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REVIEW

PAPER CHROMATOGRAPHY OF CHLOROPLAST PIGMENTS

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The classical method of chromatography of chlorophylls and carotenoids on an adsorbent column is somewhat time-consuming and is more suitable for separating larger quantities of pigments. Therefore many authors have tried to develop a simpler method of separating these substances on paper. With the methods that have been described in the literature it is possible to analyse quantities of pigments of the order of 10^{-6} g. In recent years various modifications of these methods have been described and they have been used on a larger scale than before (more than 50 articles since 1952). Some of these articles have been reviewed in three communications[15,37,55]. In this paper we are attempting to give a more complete list of articles on paper chromatography of plastid pigments. Modifications of the methods that have been described in the literature are summarized in Tables I–V.

OLDER PUBLICATIONS

The first time a separation of plastid pigments on paper was mentioned, was in 1906. At that time the founder of chromatography, M. TSWETT[60], described a separation of pigments from an aqueous alcohol solution by capillary analysis. As a result he obtained five zones most of which were close together. At the bottom of the paper strip a zone containing a mixture of all the pigments was found, then came the following zones, in ascending order: carotenes, chlorophylls, xanthophylls. Capillary analysis, from a mixture of ethyl ether and 96% ethanol (1:1 to 1:2), was also used by KYLIN[34] for the detection of carotene, xanthophyll, phyllorhodin, fucoxanthin and chlorophyll in saponified extracts of higher plants and brown algae.

BROWN[10] experimented with the circular chromatographic method on blotting paper. The paper, 15 × 15 cm (untreated or impregnated with aluminium hydroxide), was sandwiched between two glass plates. The solution of pigments in carbon disulfide was placed on the paper through a 6 mm hole at the centre of the upper plate. The chromatogram was developed by adding the pure solvent drop by drop.

STRAIN (ref. 58, p. 77) briefly mentioned the separation of pigments on paper by developing with aqueous ethanol. The possibility of separating pigments on wide sheets of paper or on cylinders of paper was proposed by ARONOFF (ref. 3, p. 55; see[36]), the chromatograms being developed repeatedly with petroleum ether.

EVOLUTION OF THE METHOD SINCE 1952

Although MÁRKUS and ASAMI reported their experiments with paper chromatography of plastid pigments at two scientific meetings as early as 1951, and although ASAMI

published a short article in Japanese in the same year[1], the method did not begin to develop favourably until 1952. At that time five independent groups of scientists published their papers dealing with this method. These communications came from BAUER[8] in Germany, MÁRKUS[38] in Hungary, ASAMI[5] in Japan, and from two French teams specially working on the separations of carotenoids, GRANGAUD AND GARCIA[23,24] and SPITERI AND NUNEZ[56]. The methods of these authors have been modified by many further workers. One- and two-dimensional techniques in ascending, descending and circular arrangements have been described. Different commercial brands and different types of paper have been used, sometimes after a preliminary treatment, such as drying, washing or impregnation. The chromatograms have been developed with polar and nonpolar organic solvents or their mixtures. In some experiments the atmosphere in the chromatograph chamber was specially prepared, *e.g.* by saturation with water vapour or vapours of petroleum ether; in some cases the chamber was filled with an inert gas (*e.g.* nitrogen). Chromatograms have been developed at laboratory temperature or at a somewhat lower temperature. A survey of the methods that have been described in the literature is presented in Tables I to V. The developing solvents and mixtures examined, the types of paper used, the techniques of placing the extract on paper, the conditions of development (atmosphere, temperature, time of development) and plant material used are summed up in these tables. Satisfactory developing solvents are printed in bold face; the relative quantity of each solvent in the mixtures is given in parts by volume.

Communications describing the use of the method of BAUER[8], with or without slight modifications, are summarized in Table I. Good results have been obtained especially on developing with the mixture petrol–petroleum ether–acetone (10:2.5:2), in which petrol is sometimes replaced by benzene. The methods of Japanese authors (Table II), who used the Japanese paper Toyo No. 50 and developing mixtures containing toluene, occupy a somewhat exceptional position, since the quality of paper can influence the partition in a rather significant manner. Methods in which other developers than those derived from BAUER's[8] mixture and other papers than Toyo paper were used, are summed up in Table III. The remarkable article by MÁRKUS[38], in which the influence of various developers and various types of paper on the separation of pigments has been carefully examined, has rarely been cited. A specially arranged evacuated chamber and development with a five-component solvent mixture have been described by HAGER[27,28].

