

CHROM. 6041

## AN EVALUATION OF REVERSED PHASE PARTITION FOR THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION OF CHLOROPHYLLS AND DERIVATIVES\*

IVAN D. JONES\*\*, LILLIAN S. BUTLER\*\*, ELEANOR GIBBS\*\* AND RAYMOND C. WHITE\*\*\*

*North Carolina State University, Raleigh, N. C. 27607 (U.S.A.)*

(First received November 16th, 1971; revised manuscript received March 14th, 1972)

## SUMMARY

Separation and identification of chlorophyllides *a* and *b*, pheophorbides *a* and *b*, as well as chlorophylls *a* and *b* and pheophytins *a* and *b* by reversed-phase partition thin-layer chromatography, was shown to be effective. Pigment quantities in the order of 0.5 nmole could be identified. Criteria of evaluation were location, color and stability of pigment zone as viewed under white light and ultraviolet light. Investigations in which oil-impregnated thin-layers of powdered sugar, Kieselguhr G and a mixture of Kieselguhr G and Silica Gel G were used were reported.

The formation of artifacts occurred when chromatographing purified pigments on inorganic thin-layers. The location of artifact formation is considered to be on the portion of the plate coating which was not oil-impregnated. Addition of an antioxidant such as butylated hydroxytoluene to pigment solutions prior to application on inorganic layers was shown to prevent artifact formation. Identification characteristics for allomerized chlorophylls *a* and *b*; methyl chlorophyllides *a* and *b*; and methyl pheophorbides *a* and *b* on oil-impregnated plates were indicated.

## INTRODUCTION

Thin-layer chromatography (TLC) studies for identification and/or estimation of chlorophylls and their degradation products have been extensively reviewed by ŠESTÁK<sup>1</sup>. Accordingly, only limited reference will be made here to reports previously published utilizing TLC techniques in chlorophyll studies. Since the above-mentioned review was published additional reports of studies on this subject have appeared<sup>2-4</sup>.

EGGER<sup>5</sup> proposed the reversed-phase partition method for TLC separation of chloroplast pigments to avoid xanthophyll destruction encountered when using adsorption chromatography techniques involving inorganic thin layers. Phase reversal was accomplished by oil-impregnation of plate coating, preferably with vegetable oil low in acid. Partial impregnation of coated plates (Kieselguhr G) permitted application of pigment to a non-impregnated area of the plate. This favored formation of

\* Paper No. 3556 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N.C., U.S.A.

\*\* Department of Food Science.

\*\*\* Department of Chemistry.

sharp pigment bands during development. RANDEATH<sup>6</sup> cited the advantages of this method for examination of the purity of chlorophyll solutions. KIM, in an investigation of bacteriochlorophyll fractionation, has recently described the use of the reversed-phase partition method<sup>7</sup>. His procedure was obviously that of EGGER<sup>5</sup>.

The investigation to be reported was conducted in connection with the preparation and spectral characterization of zinc and copper complexes of pheophytins and pheophorbides *a* and *b*, an operation which required the use of highly purified chlorophylls and derivatives<sup>8</sup>. Accordingly, reversed-phase partition received consideration. Certain portions of the study have been previously reported<sup>9</sup>.

## EXPERIMENTAL

### Materials\*

Kieselguhr G and Silica Gel G were analytical sorbents obtained from Brinkmann Instruments, Inc. Solutions of purified chlorophylls, pheophytins and pheophorbides were prepared by the procedure described by JONES *et al.*<sup>8</sup>. Chlorophyllides *a* and *b* were prepared from purified acetone solutions of chlorophyll *a* or *b*, respectively, by incubating a crude preparation of chlorophyllase under optimum conditions for enzyme conversion<sup>10</sup>. Leaf tissue of *Ailanthus altissima*, a recognized rich source of chlorophyllase<sup>11</sup>, was homogenized and extracted until pigment-free in 80 % acetone at 0°. Portions of the undried tissue filter-cake (25 g) were suspended in 100 ml of 66 % acetone solution of the desired chlorophyll and permitted to react at 25–30°. Conversion was complete in about 3 h. Solutions of the chlorophyllides were subsequently chromatographed on a column of sugar or polyethylene.

### Methods

Testing of the EGGER method for chromatographing chlorophylls, pheophytins, chlorophyllides and pheophorbides from pure solutions and mixtures was carried out and consideration was given specifically to variations in plate-coating, oil-impregnation and developing-solvents. Plate coatings or adsorbents investigated were Kieselguhr G, Silica Gel G and mixtures of these. Oil-impregnation was with corn or peanut oil of commercial grade, dissolved in isooctane or petroleum ether (b.p. 65–110°) (v/v) at concentrations of 7, 9, 14 and 21 %. Solvents were methanol–acetone–water at ratios 20:4:4, 20:4:3, 20:4:2, 20:4:1, 20:4:0, each saturated with the triglyceride under test. 5 × 20 cm plates were used. Documentation of chromatogram zones was by means of photographs of the chromatoplates under ultraviolet light (UV) with Polaroid film. The assembly previously reported<sup>12</sup> was used for studying, characterizing and documenting chromatograms.

