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Note

Simultaneous analysis of anthocyanins and anthocyanidins on cellulose thin layers

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Methods for the analysis of anthocyanin pigments have been reviewed recently[1]. The separation of these compounds is particularly difficult owing to their instability at all but acidic pH values and to the existence of a series of equilibria linking various stable forms within fairly narrow ranges of pH values[2].

Although high-performance liquid chromatography (HPLC) has recently been applied[3], paper chromatography is still probably the most widely accepted method for the routine analysis of anthocyanins (glycosides) and anthocyanidins (aglycones). Since thin layers of cellulose[4], silica[5] and polyvinylpyrrolidone[6] have found applications in this field, and numerous variations of the basic systems exist[1], this is somewhat surprising. However, none of the existing thin-layer chromatographic (TLC) systems is adequate for samples containing both anthocyanins and anthocyanidins. As one of the main methods applied in the structural investigation of anthocyanins[1] is partial hydrolysis, which produces just such mixtures, this is a significant disadvantage.

The time required for paper chromatography and the difficulties with that technique, prompted us to pursue the search for a TLC system that could be used for the simultaneous separation of the aglycones, mono-, di- and triglycosides. Since Nybom[4] had previously proposed two different hydrochloric acid–formic acid–water mixtures for the separate analysis on cellulose layers of anthocyanins and anthocyanidins, it was decided to carry out a systematic study of this solvent as developed on cellulose layers. A mixture containing hydrochloric acid (24.9%), formic acid (23.7%) and water (51.4%) proved to be suitable for the analysis of mixtures containing all structural types. The results obtained also provide a basis for the choice of developing solvent when only some groups of anthocyanins are present.

EXPERIMENTAL

Thin-layer chromatography

All TLC was carried out on commercial 0.1-mm cellulose layers (Art. 5716, Merck, Darmstadt, F.R.G.). Development was accomplished with solvent mixtures prepared from analytical grades of hydrochloric and formic acids, and deionised distilled water. The following solvent compositions were used (concentrated hydrochloric acid–formic acid–water, by volume): system 1 (30.8:7.7:61.5), system 2

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(19.0:19.5:61.5), system 3 (7.1:31.4:61.5), system 4 (30.8:27.8:41.4), system 5 (19.0:39.6:41.4), system 6 (7.1:51.4:41.4), system 7 (30.8:47.8:21.4), system 8 (19.0:59.6:21.4), system 9 (7.1:71.4:21.4), system 10 (24.9:23.7:51.4) and system 11 (27.8:15.7:56.5). Developing tanks were lined with heavy-grade filter paper and allowed to equilibrate overnight prior to use. Anthocyanins were applied to the plates as 1.0-μl aliquots of solutions with an optical density of 1.0. Plates were developed to a distance of 15 cm.

Anthocyanins and anthocyanidins

The anthocyanins in this study were obtained from the established sources given in Table I. The pigments were extracted from fresh plant material with 1% methanolic hydrochloric acid. The extract was defatted by washing with light petroleum (b.p. 40–60°C) and dried under reduced pressure. The pigments were redissolved in methanolic hydrochloric acid[7].

Individual anthocyanins were isolated by TLC as here described, and their identities were confirmed by visible light absorption spectroscopy and by the identification of the aglycone and sugar obtained on hydrolysis[1].

Anthocyanidins were obtained by hydrolysis of the appropriate glycosides[1].

RESULTS AND DISCUSSION

Preliminary investigations were performed with the two hydrochloric acid–formic acid–water developing solvents suggested by Nybom[4] for anthocyanin pigments in a general study of TLC for fruit evaluation. The results (see Table I) show that the combination recommended for anthocyanins, system 1 here, is adequate for these compounds although the great majority of them have R_F values less than 0.50; anthocyanidins are not separated by this system. The proposed developer for anthocyanidins, system 9 here, proved to be a disappointment in our hands as only three zones, reflecting the number of hydroxyl groups present in the aglycone structures, were obtained. As expected, system 9 was unsuitable for the glycosidic compounds, for which R_F values greater than 0.60 were obtained in all cases.

