High Performance Liquid Chromatographic Separation of Chlorophyll *c* Forms from Marine Phytoplankton on Octylsilica Bonded Phases

F. Rodríguez[1]/ M. Zapata[1]*/ J. L. Garrido[2]

[1]Centro de Investigacions Mariñas, Consellería de Pesca, Xunta de Galicia, Apdo.13, 36620 Vilanova de Arousa, Spain
[2]Instituto de Investigacions Mariñas, C.S.I.C., Av. Eduardo Cabello 6, 36208 Vigo, Spain

Key Words

Column liquid chromatography
Chlorophyll *c*
Marine phytoplankton
Octylsilica phases
Photosynthetic pigments

Summary

The separation of mono- and divinyl chlorophyll *c* forms was estudied employing several octylsilica and octadecylsilica columns under isocratic conditions. In this paper we show that, using the adequate mobile phases, the monomeric C8 columns can separate the mentioned chlorophylls. A comparison between C8 and C18 columns reveals that monomeric OS phases provide always higher resolution of the MV- and DV chlorophyll *c* pairs. Such a result could be explained in terms of differences in column polarity. When adequate gradient profiles and injection conditions are used the separation is accomplished together with that of other chlorophylls and carotenoids.

Introduction

The analysis of phytoplankton pigment composition gives valuable information that constitutes the basis of a great number of studies on taxonomy, physiology, and ecology of microalgae.

The complete separation by high-performance liquid chromatography (HPLC) of the different chlorophylls and carotenoids pigments described in marine photosynthetic organisms is specially difficult, mainly in the case of chlorophylls, as these compounds cover a wide range of polarities [1] while showing very close structural similarities. In the marine environment, most chlorophylls, either acidic or esterified can appear as pairs differing only in the substitution of an ethyl group by a vinyl one. Such is the case for the chlorophylls (Chls) c_1 and c_2 and the monovinyl (MV) and divinyl (DV) forms of Chl c_3, Chl *b* and Chl *a* (Figure 1).

The resolution of isolated pairs of acidic chlorophylls has been achieved using specific methodologies such as micellar electrokinetic chromatography [2, 3] or liquid chromatography either on polyethylene [4, 5] or on octadecyl bonded polyvinyl alcohol polymer columns [6, 7]. However, the separation of the acidic chlorophylls pairs has challenged many of the methods proposed for the simultaneous analysis of polar and non polar phytoplankton pigments. Most of those methodologies employ monomeric octadecylsilica (ODS, C18) stationary phases [8–10] that do not resolve neither the acidic nor the esterified MV and DV chlorophyll pairs. Only the introduction of polymeric ODS phases, that show special selectivities towards different molecular shapes, allowed the discrimination of various of these pairs [11–15] and then the complete separation of algal chlorophylls [16, 17].

The use of monomeric octylsilica (OS, C8) phases was first introduced by Goericke and Repeta [18] for the separation of the MV- and DV Chl *a* in a method that failed in the resolution of the corresponding pairs of acidic chlorophylls. Later methodologies that employed the same stationary phase achieved worse [19, 20] or similar [21, 22] results for MV and DV-Chl *a* separation, but none of them resolved the pairs of the non esterified chlorophylls.

The exam of the operative conditions employed by most of these authors led us to the hypothesis that monomeric OS columns could separate the pairs of MV- and DV analogues of acidic chlorophylls if the correct chromatographic parameters were employed. In this paper we show that, using the adequate mobile phases, the monomeric C8 columns can separate the mentioned chlorophylls and that, when proper gradient profiles are used, the separation is accomplished together with that of other chlorophylls and carotenoids.

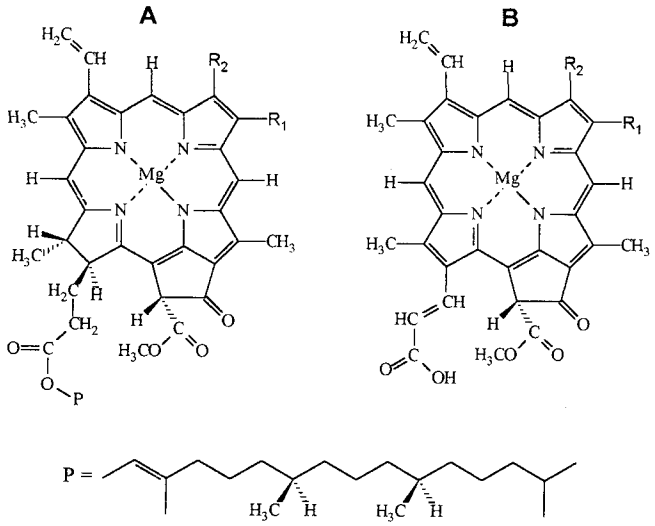
0009-5893/98/11 677-04 $ 03.00/0

Experimental

A Waters Alliance HPLC System (Milford, Massachusetts), including a 2690 separations module, a Waters 996 photodiode array detector and a Waters 474 scanning fluorescence detector interfaced by means of a Sat/in analog interface. Columns were always thermostatted at 25 °C by means of a recirculating water bath.

