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THIN-LAYER CHROMATOGRAPHY OF CHLOROPHYLLS AND THEIR DERIVATIVES ON SUCROSE LAYERS

INKERI SAHLBERG and PAAVO H. HYNNINEN*
Department of Biochemistry, University of Kuopio, PO Box 6, SF-70211 Kuopio 21 (Finland)
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SUMMARY

A simple method has been developed for the separation and identification of chloroplast pigments and their derivatives by thin-layer chromatography on self-prepared sucrose layers. When using the sucrose layers, a solvent system consisting of 1% 2-propanol in light petroleum is suitable for the separation and analysis of C-10 epimeric chlorophylls and their oxidation products as well as chloroplast carotenoids. The C-10 epimeric pheophytins can be resolved on sucrose layers using 2% pyridine in light petroleum as the eluent. Being a mild adsorbent, sucrose causes very few alterations in the pigments, and therefore this method can also be used for preparative purposes. At room temperature the recoveries for chlorophyll *a* and pheophytin *a* are 95.4% and 99.1%, respectively. The method is rapid and mild and is thus suitable for the easily degradable chloroplast pigments.

INTRODUCTION

When preparing chlorophylls and their derivatives, one must check the purity of the preparation and hence a simple and rapid method suitable for this analytical purpose is required. In the last few years, high performance liquid chromatography (HPLC) has served as the most popular method for the purity determinations[1] and for the separation of chloroplast pigments[2–10]. Thin-layer chromatography (TLC) has also been widely used for the same purposes[11–13] for several years. When comparing these two methods, there are several factors which should be noted. Methanol, which is used in the mobile phase in reversed-phase high-performance liquid chromatography (RP-HPLC) causes transformations of the chlorophylls[14] and should therefore be avoided. An HPLC technique using acetonitrile–water as eluent has been reported[15] but there are also other aspects which should be considered when choosing the method. The equipment used in HPLC is expensive and HPLC also requires large quantities of pure organic solvents, which increase the cost of the determination compared with the relatively inexpensive TLC. Several samples can be chromatographed at the same time in TLC compared with one sample in HPLC. Using high-performance thin-layer chromatography (HPTLC), the time of analysis will be further decreased, making it a far faster procedure than HPLC.

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Inorganic adsorbents used in TLC such as silica gel and Kieselguhr, however, cause chemical alterations in chlorophylls and carotenoids[12,16,17]. TLC on organic layers of sucrose[18–22], glucose[23,24] or cellulose[21,25,26] cause fewer alterations in the derivatives. According to Chan *et al.*[20] less than 5% degradation of chlorophyll occurred during sucrose TLC. Sucrose is superior to cellulose when we compare the resolution of the pigments. Chlorophylls and their C-10 epimers[27–29], with the 10(*S*) configuration, named chlorophylls *a'* and *b'* by their discoverers[30], are not separable on cellulose using the pyridine–light petroleum solvent system[25], due to the relatively rapid enolization–epimerization equilibration between the chlorophyll epimers under these conditions. Good resolution between the C-10 epimeric chlorophylls is achieved, however, even at room temperature, on sucrose layers when 1% 2-propanol in light petroleum (b.p. 60–80°C) is used as eluent.

The aim of this study was to develop an improved method for the separation of chloroplast pigments on self-prepared sucrose layers. The method developed is rapid and mild and can be used for checking the purity of the pigments as well as for semi-preparative purposes.

MATERIALS AND METHODS

Solvents

All solvents were of analytical grade and were used without further purification.

Sucrose layers

Powdered icing sugar (Finnish Sugar Co., Helsinki, Finland) was passed through an 80-mesh sieve and then mixed with 300 ml of 1% 2-propanol (2-PrOH) in light petroleum (LP, b.p. 60–80°C). The sucrose slurry was poured on glass plates (5 cm × 20 cm or 20 cm × 20 cm) and spread immediately with a commercial spreader giving layers 0.25 mm thick. Before the slurry was poured, the surface of the glass plates was moistened with a piece of wet, soft paper. The plates were allowed to dry for 30 min and their edges were trimmed before use.

