Chapter 8

Chlorophyll Analysis by New High Performance Liquid Chromatography Methods

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Summary

The improvements in high performance liquid chromatography (HPLC) analysis of chlorophylls (Chls) and bacteriochlorophylls (BChls) during the last decade rely mainly on the application of newly developed stationary phases combined with new mobile phases developed with special regard to the nature of the ion-pairing agents employed for achieving the retention of free acid forms, which is especially important for the Chls *c* group. The application of mass spectrometry (MS) as a detection technique coupled on-line with liquid chromatography (LC) has provided important structural information. All these tools have contributed to the discovery of the biosynthetic pathways of higher plant Chls, to the study of the composition and distribution of new Chls in algae, and to the characterization of complex pools of BChls. Faster, more sensitive and more specific HPLC methods are expected in the very near future, with the development of new columns and detection techniques, e.g. monolithic stationary phases and coupled liquid chromatography-nuclear magnetic resonance (LC-NMR) systems.

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I. Introduction

The chromatographic analysis of chlorophylls (Chls) is still an analytical challenge because they constitute a group of compounds that exhibit a wide range of polarities, from the very non polar phytol- and lipid-esterified Chls and Phes to the polar Chl *c* compounds, Chlides and PChlides, all of which contain a free carboxylic acid group (chapters 1, Scheer and 3, Zapata et al.).

Many of these compounds are structurally very similar, and may differ only in the position of a double bond. Recent evidence shows that Chls are formed via two routes containing di- and mono-vinyl ([DV]- and [MV])-tetrapyrrole intermediates (Ioannides et al., 1994; von Wettstein, 1995; Porra, 1997; Rüdiger, 1997; Beale, 1999; chapter 10, Grimm and Rüdiger). This biosynthetic heterogeneity explains the occurrence of various [MV]- and [DV]-derivatives of both porphyrins and/or chlorins during the greening of etiolated tissues in different plants (Shioi and Takamiya, 1992; Rebeiz et al., 1994), the variety of Chl *c* forms described in chromophyte algae (Chapter 3, Zapata et al.) and the prevalence of [DV]-Chl *a* and [DV]-Chl *b* in the euphotic zone of tropical oceans (Chisholm et al., 1992; Goericke and Repeta, 1993). Consequently, much effort in the field of analytical HPLC of Chls has recently focused on the separation of [MV]-pigments from their corresponding [DV]-analogues. The complexity of pigment extracts and the minor differences among Chls often makes separation of individual species difficult. The situation becomes more complicated when accompanying pigments (xanthophylls, carotenes) have also to be analyzed.

Shioi (1991) thoroughly reviewed the instrumental and analytical aspects of chromatographic techniques applied to Chl compounds: most have not changed, so we will concentrate on new methods, their basis and their fields of application.

Abbreviations: C_{18} – octadecylsilica; C_8 – octylsilica; DHgg – dihydrogeranylgeraniol; DV – divinyl; f – farnesol; Gg – geranylgeraniol; HPLC – high performance liquid chromatography; LC – liquid chromatography; MGDG – monogalactosyldiacylglycerol; MgDVP – Mg-[3,8-divinyl]-phytoporphyrin-13^2-methylcarboxylate (i.e. [DV]-PChlide); MS – mass spectrometry; MV – monovinyl; NMR – nuclear magnetic resonance; ODP – octadecyl-polyvinyl-alcohol polymer; ODS – octadecylsilica; OS – octylsilica; p – phytol; RP – reversed phase; THgg – tetrahydrogeranylgeraniol. Unless otherwise specified, Chls and their derivatives (i.e. Chlides, PChlides, Phes *etc.*) are [3-vinyl-8-ethyl]-(i.e. [MV]-) compounds but some Chls *c* are exceptions: Chls c_2, c_2MGDG, c_3, $c_{3(CS\text{-}170)}$ and [DV]-PChlide are all [3,8-divinyl]-(i.e. [DV]-) compounds (see Chapter 3, Figure 1).

II. New Bonded Phase Columns

The development of new HPLC methods comes from the availability of new stationary phases with general characteristics (particle size and shape, base material purity, etc.) which have greatly improved during the past decade. While monomeric octadecyl silica (ODS, C_{18}) columns remain the most frequently used stationary phases for the analysis of Chls, the need to simultaneously separate [MV]- and [DV]-forms of polar and non-polar Chls required different approaches employing polymeric and monomeric silica-based phases and octadecyl-polyvinyl-alcohol polymer (ODP) phases.

A. Silica-Based Columns

1. Polymeric Bonded Phases

Although separation of [MV]-Chl derivatives from their respective [DV]-analogues had been traditionally achieved by employing polyethylene columns (Shioi, 1991), these methods were lengthy and did not allow the simultaneous separation of polar and non polar pigments. By comparison, most of the methods achieving the separation of free and esterified pigments occurring in algae or etiolated tissues from higher plants show several multicomponent peaks corresponding to the coelution of the [MV]- and [DV]-analogues of a particular Chl derivative (Barry et al., 1991; Wright et al., 1991; Van Heukelem et al., 1992; Duke et al., 1993; Schoefs et al., 1995). Silica-based polymeric stationary phases exhibit very high selectivity towards very similar, even isomeric, compounds with rigid molecular structures, the so-called 'shape selectivity' (Sander et al., 1999). This special selectivity has been explained by the rigid nature of the polymeric layer, sometimes represented as a surface with slots into which the solute molecules penetrate during retention. The frequency and depth of this penetration (and, in consequence, the retention) depends on the overall shape of the solute molecule, its size, length and planarity (Sander and Wise, 1990). Several variables (pore size, alkyl chain length, bonding density, temperature and mobile-phase composition) can influence shape selectivity (Sander and Wise, 1990; Sander et al., 1999).

