Column Chromatographic Isolation of the Anthocyanidin-3,5-Diglucosides from Grapes

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The major pigments present in Seibel-9549 grapes, delphinidin, petunidin, malvidin, cyanidin and peonidin-3,5-diglucosides have been isolated using column chromatography on insoluble polyvinylpyrrolidone, Polyclar AT.

Column chromatography is the most frequently used technique for the isolation of flavonoid compounds. Separations have been reported on alumina (Gage *et al.*, 1948; Gripenberg, 1952; Karrer, 1936; Li, 1956), on cation exchange resins (Gage, 1951; Ice, 1951, 1953; Morris, 1950, 1953; Williams, 1952, 1953), on Magnesol (Ice and Wender, 1952), silicic acid (Li and Wagenknecht, 1956, 1958), cellulose powder (Chandler, 1958; Forsyth, 1952; Hörhammer *et al.*, 1958; Keppler, 1957; Roux and Freudenberg, 1958; Vuataz *et al.*, 1959; Werkmeister, 1954), and on polyamide (Chandler and Swain, 1959; Hörhammer *et al.*, 1957; Schmidt and Schonleben, 1957). The advantages and disadvantages of using the previously mentioned column support materials are extensively discussed by Seikel (1962). Gel filtration on Sephadex, using aqueous alcoholic hydrochloric acid (Somers, 1966) and aqueous acetone (Somers, 1968) was used for the separation of condensed wine pigments from the monomers. The condensed pigment fraction is sharply separated; however, the anthocyanins are only partially resolved, apparently because of adsorptive partition (Somers, 1966). Good resolution of the anthocyanins was reported with partition chromatography on Sephadex, using the organic phase of butanol:acetic acid:water (4:1:5) as eluent (Gombkötö, 1967). This method, however, permits the isolation of pigments only in small amounts and has the same disadvantage as cellulose powder, if macro work has to be done. Chromatography on Polyclar AT showed promise for separation of anthocyanins in larger amounts, and proved to be convenient by allowing the direct use of plant extracts without preliminary and time consuming purification procedures.

This report describes the separation, isolation, and identification of five anthocyanins present in Seibel-9549 grapes using column chromatography on Polyclar AT.

EXPERIMENTAL

The ripe grapes of the variety Seibel-9549 were processed immediately after harvesting. The grape juice was prepared by hot pressing and filtering and was held frozen until used.

A Polyclar AT column was prepared for the isolation of the anthocyanins. The Polyclar AT powder (GAF Corp., New York, N. Y.) was suspended in distilled water and allowed to stand for 10 minutes. The supernatant containing the fine particles was decanted and the sediment resuspended in distilled water. This step was repeated until the supernatant was clear. The Polyclar AT was then resuspended in fresh distilled water, poured into the chromatography column (60 × 2.5 cm) and allowed to settle. The column was washed with 1 liter of distilled water. The grape juice (1000 ml) was percolated through the column, followed by water washing until the effluent was tasteless (800 ml). The pigment material was then eluted from the column with 30% aqueous ethanol containing 1 ml of 1*N* HCl per liter. The eluent was analyzed by monitoring the transmittance at 254 nm (LKB Uvicord), collected in a fraction collector, and the single fractions analyzed on cellulose thin layer chromatograms (Carl Schleicher and Schuell Co., Keene, N. H.). The TLC plates were fumigated immediately before use with concd HCl and developed in the organic phase of *n*-butanol:acetic acid:water (4:1:5). Fractions containing the same pigment were pooled, concentrated in a rotary evaporator (40° C, N_2 atmosphere) to a small volume (30 ml), except in the case of delphinidin-3,5-diglucoside (see Results and Discussion), and rechromatographed on a Polyclar AT column (50 × 2.5 cm), using the same solvent as at the previous separation. The main pigment fractions after rechromatography showed no contamination on TLC, and were concentrated on a rotary evaporator (40° C, N_2 atmosphere) to 20 ml. One milliliter of concd HCl was added to each concentrate and the solutions were kept in a refrigerator where the pigments crystallized.

The melting points of the pigments are not corrected.

The ultraviolet and visible spectra were recorded with a Beckman DK-1 spectrophotometer, using 2 mg of pigment per 100 ml of 60% aqueous ethanolic solution, containing 0.1% HCl.

The sugar component of the pigments was determined according to the method of Kagan and Mabry (1965), by hydrolyzing 2 mg of pigment material in 25 ml of 2*N* HCl, separating the sugar fraction on a 3 × 1 cm Polyclar AT column from the aglucons, evaporating to dryness, followed by silylation and GLC analysis (Barber-Colman apparatus equipped with argon ionization detector. Column: 3% OV-1 on Gas Chrom Q; column lengths, 6-foot column; diameter: 0.25 inch; temperature, 156° C; flow rate, 65 ml per minute; carrier gas, argon). The aglucon was eluted from the Polyclar AT column with methanol, evaporated to dryness (40° C, N_2 atmosphere), dissolved in 100 ml of 60% aqueous ethanol (0.1% HCl) and the spectra were recorded immediately.

