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Note

High-performance liquid chromatographic separation of anthocyanins of *Sambucus nigra* L.

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Limitations in the use of some synthetic food colourants have renewed interest in the use of plant pigments for this purpose. One possibility is the use of anthocyanins, which are of widespread occurrence in plants and are responsible for almost all the red, blue and purple colours found in nature.

Studies evaluating the utility of anthocyanins as food colours are based primarily on extracts from the marc of grapes after wine-making. In addition to a large number of different anthocyanins, these extracts contain several other phenolic substances, especially tannins. This complex composition makes the analyses tedious, and raises questions regarding their use as a food additive.

Another source of anthocyanins for food colouring could be elderberry, *Sambucus nigra* L, a small tree growing freely in the northern hemisphere with fruits which are an extremely good source of anthocyanins of a more simple composition and with few other phenolic substances.

The use of high-performance liquid chromatography (HPLC) for the separation of anthocyanins has been reviewed by Francis[1]. Especially, the development of reversed-phase columns has greatly improved the separation performance of anthocyanins by HPLC. To our knowledge no work dealing with the anthocyanins of elderberry has previously been reported.

In this paper we describe an analytical HPLC technique based on a reversed-phase separation with linear-gradient elution which enables the resolution of the four anthocyanins in elderberry, their chalcone forms, and their common aglycone. The chalcone form and the aglycone are both intermediates of the degradation pathway of anthocyanins[2] and are therefore of interest in stability and metabolic studies of the anthocyanins.

EXPERIMENTAL

Sample preparation

The elderberries used were grown in 1980 at Pometet. The Royal Veterinary and

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Agricultural University, Tåstrup, Denmark, harvested in early October and frozen at −25°C. The juice was separated with a hydraulic press and the pomace was used for extraction. Pomace was found to be 25% of the fruit weight. A 5-g sample of pomace was macerated for 2 min in a blender with 100 ml of 0.1 *M* hydrochloric acid and filtered on a G 4 fritted glass filter. The residue was re-extracted twice with 50 ml of 0.1 *M* hydrochloric acid and filtered. The anthocyanin content of this extract was determined by the spectrophotometric pH-differential method[3], and was found to be 590 mg/l. A 20-μl aliquot of this solution was used for the analytical HPLC investigation of the red pigments. To prepare the anthocyanins in their chalcone forms the pH was adjusted to 4.5 and the sample was then heated to its boiling point and then cooled immediately. The aglycone was formed by hydrolysis by heating at pH 1 for 30 min at 100°C.

Apparatus

A liquid chromatograph consisting of a Waters solvent delivery system (Model 6000 A), a solvent programmer (LKB gradient mixer, Model 11300 Ultragrad) and a Rheodyne 7120 injection valve with a 20-μl loop was used. Detection was conducted using two detectors in series, a Waters Absorbance detector (Model 440) with fixed wavelength (254 nm) and a Perkin-Elmer Spectrophotometer (Model LC-55) with variable wavelength. Chromatograms were recorded for both wavelengths on a two-channel Omniscribe recorder (Houston Instruments). Retention and integration data for one channel were collected simultaneously on a Spectra-Physics Autolab Minigrator.

Chemicals

Tetrahydrofuran (LiChrosolv) and phosphoric acid were obtained from E. Merck, Darmstadt, G.F.R. Reference anthocyanins were obtained from Fluka, Buchs, Switzerland, and C. Roth, Karlsruhe, G.F.R.

Chromatography

For thin-layer chromatography (TLC), 20 × 20 cm cellulose-on-plastic sheets (E. Merck) were used. The chromatogram was developed in two dimensions, first with *n*-butanol–concentrated hydrochloric acid–water (5:2:1) and secondly with formic acid–concentrated hydrochloric acid–water (1:4:8)[4]. All spots could be seen directly on the plates and, in addition, the 3,5-diglycosides of cyanidin were weakly red-fluorescent in UV light (366 nm).

For column chromatography, 10 g of polyvinylpyrrolidone (PVP) (Polyclar AT, Serva, Heidelberg, G.F.R.) were packed in a 30 × 1.5-cm column which was eluted with 0.25 *M* hydrochloric acid–methanol (70:30)[5].

