plicated problems of the undesired interaction products of sugars and amino acids[27,28], and of acid reversion[29] remain to be resolved.

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Received April 15th, 1969

*J. Chromatog.*, 43 (1969) 528–530

CHROM. 4214

## Thin-layer chromatography of sugars, anthocyanins and anthocyanidins on Kieselgel G impregnated with basic lead acetate

Thin-layer chromatography has been widely used for the separation and identification of microgram quantities of sugars[1–35]. Kieselgel G and Kieselguhr G are the adsorbents most frequently used, but other substrates have also been proved satisfactory, *e.g.*, magnesium silicate[7], calcium silicate[8], plaster of Paris[30], cellulose[13,21–23,25,28,48] and polyamide[31]. To improve the separation of sugars, the adsorbent layers have been mostly prepared in buffer solutions of alkaline acetates or phosphates, and less often in borate[3,4,16,19,34,35] or sodium bisulphite[18].

Recently TLC of anthocyanins has been achieved successfully on several substrates; among them cellulose has been the one preferred[36–40], next Kieselgel G[41–45], then polyacrylonitrile–polyamide–alumina mixtures[15,46] and finally polyvinylpyrrolidone–cellulose[47]. As far as we know, the TLC separation of mixtures of sugars, anthocyanins and anthocyanidins has not been reported.

The purpose of this work was to ascertain if Kieselgel G chromatoplates impregnated with basic lead acetate would permit a separation of sugars from anthocyanins and anthocyanidins in the analysis of low and high acidity mixtures of their solutions.

*Experimental*

*Samples.* Solutions of each group of the following compounds were prepared in: (i) methanol–water–0.02 *N* hydrochloric acid (30:50:20), and (ii) methanol–water–10 *N* hydrochloric acid (30:50:20).

A: Rutin, rutinose, glucose and rhamnose.
B: Quercetin.
C: Gentiobiose, galactose, arabinose, xylose, rhamnose, pelargonin chloride, malvin chloride, peonin chloride, delphin chloride, callistephin chloride, delphinidin chloride, cyanidin chloride, pelargonidin chloride.
D: Lactose, galactose, arabinose, glucose; anthocyanins and anthocyanidins as in C.
E: Sucrose, glucose, fructose; anthocyanins and anthocyanidins as in solution C.
F: Anthocyanins of sweet cherries (*Prunus avium*).
G: Delphinidin chloride.
H: Cyanidin chloride.
I: Pelargonidin chloride.

The following final concentrations were used: sugars: 0.60 mg/ml; anthocyanins: 0.10 mg/ml; anthocyanidins: 0.10 mg/ml; rutin: 0.10 mg/ml; and quercetin: 0.05 mg/ml.

*Preparation of the plates.* 30 g of Kieselgel G "Merck" was mixed with 70.0 ml of basic lead acetate solution (40.0 ml of basic lead acetate solution $d^{15} = 1.28$, to 1 l with distilled water). The suspension was spread on five 20 × 18 cm frosted glass plates at a thickness of 0.25 cm. The plates were dried for 1 h at room temperature, then at 105° for 30 min; then stored in a desiccator over anhydrous calcium chloride.

*Application of the sample.* Aliquots (5 $\mu$l) of each sample solution were spotted on the plates at 1.5 cm intervals with a self-filling micropipette, and at 1.5 cm from the lower edge. The spots were dried at room temperature in a current of cold air.

*Solvents.* Solvents which gave the best results are listed as follows:

I *n*-Butanol–acetone–water–benzene–ammonia (28 Bé) (20:85:15:5:0.3).
II *n*-Butanol–methanol–acetone–water–ammonia (28 Bé) (70:20:100:20:0.3).
III *n*-Butanol–acetone–chloroform–water (60:25:5:10).
IV *n*-Butanol–acetone–benzene–water (100:100:3:20).
V Ethanol 95 %–acetone–ethyl acetate–ethyl ether–water (25:35:60:5:12).
VI *n*-Butanol–acetone–chloroform–water (20:90:20:10).
VII Isoamyl alcohol–ethyl acetate–pyridine–water (10:50:20:10).
VIII Isopropanol–isoamyl alcohol–acetone–ethyl acetate–chloroform–water (40:10:10:10:20:10).

*Development of the chromatoplates.* Plates were developed by an ascending tech-

nique at 20 ± 2° to a distance of 12–13 cm from the origin in closed glass tanks lined with paper saturated with the developing solvent. Development took about 40–60 min.

