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THIN LAYER CHROMATOGRAPHY OF ANTHOCYANIDINS

I. TECHNIQUES AND SOLVENTS FOR TWO-DIMENSIONAL CHROMATOGRAPHY*

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SUMMARY

Microcrystalline cellulose Avicel SF proved to be an excellent adsorbent for two-dimensional thin layer chromatography of anthocyanidins. Of the large number of solvents, those solvent combinations giving the best resolution of the common anthocyanidins on this adsorbent have been determined and are discussed. A new methanolic solvent (MHW) which resolves anthocyanidins on the basis of the number rather than the kind of substituents on the "B" ring is shown to be highly useful in aiding resolution and in avoiding fading of anthocyanidins. In general, solvent combinations based on MHW were found most valuable for resolution, reproducibility and speed.

INTRODUCTION

During our studies on the biochemistry of insect–plant interactions it was observed that anthocyanidins occurring as such in crude extracts obtained from periderm tissues of several conifers[1], and some new anthocyanidins derived from leucoanthocyanins[2] could neither be resolved by the usual paper chromatographic solvents[3,4] nor by NYBOM's TLC system[5], which uses Cellulose MN300 as the adsorbent. The observations indicated that one of the major difficulties in the resolution with NYBOM's system rested with the adsorbent. A satisfactory adsorbent was eventually found in microcrystalline cellulose Avicel SF. The advantages of Avicel SF led us to study the characteristics of a wide range of solvent combinations on this adsorbent. This paper describes several two-dimensional solvent combinations, including a new methanolic solvent, which on Avicel SF provide excellent results.

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EXPERIMENTAL

A stock solution of the six common anthocyanidins, namely pelargonidin (Pg), peonidin (Pn), malvidin (Mv), cyanidin (Cy), petunidin (Pt) and delphinidin (Dp) was prepared for this study. The concentration ratios of Pg, Pn, Cy, Mv, Pt and Dp in 45 μl of this stock solution as estimated by a "Chromoscan" densitometer were 1.1, 0.9, 1.0, 0.8, 0.5 and 0.8 μg respectively. The R_F values reported in this investigation, unless otherwise specified, are based on the concentration of respective anthocyanidins contained in 45 μl of this stock solution.

Twenty grams of Avicel SF* microcrystalline cellulose were homogenized at the "Whip" speed setting on an Osterizer for 35 sec in 80 ml water for coating five 0.25 mm thick 20 × 20 cm plates with DeSaga TLC equipment. The plates are ready for use any time after 3 h from coating.

A heavy duty kitchen dishwasher (double-wash cycle) is an appliance of choice for washing plates. The plates thus washed are stacked with alternating layers of paper and are ready for trouble-free coating.

Multiple plate development used throughout this study was achieved by means of slotted spacers made from 12 mm plate glass as illustrated in Fig. 1. The bottom spacers are matched for slot widths to avoid breakage and for slot depths to ensure levelling. Six plates were used invariably for determining R_F values. The tanks after sealing with petroleum jelly were placed in insulated containers made from discarded