Plates coated with Kieselguhr G partially impregnated with peanut oil dissolved in isooctane at 14 % concentration were found to be best. Spotting was done on the non-impregnated area. Development was with a mixture of methanol–acetone–water (20:4:3), except for certain studies to be reported later. Decreasing the water content of the solvent was found to cause the pigment zones to separate further but to be less intense and less distinct.

\* The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Experiment Station of the products named, nor criticism of similar ones not mentioned.

Freshly coated plates were found to be satisfactory if permitted to air-dry for 16 h or longer before oil-impregnation. Partial impregnation was accomplished by immersing the  $5 \times 20$  cm plate to a depth of about 16 cm in the isooctane solution of peanut oil and permitting it to drain, dipped end down, for 1 h or longer. Plates were air-dried for 72 h or oven-dried at  $100^\circ$  for 60 min, to volatilize most of the solvent. Plates containing more than a trace of residual solvent yielded chromatograms for which the  $hR_F^*$  values for the chlorophylls and pheophorbides were high; those for the pheophorbides were proportionately much higher than for the chlorophylls. Further observations on this subject will be presented later in this report.

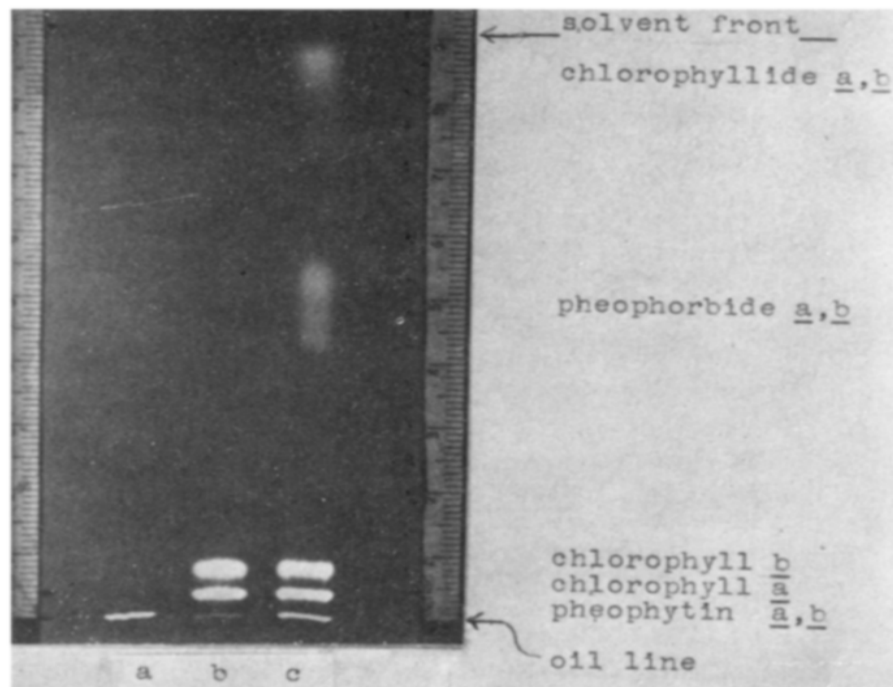


Fig. 1. Thin-layer chromatogram of chlorophylls and derivatives by reversed phase partition. Layer: Kieselguhr G, impregnated with peanut oil in isooctane (1.4% v/v); solvent: methanol-acetone-water (20:4:3) saturated with peanut oil, run 9 cm on impregnated area; pigment: (a) mixture pheophytins *a*, *b*, (b) mixture pheophytins *a*, *b*, chlorophyll *a* and chlorophyll *b*, (c) mixture pheophytins *a*, *b*, chlorophylls *a*, *b*, pheophorbides *a*, *b* and chlorophyllides *a*, *b*; application on non-impregnated area; visualization: UV 365; recording: at 10 min after development, Polacolor 108, copied in black and white.

An example of the separation of pheophytins, chlorophylls, pheophorbides and chlorophyllides under the conditions listed above is shown in Fig. 1. Presented is a black and white photograph of a Polacolor print of a chromatogram illuminated by UV. This indicates the appearance of the chromatoplate, at 10 min after development. Color fidelity of zones as photographed in Polacolor was good.