Several OS and ODS columns were purchased from the following manufacturers: Symmetry and Spherisorb form Waters (Milford, Massachusetts), Vydac from The Separations Group (Hesperia, California), Prodigy from Phenomenex (Torrance, California) and Hypersil from Shandon (Runcorn, England). All the columns employed were characterized in terms of their efficiency (N) and shape selectivity ($\alpha_{TBN/BaP}$) using the column test mixture SRM 869 (Office of Standard Reference Materials, National Institute of Standards and Technology, Gaithersburg, Maryland) as described by Sander and Wise [23]. Surface coverage values were calculated as described in [24]. The following representative bonded units were used: $CH_3(CH_2)_7Si(CH_3)_2$-O- (molecular weight 187) for OS monomeric phases, $CH_3(CH_2)_7Si(OH)_2$-O- (molecular weight 191) for OS polymeric phases, $CH_3(CH_2)_{17}Si(CH_3)_2$-O- (molecular weight 327) for ODS monomeric phases and $CH_3(CH_2)_{17}Si(OH)_2$-O- (molecular weight 331) for ODS polymeric phases.

Pigment standards were obtained from unialgal cultures of the planktonic microalgae *Isochrysis galbana* and *Emiliania huxleyi*. Peak identities were confirmed as previously described [25, 26]. Isolated pigments were dissolved in 90 % aqueous acetone. To avoid chromatographic artifacts, 0.4 mL of water was added to each mL of acetonic extract [27].



Compound (abbreviation)	Main structure	R_1	R_2
Monovinyl chlorophyll *a* (MV-Chl *a*)	A	CH_2-CH_3	CH_3
Divinyl chlorophyll a (DV-Chl *a*)	A	CH=CH_2	CH_3
Monovinyl chlorophyll *b* (MV-Chl *b*)	A	CH_2-CH_3	CHO
Divinyl chlorophyll *b* (DV- Chl *b*)	A	CH=CH_2	CHO
Chlorophyll c_1	B	CH_2-CH_3	CH_3
Chlorophyll c_2	B	CH=CH_2	CH_3
Monovinyl chlorophyll c_3 (MV-Chl c_3)	B	CH_2-CH_3	$COOCH_3$
Divinyl chlorophyll c_3 (DV- Chl c_3)	B	CH=CH_2	$COOCH_3$

Figure 1

Structures of mono- and divinyl forms of different chlorophylls (P = phytyl). The abbreviations commonly found in the literature for these pigments are also indicated.

Results and Discussion

Table I summarizes the characteristics of the different columns employed in this study. The whole set is rather heterogeneous, with columns representative of different degrees of efficiency, retention and shape selectiv-

Table I. Characteristics of the chromatographic columns employed in this study.

Columns	Bonding chemistry	Dimensions (mm)	Pore size (Å)	Particle diameter (µm)	Surface area ($m^2 g^{-1}$)	Coverage (µmol m^{-2})	Carbon load (%)	$\alpha_{TBN/BaP}$[1]	N (TBN)[2]
C8									
Hypersil C8 (MOS-2)	Monomeric	150 × 4.6	120	3	170	4.96	7.00	1.70	100024
Prodigy 5 C8	Monomeric	250 × 4.6	150	5	310	5.6	12.60	1.78	33204
Spherisorb S3 C8	Monomeric	150 × 4.6	80	3	220	3.21	6.00	1.68	138200
Symmetry C8	Monomeric	150 × 4.6	100	3.5	334	4.82	11.89	1.86	112240
Vydac 208TP54	Polymeric	250 × 4.6	300	5	60–74	8.15	4.50–5.00	1.47	59228
C18									
Hypersil ODS	Monomeric	150 × 4.6	120	3	170	3.21	10.00	1.90	112313
Prodigy C18	Monomeric	250 × 4.6	100	5	450	2.08	15.50	2.12	55888
Spherisorb C18 (ODS2)	Monomeric	250 × 4.6	80	5	220	3.08	12.00	1.75	53724
Symmetry C18	Monomeric	100 × 4.6	100	3.5	336	3.74	19.26	1.74	58540
Vydac 201TP54	Polymeric	250 × 4.6	300	5	90	4.69	8.00	0.66	43748

[1] Separation factor for Tetrabenzonaphtalene (TBN) and Benzo[a]Pyrene (BaP). [2] Number of theoretical plates per meter, measured for the TBN peak.

