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CHLOROPHYLLS AND CAROTENOIDS OF COLOURLESS CALLUS, GREEN CALLUS AND LEAVES OF *KALANCHOË CRENATA*

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Abstract—The chlorophyll content of green callus cultures of *Kalanchoë crenata* was low in comparison with *Kalanchoë* leaves. A small quantity of β-carotene was detected in colourless callus together with auroxanthin and neoxanthin. The auroxanthin content decreased when colourless callus was greened on exposure to light. The major carotenoids of *Kalanchoë* leaves, β-carotene and lutein, were also the major pigments of green callus.

INTRODUCTION

Etiolated leaf systems have been used in studies on the biochemical morphogenesis of chloroplasts but the true sequence of events in etiolated leaves may be blurred by the rapidity of the changes which occur in these tissues on exposure to light. Many plant tissue cultures develop chlorophyll in the light.[1,2] It was considered that such chlorophyllous tissues in culture would provide a useful tool in investigations on the biochemical changes which occur during the development of chloroplasts.

A clone of callus tissue cultures of *Kalanchoë crenata*, derived from stems, was used in the present preliminary work which compares the pigment content of colourless callus, green callus and leaves of *K. crenata*.

RESULTS

Chlorophylls

Freshly inoculated colourless callus cultures were transferred to a light cabinet and were subcultured at monthly intervals. These cultures became green in the light and an ether extract of fourth-generation green callus (callus grown in the light cabinet and subcultured at monthly intervals for four months) showed a similar absorption spectrum to an ether extract of *Kalanchoë* leaves (Fig. 1). The ether extract of the dark-grown colourless callus did not contain any detectable chlorophyll although there was some absorption in the blue-end of the spectrum, this absorption most probably being due to carotenoid pigments. When chlorophyll contents were expressed on a quantitative basis, it was evident that they were much lower in green callus than in *Kalanchoë* leaves (Table 1). In first-generation green callus (callus grown in the light cabinet for one month), the chlorophyll content was 70 × less than that of leaves but in fourth-generation green callus the chlorophyll content was only 18 × less than that of leaves. Whenever colourless callus was placed in the light cabinet the chlorophyll content increased with each successive subculture to reach a maximum after the fourth generation. These experimental observations are partially substantiated by the

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[1] N. Sunderland, *Ann. Botany* (*London*) **30**, 253 (1966).

[2] W. M. Laetsch and D. A. Stetter, *Am. J. Botany* **52**, 798 (1965).

fact that protochlorophyll or protochlorophyllide have not been detected in colourless callus. Accompanying the slow increase in chlorophyll content in successive generations of callus in the light, is a decrease in the ratio chlorophyll *a*/chlorophyll *b* (Table 1). This ratio was high in the first-generation green callus but had approached the ratio obtained for *Kalanchoë* leaves after four generations in the light. This suggests a time-lag in the formation of chlorophyll *b* by colourless callus on exposure to light.

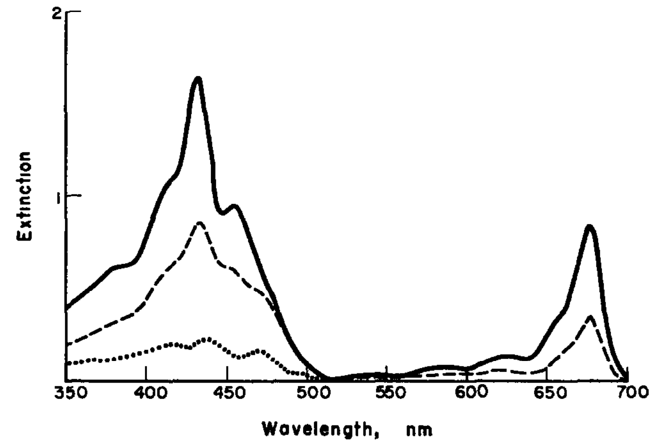

Fig. 1. Absorption spectra of ether extracts of (a) *Kalanchoë* leaves ———; (b) 4th generation green callus (callus grown in the light cabinet and subcultured at monthly intervals for 4 months) - - - - -; (c) colourless callus ·········.

