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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC SEPARATION OF 3-GLUCOSIDES, 3,5-DIGLUCOSIDES, 3-(6-O-*p*-COUMARYL)GLUCOSIDES AND 3-(6-O-*p*-COUMARYLGLUCOSIDE)-5-GLUCOSIDES OF ANTHOCYANIDINS

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SUMMARY

A high-pressure liquid chromatographic method has been developed for separation of anthocyanins from fruits and their products on a μBondapak C_{18} analytical column. Separation of 3- and 3,5-diglucosides of anthocyanidins can be achieved with aqueous acetic acid. That of the *p*-coumaryl-3 and 3,5-diglucosides requires an aqueous methanolic acetic acid solution. For the separation of complex mixtures of anthocyanins containing members of all four above mentioned pigment groups, a programmed non-linear-gradient elution between aqueous acetic acid and aqueous methanolic acetic acid solution is required. This technique enables a relatively fast separation and identification of twenty anthocyanins in one run without prior treatment of the plant extract, or derivatization of the pigments.

INTRODUCTION

Paper and thin-layer chromatographic methods are commonly used in the process of identification of anthocyanins and other flavonoid compounds[1–3]. Because of the time-consuming development for both paper (up to 36 h) and thin-layer chromatograms (2–5 h), a speedy analysis of a larger number of samples were cumbersome. Recent developments in high-pressure liquid chromatography, instrumentation and column support materials, allowed the analysis of a number of natural products whose properties did not permit derivatization and gas chromatographic detection.

Successful high-pressure liquid chromatographic separation of anthocyanidins

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was reported recently[4] on a μBondapak C_{18} column with the aid of a Schoeffel variable-wavelength UV-VIS detector. Using this, or similar instrumentation, anthocyanins and anthocyanidins can be monitored selectively at their $\lambda_{max.}$ without any interference from other compounds.

The availability of a relatively large number of anthocyanins in our laboratory (M.W. and G.H) and the lack of a speedy analytical method for detection of anthocyanins prompted us to investigate the properties of the μBondapak C_{18} column support material toward separation of anthocyanins. The data obtained for the twenty anthocyanins commonly found in *Vitis* sp. are presented here.

EXPERIMENTAL

Materials and methods

Grape juice was obtained from ripe Concord, Ives and De Chaunac (S-9549) grapes grown in the Experiment Station vineyards. Chromatograms were run on a Waters liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) using a 300 × 4 mm I.D. μBondapak C_{18} column (Waters Assoc.) and a Schoeffel SF770 UV-VIS detector. The anthocyanins were monitored at 520 nm.

Solvent systems

The following solvent systems were used for the separation of anthocyanins: A, 0.1% H_3PO_4 in acetic acid–water (10:90); B, acetic acid–water (15:85); C, water–acetic acid–methanol (65:15:20).

Authentic samples of anthocyanin pigments were obtained from our laboratory collections. Samples for chromatographic analysis were prepared by dissolving approximately 1 mg of the compound in 1 ml of distilled water. Because of differences in purity of the various pigment preparations the exact concentration of each pigment in the complex mixture was purposely varied in order to obtain chromatograms showing peaks of similar heights.

Analysis

Analysis of anthocyanidin-3,5-diglucosides. A sample of 5 μl of a mixture (containing approximately 0.1% each of the 3,5-diglucosides of cyanidin, petunidin, peonidin and malvidin) was injected into the column and eluted with solvent A at a flow-rate of 1.0 ml/min.

Analysis of anthocyanidin-3-glucosides. A sample of 5 μl of a mixture (containing approximately 0.1% each of the 3-glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin) was injected into the column and eluted with solvent B at a flow-rate of 1.5 ml/min.

Analysis of a mixture of 3- and 3,5-diglucosides of anthocyanidins. A sample of 10 μl containing 5 μl each of a mixture of 3-monoglucosides and that of 3,5-diglucosides was injected into the column and subjected to gradient elution starting with 99% of solvent B and 1% of solvent C. Solvent C was increased from 1 to 100% during a period of 40 min on program 9 of the solvent programmer. The flow-rate was 0.2 ml/min for the first 50 min of the elution and then increased to 0.3 ml/min.

