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REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY OF CHLOROPHYLL DERIVATIVES

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SUMMARY

A reversed-phase thin-layer chromatographic (TLC) procedure using layers of Kieselguhr G impregnated with triolein, castor oil or paraffin oil is described. Eighteen identified and eight unknown derivatives of chlorophylls *a*, *b* and *c* can be resolved from the parent compounds. These include allomers, isomers, pheophytins, pheophorbides and chlorophyllides. In addition, at least six derivatives of bacteriochlorophyll *a* and a variety of algal and bacterial carotenoids can also be fractionated.

Recoveries are not quantitative, ranging from 65 to 95%, but are highly reproducible (standard deviation 3% or less). Hydrated extracts can be chromatographed directly, thereby eliminating the need for time-consuming and potentially destructive drying procedures. When the pigments are applied to the layers under a nitrogen atmosphere, no degradation is observed. Pigment zones are sharp and uniform and double tailing effects do not occur. The procedure is rapid, simple and relatively insensitive to many variables that have caused difficulties in adsorption TLC, such as relative humidity and the complexity of the pigment mixture.

INTRODUCTION

In present limnological, paleolimnological and oceanographical studies on the transformations of phytoplankton chlorophyll, there is a need for a rapid, quantitative, artefact-free method by which the chlorophyll degradation products can be resolved from their parent compounds[1]. Similar analytical techniques for the chlorophyll derivatives are also needed in biochemical investigations of plant materials in soils, crops and preserved foods[2]. Column and paper chromatographic procedures with adequate resolution for these purposes have been reported[3,4], but they lack the necessary simplicity, sensitivity and speed. On the other hand, present thin-layer chromatographic (TLC) methods, although fast, sensitive and easy to use, do not provide complete resolution of the more than twenty common derivatives of chlorophylls *a*, *b* and *c*. Thus, only pheophytins *a* and *b* have been separated on layers of sucrose[5], silica gel[6] and Kieselguhr G impregnated with oil[7]. Cellulose thin layers have been used by BACON[8] to resolve the *a* and *b* pheophytins, pheophorbides and chlorophyllides, and by BACON AND HOLDEN[9] to separate the

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isomers of the *a* and *b* chlorophylls and pheophytins as well as to partially resolve allomerized chlorophyll *a* from an isomer of chlorophyll *a*. A large number of additional TLC procedures for chloroplast pigments have not been evaluated with respect to resolution of the derivatives[10–17].

In the present paper, we describe and evaluate a reversed-phase TLC method (RP-TLC) by which eighteen identified and eight unidentified derivatives of chlorophylls *a*, *b* and *c* as well as six derivatives of bacteriochlorophyll *a* can be resolved.

EXPERIMENTAL

Preparation of chlorophyll derivatives

Stocks of chlorophylls *a* and *b* in diethyl ether were obtained from crude pigment extracts of *Scenedesmus quadricauda* by column chromatography[18]. The isomeric chlorophylls *a'* and *b'* were prepared by heating chlorophylls *a* and *b* in methanol for 2 h at 90°, taking care to replace the solvent lost by evaporation during the incubation. To prepare the allomerized chlorophylls, stock solutions of the native chlorophylls were evaporated to dryness *in vacuo*, taken up in dry redistilled methanol and scrubbed with dry oxygen for 72 h. The native, allomeric and isomeric pheophytins were synthesized from their corresponding chlorophylls by the method of BROWN[4]. Pheophorbides *a* and *b* were prepared in two ways: by acidic hydrolysis of the respective pheophytins with 12 *N* HCl[10] and by acidification of the corresponding chlorophyllides[4]. The chlorophyllides were synthesized by using a crude chlorophyllase preparation from cockleburr leaves (*Xanthium pennsylvanicum*). The de-veined leaves were repeatedly homogenized in a blender with ice-cold acetone until bleached. The pulp was then incubated with chlorophyll *a* or *b* for 12 h in an acetone solution diluted with 0.1 *M* N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer[20] to give a final acetone concentration of 40% and a pH of 7.8. Allomerized chlorophyllides were prepared from the allomerized chlorophylls in the same manner. Chlorophyll *c* was extracted from *Cylindrotheca cosmariun* in a mixture of acetone, methanol and water (80:15:5) and used without further purification. This crude extract was acidified directly to provide pheophytin *c*. The 80:15:5 mixture was also used for bacteriochlorophyll *a*, obtained from *Rhodopseudomonas spheroides*, and the resultant extract was used directly.