Fading of pigment zones on this plate-coating following chromatoplate development occurred rapidly for the chlorophyllides (15–30 min) and chlorophyll *b* (30–60 min); slowly for chlorophyll *a* (3–10 h); and nearly imperceptively for pheophytins and pheophorbides. The color change is from that which is characteristic for a given

\*  $hR_F = 100 \times R_F(\text{ref. } 13)$ .

pigment as listed above to light gray to colorless. Recently-developed zones of all fluorescing pigments were seen under UV at about one tenth the concentration required for visualization by reflected or transmitted white light. As the pigment zones visibly faded, they changed in color when viewed under UV from red to pink to blue-white to invisible<sup>12</sup>, but these zones were seen for a much longer time under UV than under white light.

Under the conditions indicated in this study, pigment samples approximating 0.5 nmole each could be readily seen under transmitted or reflected white light. It was found desirable to make a single application of a suitable concentrated sample (a 5- $\mu$ l sample) concentration 75-100  $\mu$ moles per l). Repeated application of undesirably dilute solutions resulted in formation of distorted developed pigment zones.

## RESULTS

### *TLC induced chlorophyll changes*

When applied in verification of the purity of solutions of chlorophylls *a* and *b* or mixtures of these pigments which had been repeatedly purified as previously outlined, the chromatographic techniques described above yielded chromatograms indicating that the purified solutions were impure. The impurity became apparent through the presence of multiple bands of chlorophylls *a* and *b*. Such multiple banding did not occur in the chromatographing of total extracts (unpurified). Evidence of the susceptibility of a purified solution of chlorophyll *a* or *b* to form a spurious double band when chromatographed by partition as described is presented in Table I.

The data of Table I indicate that the samples of purified chlorophylls *a* and *b* were impure, due to the presence of compounds we will call "altered"\* chlorophyll *a* and *b*.

TABLE I

CHARACTERIZATION OF CHROMATOGRAM OF PIGMENT SAMPLES SEPARATED ON OIL-IMPREGNATED KIESELGUHR G.

Layer: Kieselguhr G, impregnated with peanut oil in isooctane (1.4% v/v); solvent: methanol-acetone-water (20:4:3) saturated with peanut oil, 10 cm run on impregnated area; pigments: leaf extract and chlorophylls *a* and *b* respectively from this extract following purification on sugar column, applied on non-impregnated area; appearance observations: immediately following plate development.

<i>Sample</i>	<i>hR<sub>F</sub></i> <i>values</i>	<i>Appearance in</i> <i>white light</i>	<i>Identity</i>
Leaf extract	28	Yellow	Lutein
	11	Yellow-green	Chlorophyll <i>b</i>
	5	Blue-green	Chlorophyll <i>a</i>
	0	Yellow line	Carotenes
Purified chlorophyll <i>a</i>	10	Blue-green	"Altered" chlorophyll <i>a</i>
	5	Blue-green	Chlorophyll <i>a</i>
	0	Faint gray line	Pheophytin <i>a</i> (trace)
Purified chlorophyll <i>b</i>	21	Yellow-green	"Altered" chlorophyll <i>b</i>
	11	Yellow-green	Chlorophyll <i>b</i>

\* No observations were made relative to the nature of "altered" chlorophyll *a* and *b* other than the zone color when viewed under white light and the *hR<sub>F</sub>* values of this pigment, as compared with corresponding unaltered pigments.

The "altered" chlorophyll *a* was blue-green in color with an  $hR_F$  value virtually that of chlorophyll *b*. Similarly, the "altered" chlorophyll *b*, with a decidedly larger  $hR_F$  value than that for chlorophyll *b*, was the same color as chlorophyll *b* when evaluated visually by means of white light.

In an attempt to determine the conditions under which the "altered" chlorophylls *a* and *b* were formed, and prompted by the use of sugar-coated plates by COLMAN AND VISHNIAC<sup>14</sup>, an investigation was made of oil-impregnated chromatoplates coated with powdered sugar, rather than with Kieselguhr G. The pigments applied and the techniques of pigment application and plate development were the same as for the Kieselguhr G chromatoplate which yielded the chromatogram described in Table I. Information obtained through the use of the oil-impregnated, sugar-coated plate is presented in Table II\*.

TABLE II

CHARACTERIZATION OF CHROMATOGRAM OF PIGMENT SAMPLES SEPARATED ON OIL-IMPREGNATED POWDERED SUGAR

Layer: Powdered sugar, impregnated with peanut oil in isooctane (14% v/v); solvent: methanol-acetone-water (20:4:3) saturated with peanut oil, run 10 cm on impregnated area; pigments: leaf extract and chlorophylls *a* and *b*, respectively, from this extract following purification on sugar column, applied on non-impregnated area; appearance observations: immediately following development.

