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Note

Separation of anthocyanin chalcones by high-performance liquid chromatography

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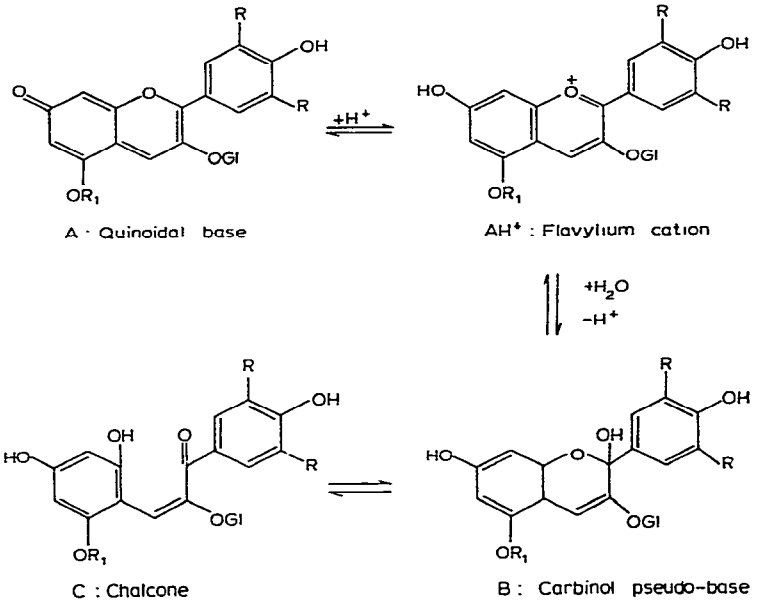
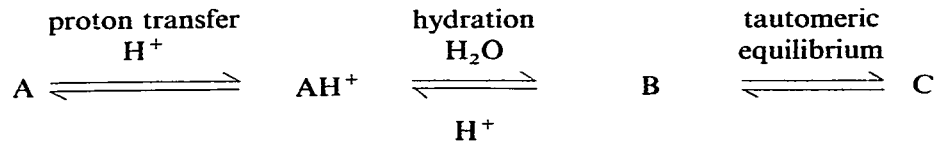
Anthocyanins such as malvidin 3-glucoside and malvidin 3,5-diglucoside can occur in four chemical structures between pH values 0–6, *viz.* the flavylium cation (AH^+), the quinoidal base (A), the carbinol base (B) and the chalcone (C) (Fig. 1)[1,2].



Malvidin 3-glucoside: Gl = glucose, R = OCH_3, R_1 = H
Malvidin 3,5-diglucoside: Gl = R_1 = glucose, R = OCH_3

Fig. 1. Anthocyanin structures.

These structural forms are inter-related according to the following scheme:



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Their proportions at equilibrium are determined by the environmental conditions (pH, temperature, etc.)[3]. Kinetically, the proton transfer and hydration equilibria can be regarded in relative terms as very fast and fast respectively, since relaxation times of malvidin 3-glucoside equilibria at 25°C are of the order of 10^{-5} sec for proton transfer and 1 sec for the hydration equilibria[4]. In contrast, equilibration between the carbinol base (B) and the chalcone (C) of malvidin 3-glucoside is slow, about 1 h at 25°C[4]. To date, the chalcone forms of malvidin 3-glucoside and malvidin 3,5-diglucosides have not been studied in isolation because of the co-occurrence of one or more of the other structural forms, usually in large excess. A method of evaluating the UV–visible absorption spectrum of the chalcone of malvidin 3-glucoside has been described based on measurements of absorbance changes occurring after rapid pH adjustment of an aqueous solution of this anthocyanin from pH 5 to pH < 1 (ref. 4). The calculated spectrum exhibited a broad peak at 350 nm, but the amount of chalcone present was small (*ca.* 10%) compared with the amount of other structures present, *viz.* the carbinol base at pH 5 and the flavylium cation at pH < 1. We have now demonstrated that the chalcone forms of anthocyanins can be separated during the time-scale of high-performance liquid chromatography (HPLC) because of the relatively slow rates of their equilibration with the carbinol forms. For the first time the chalcones of malvidin 3-glucoside and malvidin 3,5-diglucoside have been obtained practically free of the other structures and in sufficient amounts to measure their UV–visible absorption spectra directly and to follow their conversions to their corresponding flavylium cations.