Reagent-grade chemicals and glass-redistilled water were used throughout. Stocks of acetone, methanol, diethyl ether and light petroleum were stored over Na_2SO_3–Na_2CO_3 (1:1, w/w), CaO, sodium chips and anhydrous $NaSO_4$, respectively. All solvents were redistilled before use. Pigment solutions were stored, oxygen free, at −5° in the dark.

Thin-layer chromatography

Kieselguhr G (Stahl, grain size 10 µm) was slurried with distilled water (1:2, w/v) and spread on grease-free, 20 × 20 cm polished glass plates with a Camag applicator[21]. Following air drying at room temperature for 24 h, the thin layers were impregnated by hand dipping in the required oil solution to a depth of 16 cm. Triolein (technical grade, Sigma Chemical Corp. or Matheson, Coleman and Bell Corp.) and paraffin oil (N.F. viscosity 125/135, Fisher Scientific Ltd.) were dissolved for impregnation in light petroleum (boiling range 30–35°) while castor oil (pharma-

ceutical, local purchase) was dissolved in methanol. Oiled layers were dried horizontally for at least 12 h before use.

All pigment operations were carried out under dim light and at room temperature. Pigment solutions were streaked or spotted on to the unimpregnated portion of the plate with a 100-μl gas-tight syringe (Hamilton Corp.). For quantitative analyses, the layers were placed in a plexiglass spotting box through which nitrogen gas was flushed. Uniform spot sizes were most easily obtained when the syringe needle was placed in direct contact with the layer during application. The chromatograms were developed either in sandwich chambers (Brinkmann Instruments) or in twin-plate sandwiches in unequilibrated, unlined chromatography tanks.

Quantitative estimates of the pigments were made spectrophotometrically following their removal and elution from the chromatograms. After the chromatograms had developed 10 cm, individual pigment zones were outlined under UV light, scraped from the plate with a narrow razor knife and collected in "micro-vacuum cleaners". The latter consisted of disposable Pasteur pipettes packed to a depth of 2 cm with acetone-wetted fine glass wool. The pigments were then eluted from the inverted pipettes into 1-ml calibrated flasks using two 1-ml aliquots of solvent. The aliquots were evaporated to a volume of less than 1 ml under a stream of nitrogen and the solution was then brought up to volume with fresh diethyl ether. The eluate was transferred to quartz cuvettes (1 cm path length, semi-micro) using a 1-ml gas-tight syringe (Hamilton Corp.) and the absorption spectrum determined on a Cary 14 recording spectrophotometer, equipped with a 0–0.1 absorbance slidewire. Pigments were identified on the basis of colour, chromatographic sequence and visible-light absorption spectra. Recovery efficiencies were determined by comparing the absorbance of the TLC eluate with the absorbance of the original pigment solution which had been diluted with solvent but not subjected to chromatography. The absorbance at 710 nm was deducted from the absorbance at the wavelength maximum for each pigment, as a correction for any Kieselguhr particles not retained by the "micro-vacuum cleaners".

Resolution of pigment mixtures on the chromatograms was assessed with a Turner filter fluorimeter (Model 111) and TLC scanner door (Model 2). Details of the operating conditions and procedures were given by DALEY *et al.*[22]. Separation of two adjacent pigment bands was judged to be complete if the fluorescence emission returned to zero between the bands.

RESULTS AND DISCUSSION

Resolution

As the average diameter of the pigment bands on reversed-phase thin-layer chromatograms was approximately 0.5 cm, it was clear from the outset that all of the 20 or more common chlorophyll compounds could not be resolved by a single, one-dimensional, 10-cm development. Therefore, we first attempted to find several different solvent systems for use with one oil type and concentration, which would selectively resolve pigment groups of different polarities. These attempts were unsuccessful. It was noted, however, that the R_F values could be altered by changing both the type and the concentration of the impregnation oil. After evaluating many different combinations of oil types and concentrations with various developing solvents, three primary separation systems were chosen for the analysis of complex

TABLE I

THE RP-TLC SYSTEMS FOR SEPARATING CHLOROPHYLL DERIVATIVES

See text for details.