*Detection of the spots.* Developed plates were dried in a current of air, till most of the solvent had evaporated, and then returned for 15 min to a glass tank saturated with hydrochloric acid vapour. Anthocyanins and anthocyanidins show up as vivid red spots, when observed under UV light (350 nm).

The plates are then heated at 105° until the odour of solvent could no longer be detected, and then sprayed with a chromogenic reagent. The reagent is made up by mixing 22 ml of 0.20 % ethanolic naphthoresorcinol solution with 1 ml of 85 % phosphoric acid[3].

After heating at 105° for 5 min, the sugars appear against a pink background as vivid spots with the colours listed in Table I.

TABLE I

COLOURS OF SUGARS ON SPRAYING WITH NAPHTHORESORCINOL REAGENT AFTER DEVELOPMENT IN NEUTRAL (A) AND BASIC SOLVENTS (B), AND FLUORESCENCE OF SPOTS

| *Sugar* | *Colour* | | *Fluorescence* |
|---|---|---|---|
| | *A* | *B* | |
| Rutinose | Sea blue | Sea blue | none |
| Lactose | Blue-violet | Violet | none |
| Sucrose | Lilac | Violet | none |
| Gentiobiose | Gray-violet | Gray-violet | none |
| Galactose | Gray-blue | Blue-green | violet |
| Glucose | Gray-blue | Blue-green | violet |
| Arabinose | Olive | Olive | none |
| Xylose | Olive | Olive | none |
| Rhamnose | Cherry red | Red-violet | none |
| Fructose | Brick red | Brick red | none |

*Results and discussion*

In preliminary experiments it was noted that Kieselgel G layers without the addition of basic lead acetate did not permit the separation of the anthocyanins from sugars. Furthermore, increasing the concentration of basic lead acetate from 4 to 20 % (v/v) in the solution with which the plates were prepared, only kept the anthocyanins and anthocyanidins at the origin, while the sugars showed low $R_F$ values for all the solvents tried.

However, it can be observed, from the data in Table II, that if the sample solutions are slightly acid and chromatoplates impregnated with basic lead acetate are used, a satisfactory separation of the sugars from anthocyanins and anthocyanidins is achieved.

The $R_F$ values of the sugars in each solvent system listed lie between those of the anthocyanins and that of the pelargonidin. The pigments, which constitute the colouring matter of sweet cherries (*Prunus avium*) have an $R_F$ value clearly lower than the sugars.

Solvents I, II and III give a good separation of rutin from rutinose, and of this from its hydrolysis products, glucose and rhamnose.

As to the different sugars, solvents I, III, VI, VIII gave a good resolution of

TABLE II

$R_F$ VALUES (× 100) OF SUGARS, ANTHOCYANINS, ANTHOCYANIDINS, RUTIN AND QUERCETIN DISSOLVED IN METHANOL–$H_2O$–0.02 *N* HCl (30:50:20)

| *Solution* | *Compound* | *Solvents* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *I* | *II* | *III* | *IV* | *V* | *VI* | *VII* | *VIII* |
| A | Rutin | 7 | 9 | 15 | 17 | 24 | [a] | [a] | 25 |
| | Rutinose | 21 | 28 | 16 | 27 | 28 | 11 | 8 | 26 |
| | Glucose | 30 | 37 | 22 | 35 | 37 | 21 | 18 | 31 |
| | Rhamnose | 48 | 56 | 45 | 52 | 55 | 45 | 40 | 56 |
| B | Quercetin | — | 5 | 9 | — | 10 | 9 | — | — |
| C | Gentiobiose | 12 | 15 | 10 | 14 | 16 | 5 | 5 | 10 |
| | Galactose | 21 | 29 | 20 | 27 | 29 | 16 | 14 | 23 |
| | Arabinose | 30 | 37 | 26 | 33 | 36 | 26 | 22 | 33 |
| | Xylose | 42 | 49 | 36 | 45 | 50 | 35 | 32 | 47 |
| | Rhamnose | 49 | 55 | 44 | 51 | 56 | 43 | 42 | 55 |
| | Delphin<br>Cyanin<br>Malvin<br>Peonin<br>Pelargonin<br>Callistephin<br>Delphinidin<br>Cyanidin | <8 | <9 | <9 | <7 | <10 | <5 | <5 | <7 |
| | Pelargonidin | 82 | 90 | 95 | 94 | 92 | 88 | 87 | 77 |
| D | Lactose | 10 | 14 | 8 | 11 | 16 | 6 | 5 | 11 |
| | Galactose | 22 | 27 | 19 | 26 | 29 | 17 | 14 | 24 |
| | Arabinose | 32 | 34 | 21 | 32 | 34 | 27 | 23 | 30 |
| | Glucose | 28 | 37 | 24 | 37 | 38 | 22 | 18 | 34 |
| | Anthocyanins<br>Anthocyanidins | [b] | [b] | [b] | [b] | [b] | [b] | [b] | [b] |
| E | Sucrose | 26 | 31 | 18 | 31 | 31 | 13 | 12 | 24 |
| | Glucose | 29 | 38 | 25 | 37 | 39 | 23 | 21 | 31 |
| | Fructose | 21 | 23 | 21 | 23 | 27 | 19 | 14 | 27 |
| | Anthocyanins<br>Anthocyanidins | [b] | [b] | [b] | [b] | [b] | [b] | [b] | [b] |
| F | Cherry anthocyanins | 5 | 9 | 7 | 5 | 9 | 6 | 8 | 6 |
| G | Delphinidin | 3 | 0 | 6 | 4 | 3 | 4 | 0 | 4 |
| H | Cyanidin | 10 | 2 | 9 | 9 | 12 | 7 | 6 | 8 |
| I | Pelargonidin | 83 | 81 | 95 | 95 | 92 | 88 | 87 | 77 |