Shape selectivity, applied in the separation of different rigid compounds such as polycyclic aromatic hydrocarbons and carotenoids (Sander et al., 1999), was also used to separate [MV]- and [DV]-pairs of Chls. Although the [DV]-Chls are slightly more polar than the [MV]-Chls, the [DV]-forms elute *after* their [MV]-counterparts on these columns. The separation of 3-vinyl-8-ethyl (i.e. [MV]-) and 3,8-divinyl (i.e. [DV]-) substituted Chls is based on the differences in their molecular shape: in the [DV]-form, the 8-vinyl substituent and the tetrapyrrolic macrocycle are located in the same plane (as conjugated systems are greatly stabilized when the double bonds are coplanar), whereas the [MV]-forms, with an 8-ethyl substituent adopt a non-planar conformation, so that the steric hindrance is the smallest (Fig. 1). In consequence, the more planar [DV]-forms are retained longer than the corresponding [MV]-forms (Garrido and Zapata, 1997).

The difference in molecular shape, induced by an ethyl or a vinyl substituent at C-8, is reduced if a voluminous group is present at C-7 of the macrocycle; i.e. the formyl (–CHO) in Chl *b*, or a methoxycarbonyl ($-COOCH_3$) in Chl c_3. In these cases, the vinyl group appears slightly twisted from the macrocycle plane to relieve steric tension (Fig. 1), which could explain the difficulties in separating Chl *b* from [DV]-Chl *b* in certain methods that successfully resolve Chl *a* and [DV]-Chl *a* (Van Heukelem et al., 1994).

Silica-based polymeric stationary phases were first introduced in the analysis of Chls by Garrido and Zapata (1993a). Since then, several works have been published that explore the role of particle pore size (Garrido and Zapata, 1993b), column temperature (Van Heukelem et al., 1994; Van Lenning et al., 1995), bonded phase length of C_{18} *versus* C_{30} (Schmid and Stich, 1995; Van Heukelem and Thomas, 2001), and mobile phase composition (Garrido and Zapata, 1996, 1997) on the separation of Chls by HPLC.

The influence of ion-pairing agents deserves special attention. For the HPLC analysis of acidic pigments, such as some Chl *c* forms, Chlides and Pheids, either ion-suppression or ion-pairing techniques must be used to achieve sufficient retention and optimal resolution. Many reversed-phase pigment separation methods have used a gradient system where the first eluent contains ammonium acetate as the buffering and ion-pairing reagent (Tables 1, 2 and 3).

The use of pyridine (as pyridinium acetate) as an ion-pairing reagent for the analysis of Chls was introduced by Garrido and Zapata (1996). Pyridine shows several properties which make it a preferred additive in the chromatographic separation of acidic Chls: (i) It is miscible with water and most organic solvents and shows adequate viscosity and boiling point values, (ii) Although it absorbs strongly in the ultraviolet, it does not interfere with the visible pigment spectrum, and (iii) Unlike the ammonium ion, it does not react with acetone (frequently employed as an eluent in RP-HPLC separation of Chls). The planar structure of the pyridinium ion makes it suitable as a counter ion in paired-ion chromatography on polymeric bonded phases that exhibit special selectivity towards molecular shapes.

The use of pyridine-containing mobile phases improves the chromatographic behavior of acidic and esterified Chls both on monomeric and polymeric alkyl silica columns (Garrido and Zapata, 1996,

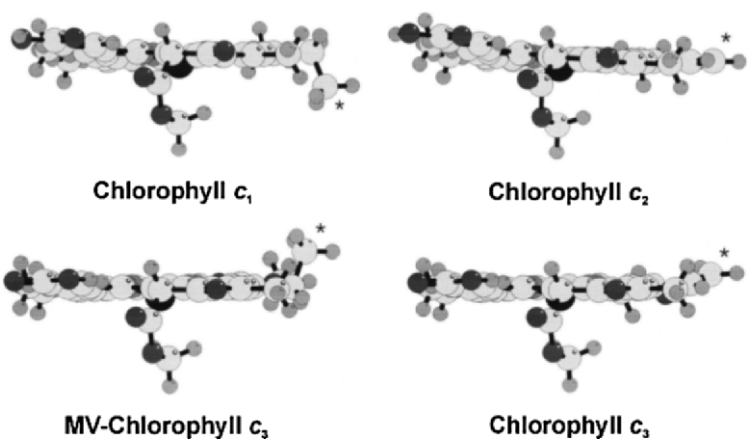

Fig 1. Lateral views (macrocycle perpendicular to the page) of the molecular models of Chl c_1 and [MV]-Chl c_3 ([MV]-forms), and Chls c_2 and c_3 ([DV]-forms). The asterisk indicates the substituent at C-8. MV and DV refer to mono and divinyl, respectively.

1997; Zapata et al., 2000) because the pyridinium ion acts both as a more hydrophobic ion-pairing reagent (increasing the retention of acidic Chls), and as a real mobile phase modifier (co-solvent), affecting the selectivity towards both neutral and charged Chls. A possible explanation for this effect could rely on π-π interactions established between the aromatic ring of pyridine and the aromatic Chl macrocycle.

The combined use of polymeric ODS columns with pyridine-containing eluents has been applied to the separation of algal Chls (Garrido and Zapata, 1997) and of Chlides and PChlide in etiolated plant tissues (Garrido and Zapata, 1997; Schoefs et al., 2000).

The simultaneous separation of polar and non polar [MV]- and [DV]-Chl pairs was achieved (Garrido and Zapata, 1997) by employing polymeric ODS phases operating at low temperature (15 °C) and using pyridine as an ion-pairing and solvent modifying agent (Fig. 2).

