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Note

Droplet counter-current chromatography of anthocyanins

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A variety of chromatographic methods is available for the analytical separation of anthocyanins[1,2]. However, isolation of these compounds on even a moderate scale has been largely limited to the use of column and paper chromatography, with thin-layer chromatography being applied in cases where the former failed[1,2]. Droplet counter-current chromatography (DCCC) has previously been applied to the separation of a number of classes of polar compounds[3,4], including flavonoid glycosides[5]. It thus seemed worthwhile to apply this powerful technique to the difficult problem of the separation of anthocyanins. The major problems associated with chromatography of these compounds are instability in any but acidic conditions and irreversible adsorption[2]. It seemed reasonable to assume that these difficulties would be minimal in the case of DCCC, because separation could be carried out in acidic media without the use of adsorbants and in the absence of oxygen.

In this paper a system containing *n*-butanol, acetic acid and water is shown to be suitable for the semi-preparative isolation of anthocyanins from blackcurrants and raspberries. Blackcurrent anthocyanins differ with respect to the aglycone and sugar present[6], while raspberries contain a mixture of four glycosides of the same aglycone[7].

EXPERIMENTAL

Droplet counter-current chromatography

DCCC was carried out using a Model A DCC chromatograph from Tokyo Rikakikai, Tokyo, Japan: the instrument was fitted with 300 glass capillaries (40 cm × 2 mm) connected in series. A mobile phase flow-rate of *ca.* 10 ml/h was maintained throughout the experiments. Individual fractions of 4 ml were collected by means of an automatic fraction-collector (ISCO, Model 1850).

Three solvent systems were used: CMH, chloroform–methanol–0.1 *N* hydrochloric acid (5:6:4); PAH, *n*-propanol–acetic acid–0.1 *N* hydrochloric acid (3:4:3); BAW, *n*-butanol–acetic acid–water (4:1:5). Direct comparison of the results obtained on developing cellulose thin layers with the two layers formed by the solvent system was used to judge which layer gave the better separation of the components present, and this layer was then used as mobile phase. (The more elegant method of Hostettmann[3] could not be relied on owing to the instability of anthocyanins on silica.). Use of this method suggested that the upper layer should be used as mobile phase for the CMH and PAH

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systems with the respective lower layer as stationary phase. There seemed to be little difference as to which layer should be used in the case of the BAW system.

Analysis

Aliquots of each anthocyanin fraction were applied to commercial thin-layer plates coated with cellulose (DC-Plastfolien Cellulose, Art. 5577, Merck, Darmstadt, F.R.G.). Plates were developed with formic acid–conc.hydrochloric acid–water (10:3:11). Relative amounts of the various components were then determined against a standard by means of densitometry using a Quick-Scan R & D densitometer (Desaga/Helena) connected to a Quick Quant II integrator (Desaga/Helena). The densitometer was fitted with a 510-nm filter.

Anthocyanins

The anthocyanin sources used in this work were European blackcurrants (*Ribes nigrum* L) and European raspberries (*Rubus idaeus* L), both of which were readily available locally.

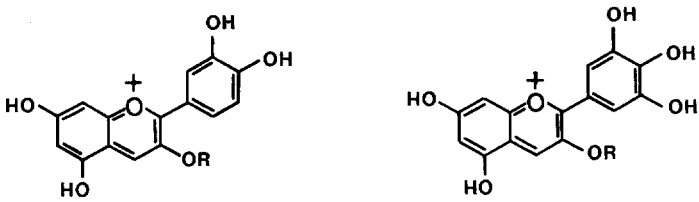
Fresh plant material (100 g) was macerated in a Waring Blendor with methanol (200 ml) containing conc. hydrochloric acid (2 ml)[8]. The suspension was transferred to a flask, which was thoroughly flushed with nitrogen, sealed and allowed to stand for 12 h at 4°C. The extract was recovered by filtration and the process repeated. The extracts were collected together and a small amount of water added. Chlorophylls and lipids were removed by washing the aqueous extract with light petroleum (b.p. 40–60°C)(500 ml). The aqueous phase was then concentrated to small volume under reduced pressure and stored under nitrogen at 4°C. Prior to DCCC, the requisite amount of concentrate was taken to dryness under reduced pressure and dissolved in a 1:1 mixture of the phases to be used in chromatography.

Anthocyanins were identified by standard procedures[2,8]. Visible light absorption maxima and the shift of these on addition of aluminium(III) chloride indicated which aglycone was present. The identity of the aglycone was confirmed by subjecting the isolated anthocyanin to hydrolysis, which was carried out by heating in 2 *N* hydrochloric acid for 30 min at 100°C[2]. Extraction of the hydrolysis mixture with amyl alcohol furnished the aglycone, which was identified by the visible light absorption spectrum and by co-chromatography with genuine aglycones (anthocyanidins). Treatment of the aqueous phase with N,N-dioctylmethylamine removed residual acid, and the freed sugars could then be identified by co-chromatography with authentic material[2].

RESULTS AND DISCUSSION

Blackcurrants are known to contain cyanidin-3-glucoside (1), delphinidin-3-glucoside (2), cyanidin-3-rutinoside (3) and delphinidin-3-rutinoside (4)[6]. Thus, 1 and 3 (2 and 4) differ in the sugar residue, while 1 and 2 (3 and 4) differ only in the aglycone (see Fig. 1).