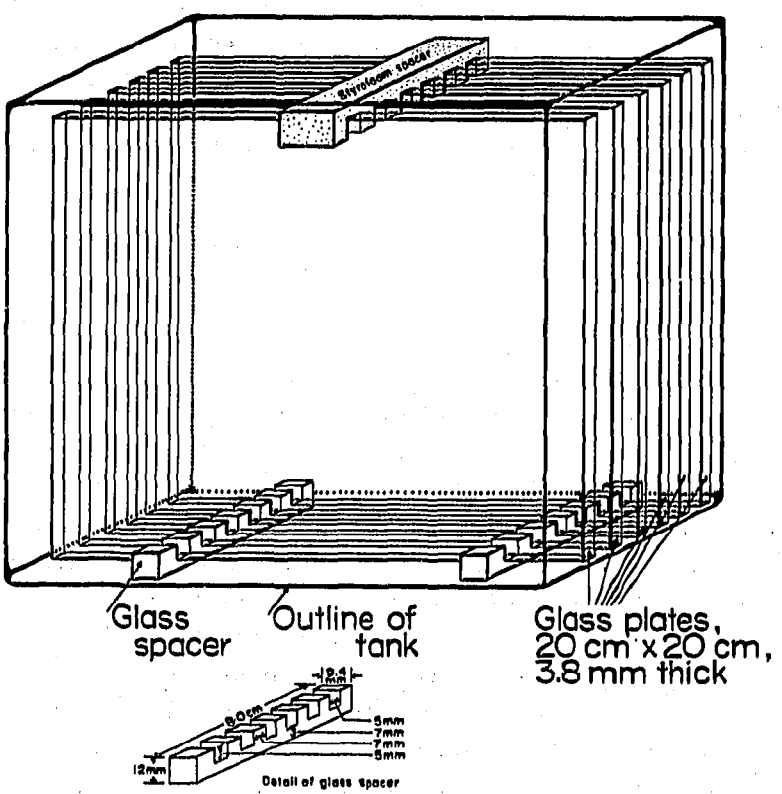

Fig. 1. Details of the spacers used for multiple plate development in DeSaga tank.

* FMC Corporation, American Viscose Division, Newark, Del, U.S.A.

styrofoam packing boxes for acid bottles, which had been shaped to approximate dimensions of DeSaga tanks. The insulation box protects anthocyanidins against photolability and minimizes R_F value fluctuations of the alcoholic solvents caused by temperature variations.

After drying the plates, run in the first direction, in an efficient fume-hood, the cellulose was scored off approximately 1 cm below the solvent front prior to chromatography in the second direction. If the plates could not be chromatographed in second direction immediately, they were left stacked after cleaning their backs. Stacking helps avoid fading of anthocyanidins even after months of storage.

Composition of a new methanolic solvent (MHW) and six other solvents is listed in Table I. Of each solvent 225 ml were used for chromatography in DeSaga tanks in the laboratory at about 21°. The solvent fronts of the MHW solvent are marked at the time the plates are being removed from the tank, because it does not leave a readily discernible front, even under U.V., after drying. The solvent fronts reach about 15–16 cm at the end of the development period stated in Table I. R_F values were measured to the leading edge of spot, which is also the point of maximum density of anthocyanidins.

TABLE I

COMPOSITION OF SOLVENTS[a] USED FOR THIN-LAYER CHROMATOGRAPHY OF ANTHOCYANIDINS

Abbreviation	*Composition*	*Development period (h)*
Non-alcoholic solvents		
Forestal	CH_3COOH–conc. HCl–H_2O (30:3:10)	7
Propionic	CH_3CH_2COOH–CHOOH–HCl–H_2O (2:5:1:6)	4½
FA–4 *N* HCl	HCOOH–4 *N* HCl (2:1)	4½
FA–Nybom	HCOOH–HCl–H_2O (10:1:3)	5
Alcoholic solvents		
MHW	MeOH–conc. HCl–H_2O (190:1:10)	2
AAW	*n*-AmOH–CH_3COOH–H_2O (2:1:1)	8
BAW	BuOH–CH_3COOH–H_2O (4:1:5) upper phase[b]	6½
BHAW[c]	*tert.*-BuOH–2 N HCl–CH_3COOH–H_2O (6:1:1:2)	11

[a] All solvents except BAW form single phase.
[b] Freshly prepared.

RESULTS AND DISCUSSION

MHW and non-alcoholic solvent combinations

Typical two-dimensional resolutions, of the six common anthocyanidins in MHW and non-alcoholic solvent combinations are illustrated in Fig. 2a, b, c. Whenever any of the non-alcoholic solvents are followed by MHW solvent in the second direction, the six anthocyanidins resolve characteristically into a triangle so that on the base lie Mv, Pt and Dp, on the vertical axis lie Mv, Pn and Pg and on the diagonal lie Dp, Cy and Pg, all well separated from one another, and so that a line joining Pn