The above results showed that the systems 1 and 9 could be adopted as outer limits in searching for a developing solvent for the simultaneous evaluation of all anthocyanin pigments. The water and hydrochloric acid contents of these systems and their mean values were thus used to produce a series of compositions, nine in all (see Fig. 1).

In practice, systems 7 and 8 had to be discarded as many compounds gave ill-defined spots at or near the solvent front, and will not be further discussed. Comparison of the figures obtained with the remaining systems showed that increasing the formic acid content at the expense of either hydrochloric acid or water led to increases in R_F values.

The results indicate that this solvent mixture is sensitive to variations in the aglycone in the glycosides. While R_F values change in response to alteration of the sugar moiety in the biosides (cyanidin-3-rutinoside, sambubioside and sophoroside), there is no similar sensitivity to the monoglycosides (cyanidin-3-glycoside, galactoside and arabinoside). Earlier work on paper chromatography with solvents of low organic content showed the same trends[13].

TABLE I

R_F VALUES FOR ANTHOCYANIN PIGMENTS

Separation was on 0.1-mm cellulose layers using various hydrochloric acid–formic acid–water mixtures as developing solvent: solvent combinations are indicated by numerals and details are given in the main text. Abbreviations used in compound nomenclature: Dp = delphinidin; Pt = petunidin; Cy = cyanidin; Mv = malvidin; Pn = peonidin; Pg = Pelargonidin; glu = glucoside; gal = galactoside; ara = arabinoside; rut = rutinoside; sam = sambubioside; sop = sophoroside; glurut = (2^G-glucosyl)rutinoside. Compound sources: (a) hydrolysis[1]; (b) blackcurrant[8]; (c) grape[9]; (d) cranberry[10]; (e) strawberry[11]; (f) plum[12]; (g) snapdragon[13]; (h) redcurrent[14]; (i) raspberry[12]; (j) foxglove[15]; (k) fuschia[16]; (l) tufted vetch[17]; (m) radish[18].

Type	*Compound (source)*	*Solvent composition*								
		1	*2*	*3*	*4*	*5*	*6*	*9*	*10*	*11*
Aglycones	Dp (a)	0.00	0.01	0.04	0.04	0.07	0.12	0.26	0.03	0.02
	Pt (a)			0.07	0.07	0.11	0.19	0.40	0.05	0.03
	Cy (a)			0.10	0.09	0.14	0.22	0.40	0.06	0.04
	Mv (a)			0.11	0.11	0.17	0.28	0.53	0.07	0.04
	Pn (a)		0.05	0.12	0.12	0.20	0.32	0.53	0.08	0.05
	Pg (a)	0.03	0.07	0.15	0.14	0.23	0.36	0.53	0.11	0.07
Monoglycosides	Dp-3-glu (b)	0.04	0.08	0.16	0.21	0.30	0.37	0.62	0.14	0.08
	Pt-3-glu (c)	0.06	0.13	0.23	0.32	0.42	0.50	0.73	0.22	0.08
	Cy-3-glu (b)	0.09	0.17	0.29	0.35	0.45	0.52	0.72	0.26	0.17
	Cy-3-gal (d)	0.09	0.17	0.29	0.35	0.45	0.54	0.74	0.27	0.18
	Cy-3-ara (d)	0.09	0.18	0.30	0.35	0.45	0.52	0.70	0.27	0.18
	Mv-3-glu (c)	0.12	0.22	0.37	0.48	0.58	0.64	0.83	0.34	0.22
	Pn-3-glu (c)	0.14	0.25	0.41	0.49	0.59	0.64	0.82	0.38	0.26
	Pg-3-glu (e)	0.19	0.32	0.48	0.50	0.61	0.65	0.80	0.41	0.31
Diglycosides Biosides	Dp-3-rut (b)	0.15	0.24	0.35	0.46	0.56	0.69	0.77	0.36	0.26
	Cy-3-rut (b)	0.22	0.35	0.49	0.57	0.68	0.68	0.83	0.48	0.38
	Pn-3-rut (f)	0.32	0.47	0.59	0.74	0.79	0.75	0.90	0.62	0.49
	Pg-3-rut (g)	0.36	0.52	0.65	0.73	0.78	0.76	0.87	0.63	0.54
	Cy-3-sam (h)	0.38	0.48	0.58	0.70	–	–	0.87	0.62	0.54
	Cy-3-sop (i)	0.55	0.62	0.72	0.78	0.87	0.81	0.87	0.73	0.70
True diglycosides	Cy-3,5-diglu (j)	0.26	0.38	0.47	0.67	0.72	0.70	0.87	0.52	0.43
	Pn-3,5-diglu (k)	0.37	0.49	0.60	0.80	0.84	0.81	0.93	0.66	0.57
	Dp-3-rha-5-glu (l)	0.43	0.52	0.61	0.80	0.81	0.77	0.87	0.68	0.59
	Pt-3-rha-5-glu (l)	0.52	0.60	0.68	0.88	0.88	0.84	0.92	0.77	0.71
	Mv-3-rha-5-glu (l)	0.63	0.69	0.76	0.94	0.94	0.90	0.96	0.86	0.80
Triglycosides	Cy-3-glurut (i)	0.77	0.80	0.83	0.94	0.93	0.86	–	0.89	0.89
	Pg-3-sop-5-glu (m)	0.87	0.88	0.90	0.99	0.96	0.92	0.87	0.95	0.94