Separation of anthocyanidin-3-(6-O-p-coumarylglucoside)-5-glucosides. A sample of 5 μl of a mixture (containing approximately 0.2% each of the 3-(6-O-*p*-

coumarylglucoside)-5-glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin) was injected into the column and eluted with solvent C at a flow-rate of 0.3 ml/min.

Separation of anthocyanidin-3-(6-O-p-coumarylglucoside)s. A sample of 7.5 μl of a mixture (containing approximately 0.2% each of the 6-O-*p*-coumarylglucosides of delphinidin, petunidin and malvidin) was injected into the column and eluted with solvent C at a flow-rate of 0.3 ml/min.

Analysis of a mixture of anthocyanidin-3-(6-O-p-coumarylglucoside)s and -3-(6-O-p-coumarylglucoside)-5-glucosides. A sample of 10 μl containing 5 μl of a mixture of acylated monoglucosides and 5 μl of a mixture of acylated diglucosides was injected into the column and eluted with solvent C at a flow-rate of 0.3 ml/min.

Analysis of a complex mixture of anthocyanins. A sample of 20 μl of a complex mixture of anthocyanins [containing 5 μl each of a mixture of 3-glucosides, 3,5-glucosides, 3-*p*-coumarylglucosides and 3-(6-O-*p*-coumarylglucoside)-5-glucosides] as described above was injected into the column and subjected to non-linear-gradient elution starting with 95% of solvent B and 5% of solvent C. The percentage of solvent C was increased from 5 to 100 during a period of 20 min at a flow-rate of 0.2 ml/min on program 9 of the solvent programmer.

Analysis of anthocyanins in grape juices. A 20-μl sample of the grape juice was injected into the column and subjected to non-linear-gradient elution starting with 95% of solvent B and 5% of solvent C and increasing the percentage of solvent C from 5 to 100 during a period of 20 min at a flow-rate of 0.2 ml/min on program 9 of the solvent programmer.

RESULTS

Separation of the anthocyanidin-3,5-diglucosides

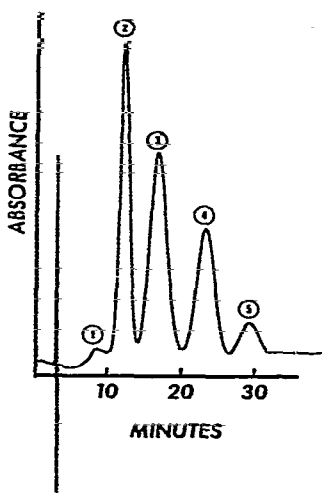
Solvent system A, consisting of 0.1% of H_3PO_4 in 10% acetic acid gave the optimal elution for the anthocyanidin-3,5-diglucosides. The separation of the five *Vitis* anthocyanidin-3,5-diglucosides occurred in the reverse order of that reported earlier on conventional columns of polyvinylpyrrollidone[5]. As shown in Fig. 1, the delphinidin derivative was the first pigment eluted (peak 1), followed by derivatives of cyanidin, petunidin, peonidin, and malvidin, showing the order of elution to be in decreasing polarity of the compounds (peaks 2, 3, 4 and 5, resp.).

Separation of the anthocyanidin-3-glucosides

Solvent A showed a slow and inferior separation of the anthocyanidin-3-glucosides. Therefore, the acidity of the solvent was increased to 15% acetic acid and H_3PO_4 was omitted. With this solvent system (solvent B) the 3-glucosides of the *Vitis* anthocyanidins separated well, as shown in Fig. 2. As is the case of the 3,5-diglucosides of anthocyanidins, the 3-glucosides gave the same elution order, delphinidin-3-glucoside (peak 6) being the first pigment eluted from the column, followed by the 3-glucosides of cyanidin (peak 7), petunidin (peak 8), peonidin (peak 9) and malvidin (peak 10).

