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High-performance liquid chromatography of red fruit anthocyanins

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ABSTRACT

Anthocyanins of the main red fruits (bilberry, blackcurrant, strawberry, blackberry, black cherry, morello, raspberry and elderberry) were analysed by high-performance liquid chromatography (HPLC). Some identifications could only be achieved after semi-preparative HPLC, partial hydrolysis and analysis of the fragments obtained. The various parameters affecting the retention were studied and, in particular, the respective influences of the constituent aglycone and sugars could be distinguished and quantified. The effect of the mobile phase composition was also studied and allowed the peak inversions observed to be explained and some of the coelutions to be solved.

INTRODUCTION

Anthocyanins are an important family of flavonoid compounds and have been thoroughly studied. Anthocyanin pigments of many fruits have been separated by paper chromatography [1–7], thin-layer chromatography (TLC) [8–10] and, more recently, high-performance liquid chromatography (HPLC) [11–29].

From the published data, very different chromatographic profiles are obtained when dealing with raspberries, strawberries and blackcurrants as starting material. This diversity is of interest when examining the problems of the purity of food products prepared from red fruits (fruit juices, sorbets, fruit wines, liquors, jams, etc.). An anthocyanin chromatographic profile could help to determine adulteration of these products with fruits other than those with which they are supposed to be prepared.

A serious drawback in the analysis of anthocyanins is their instability towards temperature, oxidizing agents and light [30]. Nevertheless, it is known from long experience that noticeable amounts of anthocyanins still remain in jams or heated food products. Sample pretreatment and clean-up are of major importance to keep the anthocyanins free from degradation.

In this study, our interest was focused only on non-acetylated monomeric anthocyanins, which constitute the main part of the red pigments in most fruits (structures are shown in Fig. 1). From the literature, reversed-phase (RP) HPLC looks promising for the separation of the different anthocyanins. For this purpose, a C_{18}

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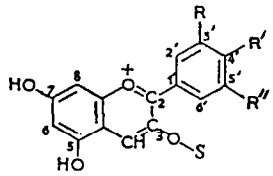
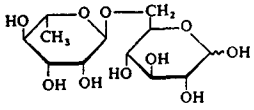
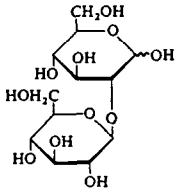


Aglycone moiety	R	R'	R"
Delphinidin	OH	OH	OH
Cyanidin	OH	OH	H
Petunidin	OH	OH	OCH_3
Pelargonidin	H	OH	H
Peonidin	OCH_3	OH	H
Malvidin	OCH_3	OH	OCH_3

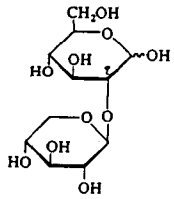
Anthocyanin
(The carbohydrate moiety S is linked to aglycone by its carbon n° 1)



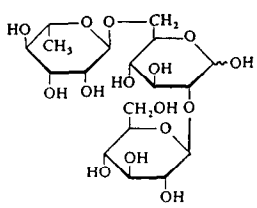
Rutinose
(α-L-Rha (1→6) Glu)



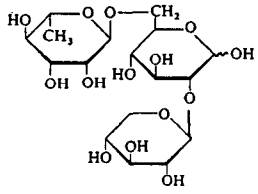
Sophorose
(β-D-Glu (1→2) Glu)



Sambubiose
(β-D-Xyl (1→2) Glu)



2^G Glucosylrutinose



2^G Xylosylrutinose

Fig. 1. Formulae of the anthocyanins and the di- and triosides present in anthocyanins.

column was selected. A large number of different mobile phases have been advocated and comparisons between published data are difficult. Some essential features are well established, as follows.

(i) As usual in RP-HPLC, the retention of the aglycone moiety is correlated with the hydrophobicity of the molecule and the observed elution order is delphinidin < cyanidin < petunidin < pelargonidin < peonidin < malvidin [11,15–17,19,20,22, 26,29,31].

(ii) With an identical anthocyanin aglycone moiety, the order of elution is 3-galactoside < 3-glucoside < 3-rutinoside < 3-arabinoside [16,19,21,31]. However, Van de Casteele *et al.* [15] pointed out that cyanidin 3-arabinoside is eluted between cyanidin 3-galactoside and cyanidin 3-glucoside.

(iii) Addition of a second carbohydrate moiety to a 3-glucoside anthocyanin increases its polarity, resulting in a decrease in retention. The presence of a methyl

group in the rhamnose molecule affects the chromatographic behaviour as cyanidin 3-rutinoside is eluted after cyanidin 3-glucoside [24,29].

The prediction of an absolute order of retention for all the anthocyanins is impossible as some polyglycosylated molecules elute among monoglycosylated compounds. For each of these two groups (mono- or polyglycosylated) the retention order is always the same, but the relative positions of these groups can change. For example, peak cross-over occurs between delphinidin 3-glycoside and peonidin 3,5-diglucoside [11,22,26]. Moreover, it is well known that the elution order may be dramatically affected by the origin of the alkyl-bonded phase or the treatment of silica prior to the alkyl-bonding reaction [32].

Peak identification is not a major problem when dealing with fruits containing monoglycosylated anthocyanins, such as bilberries or strawberries. Conversely, the situation is more difficult with redcurrants, raspberries or elderberries, which contain many polyglycosylated anthocyanins. Sometimes the recommended procedure is to perform hydrolysis and to analyse the fragments obtained.

The purpose of this work was to investigate the different parameters that affect retention in order to predict the elution order of a set of anthocyanins with no overlapping. This will facilitate unambiguous identification and permit the analysis time to be optimized.

EXPERIMENTAL

Selection of chromatographic conditions

We used a C_{18}-bonded silica stationary phase. Mobile phases advocated in the literature are usually water–methanol, water–acetonitrile or water–tetrahydrofuran mixtures together with formic, acetic acid or phosphoric acid to maintain acidity. Detection is usually carried out at 546 nm in the visible range and it is well established that the response factor is enhanced with an acidic mobile phase. Ribereau-Gayon [33] demonstrated that a change from pH 2.9 to 1 yields a sixfold increase in colour intensity. Unfortunately, pH 1 is not suitable with a C_{18}-bonded silica column and we selected pH 1.9, which corresponds to 10% formic acid in the mobile phase.

Owing to the wide polarity scale of anthocyanins, gradient elution has usually been performed in most studies. For the sake of unambiguous retention characteristics, the isocratic mode was selected. From the data, gradient elution was carried out to enhance the separation and to accelerate the elution of the strongly retained solutes.