In Tables I–III a survey of methods based on the main principles of adsorption is presented. In Table IV a summary is given of methods, which, according to their originators, are based on the principles of partition chromatography[15,16,48]. DOUIN[15,16] developed his chromatograms on paper moistened with water. He placed a perforated glass cylinder in the development chamber to ensure contact of the solvent (100% methanol) with water vapour. SERCHI, MICHI AND RAPI[48] used the circular development technique. Methods based on the principle of the so-called "reversed-phase chromatography" may also be found in Table IV. In this technique paper is impregnated with a nonpolar substance (triglycerides, vaseline, etc.), a strongly polar solvent

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being used as developer. The sequence of spots is approximately the reverse of that obtained with the usual type of paper chromatography, where at least small amounts of water (polar component) are held on the paper. The "reversed-phase" technique has been used by SPITERI AND NUNEZ[56] and STRAIN[59], and NUNEZ[40] (see Table V) has resolved carotenoids by this method. STRAIN[59] has tested different techniques of preliminary preparation of cellulose paper and glass paper, as well as different types of separation (a–d in Table IV).

Data of the separation of only carotenoids from extracts have been collected in Table V.

RESULTS OF THE CHROMATOGRAPHIC SEPARATION

Except in the case of "reversed phase" chromatography, the pigments are resolved with different solvents on one-dimensional chromatograms in the following general order (beginning from the starting point): chlorophyll b–chlorophyll a–pheophytins–carotenes. The greatest difficulties are caused by xanthophylls, which sometimes form one spot, while in other developing solvents several spots may arise (lutein–violaxanthin–neoxanthin and others). These spots lie in front of, between, or behind the spots of chlorophylls, often overlapping them, and thus interfering with the isolation of microquantities of pigments. The "R_F" values* of carotenoids depend on the number of oxygen atoms in their molecules: carotenoids containing fewer atoms of oxygen (less polar compounds) possess a greater "R_F" value[54]. Carotenes (without oxygen) run immediately behind the solvent front, followed by xanthophylls with two oxygen atoms in their molecules (*e.g.* lutein, zeaxanthin); the spot(s) of xanthophyll-epoxide(s) with four oxygen atoms (violaxanthin, neoxanthin, etc.) has (have) the smallest "R_F". Up to the present no successful developer has been found for resolving the carotene spot into α- and β-carotene. The pheophytins form one or two spots (pheophytin b and pheophytin a). Some authors[27,28,53,54] have succeeded in separating pheophorbides as one or two spots. Chlorophyllides and similar hydrophilic compounds do not run with the solvent but remain at the starting point[5,8,27,28,49,53,54]. FOUASSIN[19,20] separated Zn- and Cu-derivatives of chlorophyll and found that chlorophylls in which Mg was replaced by Zn moved quite similarly to normal chlorophylls a and b, whereas chlorophylls with Cu instead of Mg had "R_F" values similar to pheophytins. Double spots of chlorophylls have been observed on some chromatograms[5,49,57]. They have usually been regarded as isomers—chlorophylls a′ and b′, originating during the preparation of the extract or during the process of paper chromatographic separation. A colourless spot fluorescing in U.V. light has also been observed[36,49].

For better resolution of some pigments, two-dimensional chromatography has sometimes been used[8,36,39,45]. However, this technique is slower than one-dimensional chromatography, and is therefore not favourable for the unstable plant pigments.

Using "reversed-phase" chromatography the sequence of pigment spots is

* "R_F" in this paper indicates only the approximate position of the spots on the paper, no definite value as is the case in the chromatography of amino acids and other compounds. Therefore quotation marks are used.