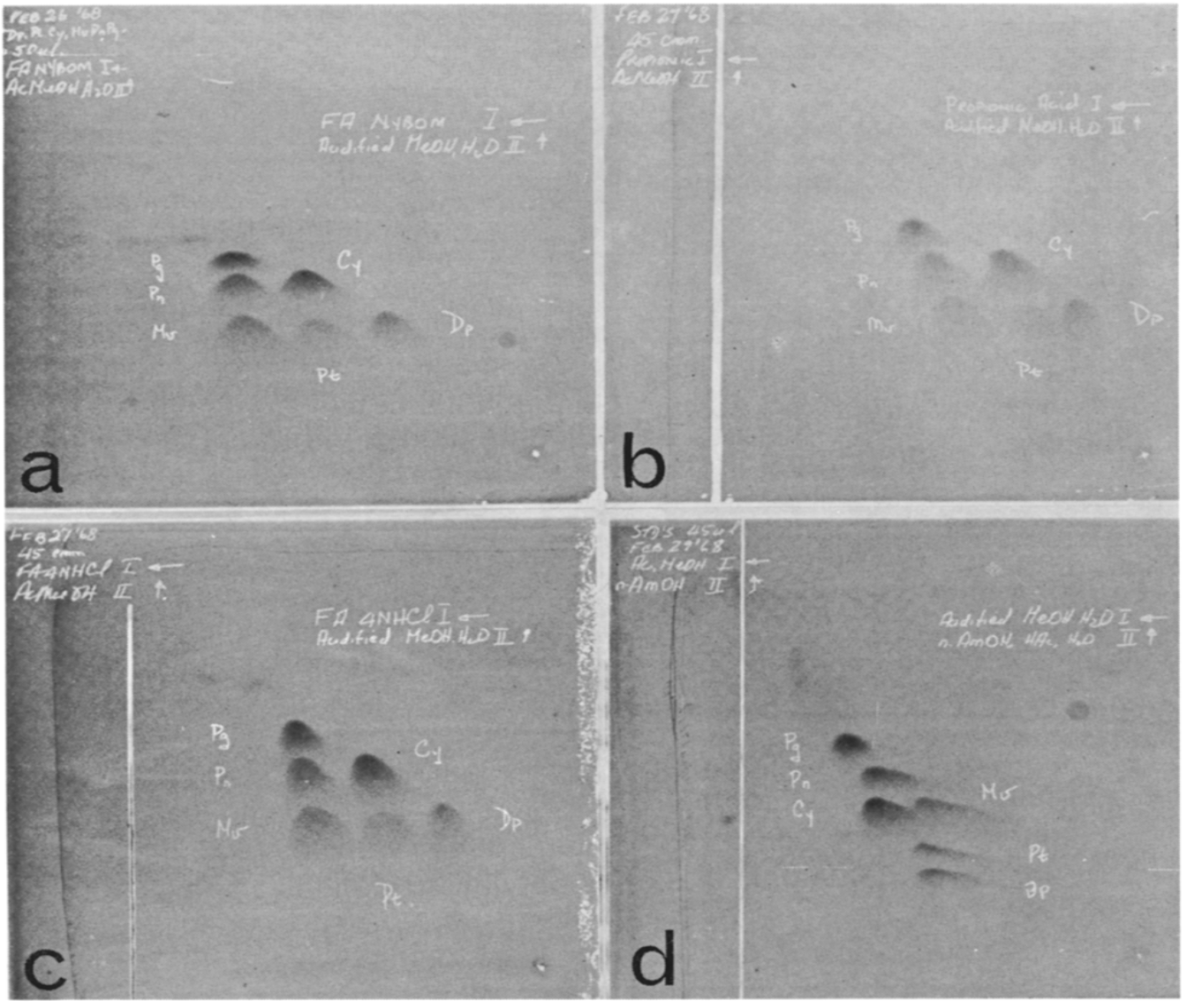

Fig. 2. TLC resolution of anthocyanidins in (a) FA–NYBOM, (b) propionic , and (c) FA–4 *N* HCl with MHW used as a second solvent in each case. The resolution in (d) was obtained by using the alcoholic solvent pair: MHW (first direction) and AAW (second direction).

and Cy is parallel to the base. The reasons for this characteristic resolution become clear from perusal of Fig. 3, which summarizes the general patterns of chromatographic mobilities of the common anthocyanidins in relation to their structure in the MHW solvent as well as in the other alcoholic and non-alcoholic solvents. A characteristic feature of the MHW solvent is that it resolves the six anthocyanidins largely on the basis of the number of hydroxyls on the B ring regardless of the state of their methylation. Thus, the R_F values of Mv, Pt and Dp, each with three substituents on the B ring are the same (see Fig. 2 and Table II). Similarly, the R_F values of Cy and Pn each with two substituents on the B ring are equal. It may be noted from Fig. 3 that whereas the MHW solvent does not differentiate the kind of substituents when the number of substituents is equal, the non-alcoholic solvents do, and thus when the