2. Monomeric C_8 Bonded Phases

Monomeric OS phases were first used by Goericke and Repeta (1993) to resolve Chl *a* and [DV]-Chl *a*. Their method, and other methods which employed the same stationary phase (Vidussi et al., 1996; Barlow et al., 1997), failed to separate Chl *c* pigments. Since then, Rodríguez et al. (1998) have shown that using adequate gradient profiles and injection conditions, monomeric C_8 columns can separate [MV]- and [DV]-pairs of acidic Chls (e.g. [DV]-Chl c_3 from [MV]-Chl c_3 or Chl c_2 from Chl c_1) simultaneously with other Chls and carotenoids. A more recent method using a C_8 column and pyridine-containing mobile phases was selective enough to resolve [MV]- and [DV]-pairs of polar Chls and [DV]-Chl *a* (a pigment marker for the prokaryote *Prochlorococcus marinus*) from Chl *a* (Zapata et al., 2000) (Fig. 3). On monomeric C_8 columns, the elution order seems to be controlled by subtle differences in the overall polarity of the molecules (Rodríguez et al., 1998), eluting the slightly more polar [DV]-Chl forms before their [MV]-counterparts.

B. Polymer Based Stationary Phases: Octadecyl-polyvinyl-alcohol Polymer (ODP)

Saitoh et al. (1993) showed that an ODP column and an acidic mobile phase can partially resolve the [MV]-/[DV]-pair constituted by Chls c_1 and c_2. To separate acidic and esterified Chls, the same authors developed a column-switching technique that combines two columns, ODP and ODS with the same mobile phase under isocratic conditions (Saitoh et al., 1995).

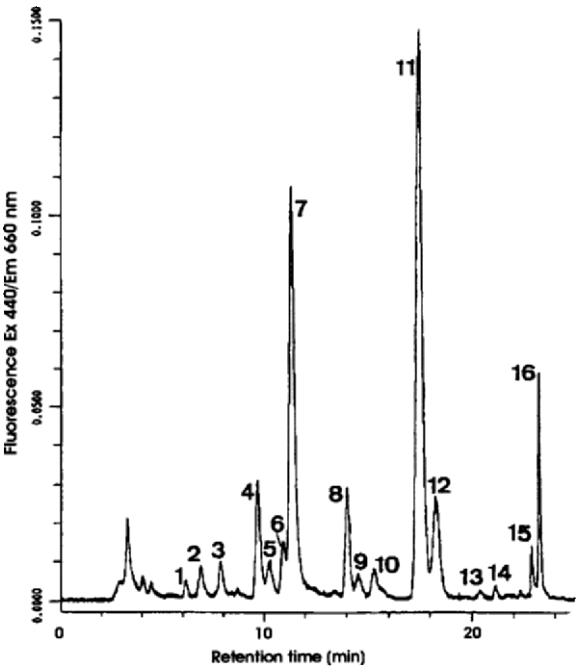

Fig. 2. Chromatogram of chlorophylls extracted from marine phytoplankton obtained with a polymeric octadecyl silica (ODS) column as described by Garrido and Zapata (1997, see Table 3). Peak identification, 1: Chl *c*-like pigment from *Pavlova gyrans*, 2. Chl *c*-like pigment from *P. gyrans*, 3: [DV]-PChlide, 4: Chl c_1, 5: [MV]-Chl c_3, 6: Chl c_3, 7: Chl c_2, 8: Chl *b*, 9: [DV]-Chl *b*, 10: allomerized Chl *a*, 11: Chl *a*, 12: [DV]-Chl *a*, 13 Chl *a*′, 14: [DV]-Chl *a*′, 15: Chl c_2-MGDG, 16: Chl c_2-MGDG. Reproduced from Garrido and Zapata (1997). MV and DV refer to mono and divinyl, respectively.

Considering the acid-labile nature of the Chls, Shioi et al. (1995) applied the neutral ammonium acetate buffered mobile phases, previously developed by Garrido and Zapata (1993b), and an ODP column for the simultaneous gradient elution and separation of BChls, and of acidic and esterified Chls of plant and algal origin: this method also achieved the separation of their [MV]- and [DV]-species.

III. Mass Spectrometry as High Performance Liquid Chromatography Detection Technique Applied to Chlorophylls

Combined LC-MS is one of the most important analytical techniques of the last decade of the twentieth century. In the early period many interfaces were