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TABL

MODIFICATIONS OF T

1 *Author*	2 *No. of dimensions*	3 *Ascend. (A) or descend. (D)*	4 *Paper, sort and dimension*	5 *Preliminary treatment of the paper*
BAUER[8], 1952	1	(A or D)	Schl. & Schüll 2043 b (strips 10–15 cm long)	dried at 105°
	2		Schl. & Schüll 2043 b (10 × 10 cm)	
SIRONVAL[52,53,54], 1953, 1954, 1957	1	D	Whatman No. 1 (45 × 5 cm)	
BLAAUW-JANSEN[9], 1954	1	A	Whatman No. 4	buffered at pH 6
ANDERSEN AND GUNDERSEN[1], 1955 GUNDERSEN AND FRIES[26], 1956	1	D	Whatman No. 1 (strip 50–70 cm long)	
RÖBBELEN[43], 1956	1	A	Schl. & Schüll 2230	
GAGE AND ARONOFF[22], 1956	1	D	Schl. & Schüll 539	
MÜLLER[30], 1957	2	A	Schl. & Schüll 2043 bM (18 × 18 cm)	

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I*

METHOD OF BAUER[8] (1952)

6 *Placing the sample on paper; solvent used*	7 *Developing solvent or solvent mixture***	8 *Conditions of development****	9 *Plants analysed*
	monochlorobenzene **toluene** **petrol–PE–Ac (10:2.5:2)** **petrol–PE–Ac–Me (10:2.5:1:0.25)** Ac petrol PE Me 1st dim.: **petrol–PE–Ac (10:2.5:2)** 2nd dim.: **petrol–PE–Ac–Me (10:2.5:1:0.25)** (in mixtures PE can be replaced by monochlorobenzene or by toluene)		*Tradescantia albiflora*
a piece of plant tissue is squeezed on the starting point	**benzene–PE–Ac (10:2.5:2)**	S.F.–S.P.: 40 cm atmosphere of PE + 23°	*Heracleum* *Pelargonia* *Fragaria*
acetone extract			
ethyl ether extract	**benzene–PE–Ac (10:2.5:2)**		*Chlorella vulgaris*
ethyl ether or acetone extract	**benzene–PE–Ac (10:2.5:2)**	15° atmosphere of PE	pigments in gyttja pith and xylem of: *Corylus avellana* *Fagus silvatica* *Salix caprea*
acetone	**benzene–PE–Ac (10:2.5:2)**		*Arabidopsis thaliana*
	benzene–PE–Ac (10:2.5:2)	atmosphere of PE	*Soja hispida*
acetone	1st dim.: **petrol–PE–Ac (10: 2.5:2)** 2nd dim.: **petrol–PE–Ac–Me (10:2.5:1:0.25)**		*Abies alba*

* Abbreviations used in Tables I to V: PE = petroleum ether; Me = methanol; Ac = acetone.

** Satisfactory mixtures are printed in bold face; other mixtures that have been tested are also given.

*** Duration of the development; distance of solvent front from the starting point (S.F.–S.P.); temperature; atmosphere in the chamber.

TABLE
METHODS OF

1	2	3	4	5
ASAMI[5,7], 1952, 1955	1	A	Toyo filter paper 50 (1.2 × 35 cm)	
CHIBA AND NOGUCHI[14], 1954 CHIBA[13], 1955	1	A	Toyo filter paper 50	
KATAYAMA AND SHIDA[32,33], 1956	1	A	Toyo filter paper 50	

TABLE
OTHER

1	2	3	4	5
MÁRKUS[38], 1952	1	A or D	**Schl. & Schüll 602 e.h.** (the following papers were also tested: Hungarian paper, no brand mentioned Schl. & Sch.: 595, 597, 598 Swedish paper, no brand mentioned Munktell OB Macherey-Nagel 640 D) (paper strips 20–25 cm long)	paper equilibrated with atmosphere of acetone
LIND, LANE AND GLEASON[36], 1953	2	A	Whatman No. 1 (23 × 23 cm)	washed with PE and dried
HARDER AND KOCH[30], 1954	1	A	Schl. & Schüll 2043 b (strip 3 cm wide)	dried at 60°

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II*

JAPANESE AUTHORS

6	7	8	9
Me–Ac (3:1)	**toluene** **carbon tetrachloride** **xylene** } **anhydrous or saturated with water** *n*-butanol isobutanol benzene chloroform } anhydrous or saturated with water Me 100% or 80% ethanol 100% or 80% phenol 80% cyclohexanol Ac lutidine collidine petrol (b.p. 45–60°) ethanol–*n*-butanol (1:1) Me–ethanol (1:1) benzene–petrol (1:1)		*Trifolium repens*
ethyl ether	**toluene** **toluene–90% ethanol (200:1)**	time: 15 to 30 min	*Trifolium repens*
Me–Ac (3:1)	**toluene–PE (2:1)**	time: 40 min + 1 to + 2°	*Oryza sativa*