Separation of mixtures of anthocyanidin-3-, and -3,5-glucosides

For the separation of the individual pigments from the mixture a non-linear-



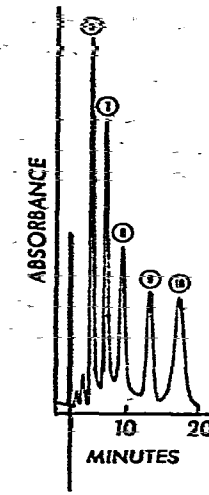


Fig. 1. Separation of the anthocyanidin-3,5-diglucoside on a μBondapak C_{18} column. Solvent A. 1 = Delphinidin-3,5-diglucoside; 2 = cyanidin-3,5-diglucoside; 3 = petunidin-3,5-diglucoside; 4 = peonidin-3,5-diglucoside; 5 = malvidin-3,5-diglucoside. Flow-rate: 1.0 ml/min.

Fig. 2. Separation of anthocyanidin-3-glucosides on a μBondapak C_{18} column. Solvent B. 6 = Delphinidin-3-glucoside; 7 = cyanidin-3-glucoside; 8 = petunidin-3-glucoside; 9 = peonidin-3-glucoside; 10 = malvidin-3-glucoside. Flow-rate: 1.5 ml/min.

gradient (program 9) elution was required. Fig. 3 shows such a separation. With the exception of malvidin-3,5-diglucoside (peak 5) and cyanidin-3-glucoside (peak 7) which overlapped in the above, and also in other solvent systems tried, all anthocyanins were separated. The separation of the individual components of anthocyanins required 70 min.

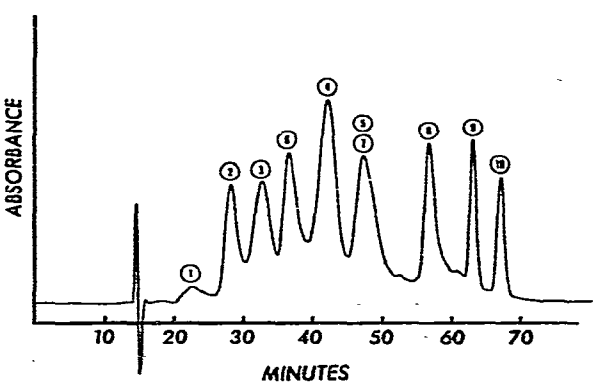


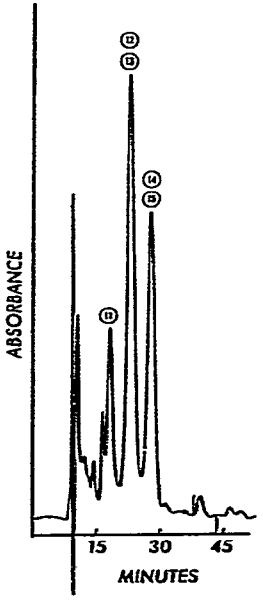
Fig. 3. Separation of mixtures of anthocyanidin-3- and -3,5-glucosides on a μBondapak C_{18} column. Solvent: non-linear gradient between solvent B and solvent C on program 9 of the Waters solvent programmer. Flow-rate 0.2 ml/min for 50 min; then increased to 0.3 ml/min. Peak numbers correspond to those in Figs. 1 and 2.

Separation of anthocyanidin-3-(6-O-p-coumarylglucoside)-5-glucosides

Purely aqueous acetic acid solutions did not separate the anthocyanidin-3-(6-O-*p*-coumarylglucoside)-5-glucosides. A good separation of these pigments could be obtained however when methanol was introduced (at 20% concentration) into the solvent system used for the separation of the 3-glucosides. The so obtained solvent system (solvent C) gave three peaks for the five anthocyanin pigments injected into the column. While delphinidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside separated from the other pigments as a single peak (peak 11), the same derivatives of cyanidin or petunidin (peaks 12 and 13) and peonidin and malvidin (peaks 14 and 15) were eluted as two pairs, shown in Fig. 4.

Separation of anthocyanidin-3-(6-O-p-coumarylglucoside)s

Solvent C at a flow-rate of 0.3 ml/min gave a good separation of all five 3-(6-O-*p*-coumarylglucoside)s of the *Vitis* anthocyanidins. The order of elution was the same as in previous separations (Fig. 5). Delphinidin derivative (peak 16) was eluted first, followed by derivatives of cyanidin (peak 17), petunidin (peak 18), peonidin (peak 19) and malvidin (peak 20).