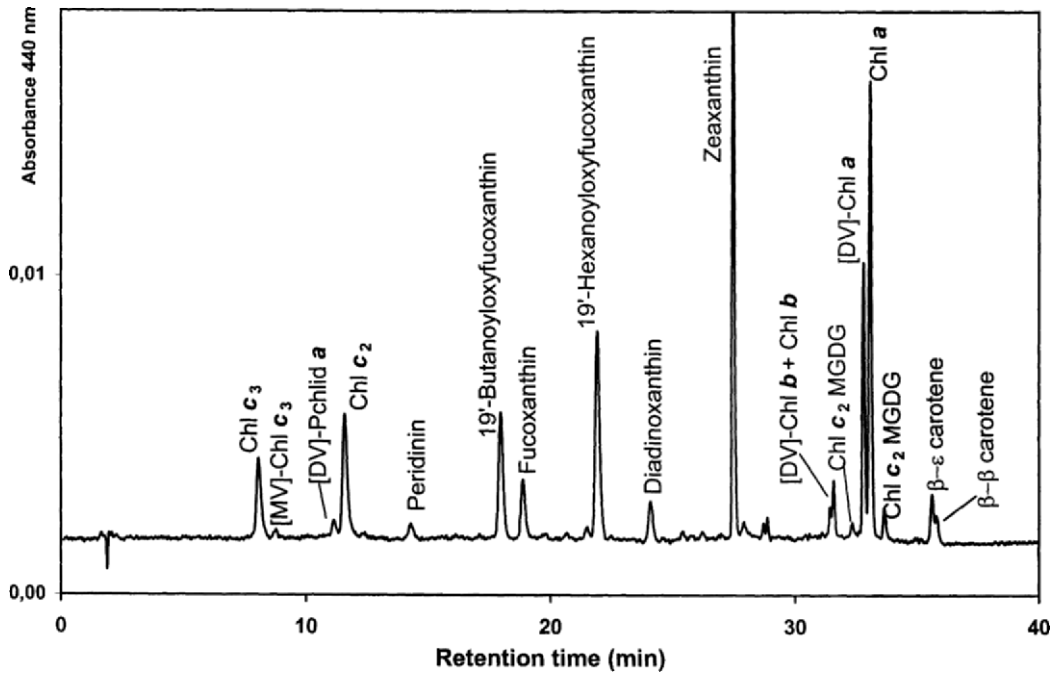

Fig. 3. Chromatogram of a pigment extract from a natural phytoplankton sample from Subtropical North Atlantic waters obtained with the method of Zapata et al. (2000) employing a monomeric octylsilica (OS) column (see Table 3). MV and DV refer to mono and divinyl derivatives.

developed, including moving belt, thermospray and others, which have gradually disappeared. The introduction of interfaces applied in combination with atmospheric pressure ion sources, such as electrospray and atmospheric-pressure chemical ionization, have improved the ease of operation, robustness, detection limits and applicability ranges of LC-MS so that it is routinely used in both industrial and research laboratories. Most LC-MS applications still employ single and triple quadrupole instruments, but other MS instruments (specially ion-trap and time of flight analyzers) are being used successfully (for comprehensive reviews on instruments and applications see Abian, 1999; Niessen and Voyksner, 1998; Niessen, 1999a,b).

Chls presented special analytical challenges to traditional MS methods because of their high mass, low volatility and thermal instability. The introduction of desorption ionization methods offered a new means of molecular ion detection (and thus direct molecular weight determination). The occurrence of additional signals in the lower mass regions enables the building of fragmentation schemes for the target compound, that can be very useful in structure determinations (Schoefs, 2001). Similar information can be obtained from atmospheric ionization methods and their combination with HPLC can provide structural information with very high sensitivity. Consequently, most modern LC-MS methods for Chl analysis employ atmospheric pressure ionization interfaces, always in combination with reversed phase chromatographic methods. The main applications are in the study of Chl alteration products (specially in natural environments like marine or lake sediments) and the characterization of newly discovered algal Chls (Table 1).

IV. Applications

A. Higher Plant Chlorophylls

Generally, a 'standard' method for the separation of higher plant Chls would employ reversed-phase columns, mainly with C_{18} monomeric chemistry, and a gradient system in which a slightly aqueous (5–20%) mobile phase is gradually substituted by an eluent containing an organic solvent of high eluotropic strength (most frequently ethyl acetate or acetone). Different authors have introduced many variations

Table 1. Liquid chromatography-mass spectrometry conditions used in the analysis of chlorophylls

Reference	Liquid chromatography	Interface	Mass spectrometry	Compounds separated
[1] van Breemen et al. (1991)	Column: RP-C_{18} polymeric Mobile phase (composition by volume): A: ethyl acetate: methanol: water: glycerol (15:65:20:0.5) B: ethyl acetate: methanol: water: glycerol (60:30:10:0.5) (binary gradient)	Continuous flow FAB	Positive ions. Double-focusing mass spectrometer	Chl *a*, Chl *b*, Chlide *a*, Chlide *b*, Phe *a*, Phe *b*, Pheide *a*, Pheide *b*, PyroPhe *a*, PyroPhe *b*
[2] Eckardt et al (1991)	RP-C_{18} monomeric Mobile phase: A: acetone; B: methanol; C: water (ternary gradient)	Thermospray	Negative ions Single quadrupole mass spectrometer	Chlorin derivatives from lake sediments
[3] Harris et al. (1995)	RP-C_{18} monomeric Mobile phase: A: acetone; B: methanol; C: water (ternary gradient)	Atmospheric pressure chemical ionization	Positive ions Single quadrupole mass spectrometer	Chlorin derivatives from marine sediments
[4] Garrido and Zapata (1998)	Column: RP-C_{18} polymeric Mobile phase (composition by volume): A: methanol: acetonitrile: 0.25 M pyridinium acetate, pH 5 (45:35:20) B: acetone (binary gradient)	Atmospheric pressure chemical ionization	Positive ions Single quadrupole mass spectrometer	Chl c_2, Chl c_3, Chl *a*, [MV]-Chl c_3
[5] Zissis et al. (1999)	RP-C_{18} monomeric Mobile phase: A: acetone; B: methanol; C: water (ternary gradient)	Electrospray	Positive ions Single quadrupole mass spectrometer	Chl *a*, Chl *a* allomers
[6] Verzegnassi et al. (1999)	RP-C_{18} monomeric Mobile phase: A: acetone; B: methanol; C: water (ternary gradient)	Atmospheric pressure chemical ionization	Positive and negative ions Single quadrupole mass spectrometer	Chlorin derivatives from lake sediments
[7] Airs and Keely (2000)	RP-C_{18} monomeric Mobile phase (composition by volume): A: 1 M ammonium acetate: methanol (25:75) B: methanol: ethyl acetate: acetonitrile: (50:30:20) (binary gradient)	Atmospheric pressure chemical ionisation Post column (pre-interface) addition of formic acid	Positive ions Ion trap mass spectrometer	Chl *a*, Phe *a*
[8] Goericke et al (2000)	RP-C_8 monomeric Mobile phase (composition by volume): A: 0.5 M ammonium acetate: methanol (30:70) B: methanol (binary gradient)	Electrospray	Positive ions Ion trap mass spectrometer	Chl c_1, Chl c_2, Chl c_3, Chl *b*, Chl *a*, [MV]-Chl c_3, [DV]-Chl *a*, [DV]-Chl *b*, [DV]-Phe *b*, [DV]-PyroPhe *a*, *b*.
[9] Verzegnassi et al. (2000)	RP-C_{18} monomeric Mobile phase: A: acetone; B: methanol; C: water (ternary gradient)	Atmospheric pressure chemical ionization	Positive and negative ions Single quadrupole mass spectrometer	Chl *a*, Chl *b*, BChl *a*, BPhe *a*, Chl *a* allomers, Chl *b* allomers
[10] Airs et al. (2001)	RP-C_{18} monomeric Mobile phase (composition by volume): A: 0.1 M ammonium acetate: methanol: acetonitrile (5:80:15) B: methanol: acetonitrile: ethyl acetate (20:15:65) C:methanol: acetonitrile: ethyl acetate (1:1:98) (ternary gradient)	Atmospheric pressure chemical ionization	Positive ions Ion trap mass spectrometer	Chl *a*, Phe *a*, Pheide *a* Pyro-Pheide *a* esters (esterified to different alcohols); BPhe *a*, *c*, *d* and *f* esters(esterified to different alcohols)
[11] Gautier-Jacques et al. (2001)	RP-C_{18} monomeric Mobile phase: A: 0.1 M ammonium acetate; B: methanol; C: acetone (ternary gradient)	Atmospheric pressure chemical ionization	Positive ions Triple quadrupole mass spectrometer	Chl *a*, Chl *b*, Phe *a*, *b*; Phe *a*, Phe *b*, Chl *a* allomers, Chl *b* allomers, PyroPhe *a*, PyroPhe b, Pyro-Pheide *a*, PyroPheide *b*