EXPERIMENTAL

Malvidin 3,5-diglucoside was a commercial sample (Fluka, Buchs, Switzerland) and malvidin 3-glucoside was isolated in this laboratory from the skins of red grapes (*Vitis vinifera*). A Spectra-Physics SP8000B high-performance liquid chromatograph was used with its detector operating at 280 nm. A Pye-Unicam LC-UV variable-wavelength detector was used for simultaneous monitoring at other wavelengths. The reversed-phase column (100 × 5 mm) was slurry-packed with Spherisorb Hexyl, 5 μm. The 20-μl samples were filtered through a 0.45-μm Millipore filter before injection. Separations were carried out at a column temperature of 35°C.

Solvent A was 0.60% perchloric acid (pH 1.5), prepared by addition of 60% $HClO_4$ to deionized and glass-distilled water. Solvent B was methanol, glass-distilled from KOH. Both solvents were filtered through a 0.45-μm Millipore filter before use.

Solutions of malvidin 3-glucoside and malvidin 3,5-diglucoside (2.0 and 1.5 mg/ml, respectively, in 0.02 *M* potassium hydrogen tartrate, pH 3.5) were left to stand overnight at room temperature in the dark to attain equilibrium before chromatography. The gradients used for separation of the chalcone glycosides from the equilibrium mixtures were as follows: for malvidin 3-glucoside, 20% of B to 30% B in 15 min; for malvidin 3,5-diglucoside, 2% B to 20% B in 10 min, then 20% B to 60% B in 40 min. Mean retention times for peaks detected at 280 nm were: malvidin 3-glucoside, chalcone glycoside form. 513 sec, flavylium form, 630 sec; malvidin 3,5-diglucoside, chalcone glycoside form 852 sec, flavylium form 1073 sec.

To identify the chalcone glycoside peaks successive separations were carried out using detectors monitoring simultaneously at 280 nm and 525 nm, then 280 nm

and 340 nm. The chalcone glycosides showed no absorbance at 525 nm, but a substantial absorbance at 340 nm. Confirmation was obtained by injecting samples immediately after heating to 100°; the chalcone glycoside peaks were now considerably enlarged, while peaks from the flavylium forms of the anthocyanins were slightly reduced.

Once identified, the chalcone glycoside peaks were collected immediately after passage through the 280-nm detector, in a silica spectrophotometer cell (10 mm pathlength, 0.5 ml capacity) and were transferred at once to the cell compartment (at 28°C) of a Pye Unicam SP8-100 spectrophotometer; their UV–visible spectra between 700 and 210 nm were recorded without delay, using a reference of 75% solvent A, 25% solvent B for malvidin 3-glucoside, and 79% solvent A, 21% solvent B for malvidin 3,5-diglucoside. The relaxation times of the chalcone glycosides were determined by scanning the UV–visible range continuously until negligible change between successive scans was observed. Scanning speed was 2 nm/sec, and the interval between the start of successive scans was 347 sec. For samples requiring minimum exposure to light, continuous scanning was not employed, and absorbance values at 340 nm and 524 nm were read directly from the spectrophotometer at 5-min intervals. Between readings the samples remained in the instrument with the incident radiation masked off.