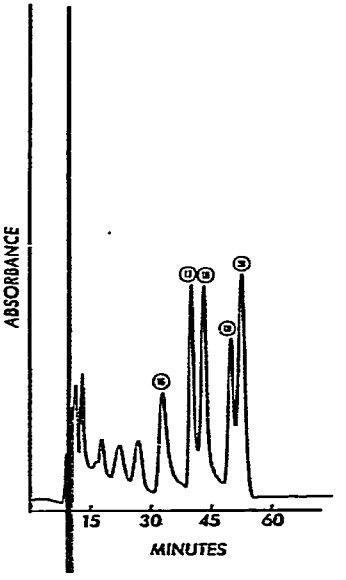


Fig. 4. Separation of anthyocyanidin-3-(6-O-*p*-coumarylglucoside)-5-glucosides on a μBondapak C_{18} column. Solvent C. 11 = Delphinidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside; 12 = cyanidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside; 13 = peninidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside; 14 = peonidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside; 15 = malvidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside. Flow-rate: 0.3 ml/min.

Fig. 5. Separation of the anthocyanidin-3-(6-O-*p*-coumarylglucoside)s on a μBondapak C_{18} column. Solvent C. 16 = Delphinidin-3-(6-O-*p*-coumarylglucoside); 17 = cyanidin-3-(6-O-*p*-coumarylglucoside); 18 = petunidin-3-(6-O-*p*-coumarylglucoside); 19 = peonidin-3-(6-O-*p*-coumarylglucoside); 20 = malvidin-3-(6-O-*p*-coumarylglucoside). Flow-rate: 0.3 ml/min.

Separation of mixtures of anthocyanidin-3-(6-O-p-coumarylglucoside)s and -3-(6-O-p-coumarylglucoside)-5-glucosides

The separation of mixtures of the acylated anthocyanidin-3 and -3,5-diglucosides appeared in the same order as that reported for the individual groups (Fig. 6) using a single solvent system (solvent C). Delphinidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside was eluted first and malvidin-3-(6-O-*p*-coumarylglucoside) was eluted last. The time required for the separation was 55 min at 0.3 ml/min flow-rate. Peaks preceding the acylated anthocyanins on the chromatogram were contaminations in the preparates by non-acylated anthocyanidin-3- and -3,5-diglucosides.

Separation of mixtures containing the 3-glucosides, 3,5-diglucosides, 3-(6-O-p-coumarylglucoside)s and 3-(6-O-p-coumarylglucoside)-5-glucosides of anthocyanidin

A complex mixture containing the above derivatives of the 5 *Vitis* anthocyanidins could not be separated by the use of one single solvent system. Therefore, a non-linear gradient between solvent systems B and C was used. As shown in Fig. 7 seventeen peaks for the twenty anthocyanins were obtained. The overlapping pigments were malvidin-3,5-diglucoside and cyanidin-3-glucoside (peaks 5 and 7), the 3-(6-O-*p*-coumarylglucoside)-5-glucosides of cyanidin and petunidin (peaks 12 and 13) and those of peonidin and malvidin (peaks 14 and 15). All other anthocyanins separated clearly. The structural relationship and retention times of the investigated anthocyanins obtained by the above described non-linear-gradient elution between solvent systems B and C are shown in Fig. 8.

