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Purification and separation of oenocyanin anthocyanins on sulphoxyethylcellulose

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A sulphoxyethylcellulose cation-exchanger (SE cellulose) has been used for chromatography of a methanolic extract of commercial oenocyanin. This support permits a good separation of anthocyanin monoglucosides from contaminants such as red polymers, polyphenols, acids, sugars and also a partial resolution among the 3-glucosides. The fractions eluted from SE cellulose were analysed by TLC and HPLC and some pigments were identified by comparison with authentic reference compounds. This methodology offers some advantages on cationic resin exchange and could be of more general use in purification of anthocyanins from other sources.

INTRODUCTION

During research on immobilization of fungal anthocyanase (β-glucosidase) to control the tonality of red wines and for removal of the anthocyanins from the juice of varieties of blood oranges (tarocco, moro and sanguinello), the need emerged to obtain, from the crude oenocyanin, pure anthocyanins as substrates of the enzyme, free of sugar inhibitors of β-anthocyanase (Blom & Thomassen, 1985; Pifferi *et al.*, 1979), acids, polyphenols and brown polymers. According to some papers (Smith & Luh, 1965; Fuleki & Francis, 1968; Maccarone *et al.*, 1983) cation-exchange chromatography was tried, but the weak cation-exchanger, Amberlite CG 50, did not fix some anthocyanins, while strong cation-exchangers, such as Amberlite IRA-120, fixed some pigments irreversibly without the possibility of any recovery (Lin & Hilton, 1980) despite use of very polar solvents such as HCl–water, HCl–methanol, formic acid and dimethylsulphoxide. The use of PVP (Van Teeling *et al.*, 1971) is not without inconvenience, because the elution of non-polar pigments occurs with more polar solvents, with the possibility of producing artefacts. To overcome this situation, chromatography of a methanolic extract of crude oenocyanin was developed on an SE cellulose cationic exchanger to obtain a satisfactory resolution of monoglucoside anthocyanins, from the red polymers, sugars, flavonoids and acids. A partial separation of the monoglucoside pigments was also achieved. The nature of the pigments eluted in the different fractions was ascertained by TLC and HPLC analysis.

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MATERIALS AND METHODS

Sample

The anthocyanin source was grapes (*Vitis vinifera*) in the form of commercial solid oenocyanin (Reggiana Antociani, Reggio Emilia, Italy).

Pigment extraction

The anthocyanins were extracted from dried oenocyanin (40 g) with three successive extractions of anhydrous methanol (160 ml) for 3 h at 25°C. The combined extracts were filtered and stored in a dark bottle at 0°C for chromatography.

Column chromatography

Preparation of the column

The adsorbent was sulphoxyethyl (SE) cellulose cation-exchanger (SE 52, Whatman Chemical Separation Ltd). The SE cellulose (1 vol.) was stirred, in sequence, with 15 vol. 0·2 N HCl in methanol, distilled water, 0·5 N NaOH, distilled water, and finally with 0·002 N HCl. The slurry of SE cellulose in 0·002 N HCl was introduced to a chromatography column, 15 mm × 400 mm, to a height of 310 mm. Then the adsorbent was equilibrated by fluxing the same solvent overnight.

Chromatography
An aliquot (0·5 ml) of extract, acidified to 0·02 N HCl, after about 40 min, was applied to the top of the column. To elute the anthocyanins the following solvents were used: (1) 0·02 N HCl (80 ml), until three bands had left the column; (2) 0·2 N HCl in EtOH/H_2O (80:20 v/v) to wash the column; (3) 0·2 N HCl in EtOH/H_2O (45:55 v/v) until three more bands had migrated down the column; and (4) 0·2 N HCl in MeOH until all the bands had left the column. To collect the fractions the LKB 2212 was used. The fraction size was usually 1·0 ml, although the volume was changed according to the band width. For subsequent separations the procedure used in the previous section was repeated.

Spectra

All the fractions were analysed at 530 nm, but only the absorption maxima of the fractions were chosen to effect TLC and HPLC analysis. The aqueous fractions were lyophilized, while the methanolic fractions were concentrated to dryness in a rotary evaporator at 35°C under vacuum. Then the fractions were dissolved in 99% HCOOH/MeOH (1:99 v/v) and stored in the dark at 0°C.

Thin-layer chromatography (TLC)

For an analytical examination the original extract and the fractions obtained from SE cellulose chromatography were deposited by CAMAG-LINOMAT IV on a glass plate cellulose-precoated, layer thickness 0·1 mm (20 cm × 20 cm, 5716 Merck). For two-dimensional chromatography, BAW (*n*-butanol/acetic acid/water 12:3:5 v/v/v) in the first direction and AHW (acetic acid/conc.HCl/water 15:3:82 v/v/v) in the second direction (Harborne, 1967) were used as solvents. For preparative isolation of anthocyanins the initial extract was deposited on Whatman filter paper No. 3 MM and on a glass plate cellulose-precoated to a layer thickness of 0·5 mm (20 cm × 20 cm, 15275 Merck). To develop the spots BAW and AHW for the first and second directions, respectively, were again used as solvents. Then, identified spots of monoglucoside, and acylated and polymeric anthocyanins were isolated and used as standards for HPLC analysis.