System	Oil type	Oil (%)	Oil solvent	Developing solvent (v/v)
Primary systems				
"Low-range" (pheophytin) system	Paraffin oil	8	Light petroleum (boiling range 30–60°)	Methanol–acetone–isopropanol–water–benzene (35:50:10:10:2)
"Mid-range" (chlorophyll) system	Triolein	4	Light petroleum (boiling range 30–60°)	Methanol–acetone–isopropanol–water–benzene (60:20:10:10:2)
"High-range" (pheophorbide) system	Castor oil	10	Methanol	Methanol–acetone–isopropanol–water–benzene (80:2.5:2.5:15:2)
Auxiliary systems				
"Broad-range"	Triolein	4	Light petroleum (boiling range 30–60°)	Methanol–acetone–benzene–water (60:20:10:10)
"1.5% triolein"	Triolein	1.5	Light petroleum (boiling range 30–60°)	Methanol–acetone–isopropanol–water (45:35:10:10)
"12% triolein"	Triolein	12	Light petroleum (boiling range 30–60°)	Methanol–acetone–isopropanol–water (75:1:1:23)

mixtures of derivatives (Table I). The "low-range" (LR) system resolves the pheophytin group of derivatives, the "mid-range" (MR) system the chlorophylls, and the "high-range" (HR) system the pheophorbides and the chlorophyllides. In all instances, Kieselguhr G was used as the sorbent. RP-TLC is essentially a partition process in which the stationary phase is a non-polar oil and the mobile phase a polar mixture of organic solvents and water[21]. Consequently, the polarity sequence of the chlorophylls is the inverse of that obtained with adsorption TLC. The non-polar pheophytins remain nearest the origin, followed by the chlorophylls, the pheophorbides and the chlorophyllides.

A fluorimeter scan of a typical MR chromatogram is shown in Fig. 1a. The six common isomeric, allomeric and native *a* and *b* chlorophylls are completely resolved. Chlorophyll *c* and pheophytin *c* separate only as single spots, not as the mixture reported by DOUGHERTY *et al.*[23]. In addition, MR chromatograms showed sufficient selectivity to separate the pheophytins, pheophorbides and chlorophyllides from the chlorophylls as unresolved groups. Thus, in routine analyses, extracts were first fractionated on MR plates to ascertain the complexity of the sample and then run on LR and HR layers only if pheophytins, pheophorbides or chlorophyllides were present.

On LR chromatograms (Fig. 1b), pheophytin *a*, pheophytin *b*, pheophytin *a'* and allomerized pheophytin *b* were well separated. Allomerized pheophytin *a* and pheophytin *b'* were only partly resolved, but in practice good estimates of each could be obtained.

Determinations of the selectivity of the HR system for the non-phytylated pheophorbides and chlorophyllides proved difficult because of unexpected problems in synthesizing chlorophyllides of known identity. The phytol moiety of the chlorophyll molecule can be replaced either by hydrogen or by alkyl groups, depending on the solvent used for preparation. These acid and alkyl chlorophyllides are spec-

