A NEW DEVELOPING SOLVENT FOR PAPER CHROMATOGRAPHY OF VARIOUS PHENOLIC COMPOUNDS, SUGARS AND AMINO ACIDS*

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INTRODUCTION

Attempts to develop a quantitative method for the estimation of each of the four anthocyanin pigments in American cranberries, *Vaccinium macrocarpon* Ait. revealed the fact that no paper chromatographic solvent system was available which was capable of separating the pigments. The pigments have been identified by SAKAMURA AND FRANCIS[1] and ZAPSALIS AND FRANCIS[2] as cyanidin-3-galactoside, peonidin-3-galactoside, cyanidin-3-arabinoside, and peonidin-3-arabinoside. This research was aimed at developing a solvent system capable of separating the above pigments and testing its suitability for much wider applicability.

MATERIALS AND METHODS

Solvent

The developing solvent was prepared by thoroughly mixing 1-butanol, benzene, formic acid (sp. gr. 1.2) and water (BBFW) in a separatory funnel equipped with a Teflon stopcock. The proportion of the components, unless otherwise specified was 100:19:10:25, v/v. The developing solvent for the R_F and R_G determinations was aged over the aqueous phase at 73 ± 1 °F for 3 days. All chemicals were of reagent grade.

Standard compounds

The source of the chemicals is given in the list of R_F values. Cranberry pigment extracts were obtained from the berries of the American cranberry, var. Early Black, by extracting the crushed berries with 1 % HCl in methanol.

Spray reagents

Bis-diazotized benzidine[3]. Three parts of 0.5 % acidified (14 ml conc. HCl/l) aqueous benzidine were mixed with two parts of 10 % $NaNO_2$, immediately before use. The sprayed chromatograms were washed in tap water for 20 min and air dried.

Ninhydrin[4]. 0.2 % ninhydrin in 95 % ethanol. The treated chromatograms were heated to 60°C.

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Aniline hydrogen phthalate[5]. 1.66 g *o*-phthalic acid and 0.91 ml aniline were dissolved in a mixture of 48 ml 1-butanol, 48 ml ethyl ether and 4 ml water. The chromatograms were dipped into the solution, air dried and heated in an oven to 105°C for 2 min.

Aluminum chloride. 5 g crystalline aluminum chloride dissolved in 100 ml 95 % ethanol.

Paper

Whatman No. 1 filter paper was used for all experiments. The sheets were cut to fit the glass frames of a Kurz-Miramon all glass chromatography tank (Kensington Scientific Corp., Berkeley, Calif.) in such a way that the direction of development coincided with the machine direction of the paper. Paper strips, 7 × 46 cm, were used for preliminary screening of solvents.

Chromatography jars

Kurz-Miramon all glass, 12 × 12 × 24 in., chromatography tanks with frames for ascending and descending development were used respectively for the R_F and R_G determination. The chromatography strips were developed in 2 l cylinders covered with parafilm. The strip was suspended from a chromatographic clip attached to a nylon line pulled across the top of the cylinder. The line, as well as the parafilm cover, was fastened with rubber bands.

Ultraviolet light source

Black-Ray 365 nm long wave, Model C-50 and Mineralight 253.7 nm short wave, Model C-51 transilluminators (Ultra-Violet Products, Inc., San Gabriel, Calif.) were used for locating the spots as indicated in the list of R_F and R_G values.

Chromatographic procedure

Ascending chromatography was employed for all experiments except the R_G determinations in which a descending technique was used. For ascending chromatography, the baseline was drawn 4.0 cm from the bottom edge and for descending development it was drawn 8.2 cm from the top edge of the paper. Solutions of the standard compounds were applied as spots 2.0 cm apart leaving 3.0 cm free from both edges of the sheet. Cyanidin-3-galactoside was applied on the sheets as a marker. The chromatograms were equilibrated overnight over the developing solvent in the bottom of the tank. The development of the chromatograms was carried out at 73° ± 1°F and it required approximately 34 h for ascending and 60 h for descending development. The developed chromatograms were air-dried at 73°F and sprayed with a color developing reagent if necessary for visualization of the spots. The spots were outlined and their center of highest concentration marked for R_F or R_G values either under visible light or over a U.V. transilluminator. The solvent front was detected with the long wave U.V. source.