HPLC analysis

Equipment
JASCO HPLC system (BIP-1, VL 164, GP-A 40, UVIDEC-100 VI) was used.

Conditions of analysis
Column: Ultramex 5 μM C-18, 250 × 4·6 mm with Ultramex guard column. Solvents: (A) 99% HCOOH/H_2O (9:91 v/v) and (B) HCOOH/MeOH/MeCN (9:79:12 v/v/v) stored in a freezer at 4°C or, alternatively, (C) HCOOH/MeOH (9:91 v/v); all the solvents were HPLC grade. The programme for the solvents A and B was: linear gradient from 0 to 24% of B from 0 to 35 min, isocratic elution to 55 min and linear gradient to 100% of B from 55 to 120 min. For the solvents A and C the programme was: linear gradient from 5 to 50% of C from 0 to 60 min, linear gradient to 100% of C to 100 min. Flow rate was 1·0 ml min^{-1} at room temperature, with detection at 520 nm. Injection volume was 20 μl.

RESULTS AND DISCUSSION

The methanolic extract of oenocyanin was separated on SE cellulose in six fractions as shown in Fig. 1. The aqueous solvent 0·002 N HCl eluted two intensive bands (I and II) and another (III), less intense and better resolved than the previous ones. The organic solvent HCl/MeOH eluted three other bands (IV–VI), which were more resolved. The initial extract of oenocyanin and the six fractions (I–VI), eluted from SE cellulose were analysed by TLC and HPLC and data were then compared. The two-dimensional chromatogram of the oenocyanin extract showed 19 spots (Fig. 2). Some spots were identified by comparing with authentic reference compounds the colour, the change in colour after spraying the plate with 5% $AlCl_3$ in 95% ethanol and the R_F values and spectra (Harborne, 1967). In this way the spots of anthocyanin monoglucosides, acylated or not, and polymeric compounds were identified. The spots group 10–12, present in traces, were under the diagonal line of the plate, with a R_F in AHW higher than 40 and in BAW lower than the R_F Mv-3 glucoside (spot No. 8); consequently they might be the 3,5-glycoside or bioside anthocyanins (Harbourne, 1967). Table 1 lists spots of the fractions which were clearly identified. The anthocyanins of fractions were

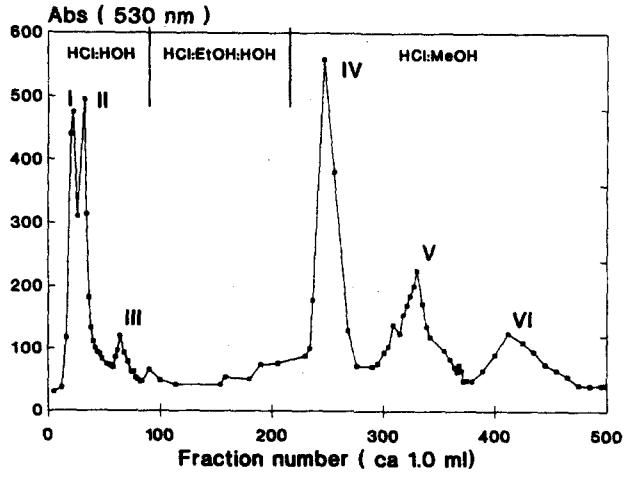

Fig. 1. Separation of oenocyanin pigments (fractions I–VI) using chromatography on SE cellulose, by increasing the organic solvent percentage of eluent.

Table 1. Identification by two-dimensional TLC of anthocyanin eluted from SE cellulose

Fraction No.	Spot No.	Compound
VI	3	Dp 3-glucoside
V	5	Pt 3-glucoside
V	6	Cy 3-glucoside
IV	8	Mv 3-glucoside
IV	9	Pn 3-glucoside
IV–VI	13–18	Acylates
I–III	19	Polymers

analysed by HPLC and identified by HPLC chromatographic comparison with authentic compounds as reference and with an HPLC chromatogram of oenocyanin extract (Fig. 3 and Table 2).