Table II. Retention factor (k) and resolution (R_s) for DV-Chl c_3/MV-Chl c_3 and Chl c_1/Chl c_2 pairs in the different columns studied. Mobile phase for C8 columns, methanol: 1M ammonium acetate 75:25 v:v. Mobile phase for C18 columns, methanol: 1M ammonium acetate 80:20 v: v. Temperature: 25 °C. Flow rate: 0.8 mL min^{-1}, except for Prodigy, Vydac and Spherisorb C18 columns where flow rate was1.2 mL min^{-1}

Columns	k		R_s	k		R_s
	DV-Chl c_3	MV-Chl c_3	DV/MV-Chl c_3	Chl c_2	Chl c_1	Chl c_2/c_1
C8						
Hypersil C8 (MOS-2)	2.53	2.90	1.41	6.38	7.09	2.13
Prodigy 5 C8	2.23	2.57	1.56	5.28	5.89	1.66
Spherisorb S3 C8	2.19	2.41	1.18	4.13	4.48	1.50
Symmetry C8	2.94	3.32	1.81	6.55	7.20	2.00
Vydac 208TP54	0.61	0.61	<0.40	1.16	1.22	1.00
C18						
Hypersil ODS	3.68	4.00	1.12	7.96	7.96	<0.40
Prodigy C18	5.89	6.55	1.99	14.17	14.63	0.83
Spherisorb C18 (ODS2)	5.19	5.19	<0.40	9.71	9.02	1.30
Symmetry C18	6.00	6.44	0.85	14.17	14.17	<0.40
Vydac 201TP54	7.27	6.83	0.82	11.52	8.76	4.32

ity. This last property was measured as it has been claimed to explain the ability for the separation of MV- and DV chlorophyll pairs showed by polymeric ODS phases [14, 16, 17].

It is well established that different bonded phases show diverse values for two main parameters: column strength (J) and column polarity (P) [28]. In consequence, the optimization of specific mobile phases is required for each case [29]. However, most of the chromatographic methods up to date proposed for the separation of algal pigments on OS columns employ very steep gradients, based on that originally developed by Wright et al. [10] *for ODS columns*, in which the aqueous part of the eluent (containing the ion- pairing and buffering agent) is quickly eliminated from the mobile phases.

As C8 columns are weaker (J = 0.00) than C18 ones (J = 0.26) [28], they require weaker mobile phases to achieve adequate retentions [29]. Under the light of that consideration such columns, that can separate MV- and DV Chl *a*, seemed to be able to discriminate the corresponding forms of the acidic chlorophylls if enough interaction of the solutes with the stationary phase were allowed. In consequence, we assayed an isocratic procedure employing eluents with adequate aqueous component content to provide increased retention of these pigments. The mobile phases were based on that previously developed by Zapata et al. [9], consisting of mixtures of methanol with a 1 M aqueous solution of ammonium acetate.

Under such conditions, all the monomeric C8 columns separate the two pairs of algal acidic chlorophylls (Table II). The elution order, with the slightly more polar divinyl forms eluting before the corresponding monovinyl counterparts indicates that, as could be expected for these monomeric phases, the retention and selectivity are governed by a partitioning process. The resolutions achieved were specially high for the three columns that showed the highest retentions, corresponding to those with the highest values of surface coverage (Table I). The only OS monomeric column showing partial resolution of both pairs of chlorophylls, Spherisorb C8, achieved baseline separation of the four pigments when the retention was further increased (data not shown). The polymeric Vydac OS maintains the coelutions of the two mono- and divinyl pairs even at high values of retention (data not shown). This behaviour could be explained considering that the shape selectivity typical of polymeric bonded phases (that accounts for a higher retention of the more planar divinylic pigments [15–17]) counteracts the partitioning mechanism, impairing the separation.