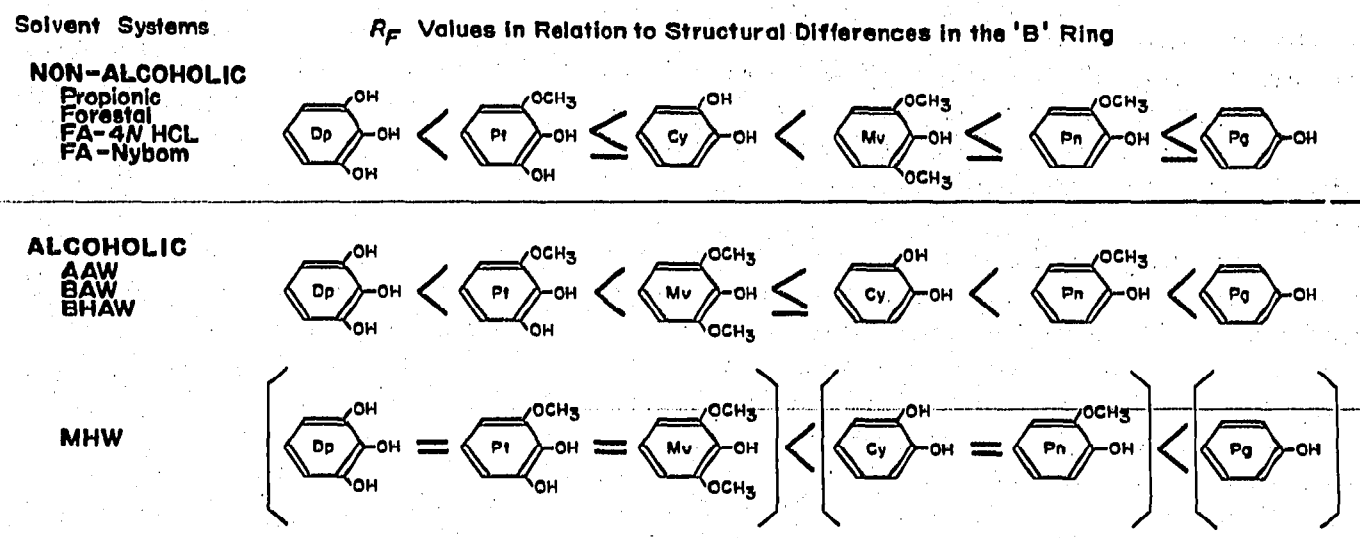

Fig. 3. The order of R_F values of the six common anthocyanidins in relation to their structure in the non-alcoholic, alcoholic and a new MHW solvent systems.

two are used in combination, good resolution with minimal fading of anthocyanidins is achieved.

The hR_F ($R_F \times 100$) values and hR_F value ranges in the first and second direction of the MHW solvent are included in Table II. When MHW is used alone or

TABLE II

AVERAGE hR_F VALUES[a] AND hR_F VALUE RANGES[b] BY TLC OF ANTHOCYANIDINS ON AVICEL SF IN MHW SOLVENT WHEN USED IN THE FIRST AND SECOND DIRECTIONS

Pigments	*hR_F values in MHW alone 60 µl*[c]	*hR_F values in MHW (second direction) First direction solvent*	
		Propionic, Forestal and FA-4N HCl 45 µl[c]	*AAW, BHAW and BAW 45 µl*[c]
Dp	50	37	44[d]
	48–53	36–41	
Pt	50	36	43[e]
	49–53	34–38	
Mv	50	37	46
	49–53	36–40	
Cy	61	49	57
	59–63	46–52	
Pn	60	50	57
	58–61	47–54	
Pg	67	58	66[e]
	63–70	55–63	

[a] First entry in each column.
[b] Second entry in each column.
[c] Volume of the reference stock solution spotted.
[d] Dp fades considerably when BAW is first direction solvent.
[e] Disappears or fades when BAW or AAW is the first direction solvent.

as the first direction solvent and is followed then by any other solvent, the R_F values are always higher than when it is used as the second direction solvent.

When MHW is used in the first direction and any non-alcoholic solvent in the second direction, the resolution is not as clear-cut as when MHW is used in the second direction; the combination is still of great value in chromatography of certain crude extracts[1].