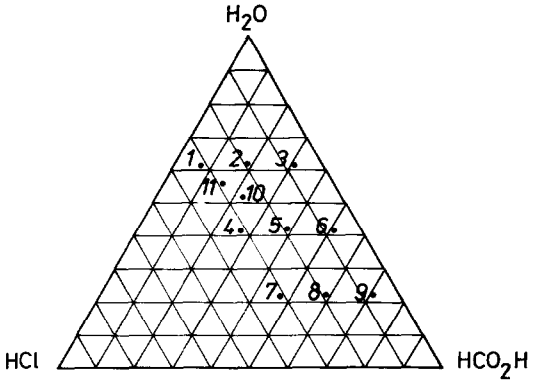

Fig. 1. Solvent compositions (by volume) for the hydrochloric acid–formic acid–water mixtures. Developing solvents for the chromatography of anthocyanins are indicated by numerals.

Development with the solvent systems 1–3 results in increasing R_F values along the series. However, if only mono- and diglycosides are considered, it is apparent that a shift rather than a spread of values has occurred. This displacement leaves room in system 3 for the adequate separation of the aglycones at the lower end of the R_F scale. On the other hand, an increasing degree of overlap between mono- and diglycosides is seen as system 3 is reached.

The systems 4–6 yield usable R_F values for the aglycones and monoglycosides, but values in excess of 0.75 for the diglycosides. A reduction of the R_F range available for the resolution of the glycosides follows from the increasing values observed for the aglycones along the series. In these systems only delphinidin-3-rutinoside crosses the mono–diglycoside boundary.

Based on the above observations, the system 4 has the advantages of resolving within a reasonable R_F range the aglycones and monoglycosides and of providing separation at the aglycone–monoglycoside and mono–diglycoside boundaries. On the other hand, the values for the diglycosides are undesirably high. To ameliorate this last drawback a solvent system of higher water content, *i.e.* closer to systems 1–3, is needed, and a system 10 corresponding to the midpoint between 4 and 2 was investigated.

System 10 did indeed have the advantages of system 4, while at the same time retaining the diglycosides comfortably behind the solvent front. In view of the very high values found for the triglycosides, a further system (system 11) halfway between 10 and 1 was examined: system 1 gives the lowest values for triglycosides in this study. System 11 had only marginal effect on the triglycoside R_F values and failed to separate adequately both aglycones and the least polar monoglycosides.

It is concluded tht system 10 is well suited for the separation of anthocyanin mixtures. Only triglycosides can present problems and, as these are relatively unusual, this is not a major disadvantage. Where the whole range of pigments is not present specific solvent compositions may be advantageous, *e.g.* system 6 for aglycones, system 5 for aglycone–monoglycoside mixtures, and system 2 for mono–diglycoside mixtures.

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