The monomeric ODS columns assayed separate partially one or both chlorophyll pairs under the isocratic conditions assayed. However, the retention requirements for this results are very high, suggesting that these phases would not be adequate for the simultaneous separation of the acidic chlorophylls along with other non polar pigments (that usually accompany them in algal extracts) even under gradient elution. As expected, the best overall results are achieved with the polymeric ODS Vydac 201 TP column. The inversion in the elution order of the MV- and DV chlorophyll forms in this phase has been explained by the differences in the planarity of their molecules [13, 14, 17].

A global comparison between C8 and C18 columns reveals that monomeric OS phases provide always higher resolution of the MV- and DV chlorophyll *c* pairs. Such a result could be explained in terms of column polarity, considering that the increased value of this parameter (P = –0.55 for C18 and P = 0.00 for C8 phases [28]) allows the discrimination of these compounds, which differences in molecular polarity are relatively small.

The enhanced shape selectivity of the polymeric ODS stationary phase (shown by its small $\alpha_{TBN/BaP}$ value) makes it much more resolutive towards the pairs of pig-

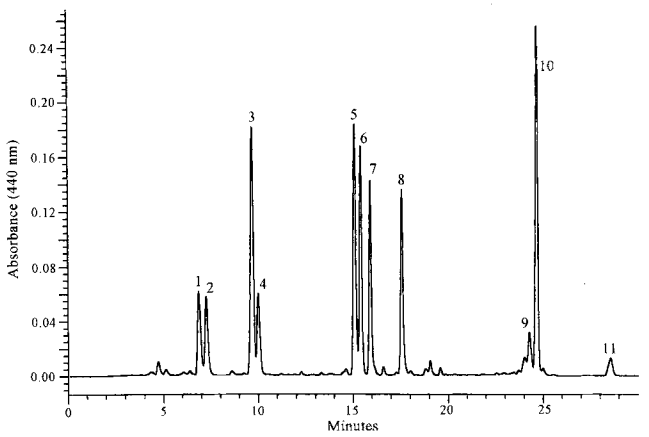


Figure 2

Chromatogram of a mixture of acetonic extracts obtained from *Emiliania huxleyi* and *Isochrysis galbana*. Column: Symmetry C8, Eluents: (A) methanol: 1M ammonium acetate, 75:25 (v:v); (B) methanol. Gradient: linear from 0 to 100 % B in 20 min., then kept at 100 % B during 10 min. Temperature: 25 °C. Flow rate: 0.8 mL min^{-1}. Peak identification: **1** = DV-Chl c_3; **2** = MV-Chl c_3; **3** = Chl c_2; **4** = Chl c_1; **5** = fucoxanthin; **6** =19'-hexanoyloxyfucoxanthin-like pigment; **7** = 19'-hexanoyloxyfucoxanthin; **8** = diadinoxanthin; **9** = apolar Chl *c* from *Emiliania huxleyi*; **10** = MV-Chl *a*; **11** = β,β-caroene.

ments under study than the corresponding polymeric C8 column.

As the acidic chlorophylls occur in algae together with other photosynthetic and/or accessory pigments, a gradient procedure for their separation was developed employing a monomeric OS column (Symmetry).The gradient profile was adjusted so the aqueous part is slowly removed from the eluent. Although an even slower gradient was employed by Goericke and Repeta [18] the method failed to resolve chlorophyll c pigments that appeared as distorted bands at the begining of the chromatogram, probably due to the injection conditions employed [27].

Figure 2 shows that using a simple linear gradient and adequate injection conditions, the resolution of non esterified chlorophylls together with that of various carotenoids, including the new 19′-hexanoyloxyfucoxanthin-like pigment recently described in *E. huxleyi* [26], can be achieved. In this column the non-polar chlorophyll *c*-like pigment, characteristic of certain haptophyte species [30] elutes immediately before Chl *a* in a part of the chromatogram where Chl *a* allomers elute and where the elution of DV-Chl *a* has been described [18–22]. In consequence, and as DV-Chl *a* is a marker for marine prochlorophytes, extreme caution is needed when inferring phytoplankton community structure from natural samples whose pigment content had been analysed employing monomeric OS columns. This caution has to be specially remarked when the presence of 19′-hexanoyloxyfucoxanthin, (usually associated to non-polar chlorophyll *c*-like pigment in phytoplankton species) is detected in oceanographic samples [21, 22].

The ability of monomeric OS columns in the resolution of MV- and DV pairs of polar and non polar chlorophylls seems to be also promising in the separation of analogous pairs of chlorophyll biosynthetic intermediates that show a wide range of polarities (MV and DV-protochlorophyllides, -chlorophyllides, -protochlorophylls, etc.) [31].