RESULTS AND DISCUSSION

Usually, anthocyanins are separated by HPLC as their coloured flavylium cations and are monitored at their peak wavelengths (*ca.* 525 nm). Peak shape is affected by solvent pH. At pH 2.5, appreciable quantities of the carbinol forms are also present, which broaden the visible peaks because of interconversion of the two pigment forms on the column[5]. Reducing solvent pH to about 1.5, this being the lower limit of pH stability of the reversed-phase packing materials in use, reduces the amounts of interfering carbinol bases present and sharpens the peaks of the cationic forms. Under such conditions anthocyanins have been separated and identified in plant materials and fruit products. But it should be emphasized that because of rapid interconversion of the various pigment forms induced by pH changes, the quantities of flavylium cations found are determined by the pH conditions pertaining during the chromatographic separation and measurement (about pH 1.5) and may be very different from those in the original plant extract or solution if this is of higher pH.

Anthocyanins also absorb in the ultraviolet region (*ca.* 280 nm). During routine HPLC examination of a solution of malvidin 3-glucoside (pH 3.5), monitoring at 280 nm as well as 525 nm, a small peak was observed at 280 nm which preceded that due to the cationic form. There was no corresponding peak at 525 nm, but a peak appeared when the wavelength of monitoring was changed from 525 nm to 350 nm. It seemed possible that the new peak was due to the chalcone form of malvidin 3-glucoside. To test this, a solution of malvidin 3-glucoside (pH 3.5) was rapidly heated to 100°C. It is known that all the reactions in the scheme from left to right are endothermic, so that heating should shift all equilibria towards the chalcone[2]. Chromatography of the hot solution resulted in the size of the new peak (at 350 nm) being more than doubled while the peak (280 nm) due to the cationic form was reduced. The heated solution was allowed to cool and was re-examined after five days. The new

peak was reduced and the cationic peak was increased to the sizes observed originally; both peaks were identical to those found in the original unheated five-day old solution. Thus the findings were consistent with those expected of the chalcone[2-4]. Similar results were found when a solution of malvidin 3-glucoside at pH 4.5 was treated in the same way.

The new peak was sufficiently well separated from the cationic peak to be collected in a micro-cell and its spectrum scanned as rapidly as possible in a separate spectrophotometer. The spectrum exhibited a broad peak near 340 nm, and a minimum at 274 nm in a solvent containing 75% of 0.60% perchloric acid and 25% methanol; this spectrum is very similar to that computed for the chalcone of malvidin 3-glucoside in aqueous hydrochloric acid by Brouillard *et al.*[4]. When the spectrum was scanned continuously, the broad peak near 340 nm slowly reduced in size with the emergence of more intense peaks at 524 nm and 276 nm, indicating that the chalcone was converting to the cationic form of malvidin 3-glucoside.

The chalcone of malvidin 3,5-diglucoside was also separated and collected after HPLC of a sample of hot solution (pH 3.5). Its spectrum was similar to that of malvidin 3-glucoside chalcone with a trough at 274 nm and a broad peak near 335 nm. The chalcone converted to the flavylium cation of malvidin 3,5-diglucoside (λ_{max} 522 nm and 274 nm) in similar fashion to that observed with malvidin 3-glucoside (Fig. 2). Re-injection of the converted solution into the chromatograph confirmed that the flavylium cation present was entirely malvidin 3,5-diglucoside and that no hydrolysis to either of the malvidin monoglucosides had occurred. The very small absorbance in the vicinity of 522 nm in the spectrum of freshly separated chalcone (Fig. 2) can be attributed to some slight conversion occurring during the time interval between its separation on the column and its initial spectral measurement.