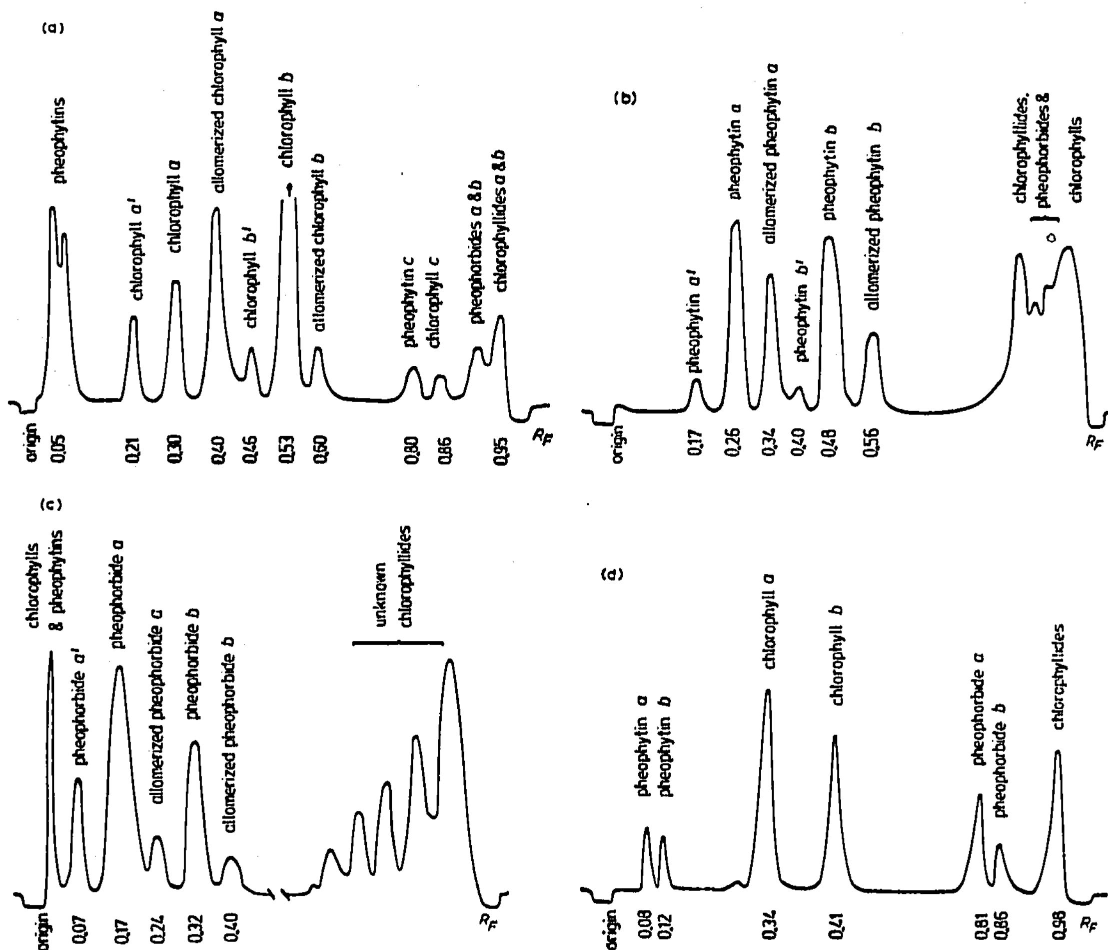

Fig. 1. Scanning fluorimeter traces of reversed-phase thin-layer chromatograms of chlorophyll derivatives. (a) "Mid-range" (chlorophyll) system. (b) "Low-range" (pheophytin) system. (c) "High-range" (pheophorbide) system, composite diagram of two scans. (d) "Broad-range" system. Details of the chromatographic systems are given in Table I.

trally similar and can be identified with certainty only from a knowledge of their chromatographic mobilities and the solvent in which they were prepared. When pure chlorophyll *a* is incubated with chlorophyllase in acetone, only one compound, acid chlorophyllide *a*, is usually formed; in methanol, only the methyl chlorophyllide should be present. However, in this study, when pure chlorophyll *a* was incubated in acetone with a chlorophyllase preparation from cocklebur and then chromatographed on HR layers, several green derivatives with spectra identical to that of chlorophyllide *a* were formed. Similar results with methanol were obtained. Repeated attempts to synthesize a single, chromatographically distinct chlorophyllide in each solvent failed. It is not clear whether the various reaction products were mixed acid and methyl forms resulting from transesterification, artefacts of chromatography, or novel, previously unresolved, derivatives. Nevertheless, tests with the prepared

derivatives showed that the HR system had considerable potential for chlorophyllide separations (Fig. 1c). In addition, small amounts of (presumably acid) chlorophyllides *a* and *b* obtained from phytoplankton samples (Lake Opinicon, Ontario) were well resolved.

The resolution of the pheophorbides on the HR thin layers was assessed more easily as authentic derivatives could be obtained by direct acidic hydrolysis of the pheophytins (Fig. 1c). Pheophorbide *b'* did not separate from pheophorbide *b*. The resolution of pheophorbide *a* from its allomer is incomplete, but was found to be adequate for quantitation. The remaining pheophorbides of the test mixture separated cleanly.

In addition to these primary separation systems designed for complex mixtures of derivatives, several other systems of use for more specialized applications were found (Table I). Mixtures of derivatives that do not contain allomers or isomers can be resolved on a single, "broad-range" chromatogram (Fig. 1d). On these layers, the *a* and *b* pheophytins, chlorophylls and pheophorbides are resolved, but all chlorophyllides remain as a single band near the solvent front. The 1.5 % triolein system (Table I) is similar to the LR system, but has proved useful in resolving a number of unknown pheophytin *a* and pheophytin *b* derivatives that are present in certain extracts of lake sediments[24]. The 12 % triolein system is similar in resolution to the HR system, but shows higher selectivity for pheophorbides. A number of unidentified sedimentary pheophorbides have been separated with this system, as have mixtures of pheophorbides and polar carotenoids.