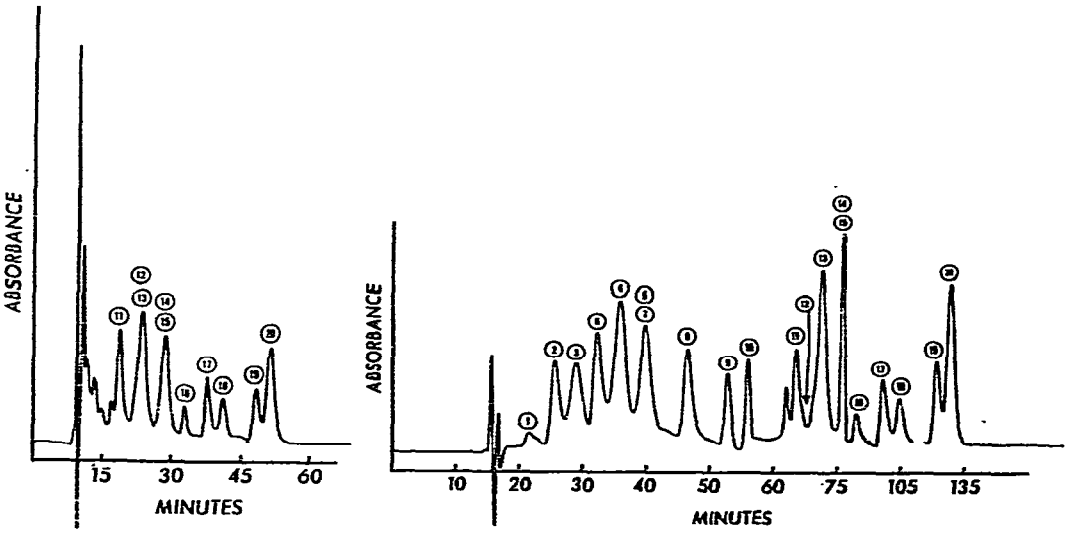
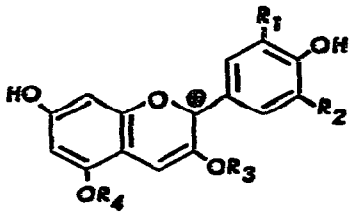
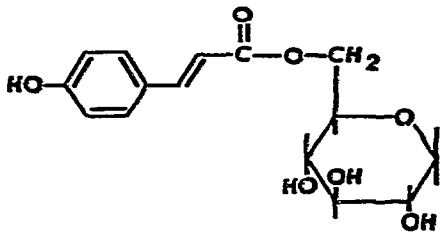


Fig. 6. Separation of mixtures of anthocyanidin-3-(6-O-*p*-coumarylglucoside)s and anthocyanidin-3-(6-O-*p*-coumarylglucoside)-5-glucosides on a μBondapak C_{18} column. Solvent C. Peak numbers correspond to those in Figs. 4 and 5. Flow-rate: 0.3 ml/min.

Fig. 7. Separation of anthocyanidin-3-glucosides, 3,5-diglucosides, 3-(6-O-*p*-coumarylglucoside)s and 3-(6-O-*p*-coumarylglucoside)-5-glucosides. Solvent: non-linear gradient between solvents B and C on program 9 of the Waters solvent programmer. Flow-rate: 0.2 ml/min. Peak numbers correspond to those in Figs. 1–5.



Anthocyanin



(6-O-p-coumarylglucoside) = (PCG)

Order of elution	Compound	Substitution R_1	R_2	R_3	R_4	Retention times t_R (min)
Diglucosides						
1	Delphinidin 3,5-diglucoside	OH	OH	Glu	Glu	22.0
2	Cyanidin 3,5-diglucoside	OH	H	Glu	Glu	26.0
3	Pentunidin 3,5-diglucoside	OCH_3	OH	Glu	Glu	30.1
4	Peonidin 3,5-diglucoside	OCH_3	H	Glu	Glu	37.8
5	Malvidin 3,5-diglucoside	OCH_3	OCH_3	Glu	Glu	42.1
Monoglucosides						
6	Delphinidin 3-glucoside	OH	OH	Glu	H	33.2
7	Cyanidin 3-glucoside	OH	H	Glu	H	42.1
8	Petunidin 3-glucoside	OCH_3	OH	Glu	H	49.3
9	Peonidin 3-glucoside	OCH_3	H	Glu	H	53.2
10	Malvidin 3-glucoside	OCH_3	OCH_3	Glu	H	55.1
Acylated diglucosides						
11	Delphinidin 3(6-O-p coumarylglucoside)-5 glucoside	OH	OH	PCG	Glu	64.5
12	Cyanidin 3(6-O-p-coumarylglucoside)-5 glucoside	OH	H	PCG	Glu	70.0
13	Petunidin 3(6-O-p-coumarylglucoside)-5 glucoside	OCH_3	OH	PCG	Glu	72.0
14	Peonidin 3(6-O-p-coumarylglucoside)-5 glucoside	OCH_3	H	PCG	Glu	79.1
15	Malvidin 3(6-O-p-coumarylglucoside)-5 glucoside	OCH_3	OCH_3	PCG	Glu	79.1
Acylated monoglucosides						
16	Delphinidin 3(6-O-p-coumarylglucoside)	OH	OH	PCG	H	87.0
17	Cyanidin 3(6-O-p-coumarylglucoside)	OH	H	PCG	H	100.9
18	Petunidin 3(6-O-p-coumarylglucoside)	OCH_3	OH	PCG	H	111.0
19	Peonidin 3(6-O-p-coumarylglucoside)	OCH_3	H	PCG	H	129.9
20	Malvidin 3(6-O-p-coumarylglucoside)	OCH_3	OCH_3	PCG	H	140.2

Fig. 8. Structure and retention times of anthocyanins on a μBondapak C_{18} column.