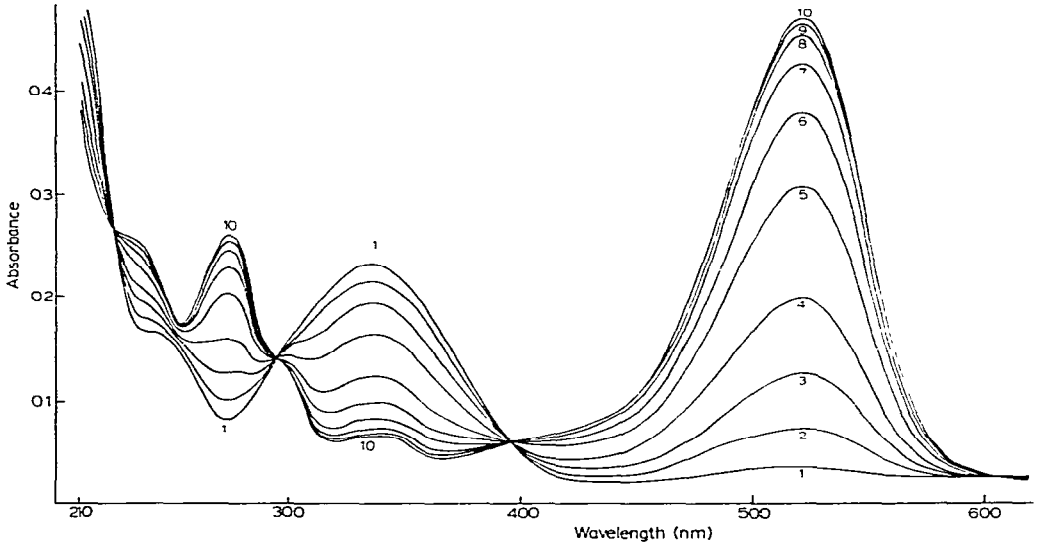
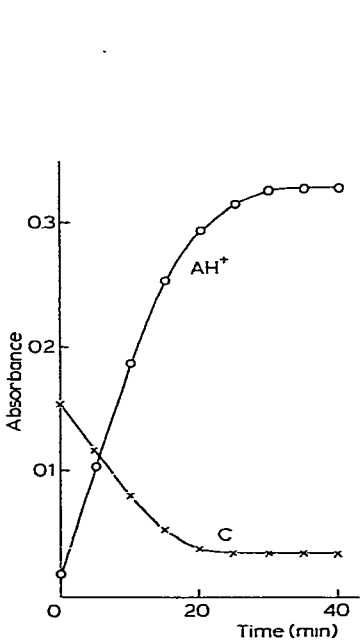


Fig. 2. Absorbance spectra of the chalcone of malvidin 3,5-diglucoside during its conversion to the flavylium cation (solvent 79% A, 21% B). Scan 1: chalcone measured immediately. Scans 2–10: chalcone conversion to the cation. Scans were at intervals of 5.8 min except the interval between scans 4 and 5 which was 7.4 min.

When the isolated chalcone of malvidin 3-glucoside was kept dark and exposed to light only during absorbance measurements made at 340 nm and 524 nm, the rate of chalcone loss (absorbance at 340 nm) and the rate of formation of flavylium cation (absorbance at 524 nm) were as shown in Fig. 3. However, the curves constructed from similar data obtained from the spectral scans (from 700 nm to 210 nm) exhibited initial delays between scans 1 and 3. This phenomenon (Fig. 4) was observed with both anthocyanins. A possible explanation is as follows. From consideration of the work of Jurd[6] and McClelland and Gedge[7] on flavylium salts, it is to be expected that the chalcone isomer from which the anthocyanin cation is derived (via the carbinol base) is the *cis*, this isomer alone having the correct configuration for cyclisation. In contrast the isomer likely to occur at equilibrium is the *trans*. Conversion of *trans* to *cis* should be endothermic. It is conceivable that heating an equilibrated solution of an anthocyanin at pH 3.5 produces a mixture containing an increased proportion of the chalcone glycoside isomers, the relative amounts of which depend upon the extent and duration of heating. Under our HPLC conditions which were chosen to separate the chalcone from flavylium form as rapidly as possible, the chalcone isomers probably would not separate but be collected as a single peak. The observed initial delay in formation of the flavylium form, referred to above (Fig. 4), could thus be due to photoisomerisation of *trans* chalcone to *cis* chalcone in the spectrophotometer, the exact rate of formation of the cationic anthocyanin depending on the proportion of



