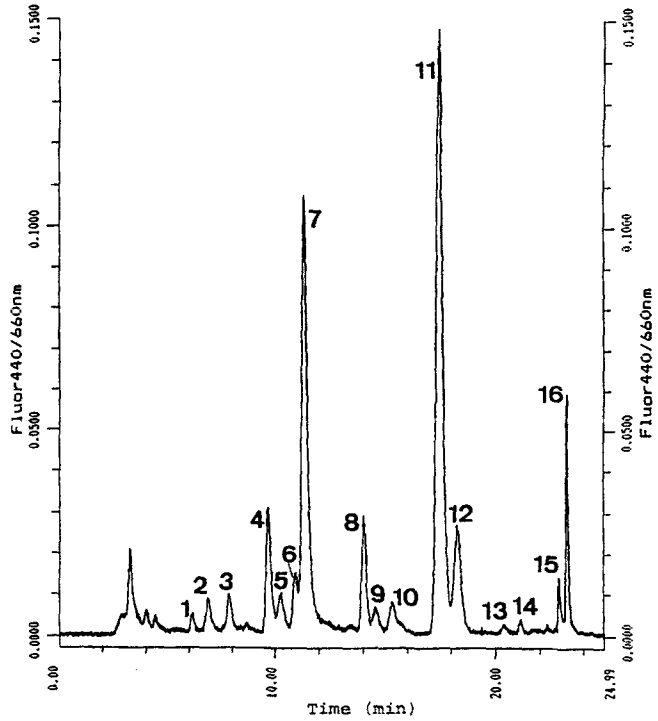

Figure 8

Chromatogram of chlorophylls from marine phytoplankton. Chromatographic conditions as in Figure 7. Peak identification: **1** = chl *c*-like pigment from *P. gyrans*; **2** = chl *c*-like pigment from *P. gyrans*; **3** = DVPchlide *a*; **4** = chl c_1; **5** = MV-chl c_3; **6** = DV-chl c_3; **7** = chl c_2; **8** = chl *b*; **9** = chl b_2; **10** = allomerized chl *a*; **11** = chl *a*; **12** = chl a_2; **13** = chl *a* '; **14** = chl a_2'; **15** = phytol-sustituted chl c-like pigment; **16** = phytol-sustituted chl *c*-like pigment.

HPLC of chlorophylls) to form hemiaminals and then imines (as ammonia, also commonly employed in pigment HPLC analysis, does).

- The planar structure of the pyridinium ion makes it specially suitable as counter ion in paired ion chromatography on polymeric octadecyl phases that exhibit special selectivities towards molecular shapes [30].

References

[1] *C. A. Rebeiz, R. Parham, D. A. Fasoula, I. M. Ioannides*, in "The Biosynthesis of Tetrapyrrole Pigments", Ciba Foundation Symposium 180, John Wiley & Sons, Chichester, 1994, p. 177.

[2] *C. A. Rebeiz, J. Lascelles*, in "Photosynthesis. Vol. I: Energy Conversion by Plants and Bacteria", Govindjee, ed., Academic Press, New York, 1982, p. 699.

[3] *Y. Shioi, K. Takamiya*, Plant Physiol. **100**, 1291 (1992).

[4] *S. W. Jeffrey*, in "The Chromophyte Algae: Problems and Perspectives", J. C. Green, B. S. C. Leadbeater and W. L. Diver, eds., Clarendon Press, Oxford, 1989, p. 13.

[5] *R. R. Bidigare, M. C. Kennicutt, M. E. Ondrusek, M. D. Keller, R. R. L. Guillard*, Energy Fuels **4**, 65 (1990).

[6] *J. L. Garrido, M. Zapata, S. Muñiz*, J. Phycol. **31**, 761 (1995).

[7] *S. W. Chisholm, S. L. Frankel, R. Goericke, R. J. Olson, B. Palenik, J. B. Waterbury*, L. West-Johnrud, E. R. Zettler, Arch. Microbiol. **157**, 297 (1992).