The chromatographic strips were treated the same way as the sheets, except that cranberry extract was applied on the middle of the baseline, 3.2 cm from the bottom edge of the strip as 1.5 cm streaks. Equilibration was not necessary because the ratio of developing solvent to tank atmosphere was such that saturation of the atmosphere occurred very quickly.

RESULTS AND DISCUSSION

Cranberry anthocyanins

More than 50 solvent systems were tested, but none of them were able to separate the four cranberry anthocyanins. BBFW and some of its modifications were the only solvent systems capable of separating these pigments. R_F data in BBFW for these four pigments together with several other anthocyanins are presented in Table I. The chromatograms developed with BBFW resulted in extremely well defined compact spots or bands. This characteristic of the new solvent is responsible primarily for the separation of cranberry anthocyanin where the R_F difference between the two pigments (peonidin-3-galactoside and cyanidin-3-arabinoside) which were not separated by BAW (1-butanol–acetic acid–water, 4:1:5) was smaller with BBFW (0.024) than with BAW (0.04). The ability to form very compact bands or spots is highly desirable for good pigment separation and also for densitometric measurements for quantitative analysis.

To select the developing solvent best suited for the separation of cranberry anthocyanins, several variants of BBFW have been tried. The proportion of the

TABLE I

R_F VALUES OF VARIOUS COMPOUNDS

Compound	*Source*[a]	$R_F \times 100$		*Detection method*[d]
		BAW[b]	*BBFW*[c]	
I. Anthocyanins				
Pelargonidin-3-glucoside	6	59 (7)	13	U.V.
Cyanidin-3-galactoside	1	37 (2)	5.3	U.V.
Cyanidin-3-glucoside	6	38 (7)	6.1	U.V.
Cyanidin-3-arabinoside	2	43 (2)	9.1	U.V.
Peonidin-3-galactoside	1	39 (2)	6.7	U.V.
Peonidin-3-arabinoside	2	44 (2)	12	U.V.
Cyanidin-3-rhamnoglucoside	8	33 (8)	4.7	U.V.
Pelargonidin-3,5-diglucoside	A	34 (7)	2.1	U.V.
Cyanidin-3,5-diglucoside	A	16 (7)	1.4	U.V.
Malvidin-3,5-diglucoside	A	22 (7)	1.1	U.V.
Delphinidin-3-*p*-coumaroyl-rutinoside-5-glucoside	9		2.5	U.V.
II. Anthoxanthines		(10)		
Apigenin	K	89	89 (t)	$AlCl_3$ spray, V
Kampferol	K	84	86 (t)	U.V.
Fisetin	K	63	70 (s)	U.V.
Robinetin	K	41	23 (t)	U.V.
Quercetin	K	66	62 (s)	U.V.
Quercetin-3-rhamnoside	K	67	57 (t)	U.V.
Quercetin-3-arabinoside	10	71	65 (s)	U.V.
Quercetin-3-galactoside	K	50	34 (s)	$AlCl_3$ spray, V
Quercetin-3-rhamnoglucoside	K	43	15	U.V.
Myricetin-3-arabinoside	10	56	42 (t)	U.V.
Myricetin-3-digalactoside	10	37	15 (t)	U.V.
Neohesperidin	L		25 (s)	U.V.
2′, 4′, 4-Trihydroxychalkone-4,4′-diglucoside	11	34 (11)	5.8	U.V.