RESULTS AND DISCUSSION

The thin layer chromatography of the grape juice on cellulose showed five major and three minor pigment components. The major pigments could be easily purified using chromatography and rechromatography on Polyclar AT.

Upon elution of the column with 30% aqueous ethanol (1 ml of 1*N* HCl per liter), the pigments were eluted and fractionated. The condensed pigment material was irreversibly adsorbed on the top of the column and could not be eluted by increasing the ethanol concentration of the eluent to 95%.

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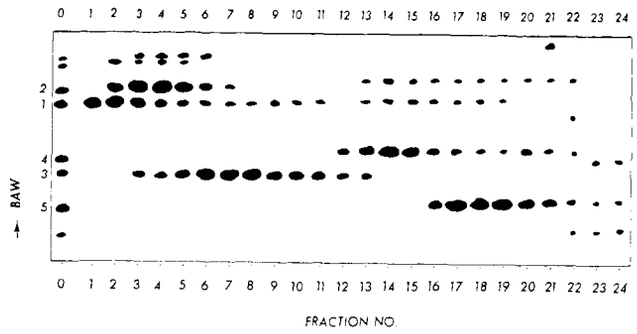

Figure 1. Cellulose TLC of the pigment fractions obtained from column chromatography on Polyclar AT

O: original grape juice; spots 1 to 5: malvidin, peonidin, petunidin, cyanidin, and delphinidin-3,5-diglucoside

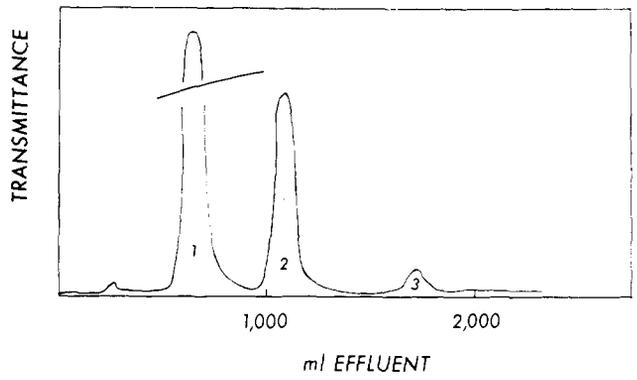

Figure 2. Rechromatography of fractions 1 to 5 on Polyclar AT with 30% aqueous EtOH containing 1 ml of 1N HCl per liter

Peaks 1 to 3: malvidin, peonidin, and petunidin-3,5-diglucoside, respectively

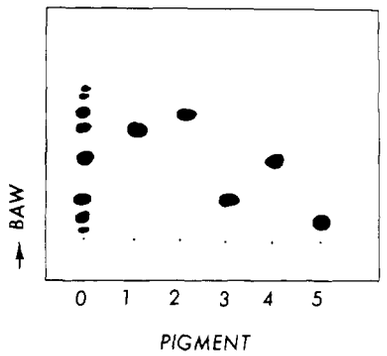

Figure 3. Cellulose TLC of the isolated pigments

O: grape juice; pigments 1 to 5: malvidin, peonidin, petunidin, cyanidin, and delphinidin-3,5-diglucoside

The thin layer chromatography of the eluate (20 ml per fraction) on cellulose showed that complete separation of the pigments was not obtained (Figure 1), because of the broad starting band on the column. Therefore, for further purification, rechromatography on Polyclar AT was required.

Upon rechromatography of the combined fractions 1 to 5 with the same solvent used for the previous separation, the analysis of the effluent by monitoring the transmittance at 254 nm (LKB Uvicord), showed a complete separation of the pigment components (Figure 2). Cellulose TLC of peaks 1 and 2 showed no impurities (Figure 3). After concentration of the two fractions to *ca.* 20 ml and addition of 1 ml concd HCl, pigment 1 crystallized in dark, red-brown colored needles with a yield of 670 mg. M.p.: 174° C (decomposition). Pigment 2 formed fine red violet colored needles. Yield: 57 mg, m.p.: 164–5° C (decomposition).