Sample preparation

Only fruit juices were considered. Prior to injection, centrifugation for 10 min at 3500 g and filtration through a 0.5-μm filter were performed. The selected detection wavelength (546 nm) is specific to anthocyanins and no compounds from other fruits can interfere. Therefore, there is no need for further sample treatment.

Chromatography

HPLC was performed on a Model SP 8000 instrument (Spectra-Physics, San Jose, CA, U.S.A.) equipped with a Valco (Houston, TX, U.S.A.) UHP valve with a 10-μl sample loop for analytical injections and a 250-μl loop for semi-preparative purposes. The analytical column was RP-18 LiChrospher (Merck, Darmstadt,

F.R.G.) (250 × 10 mm I.D.) packed with 7-μm particles. A Model SP 8310 fixed-wavelength detector (546 nm) from Spectra-Physics was used.

The solvents were of HPLC grade, filtered through a 0.45-μm filter and sparged with helium. In the analytical chromatography of anthocyanins two mobile phases were generally used: (a) water–acetonitrile–formic acid (84:6:10, v/v/v) (I) and (b) water–acetonitrile–formic acid (81:9:10, v/v/v) (II). Where specified, a less polar mobile phase (80:10:10. v/v/v) (III) or a more polar (3–6% acetonitrile gradient in 20 min) were used. Solvent I was used for preparative chromatography. In the chromatography of anthocyanidins water–acetonitrile–formic acid (75:15:10, v/v/v) was selected as the eluent.

The flow-rate was 1 ml/min in analytical chromatography and 6 ml/min in semi-preparative chromatography. Chromatography was performed at ambient temperature.

Fragmentation by acid hydrolysis. The anthocyanins isolated by semi-preparative chromatography were collected in distilling flasks and evaporated to dryness under low pressure. To prevent any degradation of the anthocyanins, the temperature must be kept below 30°C. Hydrolysis was performed either in a water-bath in test-tubes or in a drying oven directly on silica plates according to the method selected for the fractionation (HPLC or TLC).

In the water-bath hydrolysis, 1–2 ml of 2 *M* hydrochloric acid were added to the dry residue and heating at 100°C was performed for 45 min for complete hydrolysis or for 1, 2, 5, 10 or 20 min for partial hydrolysis. Products of the reaction were evaporated to dryness and the residue was dissolved in the minimum volume of slightly acidic water (0.1% hydrochloric acid) prior to injection.

Prior to TLC, hydrolysis was performed directly on the plate with subsequent fractionation according to the procedure of Andary *et al.* [34]. The dry residue of the collected anthocyanin was dissolved in methanol and spotted on the TLC plate under a cold air stream. A 2-μl volume of 3 *M* hydrochloric acid was added to the spot, which was then covered with a glass plate. Heating at 100°C for 5–30 min was then performed.

Thin-layer chromatography. Precoated silica gel TLC plates (20 × 20 cm) (Merck) were used for the identification of the sugars of polyglycosylated anthocyanins following hydrolysis. The mobile phase was acetone–butanol–phosphate buffer [0.05 *M* Na_2HPO_4–0.05 *M* H_3PO_4 (pH 5)] (50:40:10 v/v/v).

Detection was performed by staining with 2,3,5-triphenyltetrazolium chloride [a 4% solution in methanol–1 *M* sodium hydroxide solution (50:50, v/v)].

Standards. Glucose, galactose, arabinose, xylose and L-rhamnose were purchased from Merck. Cyanidin 3-glucoside, cyanidin 3-rhamnoside, cyanidin 3-rutinoside, cyanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, paeonidin 3,5-diglucoside, cyanidin, pelargonidin, paeonidin, petunidin, malvidin were purchased from Extrasynthèse (Gemay, France).

RESULTS AND DISCUSSION

Identification of anthocyanins

Bilberry, blackcurrant, strawberry, blackberry, black cherry and morello cherry

These red fruits mainly contain monoglycosylated anthocyanins, with the exception of delphinidin and cyanidin 3-rutinoside. These anthocyanins are more retained than those which are polyglycosylated and they can be rapidly separated with water–acetonitrile–formic acid (81:9:10) as the mobile phase. Fig. 2 shows the chromatograms obtained. Identification of the separated solutes was carried out by comparison with the retention of standard solutes; the observed elution order is consistent with the hydrophobicity order as described in previously published separations [11,16,17,19–22,26,29,31]. Table I lists the identified anthocyanins together with the selectivity α towards cyanidin 3-glucoside, which was selected as a reference solute, and $\log \alpha$.

It must be pointed out that replacement of one sugar by another results in the same variation of $\log \alpha$ value, whatever anthocyanin is considered. For example, a change from galactose to glucose yields the same shift in $\log \alpha$ whatever Pg, Cy or Pt are considered. In Fig. 3 $\log \alpha$ values for anthocyanins are plotted against the anthocyanidins arranged in order of increasing hydrophobicity. With this representation, the galactosides line is parallel to the glucosides line, shifted by $\Delta \log \alpha = 0.11$. This behaviour is valid for the four lines drawn and provides a valuable means of accurately predicting an individual anthocyanin retention time. Moreover, retention times of all the anthocyanins having the same glycoside can be found from the retention of one of them and from a known plot corresponding to another glycoside group. This mode of identification may only be approximate but it provides useful guidelines for further studies. Use of a gradient precludes advantage being taken of this phenomenon.

Redcurrant, raspberry and elderberry

The acetonitrile content of the above-mentioned mobile phase is too high to permit the resolution of the anthocyanins from these fruits. A mixture of lower elution strength [water–acetonitrile–formic acid (84:6:10, v/v/v)] is convenient, as shown in Fig. 4. Peak identification is more difficult as the glycoside part of the anthocyanins from these fruits is constituted by diholosides or triholosides.

Reports on HPLC separations in this area are scarce, with the exception of the work of Bronnum-Hansen and Hansen [18] on elderberries and Spanos and Wrolstad [29] on raspberries. Cyanidin 3-rutinoside is the only standard solute commercially available and we had to perform semi-preparative chromatography with subsequent acid hydrolysis of the isolated fractions. Partial hydrolysis first cleaves the carbohydrate–carbohydrate bond and then the aglycone–carbohydrate bond, while complete hydrolysis yields the aglycone moiety together with the different carbohydrates that constitute the anthocyanins.

Table II lists the fragments obtained by partial hydrolysis of the different heterosides. The number of identified fragments and the hydrolysis kinetics yield information for the further identification of the anthocyanin of interest.