Table 1. Chlorophyll contents of *Kalanchoë* leaves and green callus

Material analysed	mg chlorophyll per g dry weight			Ratio chlorophyll *a*/*b*
	Total chlorophyll	Chlorophyll *a*	Chlorophyll *b*	
Kalanchoë leaf	37·1±1·9	25·75±1·3	11·36±0·6	2·27
First-generation green callus	0·54±0·05	0·43±0·03	0·12±0·01	3·58
Fourth-generation green callus	2·04±0·08	1·46±0·05	0·57±0·04	2·56

First-generation green callus—callus grown in the light cabinet for 1 month. Fourth-generation green callus—callus grown in the light cabinet and subcultured at monthly intervals for 4 months. Each result is the mean of six analyses and is expressed ± standard error.

The chlorophylls from first-generation green callus and from *Kalanchoë* leaves were separated on the thin-layer system, Kieselguhr G—1% (v/v) propanol in light petroleum (b.p. 40–60°) to confirm their identity (chlorophyll *a*, R_f 0·56, and chlorophyll *b*, R_f 0·44). Co-chromatography of chlorophyll *a* and chlorophyll *b* from green callus with chlorophyll *a* and chlorophyll *b* respectively from *Kalanchoë* leaves gave coincidental spots. Both chlorophylls gave a positive phase test[3] when 30% (w/v) potassium hydroxide in methanol was added to an ether solution of either chlorophyll. A transient yellow changing to green coloration was

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[3] J. H. C. Smith and A. Benitez, In *Modern Methods of Plant Analysis* (Edited by K. Paech and M. V. Tracey), Vol. 4, p. 142. Springer, Berlin (1955).

observed for chlorophyll *a* and a red to brown to green colour change was observed for chlorophyll *b*. The identity of chlorophylls was further established by preparing the phaeophytins from the separated chlorophylls *a* and *b* from green callus and *Kalanchoë* leaves by standing ether solutions of the pigment over oxalic acid in the cold for 12 hr. The absorption spectra were identical, after this treatment, with published absorption spectra for phaeophytin *a* and *b*.[4]

Carotenoids

The green callus tissues, used in analyses of carotenoids, were first-generation green callus and callus which had been in the light for 2 weeks. Carotenoid extracts prepared from colourless callus, green callus or *Kalanchoë* leaves were separated on a zinc carbonate/hyflo column with light petroleum (b.p. 60–80°) containing increasing concentrations of peroxide-free diethyl ether. The separated bands of pigment were collected and purified on columns of magnesium oxide or on icing-sugar.

Fraction 0

Light petroleum (b.p. 60–80°). This was a colourless fraction collected before the carotenoids. Thin-layer chromatography of this fraction from extracts of colourless callus, green callus and *Kalanchoë* leaves on Kieselgel G, light petroleum (b.p. 40–60°) separated two compounds which were revealed by staining with iodine vapour as two brown areas, R_f 0·38 and R_f 0·18, on a yellow background. One of these compounds, R_f 0·38, was identified as squalene. The compound, R_f 0·18, might have been phytofluene as it fluoresced faintly under u.v. light and as it was stained pink by antimony trichloride.

Fraction 1

Light petroleum (b.p. 60–80°). Colourless callus, green callus and *Kalanchoë* leaf extracts contained β-carotene which was eluted as an orange band from the zinc carbonate/hyflo column and further purified on a magnesium oxide column. Values for absorption maxima and curve shape for β-carotene, from all three tissues, in several solvents, light petroleum (b.p. 60–80°) (426, 451, 477 nm), hexane (425, 450, 478 nm), chloroform (463, 493 nm) and carbon disulphide (485, 518 nm) compared with those for authentic β-carotene (Koch & Light Ltd.), recrystallized from peroxide-free diethyl ether, and with β-carotene prepared from spinach leaves. In thin-layer chromatography β-carotene from colourless callus, green callus and *Kalanchoë* leaf extracts ran coincidental with authentic β-carotene, R_f 0·73 on Kieselgel G, 20% (v/v) methylene chloride in undecane; R_f 0·01 on Kieselgel G, light petroleum (b.p. 40–60°); R_f 0·55 on Kieselgel G, 5% (v/v) peroxide-free diethyl ether in light petroleum (b.p. 40–60°) and R_f 0·08 in the reverse phase system Kieselguhr G impregnated with liquid paraffin, methanol–acetone (5:2 v/v). Chromatography in any thin-layer system did not reveal any other carotenoids in this fraction.