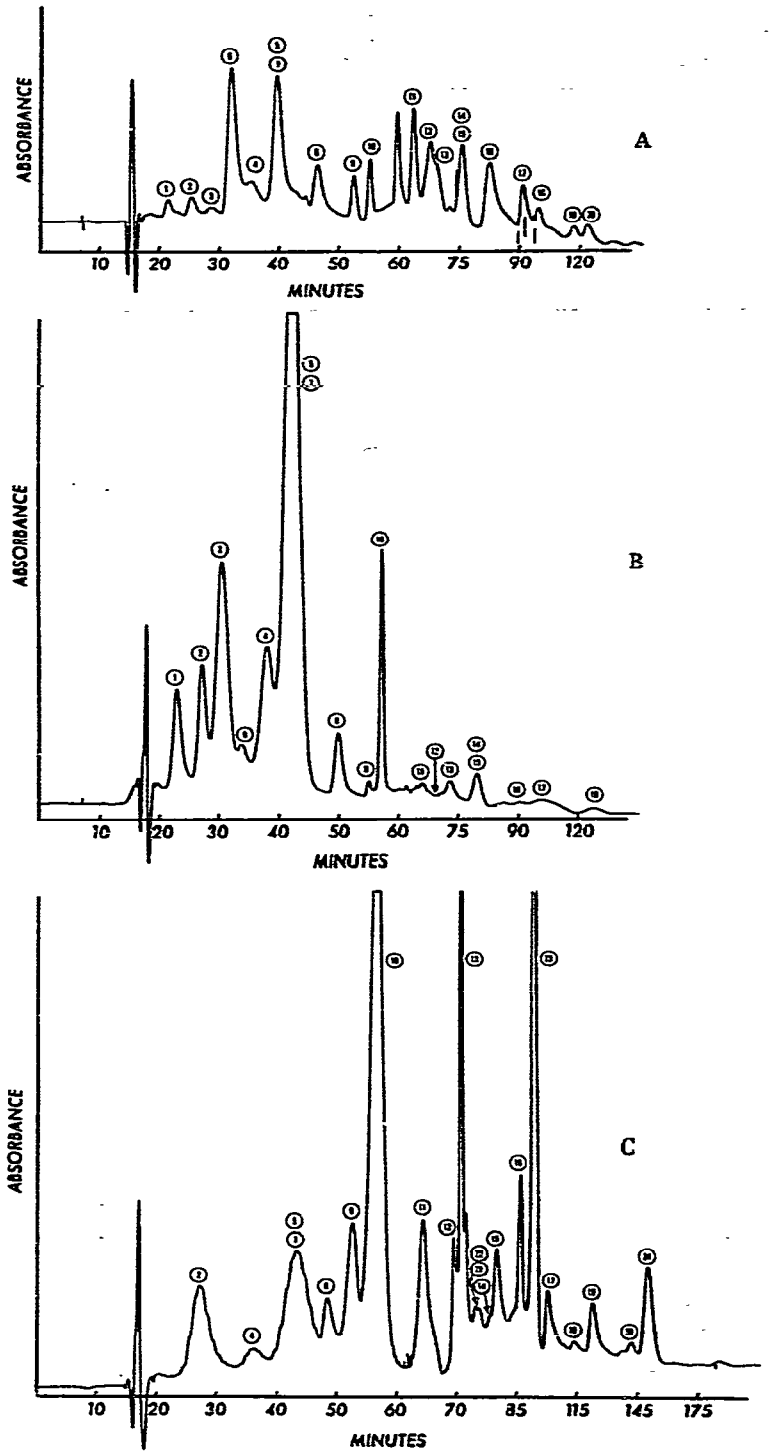


Fig. 9. Anthocyanin profile of Concord (A), De Chaunac (Seibel-9549) (B) and Ives (C) grapes. Solvent: non-linear gradient between solvents B and C on program 9 of Waters solvent programmer. Peak numbers correspond to those in Figs. 1–5. Flow-rate: 0.2 ml/min. Peaks 21–24 were not identified.