Redcurrant anthocyanins. Eleven peaks are present in the chromatogram of the

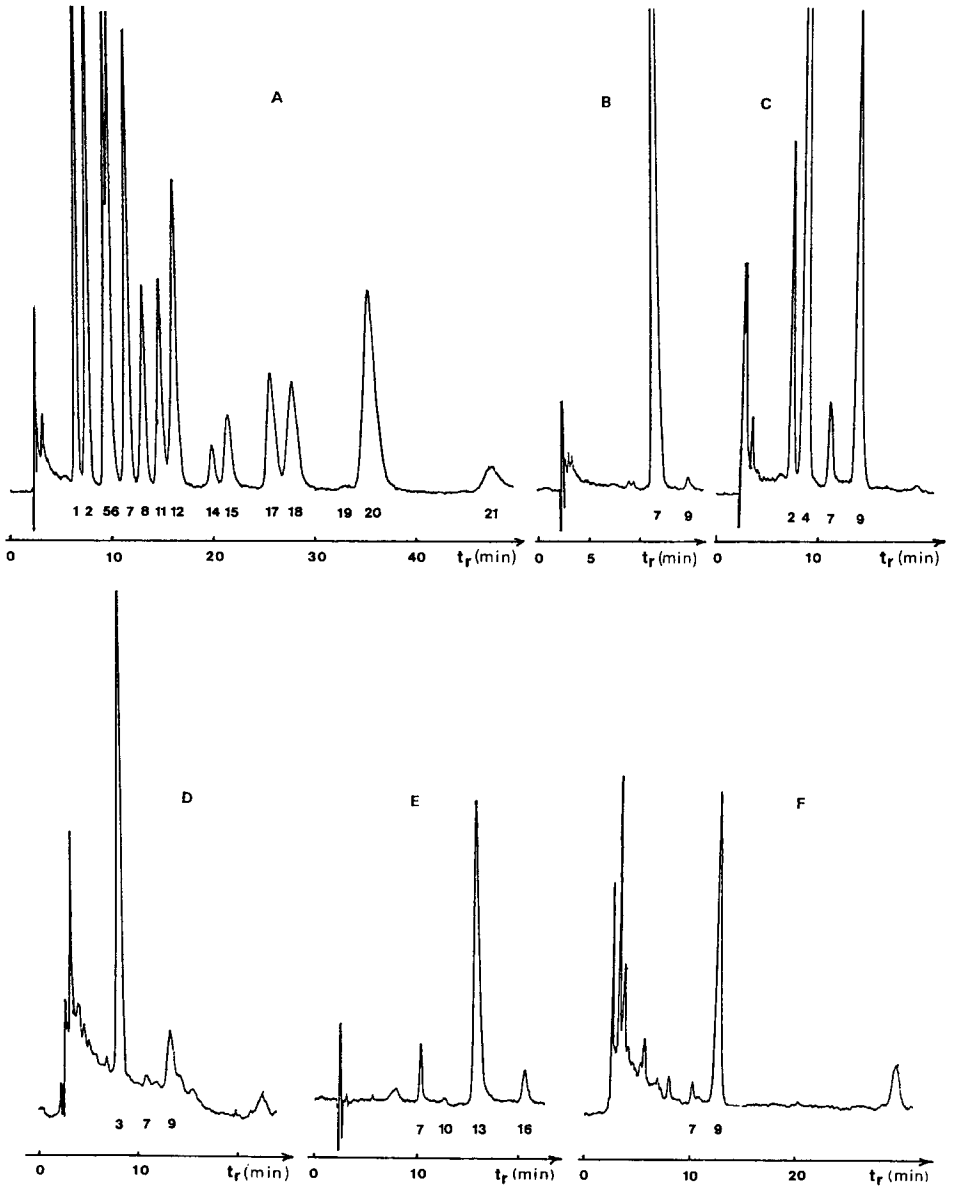


Fig. 2. Chromatograms of fruit juices. (A) Bilberry; (B) blackberries; (C) blackcurrant; (D) morello cherry; (E) strawberry; (F) cherries. Mobile phase: water–acetonitrile–formic acid (81:9:10, v/v/v). See Table I for peak identification.

anthocyanins from this fruit, but we are only interested in the five major peaks 6, 8, 9, 10 and 11. Peaks 9 and 11 were identified as cyanidin 3-glucoside and cyanidin 3-rutinoside, respectively, by comparison with authentic samples. The three others were submitted to preparative chromatography and hydrolysis. Chromatograms

TABLE I

ANTHOCYANINS IDENTIFIED IN FIGS. 2 AND 3

$\alpha = \frac{t'_r}{t'_{r(Cy\ 3\text{-}glu)}}$ with $t'_r = (t_r - t_0)$, where t_r = retention time of a solute and t_0 = retention time of an unretained compound.

Peak No.	Abbreviation[a]	α	Log α
1	Dp 3-gala	0.456	−0.341
2	Dp 3-glu	0.578	−0.238
3	Cy 3-glu-ruti	0.676	−0.170
4	Dp 3-ruti	0.706	−0.151
5	Cy 3-gala	0.772	−0.112
6	Dp 3-ara	0.814	−0.089
7	Cy 3-glu	1	0
8	Pt 3-gala	1.17	0.070
9	Cy 3-ruti	1.29	0.110
10	Pg 3-gala	1.29	0.110
11	Cy 3-ara	1.35	0.130
12	Pt 3-glu	1.51	0.179
13	Pg 3-glu	1.66	0.219
14	Pn 3-gala	1.92	0.283
15	Pt 3-ara	2.09	0.320
16	Pg 3-ara	2.27	0.355
17	Pn 3-glu	2.56	0.408
18	Mv 3-gala	2.79	0.445
19	Pn 3-ara	3.36	0.527
20	Mv 3-glu	3.61	0.557
21	Mv 3-ara	4.93	0.693

[a] Dp = Delphinidin, Cy = cyanidin, Pt = petunidin, Pg = pelargonidin, Pn = peonidin, Mv = malvidin, glu = glucose, gala = galactose, ara = arabinose, ruti = rutinose.

obtained with the anthocyanin corresponding to peak 8 are displayed in Fig. 5. To shorten the chromatographic run time, gradient elution was performed from the 25th to the 33rd minute, the acetonitrile content being increased from 9% to 25%. The anthocyanin retention times are unchanged as they are eluted before the gradient delay time.