to this scheme (Table 2) to achieve specifically-tailored methods for the separation of certain Chls. These modifications were made to both mobile and stationary phases: frequently eluents contain different buffering or ion-pairing reagents to achieve adequate retention of acidic Chls, or different organic components to modulate the required selectivity; changes to the stationary phase are mainly to affect the bonding chemistry of the silica-based materials.

Methods developed for algal pigments are versatile and have been used to resolve higher plant compounds: Schoefs et al. (2000) employed a method, originally developed to resolve the [MV]-/[DV]-pair, Chl c_1/c_2 (Garrido and Zapata, 1996), to separate [MV]-PChlide from [DV]-PChlide (also known as MgDVP); and, very recently, McGhie and Ainge (2002) determined the Chl and carotenoid composition of kiwi fruit employing a method originally developed to assess the pigment composition of marine phytoplankton (Wright et al., 1991).

B. Algal and Cyanobacterial Chlorophylls

Aquatic photosynthetic organisms have evolved a great variety of photosynthetic pigments that allowed them to succeed in changing light environments (Jeffrey, 1980). The complexity of their pigment systems has been revealed by development of better HPLC methods over the past decade to better analyze the nature and concentration of the chlorophyll components of algal and cyanobacterial photosystems. The separation of algal and cyanobacterial Chls has been pursued with HPLC (Jeffrey, 1997) using different columns with monomeric or polymeric bonded phases of various carbon chain lengths (C_8, C_{18} or C_{30}), different mobile phases developed with special regard to ion pairing agents such as ammonium, tetrabutylammonium or pyridinium ions, and column temperatures (Table 3). Advances in HPLC have been valuable for the discovery of new forms of Chl *c* in algae (Chapter 3, Zapata et al.), for the detection of Chl *d* as the predominant pigment in the cyanobacterium *Acaryochloris marina* (Miyashita et al., 1997) and for the recognition of the importance of picoplanktonic organisms containing [DV]-forms of Chls *a* and *b* in oceanic ecosystems (Goericke and Repeta, 1993).

HPLC analysis of photosynthetic pigments in phytoplankton and its importance in studies of aquatic ecology has recently been reviewed (Jeffrey et al., 1999).

C. Bacteriochlorophylls

At least six BChl chromophores are known: three are Mg-bacteriochlorins (BChls *a*, *b* and *g*), in which two rings (B and D) are reduced; and three are Mg-chlorins (BChls *c*, *d*, and *e*) in which only ring D is reduced. The esterifying alcohol may be phytol, farnesol or many others and some exist as a series of homologues with different alkyl substituents at C8 and C12 (Chapter 1, Scheer).

The HPLC analysis of BChls has been restricted to the use of monomeric ODS or ODP columns, with good results when separate families of compounds or isolated species are analyzed (Table 4). However, when natural samples have to be examined the chromatograms become extremely complex and very specific detection techniques such as MS are needed for proper identification (Airs et al., 2001).

V. Future Directions in the High Performance Liquid Chromatography Analysis of Chlorophylls

Improvement in HPLC analysis of Chls by introduction of new chromatographic columns, new mobile phases and new detection techniques will achieve shorter analysis times, better sensitivity and higher specificity.

Monomeric OS materials need development for analysis of higher plant Chls: a stationary phase which achieves good separations of [MV]- and [DV]-forms of algal Chls (Rodríguez et al., 1998; Zapata et al., 2000) will probably also separate [MV]- and [DV]-analogues of Chls *a* and *b* and their biosynthetic intermediates.

A major breakthrough can be expected with the introduction of monolithic columns which have one rod of continuous porous silica possessing large through-pores and mesopores, that results in greater permeability, giving rise to columns with high efficiencies and low column back-pressures which can be operated at high flow rates, which increase separation speed, while maintaining a high efficiency comparable with that of particulate materials smaller than 3 μm (Cabrera et al., 2000).