For HPLC an analytical column (150 × 4.6 mm I.D.), slurry packed with Nucleosil 5 C_{18} (Macherey, Nagel & Co., Düren, G.F.R.) was used. The column was operated at ambient temperature. Linear gradient elution with tetrahydrofuran in 0.05 *M* phosphoric acid (pH 1.8) going from 1% to 40% tetrahydrofuran in 15 min, was used. The solvent flow-rate was 1.2 ml/min. Detection was always performed at 254 nm; the choice of the other wavelength depended on the form of the anthocyanins. The flavylium cations were detected at 510 nm and the chalcone forms at 340 nm.

RESULTS AND DISCUSSION

The extract was applied directly to the TLC sheet, and four spots were detected, indicating the presence of one more pigment than found by Reichel and Reichwald[6,7]. The extra spot was the weakest and had R_F values of R_{FI} 0.20 and R_{FII} 0.24. It co-chromatographed with authentic cyanin (cyanidin-3,5-diglucoside).

With column chromatography on polyvinylpyrrolidone it was possible (when detecting at 280 nm) to separate the pigment into three fractions. The fractions were concentrated by evaporation *in vacuo* at 40°C and chromatographed on TLC and HPLC.

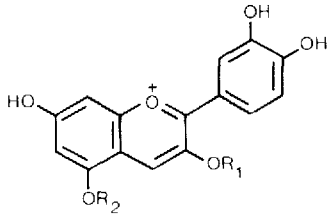
With the high-performance liquid chromatographic method, tests of several reversed-phase column materials showed Nucleosil 5 C_{18} to be suitable for separating the pigments. Tetrahydrofuran proved to be superior to both methanol and acetonitrile as the modifier in the aqueous mobile phase, as it shortened the retention time and gave better separation selectivity. The pH was adjusted to 1.8 using phosphoric acid to ensure that the anthocyanins were in the flavylium cation form[8]. This pH value is below the limit recommended by the producers of the column material, but it was noted that when the column was washed every day after use, even several months of work caused no apparent loss in column performance.

Table I lists the four anthocyanins in the raw elderberry extract that were detected by the HPLC method. Fig. 1 shows the raw extract as detected at both 254 nm and 510 nm. Rechromatography on HPLC of the three fractions from the separation on PVP showed that the second HPLC peak was found in two fractions (Fig. 2). The HPLC method when used on authentic samples of cyanin (cy-3,5-diglucoside) shows a correspondence of retention time between the smallest peak in the HPLC chromatogram of the extract (peak no. 2) and the cyanin (as also indicated by TLC).

A reference sample of cyanidin and the last peak seen in the third fraction from the PVP column had the same retention time. In fact a pronounced hydrolysis

TABLE I

STRUCTURES AND AREA PERCENTAGES OF THE FOUR ELDERBERRY ANTHOCYANIN-FLAVYLIUM CATIONS



Peak No.	*Compound*	R_1	R_2	*Area (%)*
1	Cyanidin-3-sambubioside-5-glucoside	Xylose–glucose	Glucose	1.1
2	Cyanidin-3-glucoside-5-glucoside	Glucose	Glucose	0.8
3	Cyanidin-3-sambubioside	Xylose–glucose	H	32.4
4	Cyanidin-3-glucoside	Glucose	H	65.7