Difficulty in the resolution of the six anthocyanidins in the non-alcoholic solvents which otherwise avoid fading and give reproducible R_F values, arises when Pt and Cy, or Mv and Pn, or Pn and Pg occur in a mixture (see Fig. 3). Since these difficulties occur between anthocyanidins having differing numbers of substituents, they are taken care of by using the MHW solvent in the other direction. Thus the usefulness of this new solvent is evident. Unlike the MHW solvent, the non-alcoholic solvents give about the same R_F values whether they are used as the first or second direction solvents. Their average hR_F values on Avicel SF are included in Table III.

TABLE III

AVERAGE hR_F VALUES BY TLC OF ANTHOCYANIDINS[a] ON AVICEL SF IN NON-ALCOHOLIC SOLVENTS WHEN USED IN THE FIRST AND SECOND DIRECTIONS[b]

Pigments	*FA–4 N HCl*	*Forestal*	*FA–Nybom*	*Propionic*
Dp	22	27	27	19
Pt	34	44	41	31
Cy	38	47	43	35
Mv	50	62	56	47
Pn	51	66	57	50
Pg	52	68	58	54

[a] Pigment concentration in all cases was 45 μl/spot.
[b] hR_F values are about the same, whether the solvent has been used in first or second direction.

AAW and non-alcoholic solvent combinations

NYBOM[5] had used the Cellulose MN300 instead of the Avicel SF used in this laboratory. On the basis of a very careful comparison of the two celluloses using NYBOM's TLC system[5], it is concluded that Avicel SF is superior to MN300 for anthocyanidin resolution, for R_F value reproducibility and particularly for the firm adherence of the cellulose to glass plates.

The chromatographic patterns obtained when AAW solvent of NYBOM[5] was used either in the first or second direction against one of the four non-alcoholic solvents, by our TLC system, are shown in Fig. 4. The chromatograms facing each other, for example Fig. 4a and 4b, were obtained using the same solvent pair but with the reversed sequence. The chromatograms in Fig. 4 are so arranged that AAW was used in the vertical direction, and the non-alcoholic solvents in the horizontal direction; whereas AAW was the first direction solvent in chromatograms on the right, it was the second direction solvent in chromatograms on the left. Each chromatogram in Fig. 4 was spotted with 45 μl of the stock solution. The hR_F values of AAW are summarized in Table IV and of the non-alcoholic solvents earlier in Table III.