Fraction 2

0–5% (v/v) peroxide-free diethyl ether in light petroleum (b.p. 60–80°). In the case of *Kalanchoë* leaf extracts only, a narrow light-yellow band was recovered in this fraction. After purification of this pigment on a magnesium oxide column, absorption maxima and curve shape in various solvents (hexane 425, 452, 480 nm), chloroform (434, 464, 495 nm)

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[4] J. P. Sweeney and M. E. Martin, *Food Technol.* **15**, 263 (1961).

and carbon disulphide (452, 483, 516 nm), corresponded to published values[5] for cryptoxanthin, and with those determined for authentic cryptoxanthin extracted from corn cobs.

Thin-layer chromatography confirmed the identity of cryptoxanthin as R_f values were similar to those of authentic cryptoxanthin, R_f 0·61, on Kieselgel G, 20% (v/v) ethyl acetate in methylene chloride and, in the reversed phase systems, R_f 0·94 on Kieselguhr G impregnated with liquid paraffin, methanol–acetone (5:2v/v) and R_f 0·11 on Kieselguhr G impregnated with coconut butter, methanol–acetone (5:2 v/v). On co-chromatography with authentic cryptoxanthin only a single spot was observed. No cryptoxanthin was detected in either colourless or green callus.

Fraction 3

5–15% (v/v) peroxide-free diethyl ether in light petroleum (b.p. 60–80°). A broad yellow band was collected from the zinc carbonate/hyflo column when extracts of either green callus or of *Kalanchoë* leaves were passed through it. Absorption maxima and curve shape for the purified pigment in different solvents, light petroleum (b.p. 60–80°) (420, 444, 474 nm), chloroform (428, 454, 483 nm) and carbon disulphide (446, 475, 505 nm), were similar to those published for lutein[5] and were identical to those for lutein prepared from corn cobs and spinach leaves. R_f values were the same as those for authentic lutein, R_f 0·41 on Kieselgel G, 20% (v/v) ethyl acetate in methylene chloride and R_f 0·5 on the reversed phase system Kieselguhr G impregnated with coconut butter, methanol–acetone–water (20:4:3 by vol.). Co-chromatography did not separate the pigment from authentic lutein in any thin-layer system. A narrow, light yellow band was obtained in Fraction 3 from colourless callus but was never obtained in sufficient quantity for identification.

The broad yellow band collected in Fraction 3 for green callus separated into two distinct bands on a magnesium oxide column. The first band eluted from this column was the lutein described above. The second band, orange in colour, provided absorption maxima and curve shapes in solvents, hexane (419, 445, 472 nm), chloroform (472, 504 nm), ethanol (444, 472 nm) and benzene (457, 482 nm), which corresponded to published values for isolutein.[5] An 18 nm shift of the absorption maxima to lower wavelengths was observed when one drop of 0·1 N-hydrochloric acid was added to the ethanol solution of the pigment. This shift is suggestive of the 5,6-epoxide, isolutein.[6] In thin-layer chromatography on Kieselgel G, 20% (v/v) ethyl acetate in methylene chloride, the isolutein ran as a single spot, R_f 0·12.

Fraction 4

40–80% (v/v) peroxide-free diethyl ether in light petroleum (b.p. 60–80°). A light yellow band was recovered in this fraction from colourless callus and green callus. The pigment was purified on a column of icing-sugar. Absorption maxima and curve shape in light petroleum (b.p. 60–80°) (379, 399, 423 nm), chloroform (385, 410, 435 nm) and carbon disulphide (401, 423, 451 nm) were similar to published values for auroxanthin isolated from yellow pansy petals (*Viola tricolor*). Co-chromatography of auroxanthin from yellow pansy petals, colourless callus and green callus in various thin-layer systems produced only a single spot, R_f 0·52 on Kieselguhr G, 5% (v/v) methanol in benzene and R_f 0·36 on Alumina, 5% (v/v) methanol in benzene. No auroxanthin was detected in extracts of *Kalanchoë* leaves.

