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THE USE OF POLYVINYLPYRROLIDONE IN THE THIN LAYER CHROMATOGRAPHIC SEPARATION OF FLAVONOIDS AND RELATED COMPOUNDS

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SUMMARY

A method is described for the separation of phenolic acids and flavonoids by thin layer chromatography using poly-N-vinyl pyrrolidone as adsorbent. The properties and pre-treatment of the adsorbent are described and the R_F values of eighteen naturally occurring phenolic acids and flavonoids in three solvent systems are given.

Paper chromatography has been very widely used in separating and identifying plant phenolics[1-3]. Much of the preliminary work has been done in this field and interest is now turning towards more specific studies, for example, the importance of flavonoids in chemical taxonomy and fungal toxicology, and plant decomposition studies. These entail the handling of large numbers of samples and thin layer chromatography offers a rapid method of identifying the major flavonoids in such samples. Silica gel, cellulose and mixed layers of silica gel and cellulose have been used and more recently various polymers mainly of the nylon type, have been introduced as adsorbents. Such adsorbents are generally referred to in the literature as 'polyamides'[4]. Unfortunately silica gel quenches the characteristic fluorescence of many phenolics although reasonable separations are obtained. Cellulose on the other hand does not affect the fluorescence but poor separations frequently result if a plant extract is used without some form of purification. Polyamide is a difficult material to handle in the form of a thin layer.

Cross linked poly-N-vinyl pyrrolidone (PVP) has been used for a number of years in the clarifying of beer and wines[5] and more recently in chromatographic columns to remove polyphenolic contaminants from plant enzyme preparations[6] and for the purification of anthocyanins[7]. PVP has an adsorption capacity three or more times greater than polyamide, and initial tests showed it to be suitable for the separation of polyhydroxyphenols. It was also found that the ultra-violet fluorescence of flavonoids adsorbed on PVP was frequently considerably greater than if adsorbed on silica gel or cellulose. The range of fluorescent colours produced was also extended. These properties should result in a lower limit of detection and easier recognition of many flavonoids than with previously used adsorbents.

The present work describes the use of PVP in the separation of flavonoids by thin layer chromatography (TLC).

EXPERIMENTAL

Initial investigations into PVP as a TLC adsorbent

Poly-N-vinyl pyrrolidone (PVP) in its cross linked form is insoluble in water, organic solvents, strong acids and alkali. This is in contrast to the straight chain polymer known under various trade names* which is soluble in water and alcohols.

Commercial PVP** was found to consist of particles varying in size from greater than 60 B.S. mesh (250 μ) to less than 300 B.S. mesh (53 μ). The particles swelled slightly in water so that after spreading the plates, drying resulted in the formation of a fine network of cracks throughout the layer. Spots developed on such layers tended to be irregular in shape and the solvent front did not move as a straight line. In an attempt to produce better layers the following modifications were tried.

(i) Organic solvents

Methanol, acetone, chloroform and ethyl acetate were each used to suspend PVP. Much of the layer was smooth but a few deep and wide cracks inevitably developed which disrupted the separation completely. The layers were also extremely fragile.

*(ii) Admixture with Celite 545****

PVP and Celite were mixed 1:1 with water as the suspending medium. A very satisfactory layer was obtained free from cracks. Separation was very poor however, the spots being very large and diffuse, and the characteristic fluorescence of many substances was largely quenched.

(iii) Binding agents

Two binding agents which have been successfully used with silica gel were investigated. Anhydrous calcium sulphate–PVP (1:7) was spread as a suspension in water. The resulting layer was stronger than with PVP alone but separations were not improved. In a second experiment PVP was slurried with a 2.5 % solution of polyvinyl alcohol in water[8]. The resulting layer was extremely strong, but with aqueous solvents it peeled from the plate.

(iv) Particle fractionation

A sample of PVP was fractionated using sieves of 60, 100 (149 μ), 170 (88 μ), 240 (66 μ) and 300 B.S. mesh. A sample of each particle size was suspended in water, spread on to a number of plates and these were examined for appearance, rate of solvent travel and the ability to separate a synthetic mixture of flavonoid compounds. The following qualitative observations were made:

(*a*) *Greater than 60 and 60–100 mesh.* The layers were fissured as before with the cracks sharply defined. Solvents travelled extremely rapidly with a straight front, but separated spots were large and diffuse. The dried layer was very powdery making spraying difficult.