At the very beginning of the hydrolysis, cyanidin and three intermediate anthocyanins are formed, which could be easily identified as (in order of elution) cyanidin 3-sophoroside, cyanidin 3-glucoside and cyanidin 3-rutinoside. According to Table II, the structure of the anthocyanin which yields three intermediates by hydrolysis and the aglycone moiety of which is cyanidin, corresponds to

$$\begin{array}{l} \quad S_2 \\ \quad | \\ \text{Cy 3-}S_1 \\ \quad | \\ \quad S_3 \end{array}$$

Therefore the fragment Cy 3-S_1 is obviously cyanidin 3-glucoside, Cy 3-S_1–S_2 is

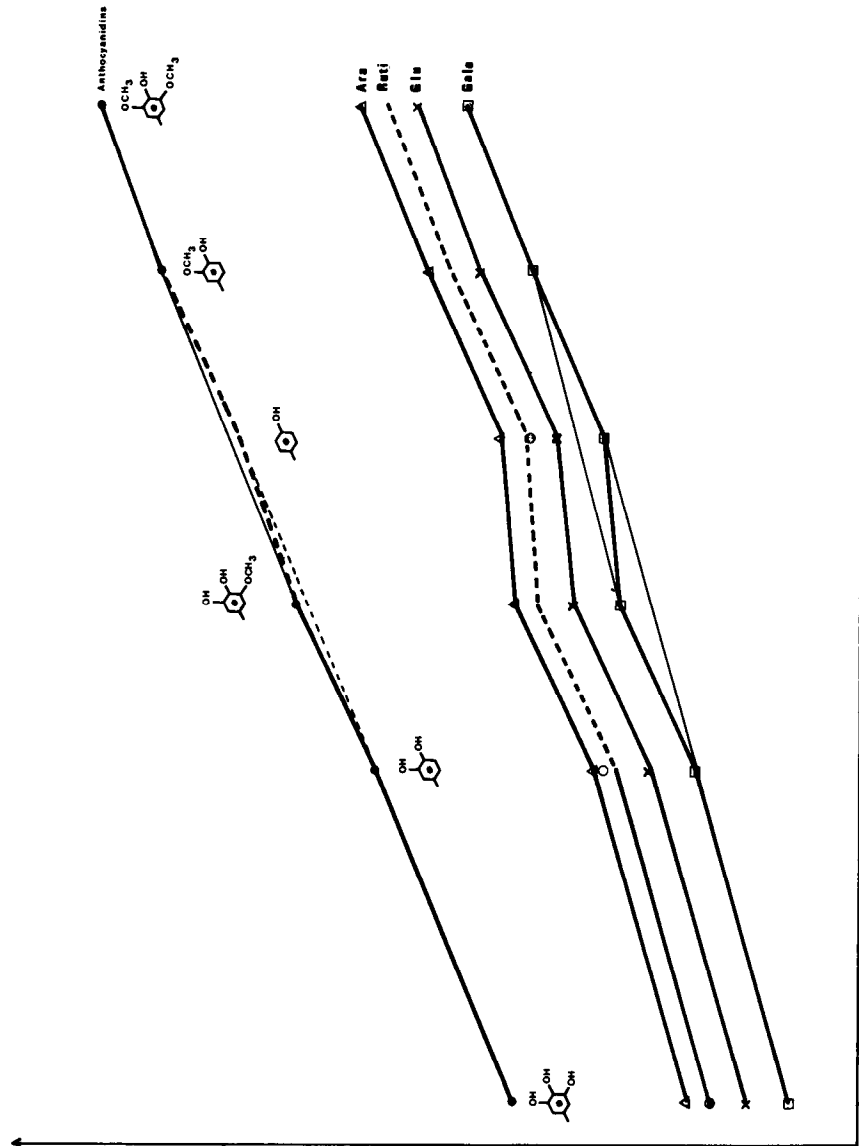


Fig. 3. Log α of anthocyanins obtained by associating each glycoside with each anthocyanidin encountered. Abscissa: 2 units correspond to an OH loss and 1 unit to an OCH_3 gain. Mobile phase: water–acetonitrile–formic acid (81:9:10, v/v/v). Dp = delphinidin (R = R′ = R″ = OH); Cy = cyanidin (R = R′ = OH, R″ = H); Pt = petunidin (R = R′ = OH, R″ = OCH_3); Pg = pelargonidin (R = R″ = H, R′ = OH); Pn = peonidin (R = OCH_3, R′ = OH, R″ = H); Mv = malvidin (R = R″ = OCH_3, R′ = OH).