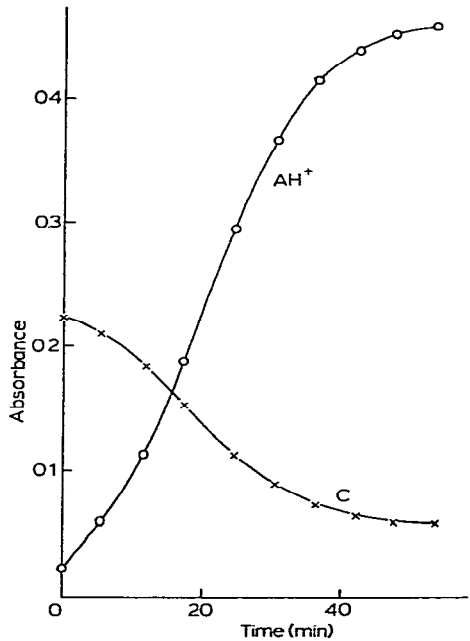


Fig. 3. Variation in the absorbance at the $\lambda_{max.}$ of the chalcone (C, 340 nm) and flavylium cation (AH^+, 524 nm) of malvidin 3-glucoside (solvent 75% A, 25% B). Solution exposed to light only during brief measurements at these wavelengths.

Fig. 4. Variation in the absorbance at the $\lambda_{max.}$ of the chalcone (C, 335 nm) and flavylium cation (AH^+, 522 nm) of malvidin 3,5-diglucoside during the spectral scans illustrated in Fig. 2.

trans chalcone in the mixture. Jurd[6] has reported such a process for the formation of certain flavylium salts in acid solutions and has found that in the absence of light, isomerisation of the *trans* chalcone is acid-catalysed and much slower. Consequently, it seems probable that the overall formation rate of flavylium cations of anthocyanins in acid solutions of the corresponding chalcones isolated by HPLC, depends on the relative proportions of the chalcone isomers in the mixture and on the degree of their exposure to light subsequent to their isolation. In the absence of light, the cationic structure should be formed initially almost entirely from the *cis* chalcone, and its rate of formation should thus be almost constant in the early stages. Measurements of the formation rate of the cationic form of malvidin 3-glucoside made with minimum exposure of the sample to light and UV radiation showed this to be so (Fig. 3).

The conversion of the chalcone was accompanied by a gradual change of the $\lambda_{max.}$ of the peak in the region of chalcone absorbance from 340 to 345 nm for malvidin 3-glucoside and from 335 to 340 nm for malvidin 3,5-diglucoside. The minor peaks remaining at the longer wavelengths after completion of the conversion must be attributed to the flavylium cationic forms of these anthocyanins rather than to small amounts of unconverted *trans* chalcone, since the chalcone could not be detected by HPLC in an equilibrated anthocyanin solution at pH 1.5.

Well-defined spectral isosbestic points at 395 and 295 nm were observed during the conversion of malvidin 3,5-diglucoside to its cationic form (Fig. 2). On two occasions all the spectral scans passed through these points; on the third occasion, the initial scans 1 and 2 did not pass through these isosbestics. In this respect similar behaviour was observed with the malvidin 3-glucoside chalcone-flavylium cation conversion. Scans 3–8 invariably passed through isosbestic points at 398 and 299 nm but this was not always so with scans 1 and 2. The inconsistency of the spectral scans 1 and 2 is in accord with the proposed photoisomerisation of the chalcone, but the significance of the absence or presence of isosbestic points will not be pursued because of the complexity of the system and the conclusions drawn from a previous study[8].

It is likely that chalcones of other anthocyanins may be separated by HPLC in a similar way. Indeed, during HPLC of *V. vinifera* grape skin extracts we have observed in addition to the chalcone of malvidin 3-glucoside, two peaks which may represent the chalcones of malvidin 3-acetylglucose and malvidin 3-*p*-coumaroylglucoside, these being the other anthocyanins present in greatest amounts in the samples studied. The peaks were not evident at 525 nm but were characterised by absorbance at 340 nm greater than at 280 nm. Peak sizes were increased on heating. We suggest that these criteria may be useful for detecting anthocyanin chalcones in other systems.

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