III*

METHODS

6	7	8	9
acetone	**petrol–Ac–Me (30:1:0.03)** **PE–Ac (20:1)** PE (b.p. 35–40°) PE–Me (20:1) Pe–Ac–toluene (20:1:1) PE–Me–benzene (60:3:1) petrol (b.p. 60–70°) petrol–Ac (20:1) petrol–chloroform (20:1) petrol–ethyl ether (20:1) petrol–Ac–benzene (40:2:1) petrol–Me–benzene (40:1:1) petrol–Ac–*n*-butanol (20:1:0.3)	time: 2–3 h S.F.–S.P.: 20–25 cm 20° atmosphere of PE	*Solanum lycopersicum* *Spinacia oleracea*
PE	1st dim.: first **acetone** then **PE** finally **PE–propanol (99:1)** 2nd dim.: **PE–chloroform (3:1)**	S.F.–S.P.: 1 cm 20 cm 20 cm 16 cm time: 1 + 1 h	*Soja hispida*
PE (xanthophylls have previously been eluted with methanol)	First **toluene** then **PE–isobutanol (100:15)**	S.F.–S.P.: 8 cm time: 15 + 3 min	*Pedinomonas tuberculata* (Chlorop

* For abbreviations and column headings see Table I.

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TA

1	2	3	4	5
[illegible] 1954	1			
[illegible], 1954	1	D	Whatman No. 1 (40 cm long)	
SPORER, FREED AND SANCHER[30], 1954 FREED, SANCHER AND SPORER[21], 1954	1	A	Whatman No. 1 (strip 3 cm wide)	impregnated with a so sucrose (0.18 g/ml in di water) and dried at 100
SAPOZHNIKOV, BRONSHTEIN AND KRASOVSKAYA[45], 1955	1	A	Chromatographic paper No. 1, manufactured in U.R.S.S. or usual filter paper (cylinder from roll 16 × 16 cm)	washed with PE
	2			
LEFORT AND SIGNOL[35], 1955	1	A	Whatman No. 1 (2 × 26 cm)	dried at 105°
HAGER[27,28], 1955, 1957	1	A	Schl. & Schull 2071 (cylinder)	dried at 50°
SILYK[50], 1956	1	A	normal filter paper or paper No. 109-71 manufactured by Leningrad papermill No. 2	
JIRÁČEK[31], 1957	1	A	Whatman No. 1 (25 × 46 cm or 6 × 46 cm)	
ŠESTÁK[39], 1958	1	D (A also)	Whatman No. 1	no special treatment, o nated with sucrose (0.1 and dried

(*Continued*)

acetone	**PE** **benzene**		*Capsicum*
PE	**PE–benzene–Ac (10:1.5:1)**	time: 3–4 h 21 S.F.–S.P.: 35 cm	*Spinacia oleracea* *Rumex* sp. *Anthriscus* sp.
	n-hexane–n-propanol (99.5:0.5)	4–5 atmosphere of N_2	
ethanol PE ethanol–Ac	**benzene–PE (2:1)** **PE–96 % ethanol (14:1)** PE benzene–PE (3:1) 1st dim.: **benzene–PE (3:1)** 2nd dim.: **PE–96 % ethanol (14:1)**		
a piece of tissue is squeezed on the starting point acetone extract or PE extract	**PE–Ac–benzene (8.5:1:0.5)**	time: 1 h S.F.–S.P.: 18 cm	*Solanum lycopersicum* *Vitis vinifera* *Hordeum* sp. *Zea mays*
chloroform	**petrol (b.p. 100–140°)–benzene–chloroform–Ac–isopropanol (50:35:10:0.5:0.17)**	time: 24 h vacuum	*Avena* *Veratrum* *Buddleia* *Hedera* *Parthenocissus* *Corylus*
	Me–PE PE (b.p. ... 80) Me		
acetone extract	**toluene–carbon disulfide (10:3)** **toluene–carbon tetrachloride (1:1)** **PE–xylene–Ac (10:1.5:1)** PE–toluene–Ac (10:1.5:1) benzene–glycerol–chloroform (5:3:1) petrol–ethylene glycol–Ac (5:3:1) petrol–ethylene glycol–Me (3:2:1) PE–benzene–dioxan (10:1.5:0.5) PE–carbon tetrachloride–Ac (10:1:1)	S.F.–S.P.: 20–25 cm	[illegible]
PE	**PE–benzene–chloroform–isopropanol (89:5:5:1)** **PE–benzene–n-propanol (94:5:1)** **benzene–PE–Ac (10:2.5:2)** **hexane–n-propanol (99.5:0.5)** **PE–chloroform–n-propanol (3:1:0.01)** benzene–PE–Ac–methanol (10:2.5:1:0.25) PE–benzene–n-propanol (88:10:2) hexane–n-propanol (99:1) PE–n-propanol (99.5:0.5) PE–chloroform (3:1)	5	*Triticum vulgare* *Secale cereale* *Pelargonium zonale* *Stellaria neglecta* *Lamium album* *Urtica urens* *Parthenocissus cuspidata*