Chlorophylls and derivatives

Chlorophylls *a* (Chl *a*) and *b* (Chl *b*) were prepared as described previously[31]. Chlorophyll *a'* (Chl *a'* = 10(*S*)-Chl *a*) was prepared from Chl *a* and chlorophyll *b'* (Chl *b'* = 10(*S*)-Chl *b*) from Chl *b*[32]. The 10(*R*)- and 10(*S*)-pheophytins *a* and *b* (Pheo *a* and *b*, Pheo *a'* and *b'*) were prepared according to Lötjönen and Hynninen[33]. 10-Hydroxy chlorophylls and 10-hydroxy- or 10-methoxy-lactone derivatives of Chl *a* were isolated from impure chlorophyll preparations by column chromatography on sucrose[14].

Plant extract

Frozen clover leaves (1.0 g) were homogenized with an Ultra-Turrax homogenizer in cold (−20°C) acetone (2.0 ml) for 1 min and the homogenate was immediately used for a TLC run.

Thin-layer chromatography

The plant extract or chlorophyll derivatives in acetone or tetrahydrofuran

(THF) were spotted 2 cm from the lower edge of the plates. The chromatograms were developed in a thin-layer chamber (22 × 10 × 25 cm) lined with chromatography paper (Whatman No. 1) and previously saturated with the eluent. The developing solvents used were (I) 1% 2-PrOH in LP, (II) 1% 1-PrOH in LP, (III) 1% pyridine (Pyr) in LP, (IV) 2% Pyr in LP and (V) 1.5% 2-PrOH in LP.

After the solvent front had ascended 16–17 cm (*ca.* 25 min), the plates were allowed to dry (*ca.* 1 min). The spots were immediately detected visually, according to colour, and further analysed by UV fluorescence detection (302 nm).

Preparative TLC and recoveries of Chl a and Pheo a

The pigments were chromatographed on a preparative plate (20 cm × 20 cm) and after development, the bands were scraped off the plate and mixed with diethyl ether or THF. Sucrose was removed by centrifugation. Absorption spectra of the pigments were measured with a Cary Model 219 spectrophotometer, and the calculated amount of the pigment recovered was compared with the actual amount put on the plate.

RESULTS AND DISCUSSION

A typical thin-layer chromatogram of a freshly prepared acetone extract of clover leaves, developed in 1% 2-PrOH in LP, is shown in Fig. 1, No. 1. The pigments are clearly separated from each other and they can also be distinguished by their colour. The rapidity of this method provides an ideal way to examine the components of leaf chloroplasts because very few or no alterations in the pigments occur during the analysis.

A chromatographic separation of chlorophylls *a* and *b* and their epimers *a'* and *b'* is demonstrated in Fig. 1, No. 2. The resolution is good and there is no difficulty in detecting any impurities in Chl *a* or Chl *b* preparations caused by the 10(*S*)-derivatives. This is an important improvement in the TLC method, because the 10(*S*)-isomers are easily formed during the isolation and purification of Chls and previously the detection of these impurities by TLC was inadequate[25]. Although pyrochlorophyll *a* differs from Chl *a* only in that instead of having a methoxycarbonyl group in the C-10 position it has a hydrogen atom, these two derivatives are clearly separated and the same holds for their magnesium-free derivatives pyropheophytin *a* and pheophytin *a* (Fig. 1, No. 3 and No. 4). The movement of the chlorophyll derivatives on sucrose thin-layers greatly depends on the self-aggregation tendency of the pigments. The magnesium-free chlorophylls (pheophytins) have the highest R_F values. The central magnesium atom, which coordinates to the 9-C=O group of another chlorophyll molecule when self-aggregation occurs, is absent from pheophytins. In Chl *a'* the methoxycarbonyl group is on the same side of the ring plane as the phytylpropionic acid residue at C-7. Since there is not enough room for both, the methoxycarbonyl group is likely to become pushed closer to the 9-C=O group. Owing to the resulting steric hindrance, Chl *a'* seems to have a reduced coordination tendency in comparison to that of Chl *a*. In pyrochlorophyll *a* there are only two hydrogen atoms in position C-10 and therefore no steric hindrance exists for the self-aggregation. Consequently, pyrochlorophyll *a* has a lower R_F value than Chl *a*.