Consequently, separations of Chls that required long analysis periods with traditional packed columns can be achieved in very short analysis times (Garrido et al., 2003). Fig 4 shows the separation of Chls *a* and *b* and their demetallated and dephytylated

Table 2. High performance liquid chromatography methods used for the analysis of higher plant chlorophylls (nr: not reported)

Reference	HPLC method characteristics			Compounds separated
	Stationary phase	Mobile phase		
	Type Brand	Elution type t (run time, min)	Solvent composition (by volume) F (Flow rate, ml min^{-1}), T (Temperature, ºC)	
[1] Canjura and Schwartz (1991)	DP-silica, 4.6 × 250 mm MacMod Analytical	Binary gradient t=28	A: hexane: 2-propanol (98.3:1.7) B: hexane: 2-propanol (75:25) F: 1.6, T= nr	Chl *a*, Chl *b*, Chlide *a*, Chlide *b*, Phe *a*, Phe *b*, Pheide *a*, Pheide *b*
[2] Canjura and Schwartz (1991)	RP-C_{18} monomeric, 5 µm, 4.6 × 250 mm MacMod Analytical	Binary gradient t=22	A: ethyl acetate: methanol: water (15:65:20) B: ethyl acetate: methanol: water (60:30:10) F: 1.3, T= nr	Chl *a*, Chl *b*, Chlide *a*, Chlide *b*, Phe *a*, Phe *b*, Pheide *a*, Pheide *b*
[3] Duke et al. (1993)	RP- C_{18} monomeric 5 µm, 4.6 × 250 mm Spherisorb ODS 1	Binary gradient t=40	A: 0.1 M $(NH_4)_3PO_4$ (pH 5.8) : methanol (20:80) B: methanol F: 1.4, T= nr	PChlide, protoporphyrins
[4] Drexler and Ballschmitter (1994)	DP-diol Serva Polyol Si 100 i)5 µm, 4.0 × 120 mm ii)5 µm, 4.0 × 250 mm	Isocratic t(i)=10 t(ii)=20-30	ethanol: hexane (2:100), column (i) (for chlorophylls) F: 1.5 ethanol: hexane (0.375-0.5:100), column (ii) (for metalloporphyrins) F: 1.0 or 2.0, T= nr	Chl *a*, Chl *a´*, Chl *b*, Phe *a*, Phe *b*, metalloporphyrins
[5] Schoefs et al. (1995)	RP- C_{18} monomeric 4.65 µm, 4.6 × 250 mm Zorbax	Binary gradient t=35	A: acetonitrile: methanol (70:30) B: dichloromethane F: 1.0, T= 20	[MV]-PChlide, Chl *a*, Chl *b*, Chlide *a* and PChlide *a* esterified to gg, DHgg, THgg and p, carotenoids
[6] Shioi et al. (1995)	ODP 5 µm, 4.6 × 250 mm Asahipak ODP 50	Binary gradient t=35	A: 1 M ammonium acetate: methanol (20:80) B: acetonitrile: acetone (70:30) F: 1.0, T= 27	[MV]- and [DV]- forms of Chlides, PChlides, Phes, Ppheids, Chl *a* and Bchl *a* esterified with gg, DHgg, THgg and p
[7] Eijckelhoff and Dekker (1995)	RP-C_8 monomeric 5 µm, 4.6 × 250 mm Spherisorb	Isocratic t=15	Methanol F: 0.7, T= nr	Chl *a*, Chl *a´*, Phe *a*, β,β-carotene
[8] Nakamura and Watanabe (1998)	RP- C_{18} monomeric 5 µm, 4.6 × 250 mm Spherisorb ODS 2	Isocratic t=40	acetone acetonitrile methanol water (43:32:15:10) F: 1.0, T= 20	Chlide *a*, PChlide *a*, Chl a_{gg}, Chl a_{DHgg}, Chl a_{THgg}, Chl *a*, Chl *a´*, Chl *b*
[9] Lebedev and Timko (1999)	RP- C_{18} polymeric 5 µm, 4.6 × 250 mm Vydac	Isocratic t=4	ethyl acetate: methanol: water (36:55:9) F: 1.0, T= nr	Chlide *a*, PChlide *a*
[10] García-Plazaola and Becerril (1999)	RP-C C_{18} monomeric 5 µm, 4.6 × 250 mm Spherisorb ODS 1	Binary gradient t=16	A: acetonitrile: methanol: water (84:9:7) B: ethyl acetate: methanol (32:68) F: 1.2, T= nr	Chl *a*, Chl *b*, Phe *a*, Phe *b*, carotenoids, tocopherols
[11] Darko et al. (2000)	RP- C_{18} monomeric 4.65 µm, 4.6 × 250 mm Zorbax	Ternary gradient t=35	A: acetonitrile B: methanol C: dichloromethane F: 1.0, T= 25	Chl *a*, Chl *b*, Chl *a´*, Chl *b´*, Phe *a* carotenoids
[12] Schoefs et al. (2000)	RP- C_{18} polymeric 5 µm, 4.6 × 250 mm Vydac	Binary gradient t=32	A: methanol: 0.025 M pyridine -pH 5 with acetic acid- (80:20) B: acetonitrile:acetone (70:30) F: 1.2, T= nr	Chlide *a*, PChlide *a*
[13] Nakamura and Watanabe (2001)	RP- C_{18} monomeric 4.6 × 150 mm Nucleosil ODS	Binary (step) gradient t=80	A: acetonitrile: methanol: ethanol: water (85:9:3:3) B: acetonitrile: ethanol (85:15) F: 1.2, T= 20	Chl *a*, Chl *a´*, Phe *a*, phylloquinone
[14] Nakamura et al. (2001)	DP-silica 6 × 150 mm Senshupack	Isocratic t=45	Hexane: toluene: methanol (100:4:0.8) F: 1.0, T= 4	Chl *a*, Chl *a´*, PChl and Phe *a* forms esterified with gg, DHgg, THgg and p
[15] McGhie and Ainge (2002)	RP- C_{18} monomeric 5 µm, 4.6 × 250 mm Spherisorb ODS 2	Ternary gradient t=24	A: methanol : 0.5 M ammonium acetate (80:20) B: acetonitrile: water (90:10) C: ethyl acetate F: 1.0, T= nr	Chl *a*, Chl *b*, carotenoids