Qualitative analysis of grape juices for anthocyanin content

The following figures show the separation of anthocyanins in the untreated grape-juice samples. The pigment profile of Concord grape juice (Fig. 9A) was obtained by injection of 20 μl sample into the column. It has been reported earlier that this grape contains the derivatives of delphinidin and cyanidin in the largest amount[3]. Clearly, the separation shows that the 3-glucosides (peaks 6 and 7) and 3-(6-O-*p*-coumarylglucoside)s (peaks 16 and 17) of delphinidin and cyanidin are the major pigments present. Peak 5/7 is caused by about a 20:1 ratio of cyanidin–malvidin-3,5-diglucoside and not by large amounts of malvidin-3,5-diglucoside. In Fig. 9B the individual anthocyanins of De Chaunac (S-9549) grapes are shown. As is the case with the Concord sample, 20 μl untreated grape juice was directly injected into the column. This grape variety contains the 3,5-diglucosides of the five *Vitis* anthocyanins in large amounts[5]. The third grape variety investigated was that of Ives (Fig. 9C). This grape contains in accord with earlier reports[2] larger amounts of anthocyanins acylated with *p*-coumaric acid. Major anthocyanins present in this grape sample were the 3,5-diglucosides of cyanidin (peak 2) and malvidin (peak 5), the 3-glucoside of malvidin (peak 10), the 3-(6-O-*p*-coumarylglucoside)-5-glucosides of delphinidin (peak 11), petunidin (peak 13) and malvidin (peak 15), and also the 3-(6-O-*p*-coumarylglucoside)s of delphinidin (peak 16), cyanidin (peak 17) and peonidin (peak 19). In addition to the known anthocyanins, two other unknown major pigment peaks (peaks 23 and 24) and some minor unidentified anthocyanins (peaks 21 and 22) were observed.

DISCUSSION

The result shows that with a μBondapak C_{18} column separation and analysis of anthocyanin pigments can be achieved by using acetic acid solution for the non-acylated glucosides and an acetic acid solution containing methanol for the acylated glucosides. The reproducibility of the chromatographic data largely depends upon the pH of the eluting solvent system, the operating pressure and the temperature at which the chromatography is performed. Thus chromatograms obtained for the same sample, containing non-acylated and acylated pigments, run on different days at a slightly different temperature and a very slight variation in the pH of two eluting solvent systems, resulted in minor differences in the retention times. Therefore, a reference mixture should be analyzed first or an appropriate internal standard must be incorporated in the sample.

The results also show that the polarity of the compounds play a vital role in the determination of the retention times. On this basis the non-acylated diglucosides, being the most polar, are the least retained by the column, followed by the non-acylated monoglucosides, acylated diglucosides and finally the acylated monoglucosides. Within the individual groups the substitution in the B-ring is a key factor in the determination of the retention times. With increasing hydroxylation in the B-ring the retention time of the compounds decrease. Thus delphinidin with three hydroxy groups in the B-ring is less retained than cyanidin with its two hydroxy groups. Methylation of the hydroxy groups in the B-ring increases the retention time of the anthocyanins. Among the methylated anthocyanins, derivatives of petunidin with two hydroxy groups and one methoxy group are retained for a shorter time than

derivatives of peonidin which has one hydroxy and one methoxy group. Malvidin derivatives in all groups of the investigated anthocyanins showed the longest retention times.

Since anthocyanins can be selectively detected in the region of 520 nm, where few other compounds absorb, this method does not require a preliminary treatment of samples. The samples (aqueous plant extracts) can be directly injected into the column, and their pigment constituents so determined. During preliminary investigation with anthocyanidin-3-bioside-5-glucosides and their acylated derivatives we found that these compounds gave an unsatisfactory separation in the solvent systems described above. Column and solvent conditions for these latter compounds apparently will have to be modified.

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