Pheophytins *a* and *b* are clearly separated from each other (Fig. 1, No. 5). In

contrast, Pheo *a'* has the same R_F value as Pheo *a* in solvent system I and the same holds for Pheo *b'* and Pheo *b* (Table I). Pheophytins and their C-10 epimers are not separated on sucrose layers using 1% 2-PrOH in LP as the solvent system due to self-aggregation which involves a π–π-interaction between two macrocycles. It should be noted, however, that the resolution of pheophytins and their epimers depends greatly on the amount of pigment put on the plate and also on the temperature. The C-10 epimeric pheophytins are separated if the quantity of the pigment does not exceed 0.2 μg and the temperature is +4°C. The following pigments also move in the same spot when chromatographed in solvent system I: Pheo *b* and Chl *a'*, 10(*S*)-methoxy-lactone derivative of Chl *a*, lutein and Pyrochl *a*, 10-hydroxy Chl *a* and violaxanthin.

Altogether, twelve leaf pigments could be separated with 1% 2-PrOH in LP (Fig. 1, no. 7). The pigments were identified by their R_F values, UV–VIS spectra or colour. The 10(*R,S*)-methoxy-lactone derivatives of Chl *a* were scraped off the plate and identified by their spectroscopic properties[34]. The component moving between

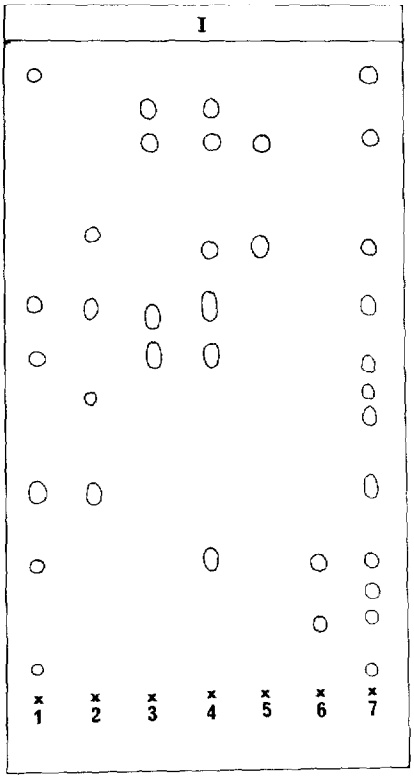

Fig. 1. Thin-layer chromatogram of chloroplast pigments and derivatives on a sucrose layer. Solvent: 1% 2-propanol in light petroleum (I). 1 = Acetone extract of chloroplast pigments (neoxanthin, violaxanthin, Chl *b*, lutein, Chl *a*, β-carotene); 2 = Chl *b*, Chl *b'*, Chl *a*, Chl *a'*; 3 = Pyrochl *a*, Chl *a*, Pheo *a*, Pyropheo *a*; 4 = 10-hydroxy Chl *a*, Pyrochl *a*, Chl *a*, Chl *a'*, Pheo *a*, Pyropheo *a*; 5 = Pheo *b*, Pheo *a*; 6 = 10(*R,S*)-hydroxy Chl *b* and 10(*R,S*)-hydroxy Chl *a*; 7 = neoxanthin, 10(*R,S*)-hydroxy Chl *b*, 10(*R,S*)-hydroxy-lactone derivative of Chl *a*, 10(*R,S*)-hydroxy Chl *a* and violaxanthin, Chl *b*, 10(*R*)-methoxy-lactone derivative of Chl *a*, Chl *b'*, 10(*S*)-methoxy-lactone derivative of Chl *a* and lutein, Chl *a*, Chl *a'* and Pheo *b*/*b'*. Pheo *a*/*a'*, β-carotene.

TABLE I

R_F AND R_c VALUES OF LEAF PIGMENTS IN SOLVENT SYSTEM I (1% 2-PROPANOL IN LIGHT PETROLEUM) AND R_F VALUES IN SOLVENT SYSTEM II (1% *n*-PROPANOL IN LIGHT PETROLEUM)