Table 3. Selected [(1)] high performance liquid chromatography methods used in the analysis of algal chlorophylls

Reference	HPLC method characteristics			No. of fractions separated for each Chl type			Composition of each Chl fraction (coeluting compounds indicated in brackets)
	Stationary phase		Mobile phase				
	Type Brand	Elution type t (run time, min)	Solvent composition (by volume) F (Flow rate, ml min^{-1}), T (Temperature, ºC)	*c*	*b*	*a*	
[1] Garrido and Zapata (1993b)	RP-C_{18} Polymeric 5µm. 4.6 × 250 mm Vydac 201TP 54	Binary gradient t=35	A: methanol: 1 M ammonium acetate (8:2) B: acetonitrile: acetone (7:3) F=1.0, T=27	7	1	1	[MV]-Chl c_3, Chl c_3, Chl c_1, [DV]-PChlide, Chl c_2, Chl *b*, non polar Chl c_2-like *E. huxleyi*-type (2 fractions), Chl *a*
[2] Van Heukelem et al. (1994)	RP-C_{18} Polymeric 5µm. 4.6 × 250 mm Vydac 201TP 54	Binary gradient t=31	A: methanol: 0.5 M ammonium acetate (8:2) B: methanol: acetone (8:2) F=1.0 (min 0-17), 1.5 (min 22), 2.0 (min 27), T=10	6	1	2	Chlide *a*, Chl c_3, Chl c_1, [DV]- PChlide, Chl c_2, (Chl *b* + [DV]-Chl *b*), Chl *a*, [DV]-Chl *a*, non polar Chl c_2-like *E. huxleyi*-type (2 fractions)
[3] Garrido et al. (1995)	RP-C_{18} Polymeric 5µm. 4.6 × 250 mm Lichrospher PAH	Binary gradient t=40	A: methanol: 1M ammonium acetate 8:2 B: acetone F=0.8, T=27	8	1	1	[MV]-Chl c_3, Chl c_3, Chl c_1, [DV]- PChlide, Chl c_2, Chl *b*, Chl *a*, non polar Chl c_2-like *E. huxleyi*-type, non polar Chl c_1-like *Prymnesium parvum*-type, non polar Chl c_2-like *E. huxleyi*-type
[4] Van Lenning et al. (1995)	RP-C_{18} Polymeric 5µm. 4.6 × 250 mm Lichrospher PAH	Binary gradient t=48	A: methanol: 1 M ammonium acetate (8:2) B: acetone F=0.8, T=Temperature gradient, t_0-t_{15}=31ºC t_{17}=8 ºC	7	2	2	[MV]-Chl c_3, Chl c_3, [DV]- PChlide, Chl c_2, Chl c_1, Chl *b*, [DV]-Chl *b*, Chl *a*, [DV]-Chl a, non polar Chl c_2-like *E. huxleyi*-type (2 fractions)
[5] Garrido and Zapata (1997)	RP-C_{18} Polymeric 5µm. 4.6 × 250 mm Vydac 201TP 54	Binary gradient t=25	A: methanol: acetonitrile: 0.25 M aqueous pyridine pH=5.0 (4.5:3.5:2) B: acetone F= 1.2, T=15	10	2	2	Chl c_2-like *P. gyrans*-type (2 fractions), [DV]-PChlide, Chl c_1, [MV]-Chl c_3, Chl c_3, Chl c_2, Chl *b*, [DV]-Chl *b*, Chl *a*, [DV]-Chl *a*, non polar Chl c_2-like *E. huxleyi*-type, non polar Chl c_1-like *P. parvum*-type, non polar Chl c_2-like *Chrysochromulina*-type
[6] Zapata et al. (2000)	RP-C_8 Monomeric 3.5µm. 4.6 × 150 mm Waters Symmetry	Binary gradient t=40	A: methanol: acetonitrile: 0.25 M aqueous pyridine pH=5.0 (5:2.5:2.5) B: methanol: acetonitrile: acetone (2:6:2) F=1.0, T=25	11	1	2	Chl $c_{CS\text{-}170}$, Chl c_3, Chl c_2-like *P. gyrans*-type, [MV]-Chl c_3, Chl c_1-like *Kryptoperidinium*-type, [DV]- PChlide, Chl c_2, Chl c_1, ([DV]-Chl *b* + Chl *b*), non polar Chl c_2-like *E. huxleyi*-type, non polar Chl c_1-like *P. parvum*-type, [DV]-Chl *a*, Chl *a*, non polar Chl c_2-like *Chrysochromulina*-type

[1] The above six methods separate six or more chlorophyll fractions. Eighteen lesser methods for resolving algal Chls *c* are described in the following papers: Wright et al. (1991), Kraay et al. (1992), Van Heukelem et al. (1992; 1994), Garrido and Zapata (1993a; 1996; 1998), Goericke and Repeta (1993), Saitoh et al. (1993; 1995), Vidussi et al. (1996), Barlow et al. (1997), Miyashita et al. (1997), Rodriguez et al. (1998), Goericke et al. (2000) and Van Heukelem and Thomas (2001).