[a] Streaks.
[b] $R_F$ values as in C.

mixture C, but with no solvent, except for VIII, could a fair separation of mixture D be achieved.

Sucrose, glucose and fructose are only resolved with the solvent systems I and IV; while lactose, glucose and galactose with solvents II, IV, V and VIII.

In the case of the anthocyanidins, $R_F$ value increases from delphinidin, to cyanidin, to pelargonidin, as their adsorption is strictly related to the number of

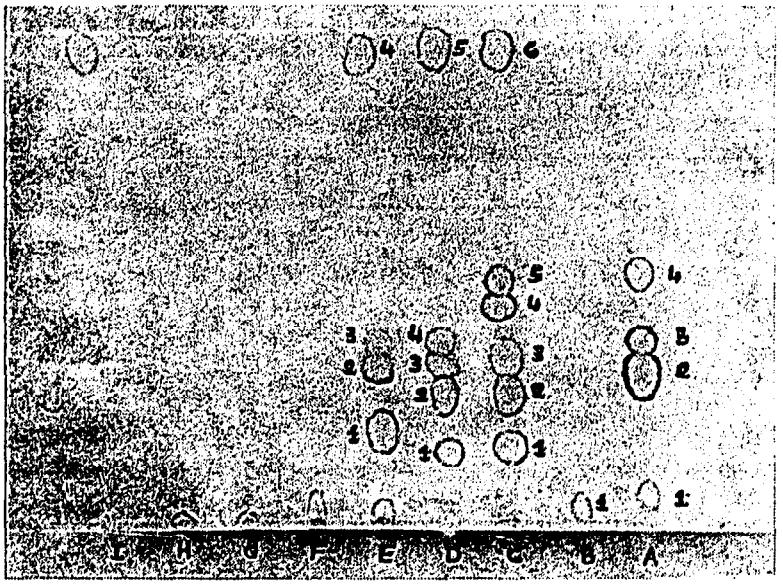

Fig. 1. Chromatogram of different mixtures dissolved in methanol–water–0.02 *N* hydrochloric acid solution and spotted onto Kieselgel G plates impregnated with basic lead acetate. A — rutin (1); rutinose (2); glucose (3); rhamnose (4). B — quercetin. C — pelargonidin, malvin, peonin, callistephin, delphinidin and cyanidin at origin; gentiobiose (1); galactose (2); arabinose (3); xylose (4); rhamnose (5); pelargonidin (6). D — anthocyanins and anthocyanidins as in C, at origin; lactose (1); galactose (2); arabinose (3); glucose (4); pelargonidin (5). E — anthocyanins and anthocyanidins as in C at origin; fructose (1); sucrose (2); glucose (3); pelargonidin (4). F — anthocyanins of sweet cherries (*Prunus avium*). G — delphinidin. H — cyanidin. I — pelargonidin. Developing solvent: *n*-butanol–methanol–acetone–water–ammonia (28 Bé) (70:20:100:20:0.30).

hydroxyl groups on the phenyl ring of their molecule. Solvents I and V, and especially III, gave well-defined and sharp spots.