In HPLC, the pigments eluted in order of their polarity according to the data of other authors (Wulf & Nagel, 1978; Hale *et al.*, 1986; Hong & Wrolstad, 1990); the anthocyanin 3-glucosides eluted before the acylates and red polymers, and among the 3-glucosides the elution order was Dp, Cy, Pt, Pn and Mv (Lea, 1988). Regarding the pigment separation on SE cellulose cation-exchanger (Fig. 1), we observe that fractions I–III, eluted with 0·002 N HCl, contained (overall) polymeric compounds; these appeared in the TLC as spot 19 (Fig. 2) and, by comparison with HPLC chromatograms (Figs 3 and 4), as peaks 18–20; see also Tables 1 and 2. The fractions I–III also contained traces of unidentified pigments, that are definitely not anthocyanin monoglucosides, because TLC R_F and HPLC retention-

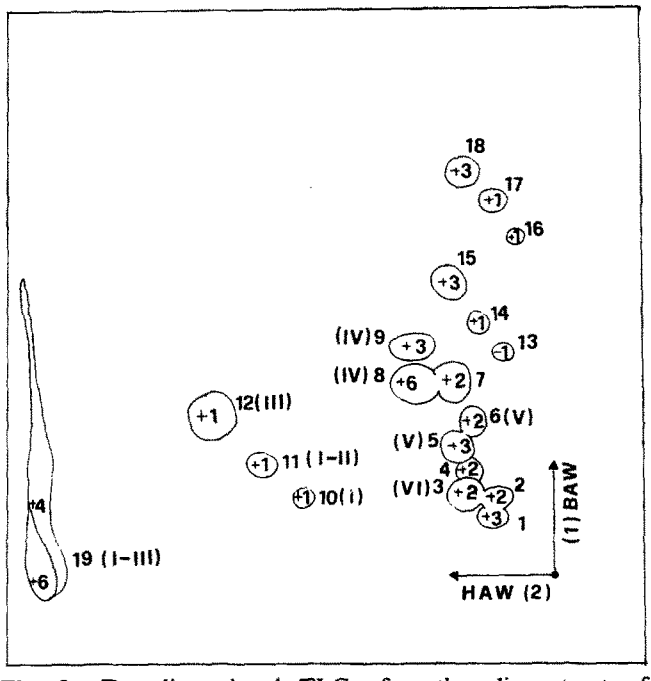

Fig. 2. Two-dimensional TLC of methanolic extract of oenocyanin using BAW and AHW solvents. Numbered spots are described according to colour intensity (+1–6) and elution order (I–VI) from SE cellulose (Fig. 1).

Table 2. Distribution of anthocyanins and red polymers in fractions I–VI (Fig. 1) and identification by HPLC chromatography

Fraction No.	Peak No.	Compound	3-Glucoside (%)
VI (V)	9	Dp 3-glucoside	4
V	10	Cy 3-glucoside	8
V	11	Pt 3-glucoside	26
IV	12	Pn 3-glucoside	14
IV	13	Mv 3-glucoside	48
			100
IV–VI	15–17	Acylates	
I–III	18–20	Polymers	

time values exclude this possibility. The fractions IV–VI, eluted with HCl/MeOH, contained the monoglucosides and some acylated anthocyanins. By HPLC and TLC we have investigated only the distribution of monoglucosides, as follows (Tables 1 and 2): fraction IV, Mv and Pn; fraction V, Cy, Pt and traces of Dp (only HPLC analysis); fraction IV, Dp. The separation on SE cellulose cationic exchanger is essentially based

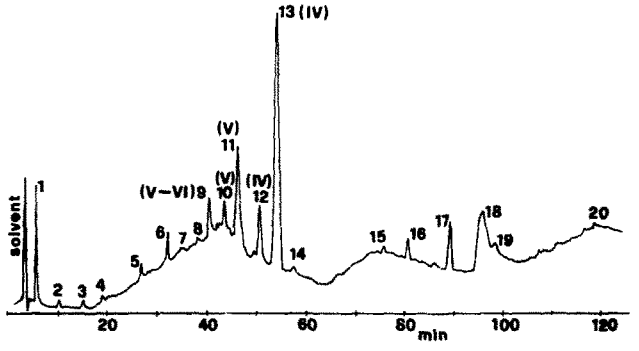

Fig. 3. HPLC chromatogram peaks of methanolic extract of oenocyanin. I–VI indicate the fractions from SE cellulose (Fig. 1); the peaks were ascertained by HPLC. Column: C-18 Ultramex. Solvents: (A) $HCOOH/H_2O$ and (B) HCOOH/MeOH/MeCN. Detection at 520 nm.