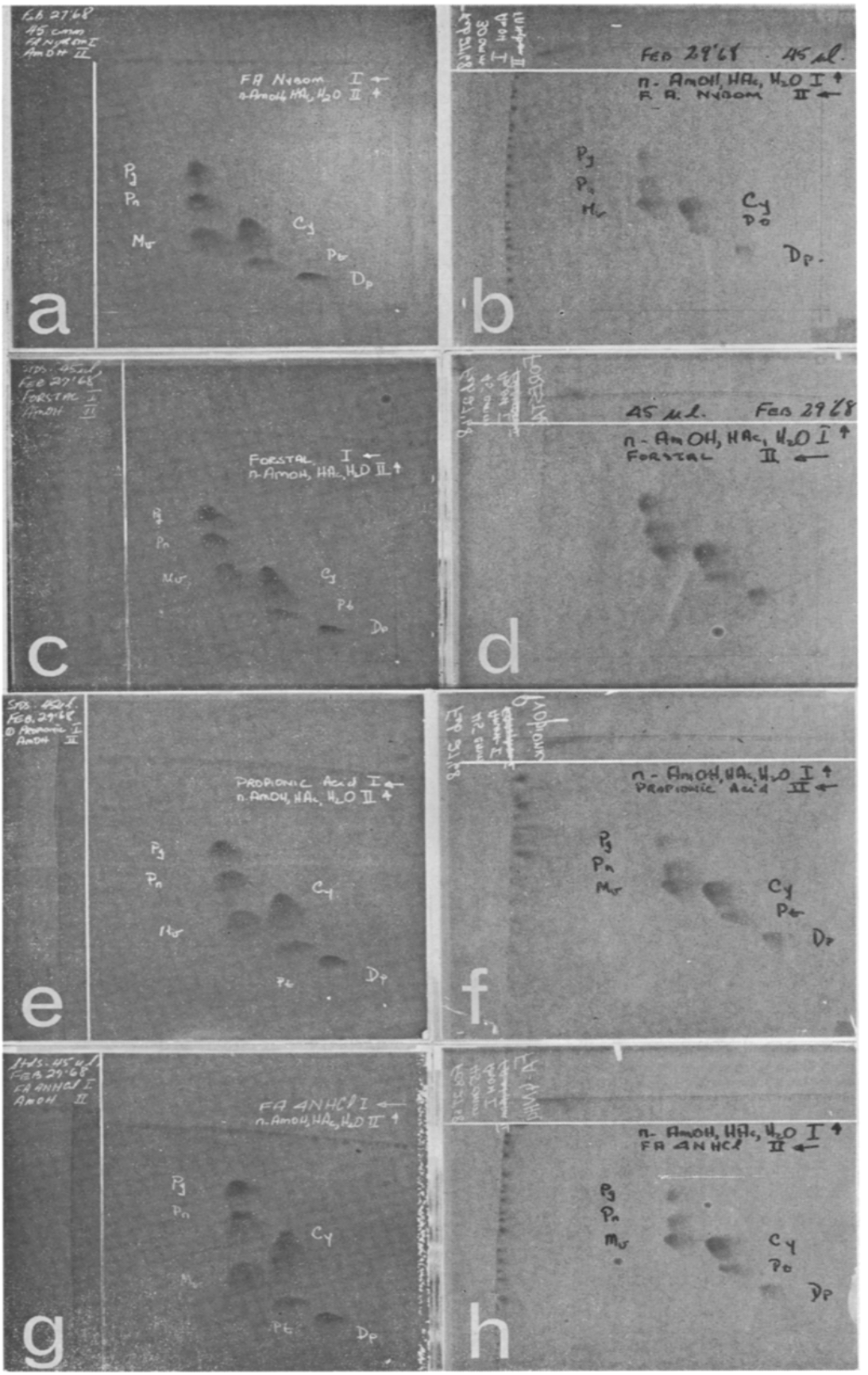

Fig. 4. The effect of reversing the sequence of the same solvent pair on TLC resolution of anthocyanidins. Beginning from left side top to bottom (a) FA–Nybom, (c) Forestal, (e) Propionic and (g) FA–4 *N* HCl with AAW used as the second solvent in each case. The resolution in the figures on the right, namely, (b) (d), (f) and (h) is given by the same solvent pair as that used in the corresponding left figure except that AAW was used as the first solvent in each case. The chromatograms are so arranged that AAW was run in the vertical and non-alcoholic solvents in the hori-

TABLE IV

AVERAGE hR_F VALUES[a] AND hR_F RANGES[b] OF ANTHOCYANIDINS[c] BY TLC ON AVICEL SF IN AAW SOLVENT WHEN USED IN THE FIRST AND SECOND DIRECTIONS

Pigments	*hR_F values in AAW (second direction) First direction solvents*					*hR_F values[e] in AAW alone*
	Propionic	*FA–4 N HCl*	*FA–Nybom*	*Forestal*	*MHW[d]*	
Dp	20	19	18	21	24	27
	18–23	17–22	15–22	17–24	24, 24	
Pt	26	24	24	28	32	34
	25–27	22–28	21–27	24–33	32, 32	
Mv	40	42	38	40	43	46
	37–38	40–44	33–44	33–45	43, 44	
Cy	47	51	42	44	43	46
	43–52	46–57	37–47	41–46	43, 44	
Pn	57	65	51	53	52	54
	53–60	62–67	47–56	50–56	52, 52	
Pg	70	80	64	63	60	63
	64–76	77–82	59–73	59–68	60, 61	

[a] First entry in each column.
[b] Second entry in each column.
[c] Pigment concentration in all cases was 45 µl/spot.
[d] Data was calculated from two plates only.
[e] Pigments fade: hR_F values are generally difficult to calculate.

The following major features of the AAW solvent were observed. Whenever AAW is the first direction solvent (all chromatograms on the right of Fig. 4), marked fading of anthocyanidins, especially Pg and Pn, occurs. However, fading is minimal when AAW is used in second direction and non-alcoholic solvents in the first direction. It was mentioned earlier that the R_F values of all anthocyanidins in the MHW solvent were higher in the first than the second direction. In the AAW solvent, however, the R_F values of Dp, Pt and Mv are higher, those of Cy are about the same and those of Pg and Pn lower in the first direction than the second direction. Whereas the R_F value of Cy and Mv are about the same in the first direction of AAW, they vary markedly in the second direction, particularly when the first direction solvents are non-alcoholic; the variation, however, is minimal when the first direction solvent is MHW (Table IV). This variation is illustrated clearly in Fig. 4 (compare the R_F values of Cy and Mv in the first and second direction of AAW). Especially, when FA–4 *N* HCl is the first direction solvent, the R_F value patterns of Cy in relation to Mv in the second direction of AAW are sometimes similar to those obtained in Fig. 4g, while at other times they are similar to those obtained in Fig. 4a, 4c and 4e.