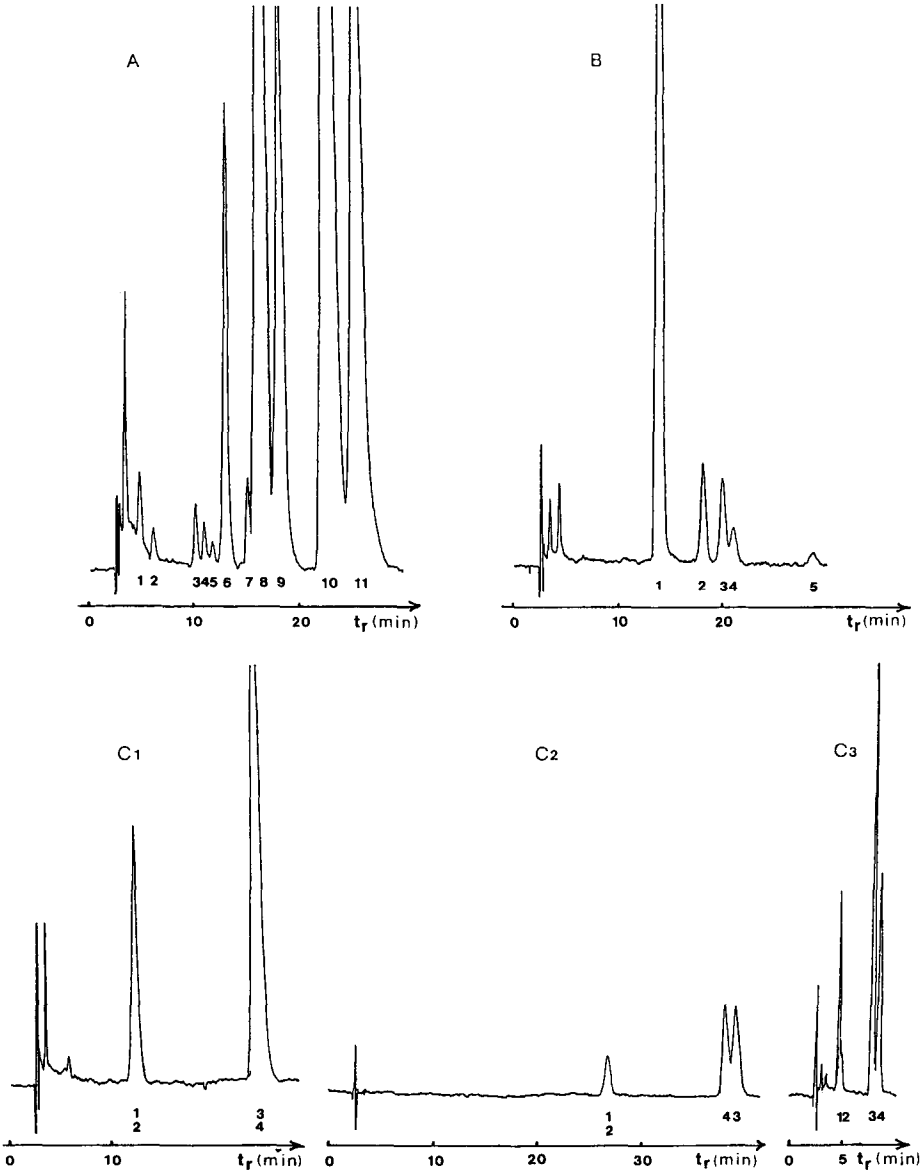


Fig. 4. Chromatograms of fruit juices. (A) Redcurrant; (B) raspberry. Mobile phase: water–acetonitrile–formic acid (84:6:10, v/v/v). (C) Elderberry: (C_1) elution with the mobile phase used for A and B; (C_2) elution with a linear gradient from 3 to 6% acetonitrile in acidic medium (10% formic acid) in 20 min and 10 min isocratic with 6% acetonitrile; (C_3) isocratic elution with 10% acetonitrile in acidic medium (10% formic acid). See Table III for peak identification.

TABLE II

FRAGMENTS OBTAINED BY PARTIAL ACID HYDROLYSES OF THE DIFFERENT HETEROSIDES

Heteroside	Anthocyanidic fragments obtained	No. of intermediates
3-Monoside Ag 3-S[a]	Ag	0
3-Bioside Ag 3-S_1S_2	Ag 3-S_1 + Ag	1
3,5-Bioside Ag 3-S_1, 5-S_2	Ag 3-S_1 + Ag 5-S_2 + Ag	2
3-Trioside Ag 3-$S_1S_2S_3$	Ag 3-S_1S_2 + Ag 3-S_1 + Ag	2
3-Trioside Ag 3-S_1 (S_2, S_3)	Ag 3-S_1S_2 + Ag 3-S_1S_3 + Ag 3-S_1 + Ag	3
3-Bioside, 5-monoside: Ag 3-S_1S_2, 5-S_3	Ag 3-S_1, 5-S_3 + Ag 3-S_1S_2 + Ag 5-S_3 + Ag 3-S_1 + Ag	4

[a] Ag = aglycone; S = sugar.

cyanidin 3-sophoroside and Cy 3-S_1–S_3 is cyanidin 3-rutinoside. Peak 8 in the redcurrant chromatogram is

glu
|
Cy 3-glu
|
rha

The amount of Cy 3-sophoroside produced is higher than that of Cy 3-rutinoside, which indicates that the 1–6 glu–rha bond is easier to cleave than the 1–2 glu–glu bond.

Further, the small amount of Cy 3-rutinoside produced decreases much faster than the amount of sophoroside and the corresponding peaks on the chromatograms remain small. TLC of the hydrolysis products reveals the presence of glucose and rhamnose and makes the identification complete.

TLC was utilized for the identification of redcurrant peak 10 and elderberry peak 1. Redcurrant peak 10 yields two intermediates, cyanidin 3-glucoside and cyanidin 3-rutinoside. Conversely, from TLC data three carbohydrates were identified: glucose, xylose and rhamnose. These results come from the fact that cyanidin 3-glucoside and cyanidin 3-sambubioside are eluted simultaneously. With a mobile phase of lower elution strength (3–6% acetonitrile gradient within 20 min) these two solutes were separated and the peak 10 was identified as:

xyl
|
Cy 3-glu
|
rha

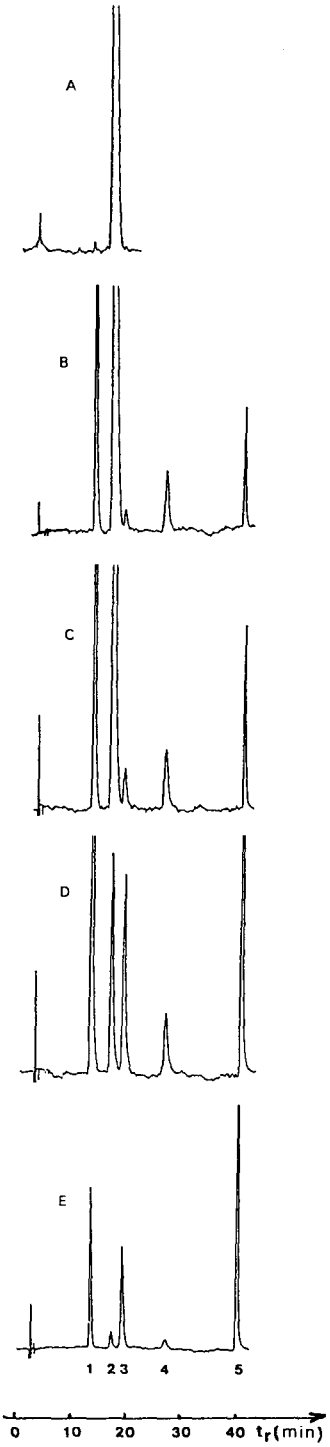


Fig. 5. Results of partial hydrolysis of anthocyanin corresponding to peak 8 of redcurrant. (A) Initial product; (B) hydrolysis time 1 min; (C) 2 min; (D) 5 min; (E) 10 min. Mobile phase: formic acid content constant (10%); from 0 to 25 min, water–acetonitrile–formic acid (84:6:10); from 25 to 35 min, linear gradient from 6 to 25% acetonitrile; from 35 to 45 min, water–acetonitrile–formic acid (65:25:10). Peak identification: (2) Cy 3-glu(glu)(rha) → (1) Cy 3-glu–glu + (4) Cy 3-glu–rha → (3) Cy 3-glu → (5) cyanidine.

The above mobile phase was used when xylose was evidenced by TLC.

All anthocyanins identified from redcurrant, raspberry and elderberry are listed in Table III.

Raspberry and elderberry anthocyanins. The same approach as above allowed the identification of cyanidin 3-sophoroside from raspberry. This result is consistent with the report of Spanos and Wrolstad [29]. Peak 4 could not be separated in a sufficient amount by preparative chromatography to allow its identification. Nevertheless, considering Spanos and Wrolstad's studies, it was assumed to be pelargonidin 3-sophoroside. This assumption agrees with retention diagrams, which are discussed below.