[8] *R. Goericke, D. J. Repeta*, Mar. Ecol. Prog. Ser. **101**, 307 (1993).

[9] *I. M. Ioannides, K. R. Robertson, C. A. Rebeiz*, Plant Physiol. (Life Sci. Adv.) **11**, 79 (1992).

[10] *S. W. Jeffrey*, in "Primary Productivity in the Sea", P. G. Falkowski, ed., Plenum Publishing Corporation, New York, 1980, p. 33.

[11] *K. S. Rowan*, "Photosynthetic Pigments of Algae". Cambridge University Press, New York, 1989, p. 66.

[12] *D. F. Millie, H. W. Paerl, J. P. Hurley*, Can J. Fish. Aquat. Sci. **50**, 2513 (1993).

[13] *E. J. H. Head, L. R. Harris*, Mar Ecol Prog. Ser. **86**, 229 (1992).

[14] *Y. Shioi, S. I. Beale*, Anal. Biochem. **162**, 493 (1987).

[15] *B. J. Whyte, W. T. Griffiths*, Biochem. J. **291**, 939 (1993).

[16] *S. W. Wright, S. W. Jeffrey, R. F. C. Mantoura, C. A. Llewellyn, T. Bjornland, D. Repeta , N. Welschmeyer*, Mar. Ecol. Prog. Ser. **77**, 183 (1991).

[17] *L. Van Heukelem, A. J. Lewitus, T. M. Kana, N. E. Craft*, J. Phycol. **28**, 867 (1992).

[18] *Y. Shioi, R. Fukae, T. Sasa*, Biochim. Biophys. Acta **722**, 72 (1983).

[19] *N. Suzuki, K. Saitoh, K. Adachi*, J. Chromatogr. **408**, 181 (1987).

[20] *P. Barry, A. J. Young, G. Britton*, J. Exp. Bot. **42**, 229 (1991).

[21] *S. O. Duke, M. V. Duke, H. J. Lee*, Z. Naturforsch. **48 c**, 317 (1993).

[22] *B. Schoefs, M. Bertrand, Y. Lemoine*, J. Chromatogr. A **692**, 239 (1995).

[23] *J. L. Garrido, M. Zapata*, J. High Resolut. Chromatogr. **16**, 229 (1993).

[24] *J. L. Garrido, M. Zapata*, Chromatographia **35**, 543 (1993).

[25] *Y. Shioi, K. Watanabe, K. Takamiya, J. L. Garrido, M. Zapata*, Anal. Biochem. **231**, 225 (1995).
[26] *L. C. Sander, S. A. Wise*, Anal. Chem. **61**, 1749 (1989).
[27] *L. Van Heukelem, A. J. Lewitus, T. M. Kana, N. E. Craft*, Mar. Ecol. Prog. Ser. **114**, 303 (1994).
[28] *K. Van Lenning, J. L. Garrido, J. Aristegui, M. Zapata*, Chromatographia **41**, 539 (1995).
[29] *M. Zapata, A. M. Ayala, J. M. Franco, J. L. Garrido*, Chromatographia **23**, 26 (1987).
[30] *J. L. Garrido, M. Zapata*, J. Chromatogr. A **738**, 285 (1996).
[31] *C. M. Hanamoto, P. A. Castelfranco*, Plant Physiol. **73**, 79 (1983).
[32] *M. Zapata, J. L. Garrido*, J. Phycol. (submitted)
[33] *L. C. Sander, S. A. Wise*, J. Chromatogr. A **656**, 335 (1993).
[34] *J. L. Garrido, M. Zapata*, J. Phycol. (submitted)
[35] *M. W. Fawley*, J. Phycol. **25**, 601 (1989).
[36] *S. Budavari* (editor), "The Merck Index. Centennial Edition", Merck, Rahway, 1989, p.7984.
[37] *L. R. Snyder, J. J. Kirkland*, "Introduction to Modern Liquid Chromatography. Second Edition", John Wiley and Sons, New York, 1979, p. 249.

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