References

[1] *H. Scheer*, in H. Scheer (Editor), Chlorophylls, CRC Press, Boca Raton, 1991, p.3.
[2] *K. Saitoh, H. Kato, N. Teramae*, J. Chromatogr. A **687**, 149 (1994).
[3] *H. Kato, K. Saitoh, N. Teramae*, Chem. Lett. (**7**), 547, (1995).
[4] *Y. Shioi, S. I. Beale*, Anal. Biochem. **162**, 493 (1987).
[5] *S. W. Jeffrey*, in J.C. Green, B.S.C. Leadbeater and W. L. Diver, (Editors), The Chromophyte Algae: Problems and Perspectives, Clarendon Press, Oxford, 1989, p.13.
[6] *K. Saitoh, I. Awaka, N. Suzuki*, J. Chromatogr. A **653**, 247 (1993).
[7] *K. Saitoh, I. Awaka, N. Suzuki*, J. Chromatogr. A **693**, 176 (1995).
[8] *R.F.C. Mantoura, C. A. Llewellyn*, Anal. Chim. Acta **151**, 297 (1983).
[9] *M. Zapata, A. M. Ayala, J. M. Franco, J. L. Garrido*, Chromatographia **23**, 26 (1987).
[10] *S. W. Wright, S. W. Jeffrey, R. F. C. Mantoura, C. A. Llewellyn, T. Bjørnland, D. Repeta, N. Welschmeyer*, Mar. Ecol. Prog. Ser. **77**, 183 (1991).
[11] *G. W. Kraay, M. Zapata, M. J. Veldhuis*, J. Phycol. **28**, 708 (1992).
[12] *L. Van Heukelem, A. J. Lewitus, T. M. Kana, N. E. Craft*, J. Phycol. **28,** 867 (1992).
[13] *J. L. Garrido, M. Zapata*, Chromatographia **35**, 543 (1993).
[14] *J. L. Garrido, M. Zapata*, J. High Resolut. Chromatogr. **16**, 229 (1993).
[15] *L. Van Heukelem, A. J. Lewitus, T. M. Kana, N. E. Craft*, Mar. Ecol. Prog. Ser. **114**, 303 (1994).
[16] *K. Van Lenning, J. L. Garrido, J. Aristegui, M. Zapata*, Chromatographia **41**, 539 (1995).
[17] *J. L. Garrido, M. Zapata*, Chromatographia **44**, 43 (1997).
[18] *R. Goericke, D. Repeta*, Mar. Ecol. Prog. Ser. **101**, 307 (1993).
[19] *G. B. McManus, R. Dawson*, Mar. Ecol. Prog. Ser. **113**, 199 (1994).
[20] *R. G. Barlow, R. F. C. Mantoura, D. G. Cummings, T. W. Fileman*, Deep-Sea Res. II, **44**, 833 (1997).
[21] *F. Vidussi, H. Claustre, J. Bustillos-Guzmàn, C. Cailliau, J-C. Marty*, J. Plankton Res. **18**, 2377 (1996).
[22] *R. G. Barlow, D. G. Cummings, S. W. Gibb*, Mar. Ecol. Prog. Ser. **161**, 303 (1997).
[23] *L. C. Sander, S. A. Wise*, LC.GC. **8**, 378 (1990).
[24] *L. C. Sander, S. A. Wise*, Anal. Chem. **56**, 504 (1984).
[25] *J. L. Garrido, M. Zapata, S. Muñiz*, J. Phycol. **31**, 761 (1995).
[26] *J. L. Garrido, M. Zapata*, J. Phycol. **34**, 70 (1998).
[27] *M. Zapata, J. L. Garrido*, Chromatographia **31**, 589 (1991).
[28] *P. E. Antle, L. R. Snyder*, LC Mag. **2**, 840 (1984).
[29] *L. R. Snyder, J. L. Glajch, J.J. Kirkland*, Practical HPLC Method Development, John Wiley & Sons, New York, 1988.
[30] *M. Zapata, J. L. Garrido*, J. Phycol. **33**, 209 (1997).
[31] *C. A. Rebeiz, R. Parham, D. A. Fasoula, I. M. Ioannides* in D.J. Chadwick and K. Ackrill (Editors), The Biosynthesis of the Tetrapyrrole Pigments, John Wiley & Sons, Chichester, 1994, p.177.

Received: May 11, 1998
Accepted: Jul 2, 1998