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TABLE

METHODS BASED ON THE PRINCIPLES AND "REVERSED-PHASE"

	2	3	4	5
[illegible]3, 1954	1	A	Whatman No. 4; Durieux 147 (25 × 3 cm)	moistened with water to 10% water content (8–15%)
SERCHI, MICHI AND RAPI[48], 1953	disc method		W 1 pressed filter paper	
SPITERI AND NUNEZ[56], 1952	1			impregnated with triglycerides, chloro- or bromonaphthalene
STRAIN[59], 1953	1	A	cellulose paper; Eaton-Dikeman 301, 0.075 or 0.125 cm thick; glass paper	(a) **adsorption on the surface of the glass or cellulose** without special treatment or dried at 100° (b) **separation on the surface of a fixed liquid:** moistened with water or with 10% solutions of polyhydroxy compounds (glycerol, sorbitol) in water; or moistened with glycerol or sorbitol in methanol, or with glycerol + glycine + urea, or glycerol + 10% methanol (c) **separation by partition between immiscible solvents** sprayed with 70, 80 or 90% methanol or dipped in these solutions and blotted (d) **reversed-phase method:** impregnated with 5% solution of vaseline

6	7	8	9
PE	**methanol 100°** ethanol propanol butanol monochlorobenzene chloroform benzene PE carbon disulfide carbon tetrachloride xylene	time: 20–30 min S.F.–S.P.: 12–15 cm spot distribution not influenced by temperature within the range 5–20° atmosphere saturated with H_2O	*Carex remota* *Tilia silvestris* *Anabaena cycadeae* *Bryales*
	stationary phase / *mobile phase*		
petrol–benzene–Me (9:1:3)	**H_2O** / **Ac** **petrol–Ac (7:3)** **Ac–petrol (9:1)** **benzene–chloroform (7:3)**		*Spinacia oleracea*
	ethyl ether / **Ac–petrol (9:1)** **Ac–petrol–H_2O (7:3:1)**		
	H_2O / chloroform benzene petrol butanol petrol–benzene saturated with ethanol (9:1) petrol–benzene (9:1) Ac–petrol–H_2O (7:3:1) petrol–benzene–Ac (7:1:2)		
	ethyl ether / ethanol–H_2O (4:1) alcohol (+ 3 drops of HCl)		
	ethyl acetate, pyridine, ligroin / chloroform, benzene, butanol		
	methanol **ethanol** **propanol**		
PE	(a) **PE–propanol (99.5:0.5)** PE	time: 20–40 min	various grasses (Poaceae)
	(b) **PE–propanol (99.5:0.5)** PE		
	(c) PE		
	(d) 80% methanol		

TABLE

CHROMATOGRAPHIC

	2	3	4	5
[illegible] AND GARCIA[23,24], [illegible]	1	A	Whatman No. 1 (50 × 15 cm)	
[illegible][25], 1954	1			impregnated with a mixture of triglycerides (0.5–4% olive oil in benzene), then dried at laboratory temperature
SAPOZHNIKOV, BRONSHTEIN-POPOVA, KRASOVSKAYA AND MAYEVSKAYA[16], 1956	1	A	chromatographic paper manufactured in U.R.S.S. (18 × 16 cm)	

reversed as follows (beginning from the original spot): carotenes–chlorophyll a–chlorophyll b–lutein–zeaxanthin–violaxanthin–neoxanthin[59].