Sample	$hR_F$ values	Appearance in white light	Identity
Leaf extract	30	Yellow	Lutein
	14	Yellow-green	Chlorophyll <i>b</i>
	8	Blue-green	Chlorophyll <i>a</i>
	0	Yellow line	Carotenes
Purified chlorophyll <i>a</i>	8	Blue-green	Chlorophyll <i>a</i>
Purified chlorophyll <i>b</i>	15	Yellow-green	Chlorophyll <i>b</i>

In the record in Tables I and II for the sample designated leaf extract, recognition is given to the carotenes which appeared at the oil line,  $hR_F = 0$ , and to a xanthophyll, presumably lutein, which exhibited an  $hR_F$  value of 28. Generally, in total pigment extracts of green plant tissue there were seen three additional xanthophylls separating above the one presumed to be lutein. Such pigments were usually rather weakly colored zones which faded rapidly, especially on a Kieselguhr G plate following development.

The data in Tables I and II provide evidence that the plate-coating was the factor in the formation of spurious second or multiple bands in the chromatographic checks on purity of purified chlorophyll *a* and *b*. From such evidence, it appeared that some component(s) in the total pigment extract protected against "altered" chlorophyll formation in this sample when chromatographed on a Kieselguhr G-coated plate. Such component(s), possibly carotenoid in nature, was (or were) removed during the

\* Oil-impregnated, sugar-coated plates yielded pigment zones which were slightly larger, more diffuse than with similar Kieselguhr G plates. Agitation of the solvent during development should be avoided to prevent loss of the immersed portion of sugar-coating from the plate. Development proceeds satisfactorily in spite of such loss, but more slowly.

chromatographic steps entailed in the separation of pure chlorophylls *a* and *b* on powdered sugar columns.

It was recognized during this investigation that little or no "altered" chlorophyll formation occurred on Kieselguhr G plates in a sample of purified chlorophyll *a* and/or *b*, for which the delay between pigment application and plate development was negligible. This observation suggested that the "altered" chlorophyll formation occurred as the result of pigment oxidation; that it was time-related; and that it probably occurred during the period the pigment was in contact with the Kieselguhr G coating of the non-oil-impregnated portion of the plate-coating. Accordingly, the use of an antioxidant which would exert protective action during the period of pigment application and early plate development was indicated.

Tests were made of the effect of adding small amounts (about 500 p.p.m.) of butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) to solutions of pigments to be chromatographed on oil-impregnated layers of Kieselguhr G, Kieselguhr G-Silica Gel G (mixture 3:2) or powdered sugar. These tests demonstrated the effectiveness of these antioxidants in the prevention of the formation of "altered" chlorophyll *a* and *b* by the inorganic plate coatings mentioned. Shown as an example of such evidence is Fig. 2.

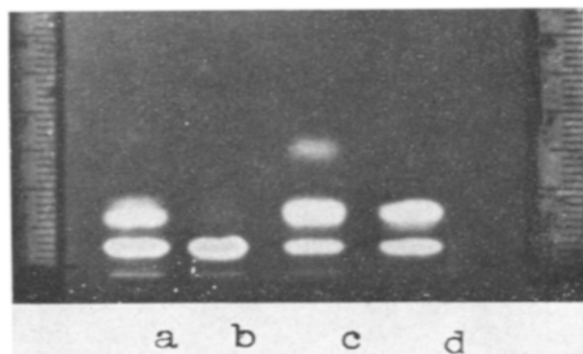


Fig. 2. Thin-layer chromatogram of chlorophyll solutions to which antioxidant (BHT, 500 p.p.m.) had and had not been added. Layer: Kieselguhr G, impregnated with peanut oil in isooctane (14% v/v); solvent: methanol-acetone-water (20:4:3) saturated with peanut oil, run 10 cm on impregnated area; pigment: sample (a) purified chlorophyll *a* without BHT, sample (b) is sample (a) with BHT, (500 p.p.m.), sample (c) mixture purified chlorophyll *a*, *b* without BHT, sample (d) as sample (c) with BHT (500 p.p.m.).

Identity and color of separated pigment zones (zones in ascending order): (a) trace pheophytins — gray, chlorophyll *a* — blue-green, "altered" chlorophyll *a* — blue-green; (b) trace pheophytin — gray, chlorophyll *a* — blue-green; (c) trace pheophytin — gray, chlorophyll *a* — blue-green, mixture "altered" chlorophyll *a* + chlorophyll *b* — blue-green lower + yellow-green upper, "altered" chlorophyll *b* — yellow-green; (d) chlorophyll *a* — blue-green, chlorophyll *b* — yellow-green; visualization and recording; see Fig. 1.

The antioxidant BHT was considered to be more satisfactory than BHA. A study of minimal amounts of BHT to be employed indicated the necessity for concentrations of 100 and 20 p.p.m. for protection of chlorophylls *a* and *b*, respectively. Routinely, BHT in diethyl ether (150 mg/100 ml) was added to a working volume of the sample for spotting at the rate of 0.1 ml per 1 ml. It was found to be desirable to remake the antioxidant after about 30 days.

