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Polyvinylpyrrolidone column chromatography of strawberry, rhubarb, and raspberry anthocyanins

We have been interested in isolating substantial quantities of pure anthocyanin pigments for use in study of reactions involved in their degradative reactions. Using column chromatography on insoluble polyvinylpyrrolidone (PVP), Polyclar AT, HRAZDINA[1] successfully separated the 3,5-diglucosides of peonidin, petunidin, cyanidin, malvidin, and delphinidin from grapes. These 5 pigments differ in number of methoxyl and/or phenolic groups and therefore are particularly well suited for a separation system employing Polyclar AT, which has a high affinity for phenolic groups. BLUNDSTONE AND CREAN[2] used column Polyclar AT chromatography for isolating the anthocyanins of strawberry, raspberry, cherry, black currant and plum which they subsequently identified by thin-layer chromatography. The degree to which the different anthocyanin systems were resolved was not reported.

This communication reports the conditions used for Polyclar AT column separation of strawberry, rhubarb, and raspberry anthocyanins, the latter two containing anthocyanins having the same phenolic pattern and differing in the glycosidic moiety.

Experimental

Column chromatography. Columns were prepared according to the procedure of HRAZDINA[1]. The Polyclar AT (GAF Corp., New York, N.Y.) was sieved and particles larger than 80 mesh were suspended in 10% aqueous HCl, boiled 10 min, let settle for 7½ min, and the supernatant decanted. The PVP was resuspended in distilled water, allowed to settle and this procedure repeated until the supernatant was clear (30–40 times). The following column sizes were employed: 50 × 2.4 cm, 36 × 5.0 cm, 35 × 1.5 cm, and 70 × 1.5 cm. Columns were water-jacketed (23–25°) and all had flow rates of 2.7–3.0 ml/min when washed with water. Both gravity flow and a pump (LKB miniflow precision micropump) were employed.

Thin-layer chromatography. The procedure of QUARMBY[3] was followed in preparing 5 × 20 cm PVP plates. Modifications in his procedure included using 100–200 mesh dry-sieved PVP and elimination of the water sieving step.

Cellulose plates of 0.25 mm thickness were also utilized.

Solvent systems. PVP column and thin-layer chromatography: The solvent used by HRAZDINA[1] (30% aqueous ethanol containing 1 ml of 1 *N* HCl/l) was used. Numerous other systems were investigated employing methanol (MeOH) and/or ethanol (EtOH), HCl, and water. MeOH systems varied from 30–100%, HCl from 0.01–4%, the remaining percentage being water. EtOH systems varied from 30–100% and HCl from 0.0083–0.1%. Cellulose TLC: (1) glacial acetic acid–water–HCl (15:82:3) (AWHCl); (2) *n*-butanol–water–HCl (5:1:2) (BuWHCl 512); (3) water–formic acid–HCl (8:1:4) (WFHCl 814); (4) *n*-butanol–water–acetic acid (6:1:2) (BuWHAc 612); (5) propanol–water–HCl (3:10:2) (PrWHCl 3102); (6) upper phase of *n*-butanol–benzene–formic acid–water (100:19:10:25) (BuBFW).

Pigment isolation. Strawberries (Northwest variety), rhubarb (crimson cherry), and red raspberries (Willamette) were obtained from the OSU Horticulture Department. Anthocyanins were extracted with water, filtered through celite, and further purified by adsorption onto PVP (WROLSTAD *et al.*[4,5]). The methanolic–HCl antho-

cyanin concentrate was precipitated with diethyl ether three times (1 vol. methanol to 10 vol. ether) and the precipitate air dried and stored in a desiccator.

Results and discussion

The anthocyanins of strawberry, pelargonidin-3-glucoside (pgd-3-glu) and cyanidin-3-glucoside (cyd-3-glu), differ in number of phenolic groups and should be easily separated using PVP column chromatography. 1 l of clarified strawberry juice was applied to the column and separated using HRAZDINA's procedure. Most fractions were contaminated, however, and rechromatography was necessary to obtain pure fractions. We found two procedural changes which resulted in improved separation, eliminating the need for rechromatography. We could obtain a very compact band by applying a dried anthocyanin isolate to the column bed and then washing with water to adsorb the pigments. Using 0.1% HCl in MeOH as a developing solvent gave better resolution, faster development time, and speeded concentration.