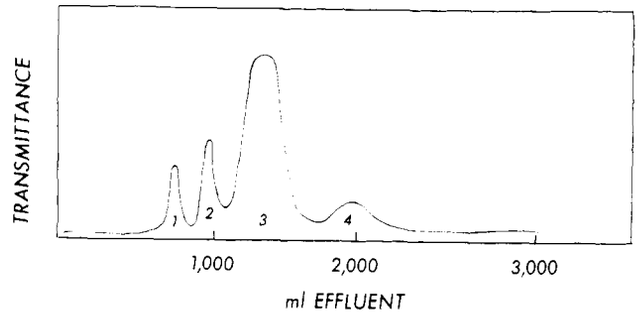

Figure 4. Rechromatography of fraction 6 to 12 on Polyclar AT

Peaks 1 to 4: malvidin, peonidin, petunidin, and cyanidin-3,5-diglucoside

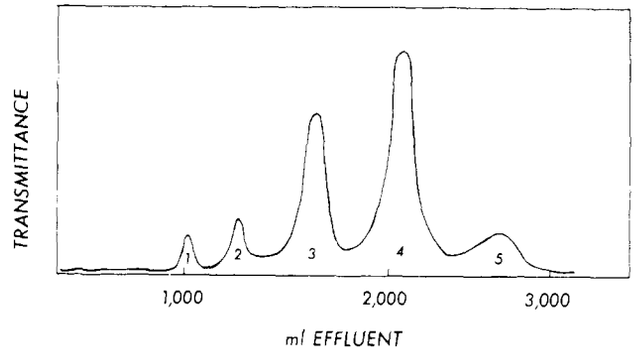

Figure 5. Rechromatography of fractions 13 to 15 on Polyclar AT

Peaks 1 to 5: malvidin, peonidin, petunidin, cyanidin, and delphinidin-3,5-diglucoside

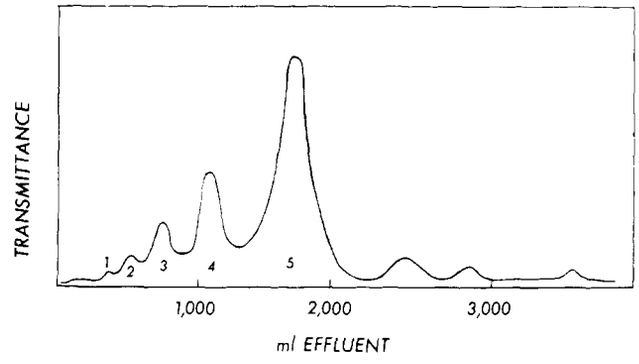

Figure 6. Rechromatography of fractions 16 to 21 on Polyclar AT

The major pigment fraction (peak 5) is delphinidin-3,5-diglucoside

Upon rechromatography of the combined fractions, 6 to 12, from the first Polyclar AT column separation (Figure 4) the main fraction (pigment 3) yielded after crystallization 171 mg of violet, rectangular tablets with the melting point of 167–8° C (decomposition). From the rechromatography of fractions 13 to 15 containing pigment 4 as described previously (Figure 5), 24 mg of brown-red colored crystals were obtained. M.p.: 189–90° C (decomposition).

Fractions 16 to 21, containing pigment 5 as a major compound, were evaporated to 80 ml. Further concentration was not possible because of the low solubility of the pigment in the 30% aqueous ethanol solution. The rechromatography

Table I. Spectral Characteristics of Isolated Pigments in 60% Ethanol (0.1% HCl)

Pigment	λ_{max} Pigment	λ_{max} Aglucon	λ_{max} Reported 3,5-Diglucoside	λ_{max} Aglucon
1	537	559	Malvidin 537	559
2	526	548	Peonidin 527	549
3	539	554	Petunidin 539	554
4	528	551	Cyanidin 528	551
5	541	561	Delphinidin 541	561

of this fraction was carried out on a 25 × 2.5 column (Figure 6). The pigment material was strongly adsorbed on the Polyclar and the use of a longer column resulted in slow elution. After concentration of peak 5 to 20 ml and addition of 1 ml of concentrated HCl, pigment 5 yielded 74 mg of red-blue colored crystalline powder. M.p.: 204–5° C (decomposition). Thin layer chromatography on cellulose (BAW/4:1:5) showed that the pigments were chromatographically pure (Figure 3).

The purity of the pigments was confirmed also by comparison of their spectral characteristics (as shown in Table I) with reported literature data (Hayashi, 1962), reaction with $FeCl_3$, and by gas chromatographic determination of the sugar residues (Kagan and Mabry, 1965). However, it was noted that by the hydrolysis of the diglucosides with 2*N* HCl and by separation of the sugar residues from the aglucons on the Polyclar AT column, a partial decomposition of the pigments occurred. The destruction increased with increasing number of OH substitution on the flavylium skeleton—*e.g.*, malvidin-3,5-diglucoside showed the least, delphinidin-3,5-diglucoside the most pronounced decomposition.

The elution pattern of the pigments from the Polyclar AT column with 30% aqueous ethanol suggest that the adsorption and separation is based on hydrogen bond formation between the pigments and the column support material. Malvidin-3,5-diglucoside having two OMe groups in *ortho* position to the OH was the first pigment eluted from the column, followed by pigments with increasing OH and decreasing OMe substitution in the molecule as peonidin, petunidin, cyanidin, and delphinidin-3,5-diglucosides. Therefore, the use of aqueous alcoholic solvents on the Polyclar AT column destroys the hydrogen bonds and the pigments can be readily eluted and fractionated, giving a convenient method for the isolation of anthocyanins in large amounts.

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