#### *Separation conditions*

A study was made of the influence of aging of oil-impregnated Kieselguhr G

TABLE III

APPROXIMATE  $hR_F$  VALUES OF PURIFIED PIGMENTS

Influence of aging of oil-dipped plates stored at room temperatures in cabinet not tightly closed. Layer: Kieselguhr G, impregnated with peanut oil in isooctane (14% v/v), solvent: methanol-acetone-water (20:4:3) saturated with peanut oil, run 10 cm on impregnated area; pigment: purified pigments containing 100 p.p.m. antioxidant (BHT) applied on non-impregnated area; color observation: immediately following plate development.

Pigment	Color (white light)	Age in days				
		3	10	28	45	67
Pheophytin <i>a</i>	Black	0	0	0	0	2
Pheophytin <i>b</i>	Brown	0	0	0	0	2
Chlorophyll <i>a</i>	Blue-green	7	4	5	5	6..
Chlorophyll <i>b</i>	Yellow-green	13	9	9	10	11
Pheophorbide <i>a</i>	Gray	74	42	34	30	30
Pheophorbide <i>b</i>	Yellow-green	79	51	39	34	33
Chlorophyllide <i>a</i>	Blue-green	93	90	81	84	78

plates in the separation and identification of seven pigments, namely: chlorophylls *a*, *b*, pheophytins *a*, *b*, pheophorbides *a*, *b* and chlorophyllide *a*. The observations, recorded in Table III, were made immediately after chromatoplate development.

The data of Table III indicate that the aging of chromatoplates impregnated with an isooctane solution of peanut oil is responsible for marked change of  $hR_F$  values of the pigments listed, especially those of the pheophorbides. They further indicate that dipped plates stored at room temperature in cabinets without special treatment exhibit only relatively small changes from the tenth through the forty-fifth day. Such aging is considered to be due largely to solvent volatilization. It was also observed that freshly-dipped plates might be used without delay if subjected to oven-drying at 100° for a period of about 60 min.

Table III demonstrates that by the procedure outlined the four groups of purified pigments, namely the pheophytins, chlorophylls, pheophorbides and chlorophyllides, can be clearly separated and further, that the chlorophylls may not only be isolated from other groups but that they can be separated from each other. The pheophorbide *a* and *b* zones, though they appear to overlap according to the values shown in the table, actually are nearly separated and exhibit distinctly different colors when viewed either under white light or on UV.

Separation of the chlorophyllides sufficient for identification was accomplished, utilizing oil-dipped, sugar-coated plates developed with methanol-acetone-water (20:4:6). Chromatography was terminated when the front had ascended the impregnated area only about 5 cm, in order to retain clear definition of the chlorophyllide zones. Evidence of the effectiveness of this procedure is presented in Fig. 3. The difference in color of the zones for chlorophyllides *a* and *b* is a distinguishing feature whether the plate be viewed under light or UV. As shown in Fig. 3, the procedure described provides an exceptionally distinct separation and identification of the pheophorbides. The chlorophylls, pheophytins, carotenes and possibly some other pigments would all be components of the band at the oil line (sample (a) in Fig. 3) when developing solvent methanol-acetone-water (20:4:6) would be used with an oil-impregnated plate.

With mixtures of pheophytins *a* and *b*, very good results in pigment separation were obtained using the Kieselgultir G plate prepared as indicated, but developed with a solution of methanol-acetone (20:4:0), where 0 indicates the absence of water. Although the distance between the bands by such separation of the pheophytins was relatively small, the zones were sharp lines and the color when viewed by transmitted white light provided identification. Pheophytin *a* is gray or black and pheophytin *b* is brown.

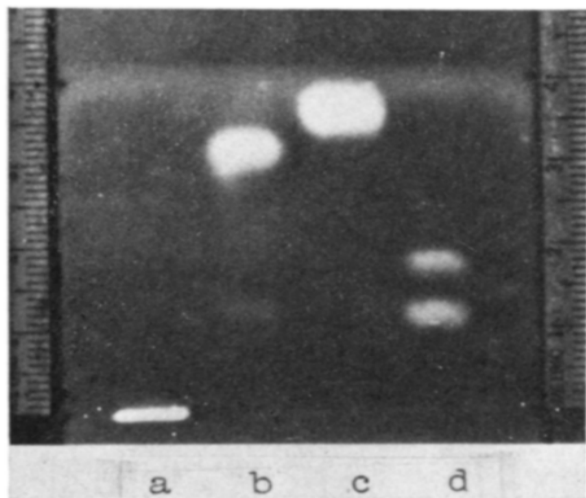


Fig. 3. Thin-layer chromatogram of chlorophylls, chlorophyllides and pheophorbides by reversed-phase partition. Layer: powdered sugar, impregnated with peanut oil in isooctane (14% v/v); solvent: methanol-acetone-water (20:4:6) saturated with peanut oil, run about 4 cm on impregnated area; samples: (a) mixture chlorophylls *a* and *b*, (b) chlorophyllide *a*, (c) chlorophyllide *b*, (d) mixture pheophorbides *a*, *b*; pigment zone identity and color: (a) chlorophylls *a* and *b* — green; (b) chlorophyllide *a* — blue-green; (c) chlorophyllide *b* — yellow-green; (d) lower, pheophorbide *a* — gray; upper, pheophorbide *b* — yellow-green; visualization and recording: see Fig. 1.

