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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF TETRAPYRROLE PIGMENTS

## PHEOPHYTINS ESTERIFIED WITH DIFFERENT DITERPENE ALCOHOLS, ISOMERIC BILIVERDINS AND SYNTHETIC BILINS

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SUMMARY

Complex mixtures of pheophytins esterified with different C-20 diterpene alcohols have been separated by high-performance liquid chromatography in a reversed-phase system. The isomers of some monomeric and dimeric biliverdins have also been studied. Analytical and semi-preparative work is reported.

## INTRODUCTION

Because of its speed, ease of quantitation and the wide variety of adsorbents, high-performance liquid chromatography (HPLC) has become increasingly important in the separation of non-volatile compounds. We wish to report the application of HPLC to the separation of tetrapyrrole pigments encountered in studies on the biosynthesis[1] and reactivity[2] of photoactive plant tetrapyrrole pigments. The HPLC of complex mixtures of plant pigments involving an elaborate program on a single column has been reported recently[3]; this paper focuses on the analysis of mixtures of compounds very similar to each other, *e.g.*, of pheophytins esterified with different diterpene alcohols, and of isomers of mono- and dimeric bile-pigments. From a similar point of view, Evans *et al.*[4] have investigated the separation of isomeric porphyrins.

## EQUIPMENT AND METHODS

### *Liquid chromatography*

HPLC was carried out with a system consisting of two Waters Model 6000 A pumps, an injector (Model U6K) and an LDC Model 1202 variable-wavelength double-beam detector fitted with a 8-$\mu$l flow-through cell (10 mm path-length). For maximum sensitivity, the detector was set to the maxima of the pigment's main absorption bands; for positive identification, the monitor was also set to other characteristic wavelengths, *e.g.*, to 667 nm for pheophytins of the *a* series. Runs were

made at room temperature with the solvent compositions given in the text or in the legends to the figures.

*Gas chromatography of pheophytin-esterifying alcohols*

Gas chromatography (GC) of the pheophytin-esterifying alcohols was carried out with a GI gas chromatograph (Carlo Erba, Milan, Italy), equipped with a WG 11 glass capillary (25 m × 0.3 mm I.D.) (Werner Günther, Düsseldorf, G.F.R.). The carrier gas was hydrogen (4 ml/min), the injection was splitless at 160° oven temperature and the temperature was raised in 2 min to 180°. Under these conditions, retention times for the alcohols were 10.5 min (P)*, 14.5 min (THGG), 17.5 min (DHGG) and 22.5 min (GG). The sensitivity was 0.06 nmole of alcohol in 2 $\mu$l of hexane injected.

Both the gas and the liquid chromatograph were connected to an Autolab system IV integrator (Spectra-Physics, Darmstadt, G.F.R.).

*Pigments and reagents*

*Pheophytins.* 7 days old aetiolated oat seedlings (*Avena sativa* L.; Bayerische Futter und Saatbau) were harvested, and the leaves were irradiated with white fluorescent light (2000 lux) for 1 min. After a dark period (*ca.* 20 min at 23–24°), the mixture of esterified pheophytins was isolated by standard procedures as described earlier[1].

*Alcohols.* The esterifying alcohols were obtained by the usual method of saponification of the pheophytins[1].

*Bile pigments.* Biliverdins (IX$\alpha$–IX$\delta$) were obtained by oxidative degradation of hemin (Sigma)[5], the octaethylbilins by cleavage of octaethylchlorin[6]. The dimers 1–4 were obtained photochemically[7]. All solvents were distilled before use.