The comparison between the data in Tables II and III shows that the increase of the acidity of the sample solution (Table III), modifies the results of the Table II considerably, mostly affecting the separation of the sugars from the anthocyanins and anthocyanidins. This separation is now only possible with solvent V and partially with solvents IV and VIII; however, solvents I and III gave a good resolution of the anthocyanins and anthocyanidins with well-shaped spots.

The increase of acidity almost always raises the $R_F$ value of the sugars above that in Table II; nevertheless the resolution of the sugars in mixture A, remains discrete in solvents, I, II and IV, and of mixture C in almost all the solvents, but the separation of the sugar in mixtures D and E is worse.

The high $R_F$ value of pelargonidin in all solvents can be attributed to the absence in its molecule of vicinal free hydroxyl groups, able to chelate with $Pb^{2+}$.

In conclusion it may be said that Kieselgel G chromatoplates impregnated with basic lead acetate allows the separation of sugars from some anthocyanins and anthocyanidins, if the sample solution is slightly acid.

With a solution of greater acidity, as is found in the analysis of the partial or total hydrolysates of anthocyanins, such a separation is still possible in some solvents, especially if the aglycon molecule possesses two vicinal free hydroxyl groups (*i.e.*: cyanidin).

TABLE III

$R_F$ VALUES (× 100) OF SUGARS, ANTHOCYANINS, ANTHOCYANIDINS, RUTIN AND QUERCETIN DISSOLVED IN METHANOL–$H_2O$–10 *N* HCl (30:50:20)

| *Solution* | *Compound* | *Solvents* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *I* | *II* | *III* | *IV* | *V* | *VIII* |
| A | Rutin | 7 | 10 | 30 | 15 | 30 | 16 |
| | Rutinose | 21 | 36 | 16 | 36 | 27 | 08 |
| | Glucose | 30 | 43 | 26 | 48 | 35 | 16 |
| | Rhamnose | 55 | 60 | 55 | 63 | 61 | 45 |
| B | Quercetin | 17 | 20 | 12 | 18 | 15 | 12 |
| C | Gentiobiose | 13 | 27 | 14 | 26 | 22 | 09 |
| | Galactose | 24 | 36 | 22 | 41 | 31 | 14 |
| | Arabinose | 32 | 43 | 28 | 47 | 36 | 21 |
| | Xylose | 48 | 54 | 47 | 58 | 56 | 36 |
| | Rhamnose | 57 | 63 | 56 | 64 | 62 | 45 |
| | Delphin | | | [a] | | | |
| | Cyanin | | | 4 | | | |
| | Malvin | [a] | | 10 | [a] | [a] | [a] |
| | Peonin | 3 | | 26 | | | |
| | Pelargonin | 9 | [b] | 32 | 3 | 6 | 25 |
| | Callistephin | 30 | | 41 | 7 | 60 | 50 |
| | Delphinidin | 40 | | 48 | | | |
| | Cyanidin | 90 | | 54 | 84 | 81 | 75 |
| | Pelargonidin | | | 86 | | | |
| D | Lactose | 10 | 22 | 14 | 28 | 19 | 7 |
| | Galactose | 21 | 35 | 22 | 42 | 32 | 14 |
| | Arabinose | 31 | 40 | 28 | 48 | 35 | 19 |
| | Glucose | 29 | 44 | 28 | 50 | 38 | 23 |
| | Anthocyanins | [c] | [b] | [c] | [c] | [c] | [c] |
| | Anthocyanidins | | | | | | |
| E | Sucrose | 28 | 35 | 23 | 43 | 30 | 19 |
| | Glucose | 32 | 45 | 29 | 50 | 38 | 16 |
| | Fructose | 28 | 35 | 33 | 37 | 32 | 17 |
| | Anthocyanins | [c] | [b] | [c] | [c] | [c] | [c] |
| | Anthocyanidins | | | | | | |
| F | Cherry anthocyanins | 10 | 12 | 13 | 12 | 15 | 9 |
| G | Delphinidin | 9 | 0 | 8 | 9 | 6 | 9 |
| H | Cyanidin | 19 | 13 | 14 | 9 | 9 | 5 |
| I | Pelargonidin | 75 | 62 | 85 | 82 | 86 | 75 |

[a] $R_F$ values of spots resolved, but not attributed.
[b] Streak.
[c] $R_F$ values as in C.

Finally, the detection of a sugar with the chromogenic reagent is not subject to interference from the anthocyanins, even if they have the same $R_F$ value. This is because under the detection conditions described, the reagent reveals the sugars without any reaction with the glycidic moiety of anthocyanins.

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Received June 9th, 1969