Table 4. High performance liquid chromatography methods used in the analysis of bacteriochlorophylls

Reference	HPLC method characteristics					Compounds separated
	Stationary phase	Mobile phase				
	Type Brand	Elution	Solvent composition (by volume)	Flow rate (ml min^{-1})	Run time (min)	
[1] Borrego and García-Gil (1994)	RP- C_{18} Monomeric 5 µm. 4.6 × 250 mm Spherisorb S5 ODS2	Binary gradient	A: methanol: 1M ammonium acetate (7:3) B: methanol: ethyl acetate: acetonitrile (5:3:2)	0.5	60	BChl *e*1-*e*3, BChl *c*1, BChl *d*1, BChl *e*4, BChl *c*2, BChl *c*3, BChl *c*4, BChl *d*2, BChl *a*, BChl *d*3*
[2] Shioi et al. (1995)	ODP 5 µm. 4.6 × 250 mm Asahipak ODP-50	Binary gradient	A: methanol: 1M ammonium acetate (8:2) B: acetonitrile: acetone (7:3)	1.0	35	BChlide *a*, BPheide *a*, BChl a_{gg}, BChl a_{gg}, BChl a_{DHgg}, BChl a_{THgg}, BChl a_p, BPhe a_p
[3] Frigaard et al. (1996)	RP- C_{18} Monomeric 4 µm. 3.9 × 300 mm Nova-Pak C18	Binary gradient	A: methanol: acetonitrile: water (42:33:25) B: methanol: acetonitrile: ethyl acetate (39:31:30)	1.0	60	Major BChl *c* homologues, major BChl *d* homologues, major BChl *e* homologues, BChl *b*, BChl *a*
[4] Airs et al. (2001) Method A	RP- C_{18} Monomeric 3 µm. 4.6 × 150 mm Spherisorb ODS2	Ternary gradient	A: 0.1 M ammonium acetate: methanol: acetonitrile (5:80:15) B: methanol: acetonitrile: ethyl acetate (20:15:65) C: methanol: acetonitrile: ethyl acetate (1:1:98)	0.7	110	BPhe c/d_p, BPhe $c_{C\text{-}14}$, BPhe c_{HHf}, BPhe $c_{C\text{-}16}$, BPhe *a*, BPhe $d_{C\text{-}17}$, Chl BPhe $d_{C\text{-}16}$, BPhe $c_{C\text{-}17}$, BPhe $c_{C\text{-}18}$
[5] Airs et al. (2001) Method B	RP- C_{18} Monomeric 3 µm. 4.6 × 150 mm Spherisorb ODS2	Binary gradient	A: 0.1 M ammonium acetate: methanol: acetonitrile (5:80:15) B: 0.1 M ammonium acetate: methanol: Acetonitrile: ethyl acetate (!:32:15:52)	0.7	80	BChls *e*1-*e*4, BChl *e* homologues (I-XII) from *Chlorobium phaeobacteroides**
[6] Airs et al. (2001) Method C	RP- C_V Monomeric 3 µm. 4.6 × 150 mm Spherisorb ODS2	Binary gradient	A: 0.1 M ammonium acetate: methanol: acetonitrile (5:80:15) C: methanol: acetonitrile: ethyl acetate (19:1:80)	0.7	100	BPhe $c_{C\text{-}16}$, BPhe *a*, BPhe $d_{C\text{-}16}$
[7] Goericke (2002)	RP- C_{18} Monomeric 3 µm. 4.6 × 150 mm Supelco Discovery	Binary gradient	A: acetonitrile: water (6:4) B: acetone	1.5	12	BChl *a*

* The numbers for BChls *c*, *d* and *e* are not in subscript text, as in the Chl *c* series, because they refer to an HPLC order of elution rather than a precisely known chemical structure. A nomenclature system for BChls *c*, *d* and *e* of known chemistry has been proposed by Smith (1994).

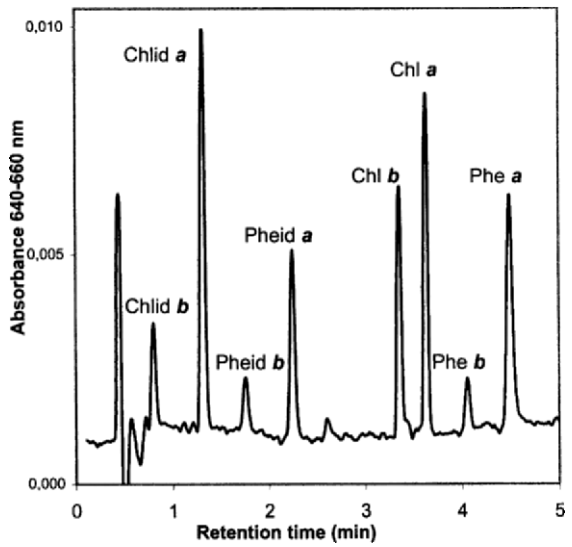

Fig. 4. Chromatogram of chlorophylls, chlorophyllides, pheophytins and pheophorbides *a* and *b* on a monolithic silica column. Chromatographic conditions as described in Garrido et al. (2003).

derivatives in less than five minutes.

The coupling of HPLC with LC-UV-photodiode array detection and LC-MS, has provided powerful tools for the identification of chlorophylls. These techniques, however, do not always allow a full identification. HPLC coupled to NMR represents a potentially interesting complementary technique for detailed on-line structural analysis. Recent progress in NMR technology has given a new impulse to LC/NMR which is now emerging as a powerful analytical tool (Vogler et al., 1998). The development of efficient solvent suppression techniques, both on-flow and stop-flow, allows the measurement of high quality LC/^{1}H-NMR spectra with reversed-phase HPLC conditions. Non-deuterated solvents such as methanol or acetonitrile can be used, while water is replaced by D_20. The concomitant development of robust automated data analysis tools has facilitated interpretation of the vast amount of NMR data generated (Stockman, 2000; Wolfender et al., 2001).

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