TABLE III

ANTHOCYANINS OF REDCURRANT, RASPBERRY AND ELDERBERRY (*cf.*, FIG. 4) WITH IDENTIFICATION OF THE FRAGMENTS OBTAINED BY ACID HYDROLYSIS WHEN NECESSARY

Fruit	Peak No.	Complete hydrolysis		Partial hydrolysis, intermediate anthocyanins[a]	Anthocyanin[a]
		Sugar (TLC)	Anthocyanidin (HPLC)		
Redcurrant	6	Glu + trace of rha	Cy	Cy 3-glu + unidentified intermediate anthocyanin	Cy 3-glu-glu (Cy 3-sopho) + unidentified minor anthocyanin
	8	Glu + rha	Cy	Cy 3-glu-glu Cy 3-ruti Cy 3-glu	glu | Cy 3-glu | rha (Cy 3–2^G glu-ruti)
	9	–	–	–	Cy 3-glu xyl
	10	Glu + rha + xyl	Cy	Cy 3-ruti Cy 3-sam Cy 3-glu	| Cy 3-glu | rha (Cy 3–2^G xyl-ruti)
	11	–	–	–	Cy 3-ruti
Raspberry	1	Glu	Cy	Cy 3-glu	Cy 3-glu-glu (Cy 3-sopho)
	2	–	–	–	Cy 3–2^G glu-ruti
	3	–	–	–	Cy 3-glu
	4	–	–	–	Pg 3-sopho
	5	–	–	–	Cy 3-ruti
Elderberry	1	Glu + xyl	Cy	Cy 3,5-diglu Cy 3-glu Cy 3-sam	Cy 3-sam, 5-glu
	2	–	–	–	Cy 3,5-diglu
	3	Glu + xyl	Cy	Cy 3-glu	Cy 3-sam
	4	–	–	–	Cy 3-glu

[a] Rha = rhamnose, xyl = xylose, sopho = sophorose, sam = sambubiose.

In contrast to Spanos and Wrolstad, we could not detect any pelargonidin 3-glucoside rutinoside and pelargonidin 3-glucoside. This may be due to the raspberry variety.

Hydrolysis and subsequent TLC of elderberry anthocyanins indicated the presence of xylose. In spite of the use of an acetonitrile gradient (3–6% acetonitrile within 20 min), we could not separate Cy 3-sam 5-glu from Cy 3,5-diglu. This separation was performed by Bronnum-Hansen and Hansen [18] with a tetrahydrofuran (THF) gradient in 0.05 *M* phosphoric acid (pH 1.8) from 1 to 40% THF within 15 min. From the solubility parameter difference between THF and acetonitrile, it seems difficult to achieve such a good separation with acetonitrile.

Retention behaviour

Table IV gives the observed retentions of the identified anthocyanins and anthocyanidins as the selectivity α towards Cy 3-glu, which was selected as a reference compound. Many solutes coelute and water–acetonitrile–formic acid (84:6:10) does not permit complete resolution.

Contribution of glycosides to anthocyanin retention

A graph similar to that in Fig. 3 was constructed from the results obtained with mobile phase I (6% acetonitrile). The lines connecting the different aglycones and those connecting the anthocyanins exhibiting the same glucoside moiety are parallel (*cf.*, Fig. 6). The slopes of the anthocyanidins lines are slightly steeper than those corresponding to the anthocyanins.

The observed data can be interpreted with the aid of the general treatment of fragmental constants derived by Rekker and Kort [35]. Retention characteristics in RP-HPLC depend on the hydrophobicity and can be determined by considering each part of the molecule of a given solute and taking in account its hydrophobic properties, f_i. The hydrophobicity of a molecule is the sum of the different hydrophobic fragment constants and the intramolecular effects f'_j. Following Rekker and Kort's theory, there should be a linear relationship between logα and the hydrophobicity of the solute.

Let P be the partition coefficient of a solute between organic and aqueous phases and k' the capacity factor:

$$\log P = \sum_i f_i + \sum_j f'_j \tag{1}$$

$$\log k' = A \log P + B \tag{2}$$

where A and B are constants for a given chromatographic system.

$$\alpha = \frac{k'}{k'_{\text{ref}}}$$

where k'_{ref} is the capacity factor of the reference solute (cyanidin 3-glucoside).