(continued on p. 407)

TABLE I (*continued*)

Compound	*Source*[a]	$R_F \times 100$		*Detection method*[d]
		BAW[b]	*BBFW*[c]	
III. Phenolic acids and simple phenols				
p-Hydroxybenzoic	F	90 (7)	90	sU.V.
2,4-Dihydroxybenzoic	L		89	U.V.
4-Hydroxy-3-methoxybenzoic	L		88	sU.V.
Chlorogenic	L	56 (13)	47 (s)	U.V.
3,4,5-Trimethoxycinnamic	Fl		91	U.V.
m-Methoxycinnamic	A		93	U.V.
p-Coumaric	A	91 (12)	88	U.V.
Gallic	F	68 (7)	51	sU.V.
Syringic	A		83 (m)	sU.V.
Caffeic	A	82 (12)	76 (t)	U.V.
Ferulic	A	89 (12)	84 (t)	U.V.
Sinapic	Fl		78 (t)	U.V.
Resorcinol	F	91 (7)	86	benzidine spray, V
Phloroglucinol	D	76 (7)	62	benzidine spray, V
IV. Amino acids		(14)		
L-Arginine	F	15	0.6	ninhydrin spray and U.V.
L-Asparagine	F	12	1.3 (m)	ninhydrin spray and U.V.
L-Glutamic acid	F	28	4.6 (m)	ninhydrin spray and U.V.
L-Histidine	F	11	0.3	ninhydrin spray and U.V.
L-Isoleucine	F	67	30 (f)	ninhydrin spray and U.V.
L-Leucine	F	70	31 (f)	ninhydrin spray and U.V.
DL-Lysine	F	12	0.3	ninhydrin spray and U.V.
L-Methionine	F	50	17 (f, m)	ninhydrin spray and U.V.
DL-Phenylalanine	F	60	28	ninhydrin spray and U.V.
L-Proline	F	34	6.9	ninhydrin spray and U.V.
L-Serine	F	22	2.3	ninhydrin spray and U.V.
L-Threonine	F	26	4.4	ninhydrin spray and U.V.
L-Tryptophan	F	50	22	ninhydrin spray and U.V.
L-Tyrosine	F	45	14	ninhydrin spray and U.V.
L-Valine	F	51	19	ninhydrin spray and U.V.

[a] Source of material: A = Aldrich Chemical Co. Inc., Milwaukee, Wis.; D = Distillation Products Industries, Rochester, N.Y.; F = Fisher Scientific Co., New York, N.Y.; Fl = Fluka A.G., Buchs, S.G., Switzerland; K = K & K Laboratories Inc., Plainview, N.Y.; L = Koch-Light Laboratories Ltd., Colnbrook, Bucks., England. A figure indicates a reference paper.

[b] The R_F values given for BAW (4:1:5) are quoted from the literature. The reference is given after R_F value in brackets if it is different from that given for the group.

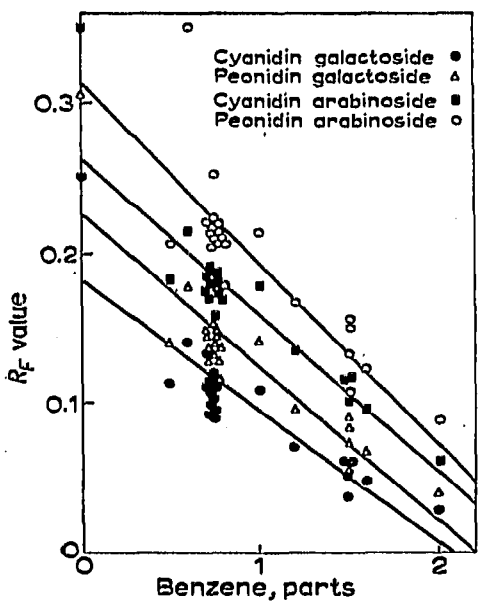
[c] The letters in brackets after the R_F values indicate the following: m = tendency to form multiple spots; s = streaking; t = tailing; f = frontal "tailing".

[d] The spot and its center of maximum concentration was located under the following light sources: V = visible; U.V. = long wavelength ultraviolet; sU.V. = short wavelength ultraviolet.

constituents was varied around the values of the solvent system first found to separate the cranberry anthocyanins. The results showed that variation of the acid content had little effect on the R_F values or on the mobility of the solvent front. The formic acid, however, is responsible for the compactness of the spots. The proportion of benzene used was more critical. The R_F values obtained for the four cranberry anthocyanins with different proportions of benzene were plotted on a scatter diagram and the lines of best fit were drawn (Fig. 1). The increase in benzene content resulted in a definite decrease in the R_F values of cranberry anthocyanins. The speed of development, as indicated by the rate of solvent flow (Fig. 2), increased with larger proportions

of benzene. The results indicated that the solvent system was not sensitive to slight variations in composition.