We were interested in separating the anthocyanins of rhubarb, cyd-3-glu and cyanidin-3-rutinoside (cyd-3-rut), as it is much richer in cyd-3-glu than strawberry (WROLSTAD AND HEATHERBELL[6]). The two pigments, having the same phenolic pattern, would presumably have the same affinity for PVP. The pigments could be resolved by PVP TLC using either methanolic or ethanolic HCl systems provided that water content was at least 50%. Best resolution was obtained with 0.1% HCl in MeOH–H_2O (60:40). The pigments were successfully separated by column chromatography (Table I) using either 0.1% HCl in 60% MeOH–H_2O or 0.1% HCl in 30%

TABLE I

PARAMETERS FOR PVP COLUMN CHROMATOGRAPHY OF ANTHOCYANINS

Sample	*Ether precipitate (mg)*	*Column size (cm)*	*Solvent*[a]	*Development time (h)*	*Resolution*
Strawberry	200	40 × 5	100% MeOH	3	Very good
	20	35 × 1.5	30% EtOH	5	Poor
Rhubarb	30–40	35 × 1.5	100% MeOH	3	Good
	30–40	35 × 1.5	30% EtOH	7	Very good
	30–40	70 × 1.5	100% MeOH	7	Very good
Raspberry	25	30 × 1.5	70% MeOH	4	Good

[a] 0.1% HCl included in all solvents.

EtOH–H_2O. The ethanolic system is preferred as a shorter column is required for resolution.

Fig. 1 is a thin-layer chromatogram showing the purity of the fractions obtained from the rhubarb column. Only 2 spots were detected when the original rhubarb anthocyanin isolate was separated by two-dimensional cellulose TLC using solvent systems 2 and 3, and 4 and 5 (NYBOM[7]). The concentration resulting from column chromatography shows presence of two additional anthocyanins, Pigment A present in fractions 1 through 7, and Pigment B present in fractions 26 through 48. FULEKI[8] using paper chromatography reported presence of 2 additional minor pigments in rhubarb, reporting one to be a cyanidin bioside.