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[5] B. H. DAVIS, In *Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. GOODWIN), p. 489. Academic Press, New York (1965).

[6] N. I. KRINSKY and T. H. GOLDSMITH, *Arch. Biochem. Biophys.* **91**, 271 (1960).

Fraction 5

80% (v/v) peroxide-free diethyl ether in light petroleum (b.p. 60–80°). A yellow band was recovered in this fraction from colourless callus, green callus and *Kalanchoë* leaves. The pigment was purified on a column of icing-sugar. Absorption maxima and curve shape in light petroleum (b.p. 60–80°) (415, 439, 467 nm), hexane (416, 437, 466 nm), chloroform (422, 449, 477 nm), carbon disulphide (466, 497 nm) and ethanol (417, 438, 467 nm) were similar to published values for neoxanthin. The addition of one drop of 0·1 N-hydrochloric acid to the ethanol solution of the pigment produced an 18 nm shift of absorption maxima to lower wavelengths, a shift characteristic of 5,6-epoxide xanthophylls[6] and suggestive of neoxanthin. Thin-layer chromatography and co-chromatography of neoxanthin from each source revealed only a single spot, R_f 0·1 on Kieselguhr G, 0·5% (v/v) acetone in light petroleum (b.p. 40–60°), R_f 0·69 on Kieselguhr G, 5% (v/v) ethyl acetate in hexane and R_f 0·89 on the reversed phase system Kieselguhr G impregnated with coconut butter, methanol–acetone–water (20:4:3 by vol.).

No violaxanthin was detected in any extract when the pigments were separated on columns. However, with a paper chromatographic method developed by Booth[7] for the separation of tocopherols, a very small quantity of a yellow pigment was detected in green callus extracts. The pigment spot was eluted from the paper with peroxide-free diethyl ether and then chromatographed on thin-layer plates of Kieselgel G, 20% (v/v) ethyl acetate in methylene chloride and three pigments separated, R_f values 0·31, 0·19 and 0·0. The only pigment obtained in sufficient quantity for identification ran with R_f 0·19. This pigment was scraped off, packed into a small column and eluted with light petroleum (b.p. 40–60°). Absorption maxima and curve shape in light petroleum (b.p. 60–80°) (420, 443, 472 nm), chloroform (423, 452, 482 nm) and ethanol (419, 441, 471 nm) corresponded to published values for violaxanthin.[5] When a drop of 0·1 N-hydrochloric acid was added to the ethanol solution of the pigment there was

TABLE 2. CAROTENOID CONTENT OF *Kalanchoë* LEAVES, GREEN CALLUS AND COLOURLESS CALLUS

	µg per g dry weight			
		Green callus		
Carotenoid	Colourless callus	Callus in light for 2 weeks	First-generation callus	leaf
β-carotene	3·2	25·6	40·4	149·7
Lutein	0	36·0	87·0	211·4
Isolutein	0	0	0·3	0
Cryptoxanthin	0	0	0	6·5
Violaxanthin	0	0	Trace	0
Auroxanthin	8·9	2·5	1·8	0
Neoxanthin	1·8	2·1	3·7	9·4
Total carotenoid	13·9	66·2	133·2	377·0

First-generation green callus—callus grown in the light cabinet for 1 month. Each value presented is the mean value of analyses on three samples except in the case of "callus in light for 2 weeks" where two samples only were analysed.

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[7] V. H. BOOTH, *Analyst* **88**, 627 (1963).

a 40 nm shift of the absorption maxima to lower wavelengths, suggesting the 5:6-diepoxide, violaxanthin. These procedures when applied to colourless callus and *Kalanchoë* leaf extracts did not provide any evidence for the presence of violaxanthin.

The quantity of carotenoids in the extracts were calculated from published extinction values.[5] The total carotenoid content of colourless callus was low in comparison to green callus and *Kalanchoë* leaves (Table 2). A fairly rapid synthesis of carotenoids occurred on exposure of the colourless callus to light, the total carotenoid content increasing with exposure to light and after 4 weeks in the light (green callus) the content was one-third that of *Kalanchoë* leaves. β-Carotene and neoxanthin were the only two carotenoids detected in all three tissues. Auroxanthin was the major pigment of colourless callus but the amount progressively decreased with exposure of the callus to light. The major pigments of *Kalanchoë* leaves, β-carotene and lutein, were also the major pigments of green callus.