(*b*) *100–300 mesh.* These layers had a similar appearance to those above. They were, however, more robust and would withstand being turned face down on to photographic paper when records of fluorescence were required. Solvent travel was slower but the migrating front was straight and the resulting separation reasonably sharp.

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* *e.g.* Periston, Plasmosan.

** Polyclar AT: Fine Dyestuffs and Chemicals Ltd., Manchester, 2, Great Britain.

*** Diatomaceous filter-aid: Koch-Light Laboratories Ltd., Colnbrook, Bucks., Great Britain.

(c) *Less than 300 mesh.* These layers were very finely reticulated but the fissures themselves appeared to be partly filled with ultra-fine particles. The layer was extremely tough and would withstand vigorous rubbing with the finger. Solvent travel was very slow however, even with volatile solvents, and investigations were therefore not continued.

Preparation of the layer

On the basis of the above work PVP with a particle size of 100–300 mesh was considered suitable for TLC. This fraction also constituted the major part of commercial PVP and further grinding was not necessary. It was found impracticable to remove the less than 300 mesh particles from a large sample by dry sieving, and the following technique was used.

200–500 g of PVP were slurried with water to give a thick paste and washed through a 100 mesh sieve in small portions using a fine but powerful jet of water. The filtrate was then washed through a 300 mesh sieve in a similar manner, allowing the filtrate to run to waste. When all the original material had been treated, the fraction retained on the 300 mesh sieve was washed several times with a large volume of de-ionised water and filtered at the pump. The filter cake was washed with acetone, spread on filter paper and air-dried. The material obtained after this procedure gave uniform layers free from U.V. fluorescent impurities. Layers were prepared using a Shandon 'Unoplan' spreader. 15 g PVP (100–300 mesh) was shaken with 80 ml water; this provided sufficient slurry to coat five 20 × 20 cm plates. As the adsorbent contains no binder it was convenient to prepare the slurry in bulk and store it until required. It was found that plates must be spread with one rapid sweep of the spreader and as soon as possible after pouring the slurry; any delay resulted in water separating from the slurry and seeping out of the bottom of the spreader. As the particle size of the PVP was larger than that commonly used for TLC, a relatively large gap of 500 μ was required in the spreader gate to ensure that plates could be coated quickly and evenly. After each spreading operation, excess water was blotted from the small leading and trailing plates to prevent damage to the other plates. The layers were allowed to air-dry overnight and stored in a cabinet without desiccant. The layers should not be dried by heating at any stage, otherwise large cracks develop.

Solvent systems

All solvents were of analytical reagent grade and were used as received. The following systems were used:

(I) 90 % formic acid;

(II) ethanol–water–concentrated hydrochloric acid (6:3:1, v/v);

(III) acetic acid–water–concentrated hydrochloric acid (30:10:3, v/v) (Forestal solvent).

Detecting agents

(1) *Sprays*

(i) Diphenyl-boric acid ethanolamine complex[9], 1 % in ethanol.

(ii) Ferric chloride–potassium ferricyanide, each 1 % aqueous. The reagents were mixed in the proportions 1:1 just prior to spraying.

(2) *Radiation source*

U.V. 3660 and 2537 Å. Camag Universal U.V. Lamp Type TL/900.

Photography

Photographs of developed plates were obtained using a Rolleicord Vb camera fitted with a Rolleinar 1 dioptre close-up lens, and a Rollei 1.5 × yellow filter to absorb excessive U.V. radiation. With the Camag light source 30 cm from the plate, exposures of 2 min at *f* 5.6 for Kodak Ektachrome -X (64 ASA) and Kodachrome -X (80 ASA), and 30 sec at *f* 8 for Ilford HP3 (400 ASA) were required, using light of 3660 Å.

Preparation of standards and vegetation extracts

(1) Standards were used as obtained from the suppliers without further purification. Each was dissolved in 90 % ethanol. The concentration was 1 mg/ml.

(2) Flavonoid aglycones were obtained from fresh samples of Rhododendron (*Rhododendron* sp.: whole buds and flowers) and from Bilberry (*Vaccinium myrtillus*: aerial shoots).

The fresh tissue was minced and extracted with cold 80 % methanol for 1 h and centrifuged. The supernatant was extracted twice with petroleum ether to remove chlorophyll, and evaporated to a small bulk at 40° under vacuum. The residue was washed into an evaporating basin and dried *in vacuo* over concentrated sulphuric acid for three days.