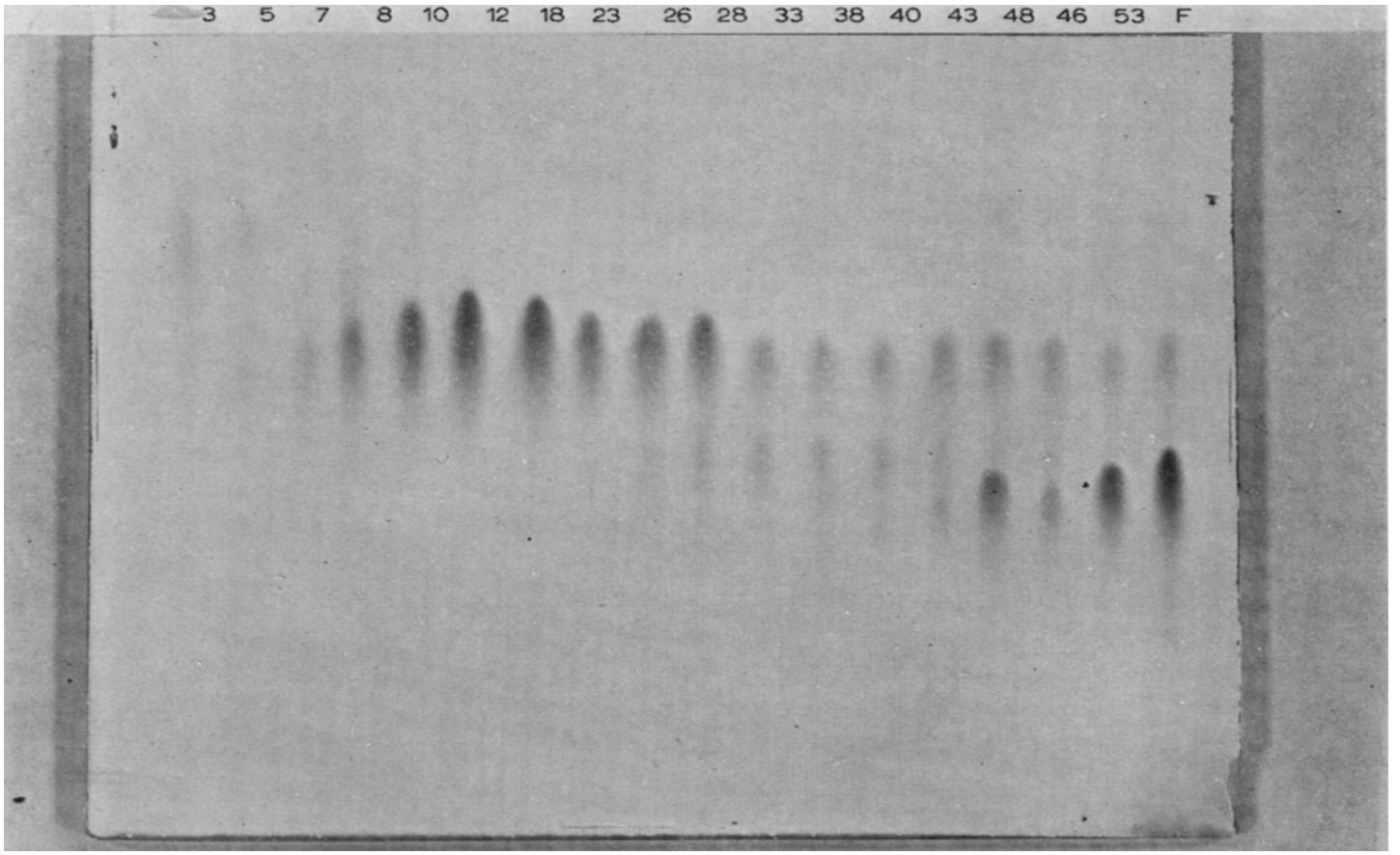

Fig. 1. Cellulose thin-layer chromatogram (developing solvent AWHCl) of fractions obtained from PVP column chromatography of rhubarb anthocyanins. Developing solvent, 30% EtOH containing 0.1% HCl; column size, 35 × 1.5 cm.

When a cellulose thin-layer chromatogram (solvent 1) of a fraction containing Pigment B was sprayed with molybdate spray (ALBACH *et al.*[9]) it gave a much more intense blue color than did the cyanidin pigments. Pigment B was extremely labile being particularly light-sensitive. We succeeded in purifying it by preparative PC. Its wavelength of maximum absorption (537 nm) and spectral shift with $AlCl_3$ (37 nm) would be more in accordance with delphinidin than with cyanidin, malvidin, or petunidin anthocyanins (HARBORNE[10]). The lack of a shoulder at 440 nm in the absorption spectrum suggests the possibility of substitution in 5th position. This could also account for its chromatographic behavior on PVP as the additional phenolic group in the B ring should give increased retention on PVP; substitution in the 5th position conceivably could compensate for this. Pigment A was present in low quantities and no spectral data was obtained. Its chromatographic properties were similar to the minor pigment in strawberries described by WROLSTAD *et al.*[11].

Red raspberries contain cyd-3-glu, cyd-3-rut, cyanidin-3-sophoroside (cyd-3-soph), and cyanidin-3-(2^G glucosylrutinoside) with some species containing the corresponding pelargonidin glycosides[12]. The anthocyanins from Willamette variety could be separated into five spots by two-dimensional cellulose TLC using solvent systems 2 and 3 and into five bands using one-dimensional chromatography on BuBFW. The best separation with PVP TLC was achieved with 0.1% HCl in MeOH–H_2O (70:30)

giving three spots. Examination by cellulose TLC (BuBFW) of different fractions obtained by PVP column chromatography (0.1% HCl in MeOH–H_2O, 70:30) revealed presence of seven different pigments. Mono-, di-, and tri-glucosides could be separated from each other; while diglucosides were not well resolved, the concentration effected would now permit separation by preparative paper or further column chromatography for identification work.

These studies show that column PVP chromatography can be utilized in separating not only anthocyanins which differ in number of phenolic groups but also those which differ in number of glycosides. PVP TLC is extremely useful in selecting suitable solvent systems for column separation of anthocyanin mixtures. Columns can be regenerated by washing with 10% HCl in MeOH, followed by distilled water to a neutral pH. The column should be prewashed with distilled water immediately before use, as in one experiment we obtained a new band which eluted very quickly from the column. Subsequent TLC analysis showed it to contain the slower moving anthocyanins and soluble PVP. PVP on a cellulose TLC plate fluoresces white when examined under UV light. Apparently some PVP was solubilized on standing in water; this adsorbed anthocyanins and eluted with the developing solvent.

PVP columns can be heavily loaded and the high recovery of pigments makes them very useful for preparative work. The concentration effect gives fractions rich enough in minor pigments to allow characterization after purification by some other method such as preparative paper chromatography.

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Department of Food Science and Technology,
Oregon State University,
Corvallis, Ore. 97331 (U.S.A.)

R. E. WROLSTAD
BARBARA J. STRUTHERS

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