DISCUSSION

Although traces of chlorophylls have been detected in dark-grown oats more commonly chlorophylls are absent in etiolated angiosperm leaves. The presence of the chlorophyll precursors, such as protochlorophyll or protochlorophyllide or both, in etiolated angiosperm leaves has been well established and on exposure to light an extremely rapid synthesis of chlorophylls occurs.[8] Chlorophylls, or the immediate precursors of chlorophylls, were not detected in colourless callus. This conclusion would appear to be partly substantiated by the observation that the colourless callus on exposure to light takes a relatively long time to synthesize detectable amounts of chlorophyll. It would seem, therefore, that the colourless callus cannot be regarded as being directly analogous to etiolated leaf systems.

There was a high value for the chlorophyll *a*/chlorophyll *b* ratio in first-generation green callus but this ratio decreased with successive subcultures of the green callus in the light until in fourth-generation green callus the ratio was similar to that of *Kalanchoë* leaves. This time-lag in the synthesis of chlorophyll *b* was in accord with the results obtained by other workers with etiolated leaf systems.[9]

The carotenoid analyses of colourless callus showed a similar picture to that reported for tissue cultures of Paul's Scarlet Rose[10] in that the colourless callus contained oxygenated xanthophylls. On exposure of the colourless callus to light, however, there was a synthesis of those carotenoids, notably β-carotene and lutein, which were the major carotenoids of *Kalanchoë* leaves. The major pigment of the colourless callus was auroxanthin (3,3′-dihydroxy-5,8,5′,8′-diepoxy-β-carotene) but the auroxanthin content decrease markedly when colourless callus was exposed to light and traces of violaxanthin (3′3′-dihydroxy-5,6,5′,6′-diepoxy-β-carotene) were detected and the neoxanthin (3,3′,5′-trihydroxy-6′-hydro-5,6-epoxy-β-carotene)[11] content had increased after 4 weeks in the light (green callus). This might possibly suggest a biosynthetic relationship between these three carotenoids. Bamji and Krinsky[12] working with *Euglena gracilis* concluded that light served as a source of reducing potential (NADPH) in carotenoid de-epoxidations. As it is known that functional chloroplasts[13] are formed when colourless callus is exposed to light it is likely that some

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[8] L. BOGORAD, In *Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. GOODWIN), p. 29. Academic Press, New York (1965).

[9] R. H. GOODWIN and O. H. OWENS, *Plant Physiol.* **22**, 197 (1947).

[10] B. L. WILLIAMS and T. W. GOODWIN, *Phytochem.* **4**, 81 (1965).

[11] B. P. SCHIMMER and N. I. KRINSKY, *Biochemistry* **5**, 1814 (1966).

[12] M. S. BAMJI and N. I. KRINSKY, *J. Biol. Chem.* **240**, 467 (1965).

[13] I. MCLAREN and D. R. THOMAS. *New Phytol.* In press.

NADPH might be available for carotenoid de-epoxidations. Such de-epoxidations induced by the availability of NADPH in developing chloroplasts might account for the lower level of auroxanthin in green callus.

The qualitative picture that has emerged from analyses of pigments in green callus have demonstrated that it is, in many ways, similar to the picture obtained for *Kalanchoë* leaves. When the results were compared on a quantitative basis it was very evident that the prime deficiency of the green callus, in comparison with *Kalanchoë* leaves, was in the chlorophyll content. It seems that to produce an autotrophic green callus it will be necessary to enhance markedly the production of chlorophylls in the light by the green callus.

MATERIALS AND METHODS

Culture Technique

The inorganic nutrients were those of a modified White's medium.[14] This medium was supplemented by the addition of thiamine hydrochloride, 0·1 mg/l; pyridoxine hydrochloride, 0·1 mg/l; nicotinic acid, 0·5 mg/l; 2,4-dichlorophenoxyacetic acid, 5 mg/l; and 25 % (v/v) coconut milk. 2 % (w/v) sucrose supplied the source of carbon and energy for growth. The medium was adjusted to pH 6, made semi-solid with 0·5 % (w/v) Oxoid No. 3 agar and sterilized in an autoclave at 15 lb/in^2. The tissue cultures were subcultured monthly onto 25 ml fresh medium, each inoculum being about 0·5 g fresh weight, contained in 100 ml conical flasks. Cultures were either grown in a dark room at 25° or in a light cabinet, which provided a 16-hr day at a light intensity of 8000 lx at 25°.