One of the problems with the AAW solvent is that it gives a wide range of R_F value fluctuation for anthocyanidins with hR_F values greater than 50 (compare R_F values of Pg and Pn in Table IV). Despite the fluctuations, the solvent combination non-alcoholic solvents (first direction)–AAW (second direction) gives a characteristic resolution. In this combination, the fading is minimised.

BAW and FA–4 N HCl combination

It is well known that the BAW solvent causes fading of anthocyanidins. NYBOM[5] found it unsuitable for TLC on Cellulose MN300. Owing to the paucity of

TABLE V

AVERAGE hR_F VALUES OF ANTHOCYANIDINS BY TLC ON AVICEL SF IN BAW WHEN USED IN THE THE FIRST AND SECOND DIRECTIONS

Pigments	*hR_F values in BAW alone*	*hR_F values in BAW (second direction)*	
		First direction solvents	
	40 µl[a]	*FA–4 N HCl (40 µl*[a]*)*	*MHW (40 µl*[a]*)*
Dp	36	24	28
Pt	—[b]	27	35
Mv	49	37	40
Cy	53	46	45
Pn	60	61	50
Pg	—[b]	88	68

[a] New stock solution. Pigment concentration in all cases was 40 µl/spot.
[b] Not visible.

suitable solvents for anthocyanidin chromatography, HARBORNE[7] has recently reported the R_F values of 19 anthocyanidins using the BAW solvent in uni-dimensional paper chromatography. HARBORNE found that the fading of the pigments can be prevented by using papers washed with 2 *N* HCl. The merits of this solvent were therefore re-appraised using two-dimensional TLC on Avicel SF. The average hR_F values in BAW are given in Table V, and those for FA–4 *N* HCl in Table III. The increases and decreases in R_F values in the first and second direction of BAW are similar to those reported for the AAW solvent. When BAW is used as the first direction solvent, pelargonidin (*ca.* 1 µg) disappears and extensive fading of Pt and Dp occurs (Fig. 5d). The fading is decreased considerably but some still occurs when BAW is used as the second direction solvent against FA–4 *N* HCl (Fig. 5c), a situation which is similar to using an acid washed paper. However, this combination owing to

TABLE VI

AVERAGE hR_F VALUES OF ANTHOCYANIDINS[a] BY TLC ON AVICEL SF IN BHAW WHEN USED IN THE FIRST AND SECOND DIRECTIONS

Pigments	*hR_F values in BHAW alone*	*hR_F values in BHAW (second direction)*	
		First Direction solvents	
		FA–4 N HCl	*MHW*
Dp	23	15	21
Pt	29	18	27
Mv	37	26	35
Cy	50	32	47
Pn	56	37	53
Pg	76	50	75

[a] Pigment concentration in all cases was 45 µl/spot.