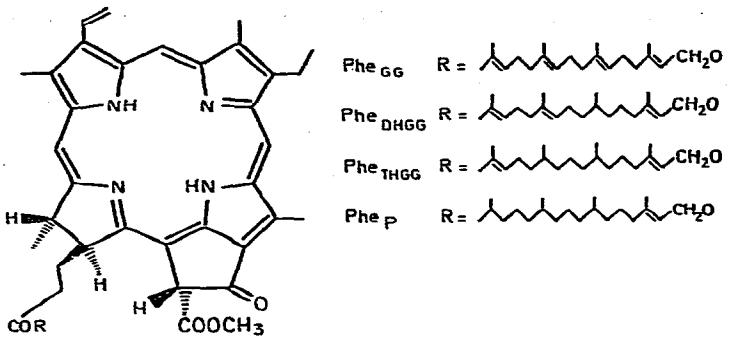
## RESULTS AND DISCUSSION

*Pheophytins*

During our research on the last steps of chlorophyll biosynthesis, we have recently detected a series of intermediates between Chlid and Chl *a* esterified with different diterpene alcohols[1]. By analysis of the kinetics by which these intermediates are formed, it was possible to formulate a sequence in which Chlid is first esterified with GG and then hydrogenated via $Chl_{DHGG}$ and $Chl_{THGG}$ to $Chl_P$. The quantitative analysis of these pigments was based on an indirect method (demetallation, thin-layer chromatography (TLC) on silica, and saponification of the resulting mixture of pheophytins; the alcohol mixture thus obtained was then analysed by GC[1]). To avoid this indirect and time-consuming method, we attempted to separate the esterified pigments at the pheophytin stage. Partial resolution was possible by TLC on silica previously buffered and impregnated with silver nitrate. By this means, $Phe_P$ and $Phe_{THGG}$ could be separated from $Ph_{GG}$ and $Phe_{DHGG}$, but separation was not

* Abbreviations: Chlid = chlorophyllid *a*; Chl *a* = Chlorophyll *a*; GG = geranylgeraniol; DHGG = dihydrogeranylgeraniol; THGG = tetrahydrogeranylgeraniol; P = Phytol; $Chl_P$, $Chl_{THGG}$, $Chl_{DHGG}$ and $Chl_{GG}$ = chlorophyllid *a* esterified with the respective alcohols; Phe *a* = pheophytin *a*; $Phe_P$, $Phe_{THGG}$, $Phe_{DHGG}$ and $Phe_{GG}$ = Pheophorbide *a* esterified with the respective alcohols.

complete, and elution of the pigments from the TLC plates gave very low yields, owing to partial formation of silver complexes[8].




Recent reports[3,4] on the separation of plant pigments prompted us to apply HPLC. As pheophytins esterified with GG, DHGG, THGG and P differ only by the number of double bonds in the hydrophobic (alcohol) part of the molecule[8,9], reversed-phase chromatography should be most suitable for this approach.

A typical chromatogram for analytical separation of the esterified mixture of pheophytins on a microparticulate $C_{18}$ bonded reversed-phase column is shown in Fig. 1. The chromatogram has five peaks, in contrast to the GLC analysis of the saponified mixture of pheophytins, which yields only the four isoprenoid alcohols, P, THGG, DHGG and GG (first column of Table I). To obtain sufficient material for identification of the peaks, the HPLC column was overloaded (80–100 $\mu$g of Phe

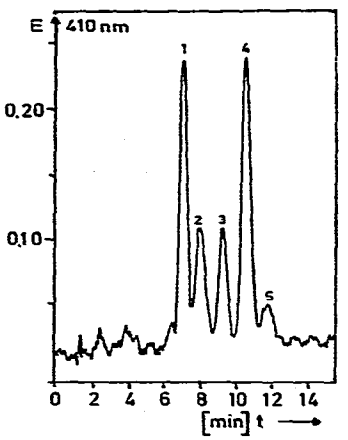



Fig. 1. HPLC of a mixture of $Phe_{GG}$, $Phe_{DHGG}$, $Phe_{THGG}$ and $Phe_P$ on a stainless-steel column (4 × 300 mm) packed with $\mu$Bondapak $C_{18}$ (Waters), with methanol–acetone (90:10) v/v) as eluent; flow-rate, 1.5 ml/min at 22°. For maximum sensitivity, the detector was set at 410 nm (Soret band; sensitivity 0.1 nmole of pigment/2 ml). A similar pattern was obtained at the characteristic wavelength of 667 nm, but the sensitivity was poorer by a factor of *ca.* 3 (decreased $\varepsilon$, increased signal-to-noise ratio).

TABLE I

GAS CHROMATOGRAPHIC ANALYSIS OF THE ESTERIFYING ALCOHOLS

All values are expressed in nmoles. The compositions shown are those of the pheophytin mixture (column 1) and the five fractions obtained from micropreparative HPLC (for conditions, see legend to Fig. 1).

| *Alcohol* | *Phe mixture (614)** | *Fraction 1 (161)** | *Fraction 2 (240)** | *Fraction 3 (67.9)** | *Fraction 4 (281)** | *Fraction 5 (67)** |
|---|---|---|---|---|---|---|
| GG | 247 | 159 | 89 | 2.8 | — | — |
| DHGG | 95 | — | 150 | 3.9 | — | — |
| THGG | 125 | — | — | 37.4 | 52 | 9 |
| P | 151 | — | — | — | 270 | 60 |

* Determined spectrophotometrically.