The effect of benzene can be explained by the fact that it reduced the water content of the organic phase. The water content of the one phase, water-saturated BAW (52:13:35) is 36.3 %, while a similar BBFW (80:15:8:10) system held only 8.8 % water. The lower water content resulted in a less polar solvent system which had a reduced affinity for hydrophylic substances such as anthocyanins, sugars and amino acids. This was advantageous in this case, because successful separation was achieved through having a large number of exchanges taking place between the stationary and the mobile phase per unit distance. Since the pigments had a short distance of travel, diffusion of the spots was also reduced.



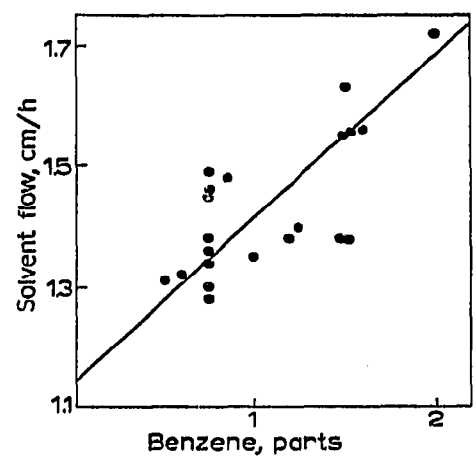


Fig. 1. Effect of benzene content of BBFW (1-butanol = 4.0; benzene = 0 to 2.0; formic acid = 0.5; water = 2.5) on the R_F value of cranberry anthocyanins.

Fig. 2. Effect of benzene content of BBFW (1-butanol = 4.0; benzene = 0 to 2.0; formic acid = 0.5; water = 2.5) on the mobility of the solvent front.

The solvent system selected on the basis of giving the best separation of anthocyanins present in cranberries was BBFW (100:19:10:25). This solvent was used for the R_F and R_G determinations as well. The results in Fig. 1 demonstrate how the polarity of the solvent system can be adjusted for specific separations.

Thirty-eight R_F values were determined under carefully controlled conditions for cyanidin-3-galactoside. The R_F values showed a mean of 0.053 and a standard deviation of $\pm$ 0.007. These results indicated that the R_F values were reliable only to two places of decimals. The R_F values included in this paper are expressed to the third decimal since effective separation can be achieved in cases where the difference in R_F values is in this range. For routine work some of the precautions applied in the R_F and R_G value determinations are not necessary. The discrepancies between the R_F values of cranberry anthocyanins reported in Table I and in Fig. 1 are due to differences in the samples applied on the chromatograms. The values reported in

Table I were obtained with pure pigments, whereas in Fig. 2 crude cranberry extracts were used.

Aging. The solvent requires two to three days aging to reach equilibrium. It could be used as soon as the organic phase became clear, which usually took about two to three hours from the preparation of the solvent. However, in this case aqueous droplets will separate during ascending developments. This results in a false front if it comes in contact with the paper. The aqueous second front is clearly visible during development since the paper loses its translucency due to the benzene-containing organic phase. The developing solvent can be used repeatedly if excessive loss of the highly volatile components due to evaporation is prevented.

Equilibration. Overnight equilibration was applied in the R_F and R_G value determinations, but this step can be omitted in routine applications. It is necessary, however, to saturate the atmosphere of the tank with the developing solvent. This can be accomplished instantaneously by lining one side of the tank with filter paper and soaking it with the solvent.