Pigment	*Solvent system*			
	I		*II*	
	R_F*	R_c*	R_F**	R_c***
β-Carotene	0.95 ± 0.01	1.00 ± 0.00	0.95 ± 0.03	1.00
Pyropheophytin *a*	0.90 ± 0.03		0.86 ± 0.04	
Pheophytin *a'*	0.86 ± 0.02			0.96
Pheophytin *a*	0.86 ± 0.02	0.89 ± 0.02	0.86 ± 0.03	0.91
Chlorophyll *a'*	0.69 ± 0.04	0.72 ± 0.03	0.78 ± 0.04	0.74
Pheophytin *b'*	0.68 ± 0.04			
Pheophytin *b*	0.68 ± 0.04	0.73 ± 0.02	0.74 ± 0.03	
Chlorophyll *a*	0.62 ± 0.04	0.64 ± 0.01	0.73 ± 0.03	0.62
Pyrochlorophyll *a*	0.60 ± 0.02		0.58 ± 0.04	
10(*S*)-Methoxy-lactone derivative of Chl *a*	0.50 ± 0.02	0.55 ± 0.01		
Lutein	0.50 ± 0.05	0.51 ± 0.04	0.56 ± 0.03	0.29
Chlorophyll *b'*	0.48 ± 0.06	0.46 ± 0.04	0.61 ± 0.04	0.46
10(*R*)-Methoxy-lactone derivative of Chl *a*	0.42 ± 0.02	0.47 ± 0.01		
Chlorophyll *b*	0.29 ± 0.07	0.28 ± 0.06	0.42 ± 0.05	0.28
10(*R,S*)-Hydroxy chlorophyll *a*	0.23 ± 0.04	0.21 ± 0.00	0.40 ± 0.04	
Violaxanthin	0.21 ± 0.04	0.20 ± 0.03	0.23 ± 0.05	
10(*R,S*)-Hydroxy-lactone derivative of Chl *a*	0.18 ± 0.02			
10(*R,S*)-Hydroxy chlorophyll *b*	0.11 ± 0.03	0.11 ± 0.03		
Neoxanthin	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	

* $n = 15$.
** $n = 9$.
*** R_c values from refs. 14 and 31.

10-hydroxy Chl *a* and *b* was 10(*R,S*)-hydroxy-lactone derivative of Chl *a*[14]. The C-10 epimers of this derivative and also the C-10 epimers of 10-hydroxy Chl *a* and 10-hydroxy Chl *b* were not separated because these components move only a short distance. The effect of 2-propanol concentration on the resolution of these epimeric chlorophylls derivatives was tested. When the concentration of 2-propanol was increased, the components moved a longer distance. Good separation between these derivatives was achieved by using 1.5% 2-PrOH in LP as a solvent (Fig. 3). These epimeric derivatives have previously been separated on a sucrose column[14].

Solvent system II was also tested for separation of the chloroplast pigments but a better resolution was achieved in solvent system I. R_F values for each solvent system are given in Table I. To separate Pheo *a* from Pheo *a'*, three solvent systems were tested (I, III and IV). In solvent system I the separation is hindered by the self-aggregation of the pheophytins. Pyridine (solvent systems III and IV) diminishes the self-aggregation tendency but the enolization becomes relatively rapid. The pyridine concentration must be chosen so that it removes the π–π interaction between the two pheophytin macrocycles but does not cause rapid enolization of the pigments. The separation of these two pigments was achieved with 2% Pyr in LP as the solvent.