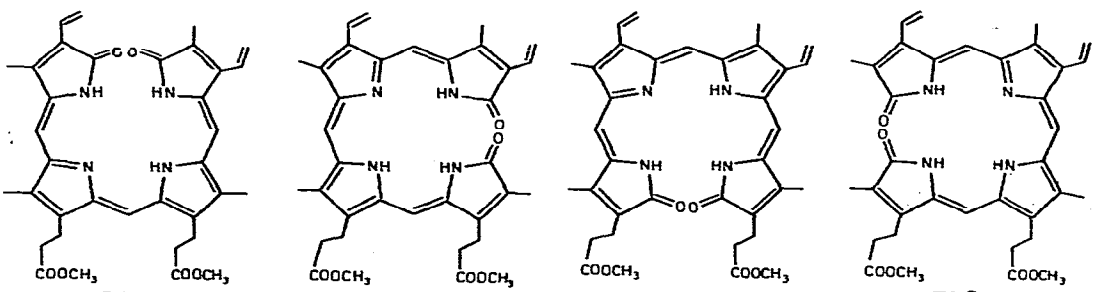
per injection), as indicated by a marked decrease in resolution, and each peak was collected. The five crude fractions (1–5) thus obtained were then re-examined by HPLC. According to this analysis, the first fraction obtained was pure, and fractions 2–5 were highly enriched. Both fractions 4 and 5 were contaminated with material from peak 3, although they were relatively well separated from the other pheophytins. Fraction 2 still contained material from peak 1, and fraction 3 of material from peaks 1 and 2. All five fractions have been identified as pheophorbide esters of the *a*-series by UV–VIS spectroscopy. As the extinction coefficients of pheophorbides esterified with different alcohols can be considered as identical, the total amount of pigment in each fraction (see column headings of Table I) was determined spectrophotometrically by using the known extinction coefficient[10] for Phe *a*.

To identify the respective esterifying alcohols, each fraction was saponified, and the alcohols were analysed quantitatively by GLC (Table I). Of the four alcohols, fraction 1 contains only GG. Thus, peak 1 of the HPLC corresponds to $Phe_{GG}$. In both fractions 4 and 5, phytol was detected as the major alcohol besides THGG. To analyse these fractions further, pheophytin *a* was isolated from green leaves and submitted to HPLC under similar conditions. The chromatogram showed a large peak 4 and a small peak 5; after saponification, phytol was the only alcohol detectable in the material from either peak. Thus, both peaks 4 and 5 are pheophytins esterified with phytol. Based on the relative amounts, peak 4 corresponds to $Phe_P$ and peak 5 then is presumably the $C_{10}$ epimer $Phe_P$ *a'* (refs. 11 and 12). If there exists an *a'* series for pheophytins esterified with other alcohols, one would always expect one large and a second smaller peak for each $Phe_{GG}$, $Phe_{DHGG}$, $Phe_{THGG}$ and $Phe_P$; the results in Table I lend support to this assumption.

With the identification of the pheophytin first eluted as $Phe_{GG}$, and the last two peaks as $Phe_P$, the HPLC mobility apparently decreases with decreasing number of double-bonds. A similar dependence on the number of double-bonds has been found for *n*-$C_{18}$ acids[13,14]. Thus, one would expect peak 2 to be $Phe_{DHGG}$, and peak 3 to be $Phe_{THGG}$. The GLC of the alcohols after saponification of the two peaks confirmed this assumption, although separation of the pheophytins was not optimum. According to these results, it is possible to separate the pheophorbide esters by HPLC according to their esterifying alcohols. Currently, this method is optimized for quantitative analysis, and to further investigate the problem of the *a'*-series pigments.