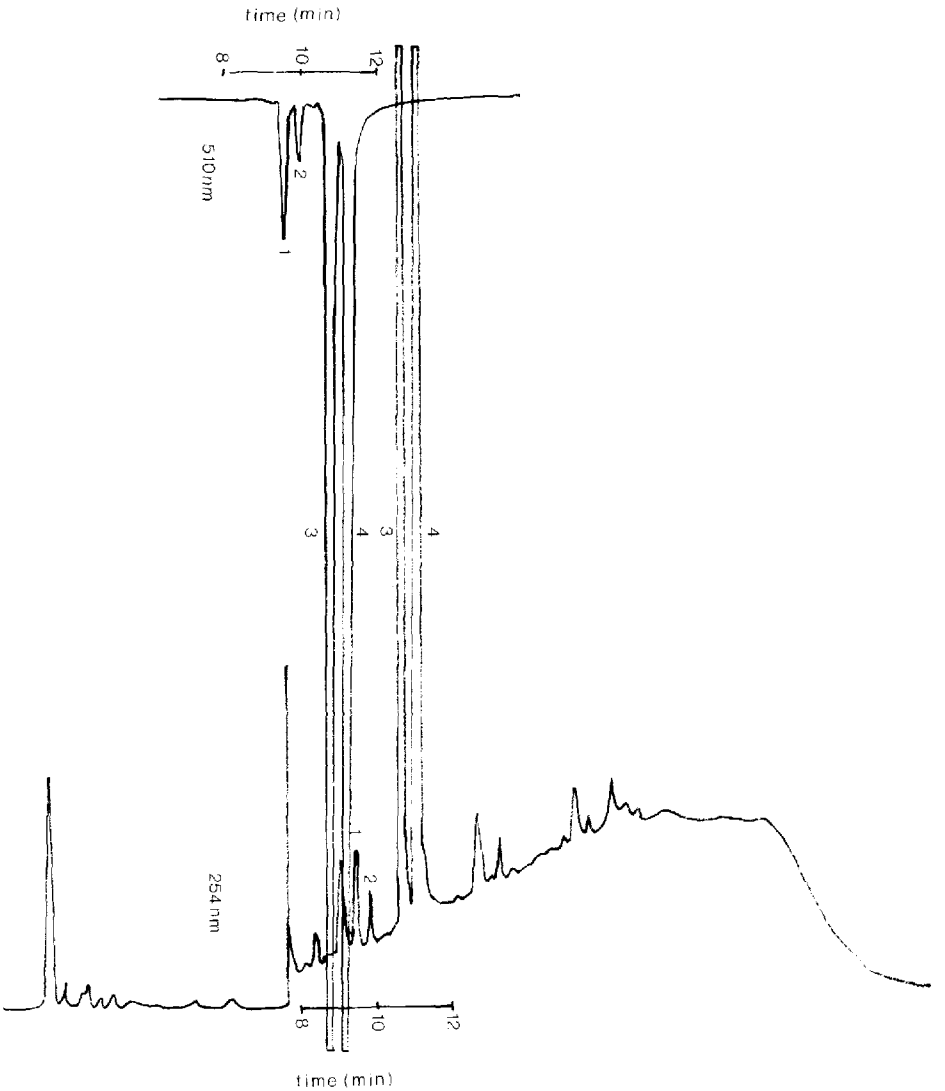


Fig. 1. Chromatogram of raw extract (for identification see Table I). Support: Nucleosil 5 C_{18}, 150 × 4.6 mm. Mobile phase: tetrahydrofuran in 0.05 *M* phosphoric acid (pH 1.8) going from 1% to 40% in 15 min. Flow-rate: 1.2 ml/min. Detection wavelengths: 254 nm and 510 nm.

of the glycosides had occurred on the PVP column. After hydrolysis of the extract, the only aglycone present had the same retention time as cyanidin, indicating that cyanidin is the aglycone present in the elderberry anthocyanins.

Table I also lists the percentage distribution of the four pigments as determined by HPLC (the average of four freshly prepared elderberry extracts), and it is noted that the two 3,5-diglycosides amount to less than 2% of the total and that the amount of cyanidin-3-sambubioside is roughly half that of cyanidin-3-glucoside.