When blackcurrant anthocyanins (300 mg) were subjected to DCCC using the CMH system with the chloroform-rich layer as the stationary phase and the aqueous layer as the mobile phase, all compounds were eluted with the first 60 ml of mobile phase and very little separation occurred. Partition was therefore too heavily in favour of the aqueous phase. As a corollary of this, the alternative system with a stationary



1 R = glucosyl
3 R = rutinosyl
5 R = sophorosyl
6 R = (2^{G}-glucosyl)rutinosyl

2 R = glucosyl
4 R = rutinosyl

Fig. 1. Structures of the anthocyanins 1–6.

aqueous phase would lead to large elution volumes and be very time-consuming. The CMH solvent system was thus rejected.

When the upper layer of the PAH system was used as mobile phase, the total elution volume increased to 240 ml. Some separation occurred but only a few of the earliest and latest fractions eluted contained a single component: compound 1 was found in fractions 20–36, 2 in fractions 25–49, 3 in fractions 35–44, and 4 in fractions 39–58. The difference between the two aglycones, one hydroxy-group, was insufficient to permit adequate separation with this system, although the mono- and diglycosides of the same aglycone were moderately separated. The PAH system was thus not discriminatory enough with respect to the functionality variations found in anthocyanins to be of general applicability and was abandoned.

The BAW solvent system was tested with the upper layer as mobile phase. The four compounds present formed obvious zones with very little overlap and the separation was regarded as completely satisfactory (Fig. 2A). When the same system (BAW) was tried in the alternative mode, *i.e.* with the lower layer as mobile phase, all the components were rapidly eluted and a poorer separation was obtained (Fig. 2B).

In order to test the preparative usefulness of the system a larger amount (3 g) of anthocyanins from blackcurrants was subjected to DCCC using BAW with the upper layer as mobile phase. The various zones were eluted in the same fractions, but the amount of contamination in each zone was increased: the first eluted zone was contaminated with 2% of compound 2, the second with 2% of 1 and 12% of 3, the third with 11% of 2 and 4% of 4, and the fourth and final zone with 4% of 3. Presumably 3 g of this mixture represents close to the saturation point for this system. However, with the relatively large amounts of pigments separated in a single chromatographic step the results must be regarded as satisfactory.

Raspberry anthocyanins (300 mg) were chromatographed using the two modes as described above with the BAW solvent system. Raspberries contained cyanidin-3-glucoside (1), cyanidin-3-sophoroside (5), cyanidin-3-rutinoside (3) and cyanidin-3-(2^{G}-glucosyl)rutinoside (6). When the upper layer was used as mobile phase, the separation achieved was, as in the case of blackcurrants, quite satisfactory (Fig. 2C). When the lower layer was the mobile phase an acceptable separation (Fig. 2D) was also achieved in spite of the relatively small elution volume. The results reported here indicate that a solvent system of *n*-butanol–acetic acid–water (4:1:5) is satisfactory for the separation

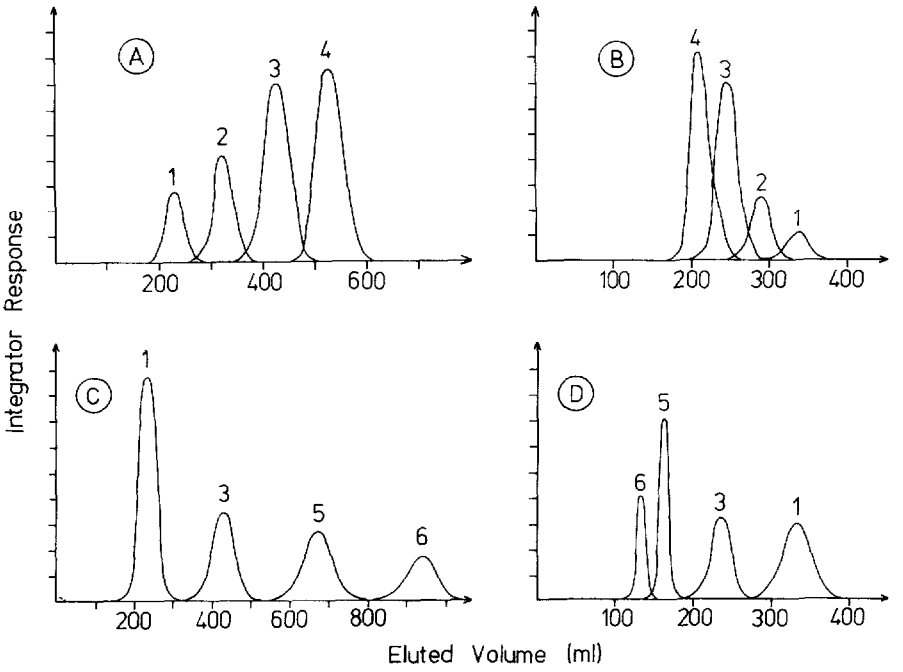


Fig. 2. DCCC of anthocyanins using *n*-butanol–acetic acid-water (4:1:5) as solvent system. Integrator response in arbitrary units is plotted against eluted volume of mobile phase (ml); *ca.* 150 ml of stationary phase displaced prior to elution of the first drops of mobile phase are not included in volumes on the chart. Chromatograms are shown for the following separations: (A) blackcurrant anthocyanins using the upper layer as mobile phase; (B) blackcurrant anthocyanins using the lower layer as mobile phase; (C) raspberry anthocyanins using the upper layer as mobile phase; and (D) raspberry anthocyanins using the lower layer as mobile phase. Peaks are identified by compound numbers as given in the text.

of anthocyanins by droplet counter-current chromatography. Our experience suggests that the upper layer of the solvent system be used as mobile phase for less polar anthocyanins, whereas the lower layer of the system may be used for more polar anthocyanins. DCCC thus provided a useful new tool in the separation, both analytical and preparative, in the difficult field of anthocyanin chemistry.

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