*Biliverdin dimethyl esters*

Biliverdins are the first stable products of enzymic and non-enzymic cleavage of the tetrapyrrole macrocycle in iron porphyrins; they are also obtainable by chemical oxidation of bilirubins[15]. In both cases, the "genuine" biliverdin IX$\alpha$ is accompanied by other isomers. The separation of these mixtures, which is also of potential medical importance, has been studied by several authors[5,15,16]. Analytical separation of the four type-IX biliverdin dimethyl esters ($\alpha$–$\delta$) have been reported by both one-[15] and two-dimensional[5] TLC on silica, but preparative separation was incomplete, especially for the IX$\beta$ and IX$\delta$ isomers[5]. By HPLC, the isomeric IX esters ($\alpha$–$\delta$) obtained from the non-enzymic cleavage of haemin[5] can be separated analytically both on silica and on a $C_{18}$ reversed-phase column, and preparative separation is possible on silica in milligram amounts per run. For analytical separation, gradient elution is unnecessary. The identification of the four isomers was carried out by co-chromatography with authentic samples by O'Carra's method[15]. On silica, the decreasing mobility (IX$\gamma$ > IX$\beta$ > IX$\delta$ > IX$\alpha$) corresponds to the sequence observed on TLC with mixtures of *n*-heptane–methyl ethyl ketone–acetic acid[5] or of carbon tetrachloride–acetone as mobile phase. The latter mixture, which is commonly used for the separation of cyclic tetrapyrroles, gave complete separation of the four isomers on HPTLC plates; however, this mixture should never be used in HPLC, as it is corrosive to stainless steel. Replacement of either of the solvent components decreased the separation of IX$\beta$ and IX$\delta$ considerably (due to peak broadening), but the resolution could be restored by addition of small amounts of pyridine, an effect common with many of the bile pigments studied; this effect is most pronounced with bilins having high p*K* values [*e.g.*, dihydrobilin (DHB)] and is therefore probably due to deprotonation of bilin cations present in equilibrium on the acidic silica surface. The best solvent system with regard to separation and freedom from corrosion is toluene–acetone–pyridine (90:10:1) (Table II).




On the $C_{18}$ reversed-phase, the four isomers were separated by water–methanol (22:78) (Fig. 2). The order of elution was inverted for the isomers IX$\alpha$ and IX$\gamma$, but the "inner pair" of isomers (IX$\beta$ and IX$\delta$) were eluted in the same order as from silica. For analytical purposes, this method is preferable to the use of silica because it gives better resolution and because the column is less contaminated by (polar) by-products. With the detector set at 335 nm, the detection limit with the equipment used is 2.5 pmoles with a signal-to-noise ratio of 10. As most bilins show a broad

TABLE II

SEPARATION OF ISOMERIC BILIVERDIN DIMETHYL ESTERS

These results were obtained on a micropreparative silica column [7 × 600 mm; 37–75 μm Bondapak packing (Waters)], with a linear gradient (40 min) from 100% to 50% toluene–pyridine (99:1, v/v), the remaining eluent being toluene–acetone–pyridine (70:30:1, v/v) (6 ml/min). For analysis on reversed-phase microparticulate column, see Fig. 2. The values are expressed as a percentage of total peak area.

| *Fraction of preparative separation* | *Isomer* | | | |
|---|---|---|---|---|
| | *IXα (11.1 min)** | *IXβ (13.3 min)** | *IXδ (14.5 min)** | *IXγ (18.8 min)** |
| 1 | — | — | — | >97 |
| 2 | 4 | 96 | — | — |
| 3 | — | 7 | 93 | — |
| 4 | >97 | — | — | — |

* Retention time on analytical column.