FACTORS AFFECTING THE DISTRIBUTION OF SPOTS ON PAPER

The sequence of spots of chlorophylls and carotenoids on paper is determined first of all by the composition of the developing solvent mixture. The ratio of polar to nonpolar solvents in the mixture seems to be of the greatest importance. (The solvents may be arranged according to their polarity-index C:O—see, *e.g.*, PROCHÁZKA[42].) In pure nonpolar solvents only nonpolar carotenes are separated from the original spot, the other pigments either not moving at all or proceeding rather slowly. In solvent mixtures containing just a small quantity of polar solvents, the movement of chlorophylls, xanthophylls and pheophytins becomes greater. The relation between perfect separation and the ratio of polar and nonpolar solvents is clearly evident, especially in mixtures containing strongly polar solvents (*e.g.* alcohols); by increasing the percentage of strongly polar solvent, the "R_F" values of chlorophylls and xanthophylls increase. With mixtures containing larger quantities of strongly polar solvents as well as with pure polar solvents (acetone[5,8], ethanol[5], butanol[5]) all pigments move with the solvent front and are not separated at all.

[illegible] evidence for this general statement may be found in the literature: *e.g.*, [illegible] NOGUCHI[14] have reported that the "R_F" of chlorophyll a is equal to 0.07–[illegible] min development with pure toluene, but with the mixture toluene–95% [illegible] 1) the "R_F" is 0.47. When using nonpolar petroleum ether only carotenes [illegible][8,45], but xanthophylls and chlorophylls move with the mixture of [illegible]ether and benzene (1:3 or 1:2)[45,46], which is further away from the non-[illegible]he table of solvents cited above[42] than petroleum ether. The influence of [illegible] polarity of the solvent used is evident from the figure in the paper of [illegible] "R_F" of chlorophyll a (similar differences in "R_F" values can be

[illegible] 7/308.

V*

SEPARATION OF CAROTENOIDS

6	7	8	9
PE	PE	atmosphere saturated with watervapours	*Tecoma radicans*
	ethanol–pyridine ethanol methanol propanol pyridine		*Solanum lycopersicum*
ethanol–acetone (1:3)	**benzene–PE (3:1)** **benzene–PE–96% ethanol (18:6:1)**	45 min	

* For abbreviations and column headings, see Table I.

observed with chlorophyll b and xanthophylls) is *ca.* 0.03 when developing with pure petroleum ether, but it is 0.27 with petroleum ether–acetone (20:1), and 0.87 (!) with petroleum ether–methanol in the same ratio (methanol being a more polar solvent than acetone). When developing with nonpolar petrol (b.p. 60° to 70°) chlorophylls remain at the starting point, while an addition of 1 part of ethyl ether (semi-polar solvent) to 20 parts of petrol brings the "R_F" of chlorophyll a to *ca.* 0.06, and an addition of 1 part of acetone (more polar than ethyl ether) raises the "R_F" to 0.44. Similarly the "R_F" of chlorophylls is increased by adding 0.5% propanol to pure petroleum ether[59]. Smaller amounts of the more polar solvent in a mixture (0.25 volume parts of methanol) produce higher "R_F" values than greater quantities (1 part) of the somewhat less polar solvent (acetone), the proportion of other solvents remaining unchanged[8]. Replacing two parts of acetone in the mixture petrol–acetone–benzene by 1 part of the more polar solvent—methanol—raises the "R_F" value of chlorophyll a from 0.38 to 0.9[38]. According to ref. 46, after addition of the polar solvent—ethanol (mixture of benzene–petroleum ether–96% ethanol in the ratio 3:1:0.33) the rate of movement of chlorophylls and xanthophylls is higher than with the mixture benzene–petroleum ether (3:1). When 0.03 volume parts of *n*-butanol, which is less polar than acetone, are added to the mixture petrol (b.p. 60–70°)–acetone (20:1) the "R_F" value of chlorophyll a decreases from 0.44 to 0.22[38].