#### *Methyl chlorophyllides and pheophorbides*

In the presentation thus far, such consideration as has been given to pigment change encountered during pigment preparation, purification and separation has been relative to formation of "altered" chlorophylls during TLC. There are other sources of pigment alteration occurring during pigment extraction and storage which may be responsible for the presence of a number of zones on the developed plate to hinder or prevent proper interpretation of the chromatogram. One such alteration is the formation of methyl or ethyl esters of the pheophorbides or chlorophyllides, occurring during the extraction of pigment from chlorophyllase-rich tissues with an extractant composed, in part, of methyl or ethyl alcohol, respectively<sup>15</sup>. Instruction for the use of such extractants has been provided by a number of investigators, as cited by STRAIN AND SVEC<sup>16</sup>.

A check into the occurrence of such impurities in pigment extracts has been conducted in this study only for the purpose of separation and identification. Pigment extracts containing free chlorophyllides, methyl chlorophyllides, free pheophorbides, chlorophylls and pheophytins in varying relative quantities were prepared by the following procedure. Samples (25 g) of freshly harvested *Ailanthus altissima* leaves were placed in flasks and covered with a specified extractant mixture. Each mixture



contained, in addition to 30 ml of 15% ammonium acetate, to maintain a pH of 6.8–7.0, one of the following solvents, namely: (1) 200 ml acetone, (2) 200 ml methanol and (3) 200 ml acetone + 20 ml methanol. The extractant-covered leaves were held in the dark at room temperature for 16 h, after which the solutions were decanted and the pigments were transferred to diethyl ether by conventional methods. The pigments separated on oil-impregnated Kieselguhr G, as is shown in Fig. 4. It will be seen that under the conditions of this test the methyl chlorophyllides were formed in relatively large amounts in samples (b) and (c), which contained methanol and, further, that in sample (b) a considerable portion of chlorophyll *a* was converted also to methyl pheophorbide *a*. The identity of methyl chlorophyllides *a* and *b* and the methyl pheophorbides *a* and *b* was indicated by the color of the respective zones when viewed by transmitted and reflected white light. Confirmation of the identity was made by acid-treatment of samples (a), (b) and (c), thereby converting the various chlorophyllides to their respective pheophorbides. The  $hR_F$  values for the acid-treated pigments were respectively, 13 and 15 for methyl pheophorbides *a* and *b*; and 38 and 47 for pheophorbides *a* and *b*. Substituting ethyl or *n*-propyl alcohol for methanol in experiments similar to that reported above yielded pigment extracts the chromatograms of which closely resembled Fig. 4. From the data presented, it was apparent