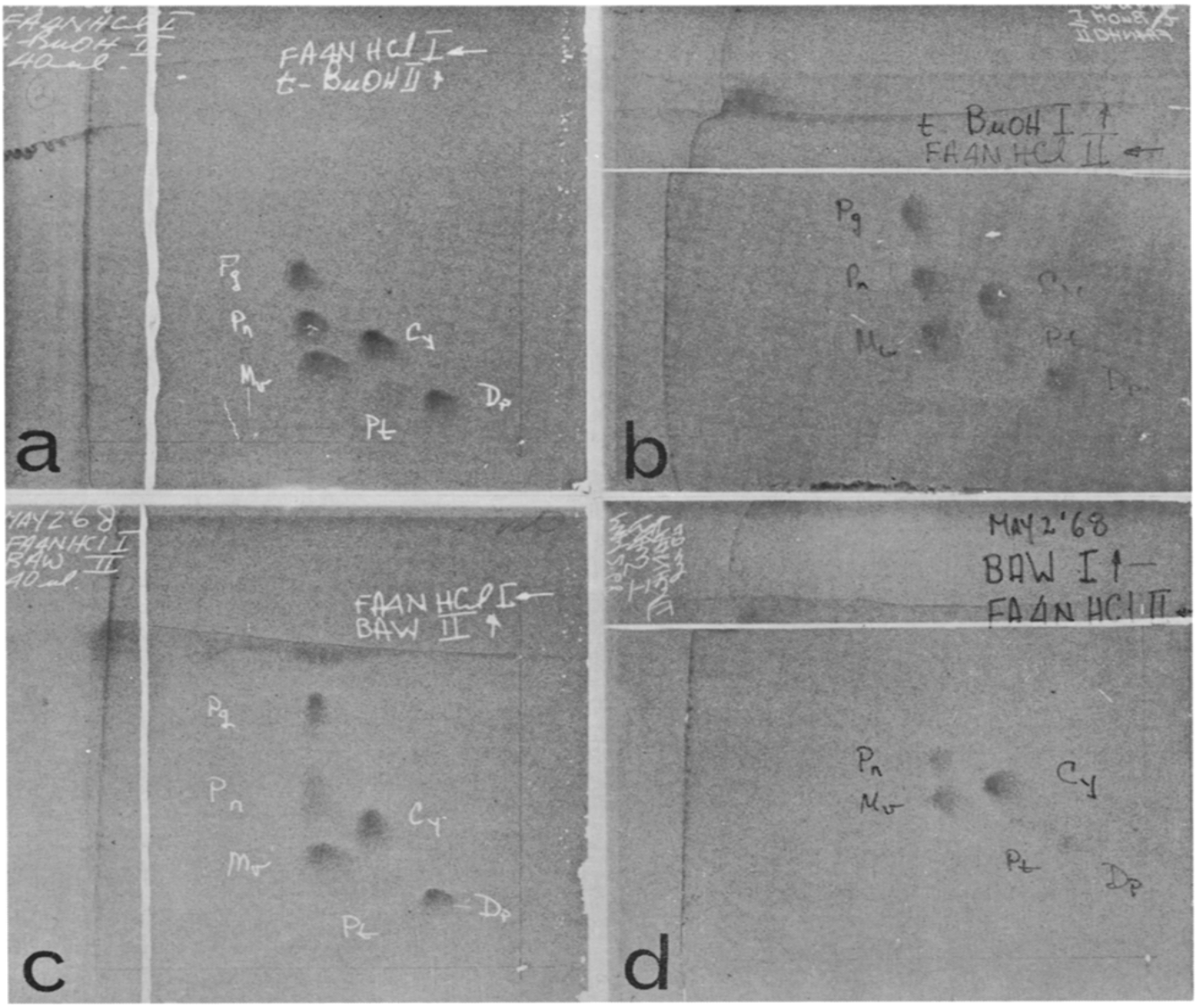

Fig. 5. TLC patterns of anthocyanidins in (a) FA–4 *N* HCl with BHAW used as the second solvent, (b) BHAW with FA–4 *N* HCl used as the second solvent, (c) FA–4 *N* HCl with BAW used as the second solvent and (d) BAW with FA–4 *N* HCl used as the second solvent. A new stock solution of anthocyanidins, which was similar to that described under the Experimental part except that petunidin was weak, was used for obtaining these chromatograms.

tailing of Pg and Pn (Fig. 5c) is not as useful as the BHAW solvent, which is described next.

BHAW and FA–4 N HCl combination

The BHAW solvent of CLEVENGER[6] gave good resolution in both directions; the separation of the spots is greater in the first direction, but the diffusion is also greater (Fig. 5a and 5b). The fading in this solvent is of the same magnitude as that in Forestal and MHW. It is our belief that the R_F value variation is minimal when BHAW is used as the first solvent rather than the second solvent. The hR_F values are included in Table VI. BHAW, like MHW, gives consistently higher R_F values for all anthocyanidins in the first direction than in the second direction.

Alcoholic solvent combinations

Of all the combinations between the alcoholic solvents listed in Table I, the one consisting of MHW (first direction)–AAW (second direction) gives very good resolution (Fig. 2d), the rest are of little use. The hR_F values are given in Table II and IV.

Application

The above solvent combinations with Avicel SF have been applied successfully to the analysis of crude extracts from periderm tissues of conifers[1]. The choice of appropriate solvent combinations have made possible the detection of new leucoanthocyanidins[2], and the detection and identification by spot tests[8] of anthocyanidins occurring as such in the periderm[1].

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