The separation of the chalcones from the four flavylium cations is possible

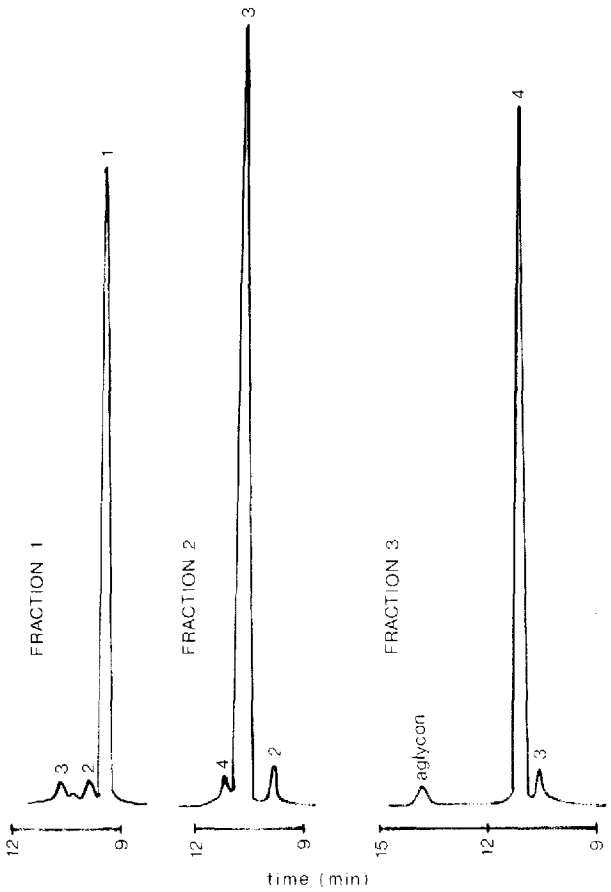


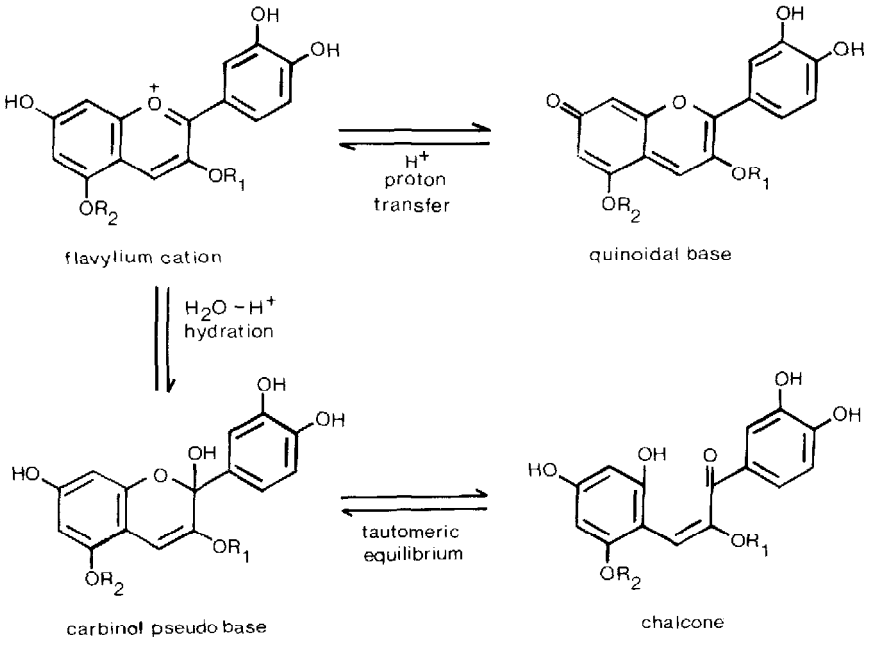
Fig. 2. Chromatograms of three fractions from the separation on PVP (for identification see Table I). Detection wavelength: 510 nm. Other conditions as in Fig. 1.

because of the slow interconversion between the chalcone and the flavylium cation[9] The structural forms are interrelated according to Scheme 1.

The reactions shown in the scheme from left to right are all endothermic which means that heating shifts all equilibria towards the chalcone. After pH adjustment and heating, the extract was chromatographed and, as seen in Fig. 3, the chalcones were eluted after their corresponding cation owing to the lower polarity of the chalcone compounds. However, this is not in agreement with the earlier findings of Preston and Timberlake[10] where the chalcones were eluted before the flavylium cations.

The two chalcone peaks corresponding to cyanidin-3-sambubioside (3A) and cyanidin-3-glucoside (4A) were collected in micro-cuvettes and their absorbance at 340 and 510 nm was measured at 5-min intervals for 1 h (Fig. 4). The absorbance at 340 nm decreased while the absorbance at 510 nm increased during this period, indicating that at pH 1.8 there was a conversion back to the cationic form.

As can be seen from the chromatogram in Fig. 3, two minor peaks (3B and 4B) follow the two chalcone peaks (3A and 4A). A possible explanation could be



Scheme 1. The four chemical structures of anthocyanins between pH values 0 to 6.