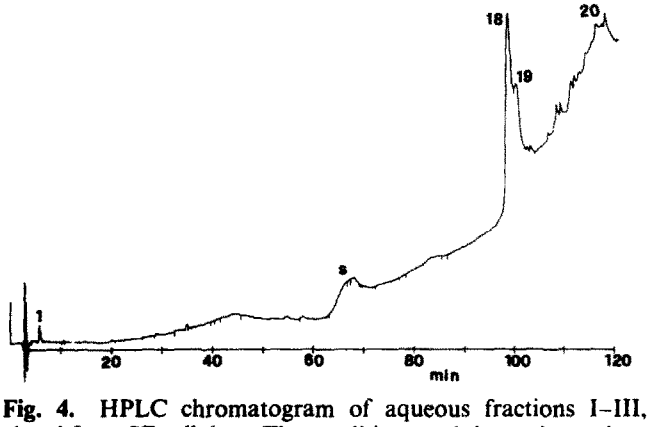

Fig. 4. HPLC chromatogram of aqueous fractions I–III, eluted from SE cellulose. The conditions and the peak numbers are the same as in the chromatogram shown in Fig. 3.

on electrostatic interaction because, at low pH, anthocyanins exist mainly in the flavylium cationic form. However, in the SE cellulose chromatography, the separation is also influenced by partition and adsorption effects. For these reasons the non-cationic compounds such as sugars, acids and polyphenols, soluble in the aqueous HCl and HCl/EtOH/H_2O, can be easily eluted from the column. The same behaviour has been observed for red polymeric compounds; this may be the effect of the low value of the positive charge/molecular weight ratio and of the probable reduction in these polymers of free hydroxyl groups, which are partially involved in intermolecular binding or are oxidized. On the other hand, in cationic exchange resin chromatography, as on Amberlite IR-120, the polymers are not eluted (Lin, 1980). The reason might be the major density of positive charges of the exchanger with increase of interaction points and the higher degree of crosslinking structure with a resulting sieve effect; in addition, the interaction possibilities between aromatic rings of the styrene–divinylbenzene matrix and the aromatic structure of polymeric pigments may contribute. The resolution of the anthocyanin monoglucosides in the eluent HCl/MeOH can be explained by the same ionic charge of 3-monoglucosides, which becomes more important than other interaction binding forces with the cellulose matrix; an increased number of hydroxyl groups on the B-ring of anthocyanins increases the polarity and the retention time. These results show that the SE cellulose cationic exchanger offers some advantages and may be an alternative to cationic resins for separation of anthocyanins. It is possible to eliminate impurities such as sugars, polyphenols, acids and overall polymeric decomposition products by recovering pure monoglucosides and acylated pigments from commercial oenocyanin with their partial resolution. This possibility might be extended to the purification and separation of anthocyanins from other natural sources.

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REFERENCES

Blom, H. & Thomassen, H. S. (1985). Kinetic studies on strawberry anthocyanin hydrolysis by a thermostable anthocyanin-β-glucosidase from *Aspergillus niger*. *Food Chem.*, **17**, 157–68.

Fuleki, T. & Francis, F. J. (1968). Quantitative methods for anthocyanins. 3: Purification of cranberry anthocyanins. *J. Food Sci.*, **33**, 266–73.

Hale, M. L., Francis, F. J. & Fagerson, I. S. (1986). Detection of oenocyanin in cranberry juice cocktail by HPLC anthocyanin profile. *J. Food Sci.*, **51**, 1511–13.

Harborne, J. B. (1967). *Comparative Biochemistry of the Flavonoids*. Academic Press, London.

Hong, V. & Wrolstad, E. R. (1990). Characterization of anthocyanin-containing colorants and fruit juice by HPLC/photodiode array detection. *J. Agric. Food Chem.*, **38**, 698–708.

Lea, A. G. H. (1988). HPLC of natural pigments in foodstuffs. In *HPLC in Food Analysis*, ed. R. Macrae, Academic Press, London, pp. 280–98.

Lin, R. I. & Hilton, B. W. (1980). Purification of commercial grape pigment. *J. Food Sci.*, **45**, 297–309.

Maccarone, E., Maccarrone, A. & Rapisarda, P. (1983). Acylated anthocyanins from oranges. *Ann. Chim. (Rome)*, **75.**, 79–86.

Pifferi, P. G., Cultrera, R., Marzocco, F., Iori, R. & Manenti, I. (1979). The structure of anthocyanins and their enzymic degradation in relation to the pH value and the phenol substrate of polyphenoloxydase. *Industria Conserve*, **54**, 105–11.

Smith, R. H. & Luh, B. S. (1965). Anthocyanin pigments in the hybrid grape variety rubired. *J. Food Sci.*, **30**, 995–1005.

Van Teeling, C. V., Consfield, P. E. & Gallop, R. A. (1971). Chromatography of anthocyanins on columns of insoluble polyvinylpyrrolidone. *J. Chromatogr.*, **9**, 505–9.

Wulf, L. W. & Nagel, C. W. (1978). High pressure liquid chromatography separation of anthocyanins of *Vitis vinifera*. *Am. J. Enol. Vitic.*, **29**, 42–9.