$$\log \alpha = \log k' - \log k'_{\text{ref}} \tag{3}$$

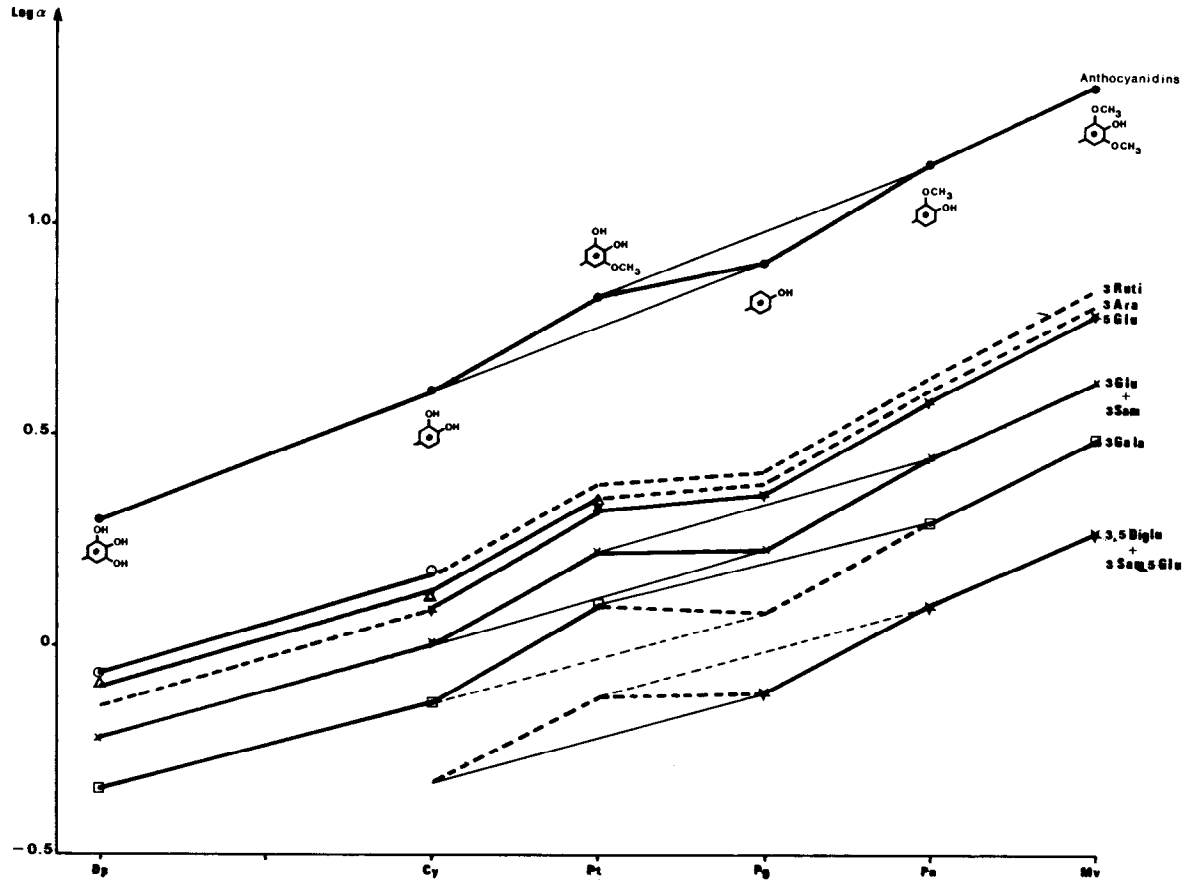


Fig. 6. Log α of anthocyanins obtained by associating each glycoside with each anthocyanidin encountered. Abscissa, 2 units corresponds to an OH loss and 1 unit to an OCH_3 gain. Mobile phase: water–acetonitrile–formic acid (84:6:10).

TABLE IV

RETENTION BEHAVIOUR OF THE IDENTIFIED ANTHOCYANINS

Mobile phase:water–acetonitrile–formic acid (84:6:10).

Anthocyanin	a[a]	b[b]	c[c]	$\alpha = \frac{t'_r}{t'_{r(Cy\ 3\text{-}glu)}}$	Log α
Dp 3-gala		×		0.45	−0.35
Cy 3,5-diglu	×			0.47	−0.33
Cy 3-sam, 5-glu			×	0.47	−0.33
Dp 3-glu		×		0.59	−0.23
Cy 3-sopho			×	0.66	−0.18
Cy 3-gala	×			0.73	−0.14
Pg 3,5-diglu		×		0.75	−0.12
Dp 3-ara		×		0.80	−0.10
Dp 3-ruti		×		0.84	−0.07
Cy 3-(2^G glu-ruti)			×	0.91	−0.04
Cy 3-glu	×			1	0
Cy 3-sam			×	1.00	0.00
Pg 3-sopho		×		1.05	0.02
Cy 5-glu	×			1.21	0.08
Pn 3,5-diglu	×			1.22	0.09
Pt 3-gala		×		1.22	0.09
Cy 3-ara			×	1.30	0.11
Cy 3-(2^G xyl-ruti)		×		1.35	0.13
Cy 3-ruti	×			1.52	0.18
Pt 3-glu		×		1.67	0.22
Pg 3-glu	×			1.66	0.22
Mv 3,5-diglu	×			1.80	0.26
Delphinidine			×	1.95	0.29
Pn 3-gala		×		1.96	0.29
Pt 3-ara		×		2.17	0.34
Pg 5-glu	×			2.30	0.36
Pn 3-glu	×			2.67	0.43
Cy 3-L-rha	×			2.68	0.43
Mv 3-gala		×		3.06	0.49
Pn 5-glu	×			3.80	0.58
Cyanidine	×			3.98	0.60
Mv 3-glu	×			4.17	0.62
Mv 5-glu	×			6.00	0.78
Petunidine	×			6.6	0.82
Pelargonidine	×			8.0	0.90
Paeonidine	×			13.4	1,13
Malvidine	×			20.4	1.31

[a] Standards and their products from partial hydrolysis.

[b] Identified through a comparison with literature.

[c] Isolated through semi-preparative HPLC and identified through a thermal fragmentation followed by analytical chromatography of the products obtained.

From eqns. 1–3, we can write

$$\log \alpha = A\left(\sum_i f_i + \sum_j f'_j\right) - A\log P_{ref} \tag{4}$$

where $A\log P_{ref}$ is constant under fixed analysis conditions.

To evaluate the hydrophobicity of a single anthocyanin, we can consider the two fragments: aglycone (Ag) and glycoside (S):

$$\log \alpha = A(f_{Ag} + f_S + f'_{Ag-S}) - A\log P_{ref} \tag{5}$$

With the aglycone,

$$\log \alpha_{Ag} = A(f_{Ag} - \log P_{ref}) \tag{6}$$

and we can write

$$\log \alpha - \log \alpha_{Ag} = A(f_S + f'_{Ag-S}) \tag{7}$$

This difference is represented in Fig. 6 by the distance between the Ag–S anthocyanin and the related aglycone Ag. It has been observed experimentally that it is constant for all the aglycones, as evidenced by the constant shift between the lines. From eqn. 7 it is obvious that the intramolecular effect f'_{Ag-S} is not related to the aglycone species. This can be explained as follows: the different anthocyanidins exhibit distinctive substituents (H, OH, OCH_3) which are far from the Ag–S bond and as a consequence it may be guessed that the aglycone–sugar interaction is not influenced by these groups. In this mode the effect is identical whatever the aglycone considered. In consequence, $f_S + f'_{Ag-S}$ is characteristic of the sugar.

We can write

$$\Delta S = A(f_S + f'_{Ag-S}) \tag{8}$$

and we can consider it as the sugar contribution to the anthocyanin retention:

$$\log \alpha = \log \alpha_{Ag} + \Delta S \tag{9}$$

The determination of ΔS from available anthocyanins allows the prediction of the retention characteristics of unknown or unavailable anthocyanins.

When many sugars S_1, S_2, S_3, etc., are involved in the glucoside structure, then $\Delta S = \Delta S_1 + \Delta S_2 + \Delta S_3 \ldots$, where ΔS_1 is the contribution of the first sugar bonded to an anthocyanidin (it is a glucose molecule in every case), ΔS_2 that of the second sugar fixed either on S_1 or in the 5-position on the anthocyanidin and ΔS_3 that of the third sugar also bonded to S_1 or in the 5-position, etc.

In Fig. 6, ΔS_1 is represented by the distance between the points corresponding to the aglycone Ag and to the anthocyanin Ag S_1, ΔS_2 by the distance between Ag glu and Ag glu S_2 and ΔS_3 by the distance between Ag glu S_2 and Ag glu S_2 S_3. As already mentioned, the aglycone line has a slightly steeper slope than the anthocyanin line and ΔS_1 is different from delphinidin to malvidin.