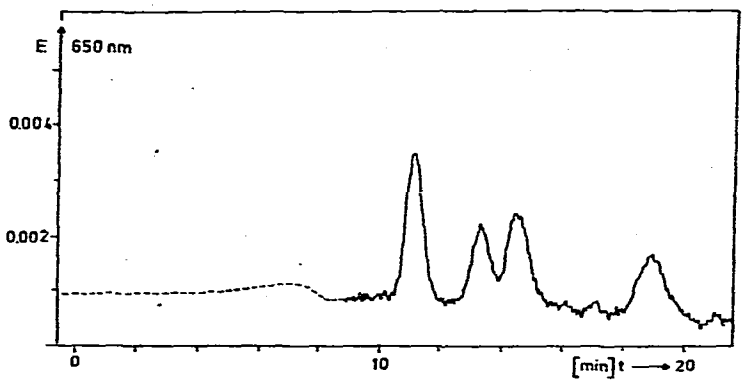


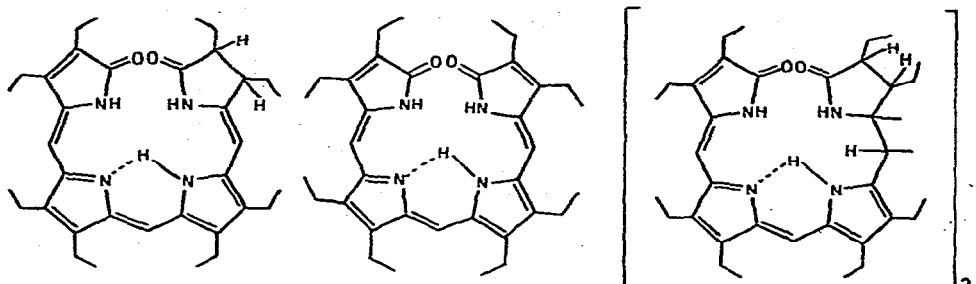

Fig. 2. HPLC of a mixture of biliverdins IXα, IXβ, IXγ and IXδ. Column as in Fig. 1, but with water–methanol (22:78) as eluent (1.5 ml/min). Elution order: IXα, IXβ, IXδ, IXγ.

absorption band in the range 300 to 350 nm, detection at 645 nm is more suitable for the selective detection of the verdins, although there is concomitant loss of sensitivity by a factor of 5–10 (decreased $\varepsilon$, increased signal-to-noise ratio). As the absorption spectra of the isomers IXα, IXβ, IXγ and IXδ are very similar, the relative peak areas were taken as being proportional to the isomer distribution.

The semi-preparative separation of the biliverdins in amounts of 1–5 mg was possible on preparative silica by gradient elution with a 50-min cycle time (Table II). Preparative separation on the $C_{18}$ reversed-phase column was less satisfactory, owing to partial irreversible absorption of the bilins. It appears that reversed-phase separations are generally more difficult to scale-up. One problem is irreversible adsorption on free OH-groups, although this can be reduced efficiently by pre-treating the column material with silylating agents (10% of BSTFA in absolute tetrahydrofuran; 2 h; 70°). However, even after this treatment, the selectivity of the preparative (37–75 μm) material differs from that of the microparticulate phase (10 μm). Similar difficulties have also been encountered with the pheophytins.

*Synthetic bilins*

2,3-Dihydro-octaethylbilindione (DHB), which has been prepared to study the reactivity of the chromophore characteristic for plant bile pigments, undergoes light-induced dimerization in the presence of one-electron oxidants[7]. The reaction mixtures containing mainly DHB, the fully unsaturated octaethylbilindione (OEB) and the dimers 1–4 (see formula) can be analysed on both silica and on reversed-phase microparticulate packings.




On silica, the best solvent system is again toluene–acetone containing small amounts of pyridine to prevent spreading. Independently of the pyridine concentration, the relative elution order of the monomers DHB and OEB, as well as that of dimers 1–4, is similar to that on silica HPTLC plates [carbon tetrachloride–acetone (9:1)]. However, although the monomers precede the dimers on plates, they are retarded by the pyridine necessary during HPLC and overlap with the dimers (Table III). Nevertheless, complete analysis is possible by making two consecutive developments with different detector wavelengths, *e.g.*, 610 nm (selective for DHB) and 570 nm (for the dimers). On reversed-phase columns, the most pronounced effect is a distinct separation of the fast-migrating monomers from the slow-migrating dimers (Table 3), which persists after changing the eluent composition and indicates a pronounced sieving effect. As the discrimination between dimers 1–4 is less pronounced (Table III), however, silica is the superior stationary phase.

TABLE III

RETENTION TIMES OF SYNTHETIC MONO- AND DIMERIC BILINS

Values are for HPLC on 10-$\mu$m microparticulate columns (4 × 300 mm). Conditions on silica: solvent A, toluene–pyridine (98.5:1.5, v/v); solvent B, toluene–acetone (9:1, v/v); ratio A:B = 9:1, increased to 1:1 after elution of dimer 3. Flow-rate 2.5 ml/min. Conditions on reversed-phase column: methanol as eluent (1.5 ml/min).

| *Column* | *Retention time (min)* | | | | | |
|---|---|---|---|---|---|---|
| | *DHB* | *OEB* | *Dimer 1* | *Dimer 2* | *Dimer 3* | *Dimer 4* |
| Silica | 3.3 | 4.8 | 2.9 | 3.3 | 6.5 | 10.0 |
| $C_{18}$ reversed-phase | 3.6 | 3.1 | 9.9 | 7.4 | 9.6 | 8.2 |

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