The RP-TLC systems reported here are also suitable for resolving bacteriochlorophyll derivatives and both algal and bacterial carotenoids. However, detailed resolution studies have not been undertaken. Non-polar carotenoids, such as α- and β-carotene, separated from each other and from algal pheophytins on both the LR and 1.5 % triolein systems. On MR layers, such polar carotenoids as myxoxanthophyll and fucoxanthin are well separated. Similar RP-TLC techniques for the carotenoids have been reviewed by RANDERATH[21]. In preliminary tests on MR layers, bacteriochlorophyll *a*, bacteriopheophytin *a* and five other unidentified bacteriochlorophyll derivatives separated cleanly, as did a number of bacterial carotenoids. KIM[25] has also reported the use of RP-TLC for bacteriochlorophyll derivatives.

Formation of artefacts

BACON[26] and STRAIN *et al.*[27] have reported the formation of chlorophyll artefacts during adsorption chromatography on unimpregnated layers of Kieselguhr G. The formation of artefacts on the oil-impregnated thin layers used here was assessed in two ways. Firstly, chromatographically pure solutions of chlorophyll *a* were applied to the oil-free portions of MR chromatograms. The layers were then exposed either to air or nitrogen for 1, 10 and 30 min and developed. Secondly, layers were spotted with the same chlorophyll solution under a nitrogen atmosphere and immediately developed until the pigment spots had just entered the oiled portion of the layers. The plates were then removed from the developing chamber, exposed to air for 1, 10 and 30 min and returned to the chamber to complete development. Rapid allomerization of chlorophyll occurred on the oil-free Kieselguhr exposed to air (25 % conversion in 30 min); no other derivatives, such as pheophytins, were detected. However, allomerization was prevented in the unoiled Kieselguhr exposed to a

nitrogen atmosphere and in the oiled Kieselguhr exposed to the air. To avoid the catalytic allomerization caused by exposure of the pigments to unoiled adsorbent, plates were routinely spotted in a closed box flushed with nitrogen and then transferred to the developing chamber without delay. No further precautions, such as the aeration of extracts or developing solvents with nitrogen, were necessary. In subsequent tests, developed chromatograms, covered by a glass plate, were stored in the dark at room temperature and at −20° for 12 h and 3 weeks, respectively, without degradation of the chlorophylls.

Serious destruction of pigments can occur when very old impregnated layers, especially of triolein, are used. However, tests showed that impregnated and unimpregnated plates could be stored for 1 and 6 weeks, respectively, without producing derivatives or affecting resolution when finally developed. In routine work, impregnated plates were used within 24–48 h of preparation.

Quantitative recovery

Table II contains representative results on the recovery of selected chlorophyll derivatives from RP-TLC layers, following collection in "micro-vacuum cleaners" and elution with an appropriate solvent. Recovery was not quantitative for any of the derivatives tested, ranging from 93% for pheophytin *a* to 63% for pheophorbide *a*. However, the statistical reproducibility was high (standard deviation 3% or less). Recovery efficiencies for a given pigment varied with the type and concentration of impregnating oil and with the elution solvent used, and must therefore be re-determined whenever any of these factors are changed.

An unknown fraction of the impregnating oil is removed from the Kieselguhr with the pigment during elution. As all of the impregnation oils adsorb exclusively in the UV region, it was found that their presence in the eluate did not alter either the absorption maxima or the fine details of the visible spectra of the derivatives. However, these oil contaminants do affect the re-chromatography of deriva-

TABLE II

RECOVERY OF SELECTED CHLOROPHYLL DERIVATIVES AFTER RP-TLC

Pigment	*Chromatographic system*[a]	*Loading level* (μg)	*Elution solvent*	*Recovery* (%)[b]
Chlorophyll *a*	MR	0.9, 2.3, 7.1	Ether	75
Allomerized chlorophyll *a*	MR	2.6	Ether	79
Pheophytin *a*	1.5% triolein	1.1, 3.2, 5.6	Ether	93
Pheophorbide *a*	12% triolein	1.8, 3.2	Methanol[c]	63
Chlorophyllide *a*	12% triolein	2.6, 3.7	Methanol[c]	62
Chlorophyll *b*	MR	5.0	Ether	79
Pheophytin *b*	1.5% triolein	5.7	Ether	82
Pheophorbide *b*	12% triolein	1.8	Methanol[c]	75
Chlorophyll *c*	MR	1.8	Methanol[c]	70
Bacteriochlorophyll *a*	MR	5.1	Methanol[c]	81

[a] See Table I for details.