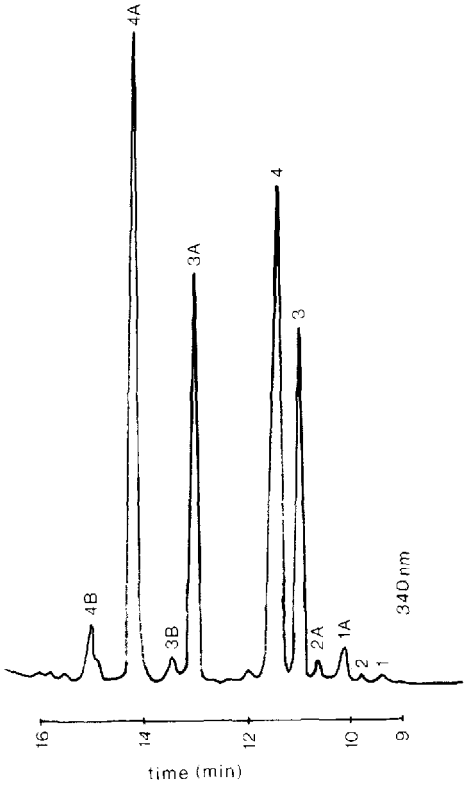


Fig. 3. Chromatogram of raw extract after pH adjustment to pH 4.5 and heating. The flavylium cation peaks are as listed in Table I. The chalcone peaks are marked as their corresponding flavylium cations, A (*trans*-chalcone) or B (*cis*-chalcone). Detection wavelength: 340 nm. Other conditions as in Fig. 1.

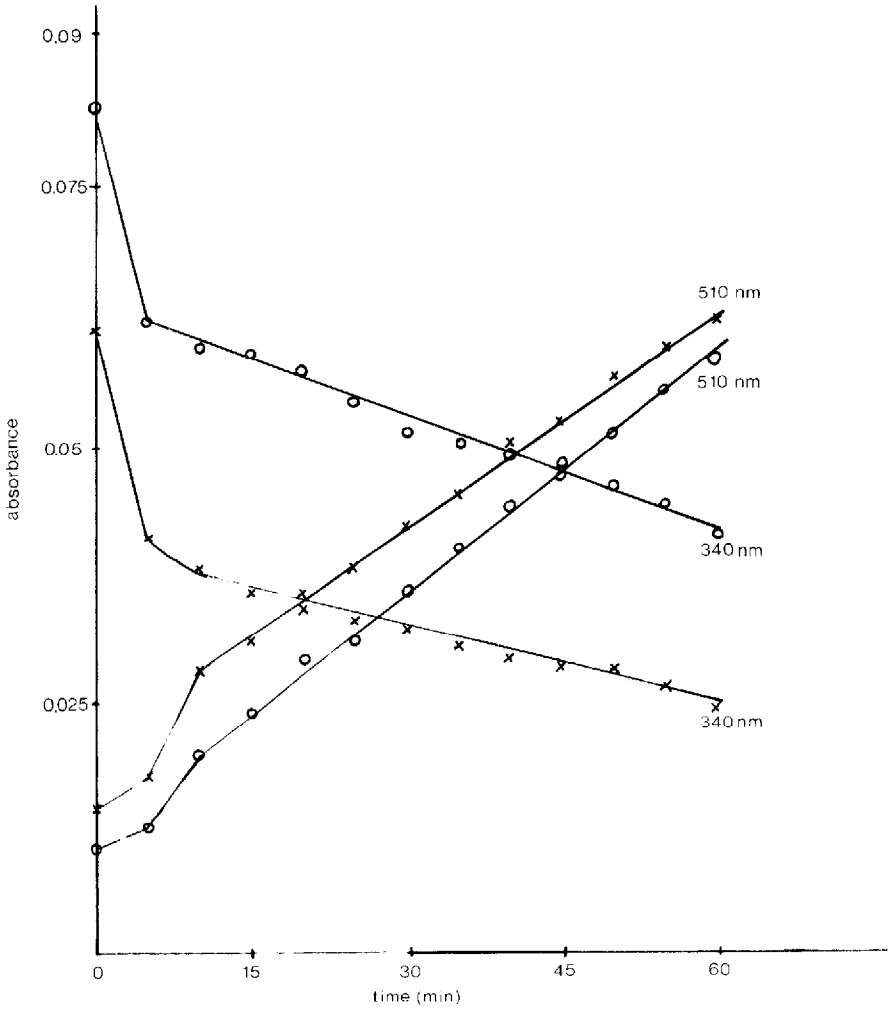


Fig. 4. Variation in the absorbance at 340 and 510 nm of the chalcones during their conversion back into the flavylium cations in solution at pH 1.8: ×, cyanidin-3-sambubioside; ○, cyanidin-3-glucoside.

that the big peak represents the *trans*-chalcone, which is the stable structure, and the much smaller ensuing peak is the *cis*-form, which is the necessary intermediate between the *trans*-chalcone and the flavylium cation[11]. This could indicate that the *cis*- and *trans*-chalcone forms may have been separated.

ACKNOWLEDGEMENT

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