To hydrolyse the glycosides present, a suitable weight of the powdered extract was heated with 5 ml *N* hydrochloric acid for 20 min. The extract was filtered into a 10 × 150 mm tube and 1 ml *sec.*-butanol added. 10 % sodium hydroxide was added dropwise with mixing until a permanent cloudiness formed. When the layers had separated more *sec.*-butanol was added to make the volume of the upper layer about 1 ml and the tube shaken again. Samples for spotting were taken directly from the upper layer without separating the two phases, using a wire loop of about 1 μl capacity.

Chromatography

1 μl of each of the standards and 5–15 μl of the sample aglycones were spotted onto 20 × 20 cm plates. Individual standards were run in one direction in each of the solvents I, II and III to obtain R_F values; mixtures of standards and the aglycones were run in two directions using the systems I followed by II and I followed by III. The plates were thoroughly dried in a current of warm air between runs and before spraying.

The plates were developed either in pairs in a small (13 × 27 × 21 cm) glass tank with filter paper saturation or in a Shandon Multiplate tank holding up to 12 plates. In the case of two-dimensional development, a small amount of residue was deposited at the solvent front by the formic acid which interfered with the development in the second direction. This was isolated by scoring two straight lines through the layer just above and below the residue mark prior to the second run.

The use of solvent III resulted in marked solvent demixing. For reasons given below, it was beneficial to extend the development in solvent III when examining the flavonoid content of vegetation. This was achieved as follows.

After running solvent I to within 2 cm of the top of the plate the layer was dried in a current of air and the solvent front isolated by scoring through the layer as

described previously. One μl of a 1 % solution of Bromocresol Green* which travels with the secondary solvent front was spotted in the narrow channel between the upper scored line and the top of the plate and in line with the row of spots separated by solvent I. A sheet 20 cm × 47 cm was cut from standard Whatman No. 4 chromatographic paper and doubled four times to give a pad 20 cm × 3 cm. This was laid over the layer at the end away from the first separation and held in place with three spring clips. The arrangement is shown in Fig. 1. The second development was then carried out, the plates being withdrawn from the tank just before the Bromocresol Green reached the pad.

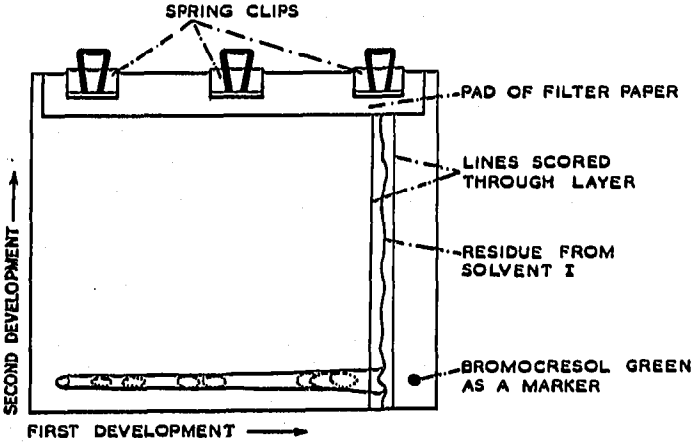

Fig. 1. Diagram of a thin layer plate prepared for extended development in the second direction.

RESULTS AND DISCUSSION

PVP adsorbs phenolic materials very strongly and a very polar solvent is required to move them from the origin. The adsorbent was also found to cause solvent demixing[10] with all solvents tested which contained two or more components. This was true even in the case of solvent I; thus the secondary (β) solvent front was located about 1 cm behind the apparent (α) front after an 18 cm run. The β front of solvent II travelled about 75 % the distance of the α front whilst for solvent III the value was about 50 %. Using solvents I and II, no fluorescent spots were observed in the region between the α and β fronts when running either the standard mixtures or extracts of vegetation. On running the standard mixture with solvent III however, the three coumarins, the glycoside naringin, and chlorogenic, sinapic, ferulic and syringic acids appeared between the α and β fronts. When these compounds were run individually the spots were discrete, but as a mixture, developed first with solvent I and then solvent III, the poor separation given by solvent I combined with the disturbing effect of the β front of solvent III resulted in spreading of the spots to an unacceptable extent. For this reason solvent III was usually used with extended development as described above. Those compounds which travelled ahead of the β front were then resolved on a second plate using solvents I and II. With solvent I a brown streak developed between the origin and the solvent front largely masking the spots present,

* Bromocresol Purple, Alizarin Red S and Sulpho Orange (Tropaeolin O) are also suitable.