[b] Mean of at least four 1-ml replicates, usually more than six. Standard deviations in all instances were less than 3%.

[c] Adsorption coefficients in methanol assumed identical to those in diethyl ether given by BROWN[4] and STRAIN AND SVEC[28].

tives. When spotting RP-TLC purified compounds on to a second plate, a ring of oil was deposited, which released the derivatives slowly during development, causing streaking. The system using 1.5 % triolein plates was the only one for which this effect was found to be negligible. In addition to the pheophytins that are normally resolved with this system, small amounts of pure chlorophylls and pheophorbides could be obtained from partially purified mixtures by using a more polar developing solvent (*e.g.*, 70:15:5:10:2 methanol–acetone–isopropanol–water–benzene).

Operational characteristics

On RP-TLC plates, the pigments are held up momentarily as they enter the impregnated portion of the plate. As a result, the pigment zones are exceptionally sharp and uniform and the double-tailing effects sometimes encountered in adsorption TLC[20] are eliminated. The carrying capacity of the layers varies to some extent with the type and concentration of impregnating oil, but in all instances is high. Hence the resolution is unaffected by the amount of pigment applied to the layers over a wide range of concentrations. Extractants and developing solvents containing up to 15 % and 20 % water, respectively, can be used without any observable deterioration in resolution or formation of artefacts. Minor variations in the following factors, which are likely to occur in routine operations, had no effect on pigment resolution: Kieselguhr brand and batch, slurrying and spreading procedures, plate thickness, oil concentration, wash liquid proportions and tank saturation. The complexity of the pigment extract and the ambient relative humidity (15–90 %) also had no effect. Pigments could be spotted on to layers from any of the common organic solvents used for extractions, thereby eliminating the need for phase transfer of extracts, a procedure which is extremely difficult to quantify. Development times were found to be rapid; 10-cm developments on LR, MR and HR plates required 30, 20 and 45 min, respectively. A detailed study of the variations in R_F values with many of the above factors has not been made, but they appear to be significantly lower than with many adsorption TLC methods.

CONCLUSIONS

Comparison of the present data on quantitative recoveries with other thin-layer methods is difficult, because recoveries are not usually quoted. With RP-TLC, recovery of the chlorophylls was not complete, as with sugar layers[14], but was comparable with those obtained by BACON[8] and SCHNEIDER[10] for cellulose adsorption TLC. As recovery values are not impractically low and the reproducibility is high, RP-TLC appears to be suitable for quantitative analysis.

The earlier observations of BACON[20] and STRAIN *et al.*[27] that artefacts can form on Kieselguhr have been confirmed in the present study. However, the presence of the impregnating oil used in RP-TLC protects the pigments from these degradative effects. When the pigments are applied under nitrogen, artefact-free separations can be achieved. Preliminary observations suggest that a similar stabilizing effect of the oil on carotenoids occurs.

The impregnating oils, which are eluted from the chromatograms with the pigments, interfere seriously with subsequent re-chromatography of the pigments, especially in mixtures of more than two compounds. Consequently, RP-TLC is unsuitable as a large-scale preparative procedure.

Improved resolution of the common algal chlorophyll derivatives is possible with the TLC method described here. A total of 18 identifiable derivatives were resolved easily and quickly without degradation. However, the unambiguous resolution of a number of derivatives remains to be achieved. The difficulties encountered in this study with the synthesis of the chlorophyllides, together with our observations of multiple isomers of pheophytins and pheophorbides from sediments, suggest that the difficulties lie more with ambiguities in the chemistry of the derivatives than with inadequate RP-TLC selectivity. Hence, it appears that future improvements in resolution will require parallel chemical and chromatographic studies. With its selectivity and flexibility, RP-TLC seems to be especially suitable for such work.

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