Extraction and Purification of Pigments

All organic solvents were purchased from B.D.H. Ltd., and before use were purified by conventional methods. All extraction procedures, chromatography etc., were conducted in dim light or, where possible, in the dark.

100 g fresh weight leaves were extracted directly but 200 g fresh weight callus tissue was frozen in liquid nitrogen and freeze-dried prior to extraction of pigments in 80 % (v/v) acetone–water in a Waring blendor. This extraction was repeated on the residues four times with a final extraction in peroxide-free diethyl ether so that the residues were white in appearance. The successive solvent extracts were combined to provide the ether solution used in subsequent analysis for both chlorophylls and carotenoids.

Separation and Determination of Chlorophylls

The ether solution was washed with water to remove traces of acetone and then dried overnight (Na_2SO_4), filtered, and the Na_2SO_4 residue washed with dry peroxide-free diethyl ether, the washings being combined with the filtrate. This ether solution was used to obtain absorption spectra and from the extinction values at 642·5 nm and 660 nm[15] the amounts of chlorophylls *a* and *b* were calculated. The ether solution was also used in the thin-layer separation of individual chlorophylls.

Separation and Determination of Carotenoids[5,10]

When carotenoids were to be examined, the ether extract was distilled to dryness under reduced pressure with a nitrogen bleeder at 15°. Successive 10 ml aliquots of ethanol were added to the residue until all substances were dissolved. For every 10 ml ethanol, 1 ml 60 % (w/v) aqueous KOH was added and the extract saponified by standing at room temperature in the dark under N_2 for 12 hr. Then, twice the volume of water was added and the pigments re-extracted into peroxide-free diethyl ether. The extract was washed with water until the washings were not alkaline to phenolphthalein. The extract was dried (Na_2SO_4) in the manner previously described for chlorophylls, and distilled under reduced pressure with a nitrogen bleeder. Residues were dissolved in light petroleum (b.p. 60–80°) and stored overnight at −20° to precipitate sterols. Centrifugation removed the sterols which were washed in cold light petroleum (b.p. 60–80°). The washings and supernatants were combined and reduced in volume to 2 ml by a jet of nitrogen. This 2 ml was put on a $ZnCO_3$/hyflo (5:2 w/w) column. Prior to the preparation of the column both adsorbents were washed thoroughly with peroxide-free diethyl ether and dried. The carotenoids were separated on and eluted from the column by increasing concentrations of peroxide-free diethyl ether in light petroleum (b.p. 60–80°). Less strongly adsorbed xanthophylls were purified on an MgO column and more strongly adsorbed xanthophylls were purified on an icing-sugar column.

The purified pigments were evaporated to dryness by a jet of nitrogen. The residues were dissolved in various solvents and absorption spectra determined.

[14] W. G. Boll and H. E. Street, *New Phytol.* **50**, 52 (1951).

[15] C. L. Comar and F. P. Zscheile, *Plant Physiol.* **17**, 191 (1942).

Chromatography

Confirmation of identity of separated, purified pigments was achieved by thin-layer chromatography[16] with authentic samples of pigment. TLC and reverse-phase TLC were done on 0·25 mm thick layers of Kieselguhr G (Merck), Kieselgel G nach Stahl (Merck) and aluminium oxide (Merck). In reverse-phase thin-layer chromatography, dried plates were immersed in either 5% (v/v) liquid paraffin in light petroleum (b.p. 60–80°) or in 5% (w/v) coconut butter in light petroleum (b.p. 60–80°) and then dried *in vacuo* over P_2O_5. After application of pigments, all plates were equilibrated with solvent before development in glass tanks, previously flushed with nitrogen, lined with solvent-soaked filter paper.

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[16] K. RANDERATH, *Thin-layer Chromatography*. Academic Press, New York (1963).