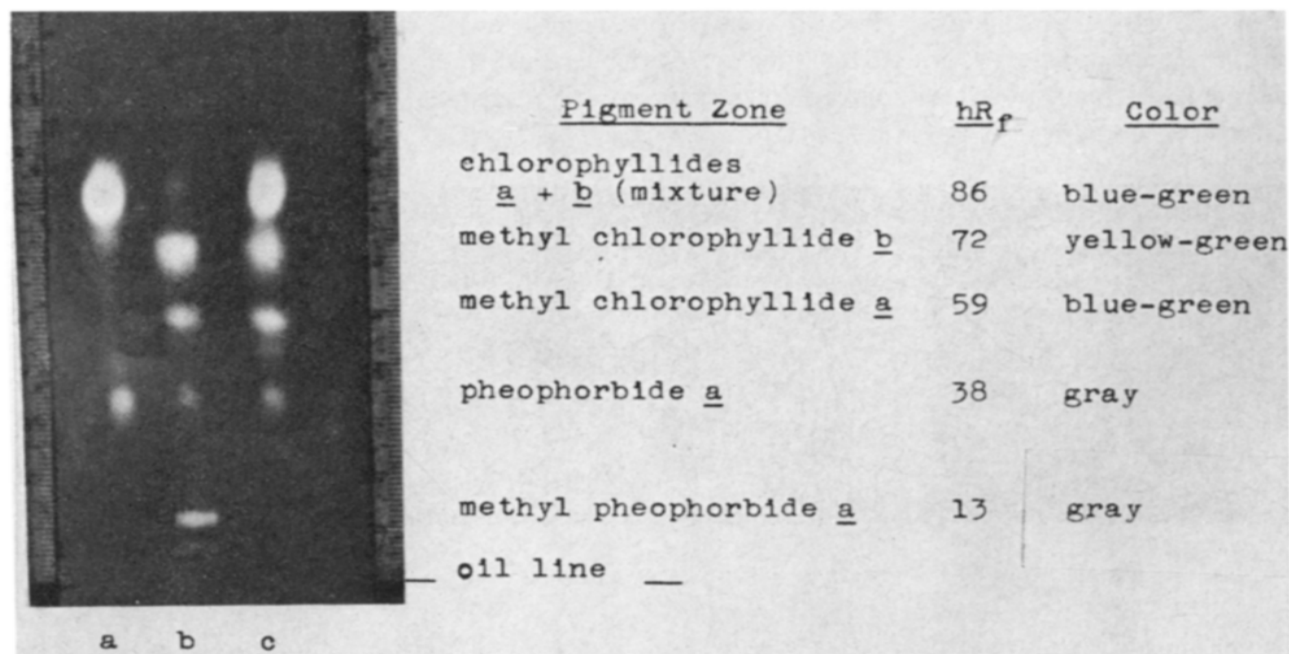


Fig. 4. Thin-layer chromatogram of the chlorophyllides, methyl chlorophyllides, pheophorbides and methyl pheophorbides by reversed-phase partition. Layer: Kieselguhr G, impregnated with peanut oil in isooctane (14% v/v); solvent: methanol-acetone-water (20:4:3) saturated with peanut oil, run 8.5 cm on impregnated area; samples: reaction products of chlorophyllase-containing leaf samples incubated with following extractants: (a) acetone + water; (b) methyl alcohol + water; and (c) acetone + methyl alcohol + water (BHT, 100 p.p.m. added to all samples).

Identity of separated pigment zones (zones in ascending order): (a) pheophorbide *a*, mixture chlorophyllides *a*, *b*; (b) trace chlorophyll *b*, methyl pheophorbide *a*, pheophorbide *a*, methyl chlorophyllide *a*, methyl chlorophyllide *b*, trace chlorophyllides; (c) pheophorbide *a*, trace pheophorbide *b*, methyl chlorophyllide *a*, methyl chlorophyllide *b*, mixture chlorophyllides *a*, *b*; visualization and recording: see Fig. 1.

that the problem of chlorophyll derivative identification is unnecessarily complicated by the use of alcohol-containing solvents in chloroplast pigment extraction.

### Allomerized pigments

Oxidative change of chlorophylls in organic solvents occurs as the result of uptake of oxygen by the pigments during extraction and storage. Such change has been termed allomerization<sup>17</sup>. The rate of allomerization may be accelerated by exposure of the extracts to oxidative enzymes, methanol, alkaline media<sup>15</sup> or to certain salts<sup>18</sup>. Allomerized chlorophylls are similar in appearance to the pigments from which they are derived, they give a negative phase test<sup>17</sup> and are slightly more strongly adsorbed than their precursors<sup>15</sup>.

Attention to allomerized or oxidized chlorophylls in this study has been limited to their recognition, primarily by means of simultaneous chromatography with reference standards. Following practices demonstrated to be effective for avoidance of the formation of "altered" chlorophylls *a* and *b* during TLC by the method under investigation, it was repeatedly found that solutions which gave a negative phase test yielded atypical pigment zones. A pure pigment which gave a strong positive phase test would yield a single characteristically shaped band or spot, while a solution of the same pigment which gave a negative phase test would yield two or more bands or spots.

Presented in Fig. 5 are chromatograms of allomerized and non-allomerized chlorophylls *a* and *b* and details of sample treatments. Non-allomerized pigments giving strong positive phase tests were used as references; pigments presumed to be allomerized gave negative or only weakly positive tests. The antioxidant BHT was added to all samples before spotting.