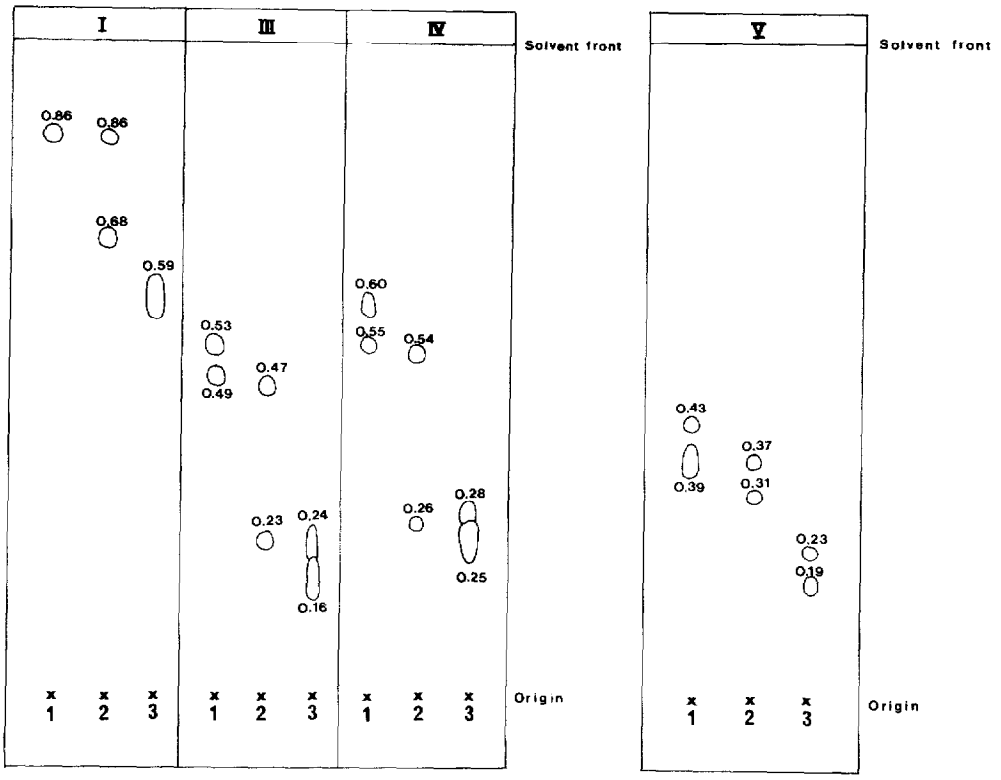

Fig. 2. Thin-layer chromatogram of pheophytin *a* and *b* and their 10(*S*)-isomers on a sucrose layer using solvent systems I (1% 2-propanol in light petroleum), III (1% pyridine in light petroleum) and IV (2% pyridine in light petroleum). 1 = Pheophytin *a* and pheophytin *a'*; 2 = pheophytin *b* and pheophytin *a*; 3 = pheophytin *b* and pheophytin *b'*.

Fig. 3. Thin-layer chromatogram of 10-hydroxy derivatives of Chl *a* and *b* using solvent system V (1.5% 2-PrOH in LP). 1 = 10(*R*)-Hydroxy Chl *a*, 10(*S*)-hydroxy Chl *a*; 2 = 10(*R*)-hydroxy Chl *b*, 10(*S*)-hydroxy Chl *b*; 3 = 10(*R*)-hydroxy lactone derivative of Chl *a*, 10(*S*)-hydroxy-lactone derivative of Chl *a*.

This is the first time that resolution of Pheo *a* and Pheo *a'* by TLC has been reported. Pheo *b* and Pheo *b'* were not completely resolved under these conditions. The chromatograms and R_F values of the pheophytins are demonstrated in Fig. 2.

The R_F values of the pigments are affected by several factors: age of the solvent, amount of the pigment spotted on the plate, thickness of the sucrose layer, pre-equilibration of the chamber and lining the chamber with chromatographic paper. It should also be noted that speed is essential during the chromatographic run because the chloroplast pigments are extremely sensitive to light and oxygen which both cause degradation of the pigments. Table I also shows the R_c values (distance moved by the pigment divided by the distance moved by β-carotene) in solvent system I. The R_c values of the pigments are almost identical with those reported for the sugar column by Hynninen[14]. The use of β-carotene as a reference compound facilitates the comparison of different TLC separations. Table I shows that standard deviations are smaller for the R_c values than for R_F values. Thus the R_c values are less sensitive

to small variations in the chromatographic conditions than the R_F values. Previously, R_F values were replaced by R_p values, which are the R_F values of the given pigment divided by the R_F of Chl *a*[35].

Using the preparative sucrose TLC, the recoveries were 95.4% for Chl *a* and 99.1% for Pheo *a*, employing 50–100 μg of pigment. The recovery of Chl *a* was lower than that of Pheo *a*, because Chl *a* decomposes more easily during the procedure. Previously, quantitative recovery of Chl *a* using thin-layer chromatography on washed cellulose was reported to be 92.0%[22]. Recovery is presumably lower than from sucrose layers because acidic carboxyl groups in cellulose cause pheophytinization. Sucrose, however, does not contain any reactive groups which degrade the pigments. Small amounts of pigments can be purified on sucrose TLC but for large scale preparations sugar columns should be used[31].

Chloroplast pigments are extremely sensitive to light and oxygen which both cause degradation of the pigments. Therefore, rapid sample handling in dimmed light is essential when analysing leaf extracts. Sucrose TLC provides a fast and mild method to investigate chloroplast pigments and to check their purity, and the method can also be used semi-preparatively. This method has been routinely used in our laboratory for many years and we are convinced of its efficiency.

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