The observations cited above seem to support the idea that a perfect separation of chlorophylls and xanthophylls would require suitable relative quantities of polar solvents in a mixture. Some results obtained by ASAMI[5] may be quoted against this opinion. He succeeded in resolving pigments with relatively nonpolar solvents—toluene or tetrachloromethane (or with xylene or benzene); the "R_F" values were essentially the same regardless of the fact whether these solvents were anhydrous or saturated with water. Some results of MÁRKUS[38] are also in opposition to the opinion mentioned above: on developing with the mixture petrol–chloroform (20:1), the "R_F" of chlorophyll a was found to be 0.15, while when chloroform was replaced by the more

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[illegible] ethyl ether, the "R_F" was only *ca.* 0.06. Contrary to expectation, addition of 1 part of toluene to the mixture petroleum ether–acetone (20:1) raised the "R_F" [illegible] of chlorophyll from 0.27 to 0.4. The situation therefore seems to be still complicated and it would be rather difficult to draw definite conclusions from [illegible] results because they are not very comparable.

Determination of constant "R_F" values of plastid pigments is impossible, for these [illegible] depend on the quantities of compounds analysed[5,7,8,14,19,27,28,32,35,39,49,50,52], [illegible] duration of development[5,13,15,16], and, in the case of impregnated paper, on the amount of compound impregnated in the paper[10]. With increasing concentration of pigments in the sample analyzed, the "R_F" values increase, while they decrease with increasing quantity of impregnation compound, and with increase in developing time. The spots of chlorophylls usually have long diffusion "tails", sometimes observable only in U.V. light. The use of samples containing approximately the same quantities of pigments has been recommended in order to obtain comparable chromatograms.

When extracts from different plant species are chromatographed using one method, the sequence of spots and degree of separation may be different. This phenomenon is sometimes imputed to the presence of colourless liposoluble compounds in the extract. In some experiments resolution became more regular if purified extracts were used or if mixtures of pure isolated pigments were analysed[5,7,15,49].

The separation is also affected by the quality of paper[38], although according to some authors[45,50] the usual filter paper is quite adequate. In most cases moderately fast running papers have frequently been used (Whatman No. 1, Schleicher and Schüll 2043 b).

Some authors recommend transferring pigments to petroleum ether prior to spotting on paper. This technique is supposed to remove the greater part of undesirable material, especially hydrophilic pigments, from the extract[15,16,30,36,49,59]. In most papers, however, it is assumed that a concentrated extract can be spotted directly on paper. Some authors[35,52] have even squeezed a piece of plant tissue on the starting point. Usually, the sample is applied as a little round spot or as a streak[15,23,27,28,46,54,57].

The decomposition of pigments during developing is sometimes prevented by keeping the temperature between + 1° and + 5°[21,32,49,57], by impregnating the paper with sucrose[21,49,57], or by making use of an inert atmosphere in the chamber[21,57]. The chamber must always be kept dark.

USE OF THE METHOD

The method described has been used (in the communications mentioned above and in other papers) for qualitative and quantitative determination of pigments in lower and higher plants[17,18,30], for identification of pigments in those plant tissues where their presence is unusual, *e.g.* in bark and xylem[25,26], for studying preparations of plant material before extraction[6], for isolation of small quantities of pigments[12], for studying various factors affecting pigment synthesis in plants[27,29,47,50,51,54], for observation of genetic effects of X-rays[43,44], for genetic survey of varieties of rice[32,33], for analysis of

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chlorophyllase activity[22,53,54,61], for studying the decomposition of pigments in sediments and muds[1], for determining the extent of smoke damage in forestry[39], as well as in food chemistry[19,20] and pharmacology[41,48].

Paper chromatography has been used in rare cases to separate the crystalline chlorophyll–lipoprotein complex into two components[2,13]. These components were separated by the ascending technique, on Whatman No. 1[2] or Toyo No. 50 paper[13], a mixture of picoline and water (1:1) being used in both cases.

Methods for separating various porphyrin pigments, some of which are precursors of chlorophylls or their degradation products are not discussed in this review.

CONCLUSION

More than 50 papers dealing with paper chromatography of plastid pigments have been published up to the present. These methods are summarized in the text or in the tables. A really critical comparison of the methods described in the literature cannot be presented in this review. Such a comparison would require a special study. All the techniques quoted above should be tested using uniform material and under constant conditions.

In spite of the lack of methods for the complete separation of all plastid pigments in a pure state, a number of techniques examined up to the present time have been used for solving diverse problems. The rapid advances that are being made in the development of this analytical procedure hold out promise that it will be very soon perfected.

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