TABLE I

R_F VALUES OF VARIOUS FLAVONOIDS AND RELATED COMPOUNDS

		Solvent system		
		I	*II*	*III*
1	Kaempferol	0.31	0.09*	0.46*
2	Quercitin	0.24	0.01*	0.20*
3	Myricetin	0.15	0.00*	0.07*
4	Umbelliferone	0.80	0.86*	0.71
5	Aesculetin	0.77	0.51*	0.57
6	Scopoletin	0.88	0.94*	0.83
7	Delphinidin chloride	0.87	0.06*	0.34*
8	Cyanidin chloride	0.83	0.22*	0.72*
9	Naringin	0.98	0.96*	0.96
10	D-Catechin	Streak 0–0.20	0.00*	0.06*
11	DL-Epicatechin	Streak 0–0.25	0.00*	0.06*
12	Chlorogenic acid	0.84	0.40*	0.57
13	Ellagic acid	0.48	0.07*	0.52*
14	Caffeic acid	0.63	0.21*	0.60*
15	Sinapic acid	0.83	0.74*	0.72
16	Ferulic acid	0.74	0.60*	0.58
17	Gallic acid	0.40	0.07*	0.21*
18	Syringic acid	0.81	0.88*	0.72

* R_F measured to β front.

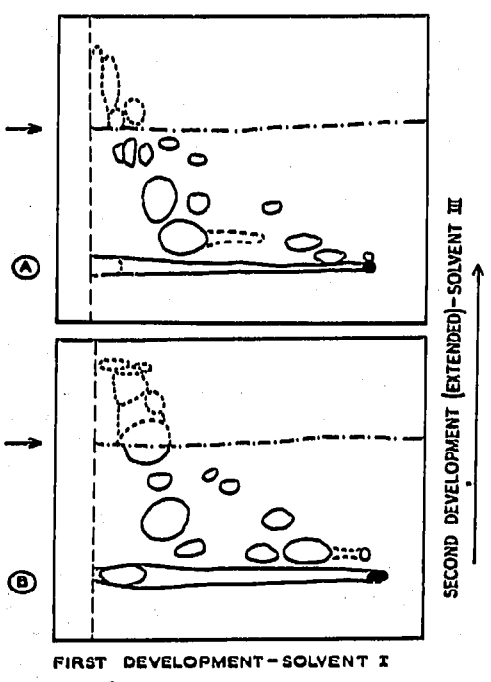

Fig. 2. Tracing of colour photographs taken under U.V. light of separations of flavonoids on PVP. The flavonoid aglycones were prepared as given in the text. Plate A: *Rhododendron* spp. Plate B: *Vaccinium myrtillus*. The small arrows give the position of the secondary (β) solvent front. The vertical broken line on each plate represents the line scored through the adsorbent to isolate the residue deposited by solvent I. The dotted areas above each β front are poorly separated components. Distinct regions can nevertheless be distinguished due to the contrasting fluorescent colours.

the bulk of this material travelling with the solvent front. On development in the second dimension with solvent II or III however, most of the spots were resolved leaving the streak at the origin. This behaviour appears to be analogous to the situation in paper chromatography where a streak develops using 6 % acetic acid but remains at the origin on development in the second direction with butanol–acetic acid–water (4:1:5, organic phase). It was also found that using crude extracts of vegetation the sequence of development of solvent I followed by II or I followed by III could not be reversed. If solvent II or III were used first, a heavy brown deposit was formed which travelled rapidly with the solvent for 3–4 cm and then suddenly stopped. This streak interfered both with the development of spots in the first direction, and subsequent development with solvent I resulted in a very broad brown streak with a disturbing effect on the solvent front. It would appear therefore that the use of formic acid as solvent in the first direction has the advantage of carrying much of the non-phenolic components of crude plant extracts with the solvent front where they can interfere least with the chromatographic separation.

The R_F values of eighteen phenolic compounds in each of the three solvents are given in Table I. Examples of typical separations of the flavonoids in vegetation are shown in Fig. 2.

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