Other anthocyanins

The separation between anthocyanin mono- and diglycosides (Table I) was also more distinct with BBFW than that with BAW. Due to their slow mobility in BBFW, the separation of anthocyanins required a fairly long period of development. The slow mobility, however, with the compactness of the spots or bands allowed the separation of chemically closely related anthocyanins by developing the chromatograms for extended periods of time. The new solvent system was found useful not only for the quantitative separation of cranberry anthocyanins[17] but for the qualitative separation and purification of various other natural anthocyanin mixtures. For example, the anthocyanin in blueberry extracts could be separated to 8 bands and those in huckleberry extract to 5 bands *vs.* the 5 and 3 bands reported with BAW[12,16]. On preparative scale chromatography peonidin-3-glucoside separated from peonidin-3-galactoside on Whatman No. 3 paper developed with BBFW in a descending direction for three days. The separation of cyanidin-3-glucoside from cyanidin-3-galactoside was more difficult, but it was accomplished by using a variant of BBFW (100:38:10:25) and five days development time.

The anthocyanidins moved well ahead of the anthocyanins but their excessive streaking prevented the measurement of accurate R_F values.

Anthoxanthines

Comparison of the R_F values (Table I) showed that the less polar flavonoids in BBFW had R_F values very similar to those in BAW. The movement of the more polar flavonoids, *e.g.* those having R_F of 0.60 or less in BAW, were more affected by the use of BBFW. The flavonoids had a tendency for streaking and tailing and for this reason BBFW is not recommended for the separation of flavonoids. It should be noted, however, that in general, the monoglycoside flavonoids and aglycones had higher R_F values than that of the corresponding anthocyanins and aglycones. This indicated that BBFW may be useful for preliminary separation of the two classes of compounds.

Phenolic acids and simple phenols

The new solvent system appeared to be well suited for the separation of these

TABLE II

R_G VALUES FOR SUGARS AND RELATED COMPOUNDS

Compound	$R_G \times 100$		*Color[b] of the spot under long U.V. light after aniline phthalate treatment*
	BAW[a]	*BBFW*	
D-Glucose	100	100	Br
D-Galactose	89	85	Br
D-Mannose	111	147	Br
D-Fructose	128	162	Br
D-Xylose	155	221	R Br
D-Arabinose	117	182	R Br
L-Rhamnose	206	409	R Br
Lactose	50	12	Br
Maltose	61	20	Br
Sucrose	78	35	Br
Raffinose	28	9	Br
Glucuronic acid[c]	67 (178)	119 (477)	Br
Galacturonic acid	78	107	Br

[a] R_G values in BAW (4:1:5) were calculated from the R_F values given by PARTRIDGE AND WESTALL[15].

[b] Abbreviations for colors: Br = brown, R = red.

[c] The value in parentheses is that of the lactone.

compounds. Although tailing occurred in some cases, it could be reduced by applying smaller quantities on the paper Those substances having high R_F values in BAW behaved similarly in BBFW. The slower moving phenolic acids in BAW had a lower R_F value when developed with BBFW.

Amino acids

The new developing solvent successfully separated synthetic mixtures of amino acids. The R_F values obtained with BBFW were considerably lower than that recorded in the literature for BAW. The separated amino acids gave compact spots, and tailing, when it occurred, was rather mild and curiously it was observed on the front of the spot. The solvent can be recommended for amino acids when the time of development is not a limiting factor.

Sugars

BBFW proved to be excellent for the separation of sugars. Examination of the R_G values (Table II) showed that the new solvent separated the sugars much better than BAW, and gave compact spots. Galacturonic acid had a tendency to give two spots, probably due to lactone formation. A similar phenomenon was reported by PARTRIDGE AND WESTALL[15] for BAW. The separation was somewhat slow as indicated by the fact that glucose, for example, had an R_F of 0.03 *vs.* R_F 0.18 reported in the literature[15] for BAW.

SUMMARY

A new developing solvent for paper chromatography is described, which is capable of separating the 3-arabinosides and 3-galactosides of cyanidin and peonidin.

It is the upper phase of 1-butanol–benzene–formic acid–water (100:19:10:25). The solvent is also applicable to many other classes of compounds and gives excellent separations of sugars in particular. R_F or R_G values are given for a number of anthocyanins, anthoxanthines, simple phenols, sugars, phenolic and amino acids.

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