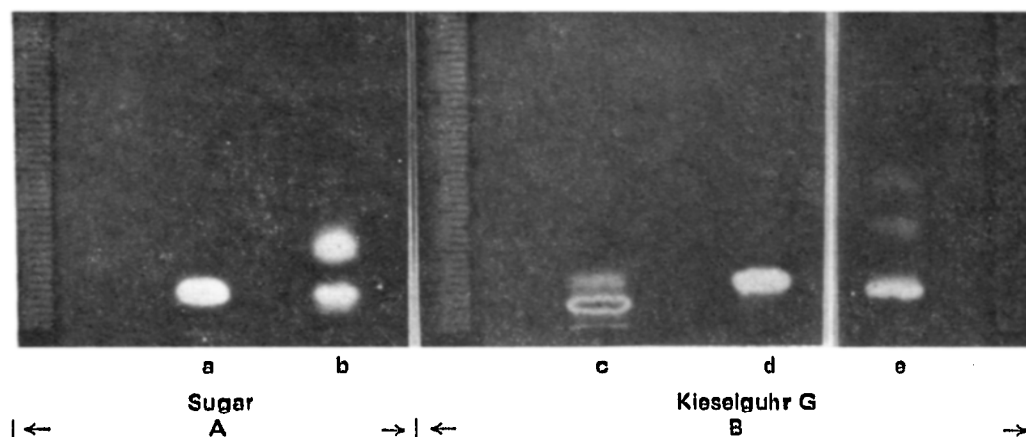


Fig. 5. Thin-layer chromatograms of allomerized and non-allomerized chlorophylls *a* and *b* by reversed phase partition. Layer: A, sugar, B, Kieselguhr G; peanut oil-impregnated in iso-octane (14% v/v); solvent: methanol-acetone-water (20:4:3), run 10 cm on impregnated area; sample details: (a) non-allomerized chlorophyll *a* reference, phase test strongly (+), (b) 10 ml sample (a) in 80% acetone + 2g  $MgCl_2 \cdot 2H_2O$  after standing 16 h at 26°, phase test weakly (+), (c) chlorophyll *a*, phase test weakly (+), (d) non-allomerized chlorophyll *b*, reference phase test strongly (+), (e) Allomerized chlorophyll *b*, phase test (-) (BHT, 100 p.p.m. added to all samples). Identity and color of separated pigment zones (zones in ascending order): (a) trace pheophytin *a*, non-allomerized chlorophyll *a* — blue-green; (b) trace pheophytin *a*, non-allomerized chlorophyll *a* — blue-green, allomerized chlorophyll *a* — blue-green; (c) trace pheophytin *a*, non-allomerized chlorophyll *a* — blue-green, allomerized chlorophyll *a* — blue-green; (d) non-allomerized chlorophyll *b* — yellow-green; (e) non-allomerized chlorophyll *b* — yellow-green, allomerized chlorophyll *b* — yellow-green; visualization and recording: see Fig. 1.

## DISCUSSION

Among the derivatives identified in detailed studies on adsorption chromatographic separation of chlorophylls were pigments from allomerized chlorophylls designated as oxidized chlorophylls by STRAIN<sup>15</sup> and as "changed" chlorophylls by BACON AND HOLDEN<sup>10</sup>. STRAIN reported that oxidized chlorophylls *a* and *b* were each more strongly adsorbed than their non-allomerized counterparts when chromatographed on a sugar column developed with the customary mixtures of petroleum ether containing acetone or alcohol. BACON AND HOLDEN similarly found that their "changed" chlorophylls *a-1*, *a-2*, *b-1* and *b-2* which yielded negative phase tests were more strongly adsorbed on cellulose thin-layers than the corresponding phase test-positive components. They showed that "changed" chlorophyll *a-1* chromatographed mid-way between the bands for chlorophylls *a* and *b* and that "changed" chlorophyll *a-2* overlapped chlorophyll *b*. The data reported in this study in Fig. 5 are seen to be in general agreement with the observation cited by STRAIN and by BACON AND HOLDEN. The band corresponding to "changed" chlorophyll *a-1* was not seen in these studies.

BACON<sup>20</sup>, in a discussion of artifacts from the chromatography of chlorophylls, has shown that chlorophylls *a* and *b* can be extensively altered during TLC separation on inorganic plate-coatings such as Silica Gel G, Kieselguhr G and Celite 545 (Johns-Manville). He concluded that the alteration was caused by the adsorbent; that it was an oxidation product and that probably most of the conversion occurred "when the pigments were spread out . . . separated from any protective substances in the sample". He pointed to the similarity of these artifacts to the "changed" chlorophylls *a-1*, *a-2*, *a-3* and "changed" chlorophylls *b-1*, *b-2*, and *b-3* of BACON AND HOLDEN<sup>10</sup>.

In the chromatograms of Fig. 5, the relationship between the principal band and the extra band for either chlorophyll *a* or *b* when evaluated by the appearance and location is very similar to such relationship between a given principal band and the corresponding "altered" chlorophyll band in chromatograms discussed earlier (Table I and Fig. 2). Such comparison suggests that from a specified pure pigment the so-called "altered" chlorophyll and the pigment from the allomerized chlorophyll are the same or are very similar products. In addition, it was concluded from data presented here with oil-impregnated plates that the extra band representing "altered" chlorophyll was an artifact but that the extra band from allomerized chlorophyll was indicative of the presence of an impurity (an allomerized product) in the solution at the time of application.

## ACKNOWLEDGEMENTS

The authors acknowledge the valuable assistance in photography given by Landis Bennett in chromatogram recording.

Study supported in part by research grant FD-0078-07 from the Food and Drug Administration D.H.E.W.

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*J. Chromatogr.*, 70 (1972) 87-98