Table V gives the values of ΔS_1, ΔS_2 and ΔS_3 obtained from the chromatographic behaviour of cyanidin. Fig. 7 displays the ΔS values of all the glycosides encountered. Parallelism of the segments corresponding to the bonding of a third identical sugar on different anthocyanins (*e.g.*, the segments sam–xyl ruti and

TABLE V

CONTRIBUTION OF SUGARS TO ANTHOCYANIN RETENTION

Sugar	ΔS_1	ΔS_2	ΔS_3
3-Glucose	−0.60	−0.18	−0.23
3-Xylose		0	−0.05
3-L-Rhamnose	−0.17	0.18	0.12
5-Glucose	−0.52	−0.34	−0.34
3-Galactose	−0.74		
3-Arabinose	−0.49		

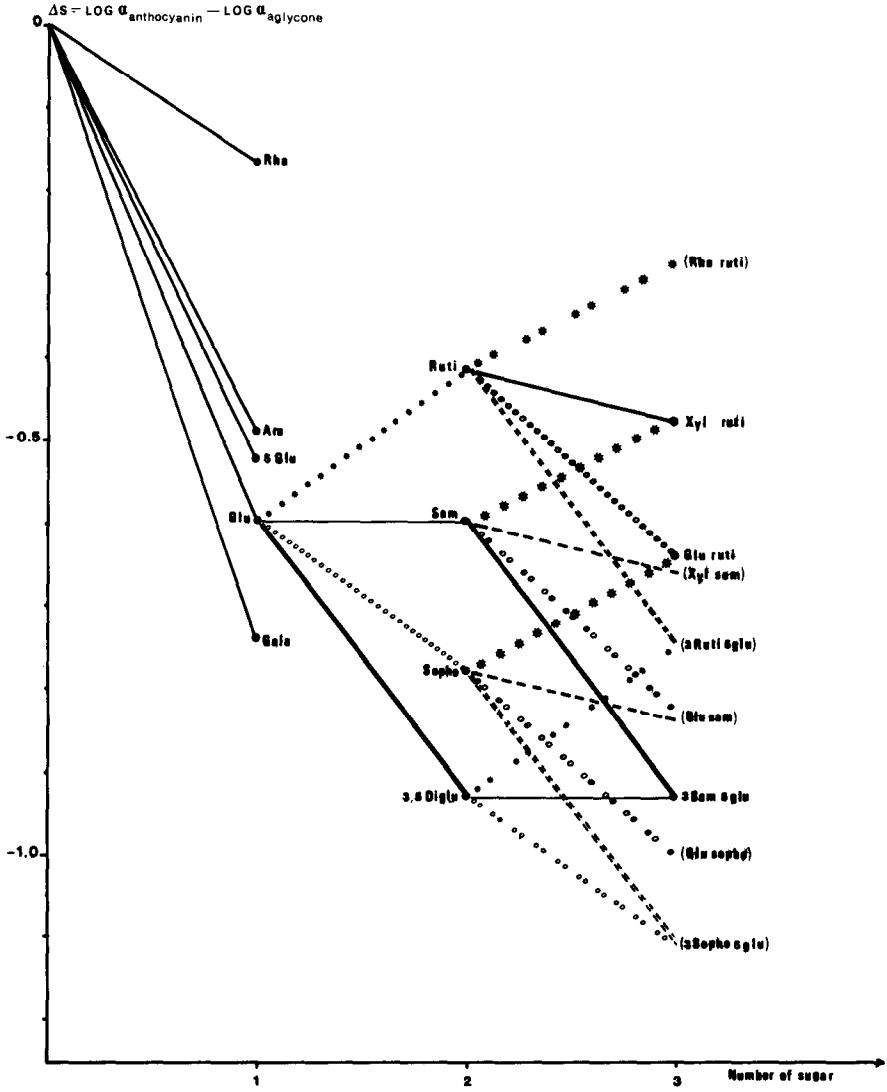


Fig. 7. Sugar contribution to the anthocyanin retention. Mobile phase: water–acetonitrile–formic acid (84:6:10). The glycosides in parentheses do not correspond to experimental points but were deduced from the other points by the dotted segments.

sopho–glu ruti). From the plots, the prediction of the retention characteristics of other triholosides not previously encountered in the experiments (glu–sopho, glu–sam, 3-ruti–5-glu, etc.) is straightforward.

From Table V and Fig. 7, the following comments can be made:

(a) With every sugar ΔS_1 is always smaller than ΔS_2, and the addition of a sugar to a monoglycosylated anthocyanin has much less effect on retention than adding a sugar to an aglycone. For rhamnose the effect is even the reverse. This phenomenon can easily be explained (see eqn. 8):

$$\Delta S_1 = A(f_{S_1} + f'_{Ag-S_1})$$

The presence of the glucoside S_1 decreases the hydrophobicity of the Ag–S_1 molecule (negative value of f_S) with the consequence of a decrease in retention. On the other hand, hydrophobicity is increased by the interactions between polar groups (positive value of f'_{Ag-S}) that increase retention and act in the reverse direction to the glucoside moiety. However, $|f_{S_1}| > |f'_{Ag-S_1}|$ and ΔS_1 is always negative (decrease in retention).

For the same sugar added to the first one:

$$\Delta S_2 = A(f_{S_2} + f'_{AgS_1-S_2}) \quad (f_{S_1} = f_{S_2})$$

The interaction AgS_1–S_2 is logically stronger than the Ag–S_1 interaction, $f'_{AgS_1-S_2} > f'_{Ag-S_1}$ and the effect of S_2 on retention is less important than that of S_1.

With rhamnose, $|f'_{Agglu-rha}| > |f_{rha}|$ and the ΔS_2 value is positive, *i.e.*, rutinosides are eluted after glucosides.

(b) On the other hand, the third sugar molecule S_3 has a slightly greater effect than the second sugar S_2 in the case of the ramified structure:

$$\begin{array}{l} \quad S_2 \\ \quad | \\ \text{Ag}\,3\text{-}S_1 \\ \quad | \\ \quad S_3 \end{array}$$

Interactions between two sugars look stronger in a diglycoside than in a triglycoside molecule.

(c) The addition of a glucose in the 5-position to an aglycone leads to a decrease in $\log \alpha$ that is greater than that given by the same addition to anthocyanin glycosylated in the 3-position (compare the values in the fourth line in Table V). Therefore, an interaction would exist between a sugar in the 3-position and glucose in the 5-position.

(d) A modification of the mobile phase such as an increase in the acetonitrile content leads to a decrease in ΔS that is greater for diosides and triosides than for monosides (cf., Figs. 7 and 8) and the elution order is changed: with 9% acetonitrile, rutinosides are eluted after arabinosides whereas with 6% acetonitrile they are eluted first.

The results are in agreement with Bitteur's work [36] on the coefficient a in the relationship $\log k' = a\varphi^n + b$, where φ represents the volume percentage of water in

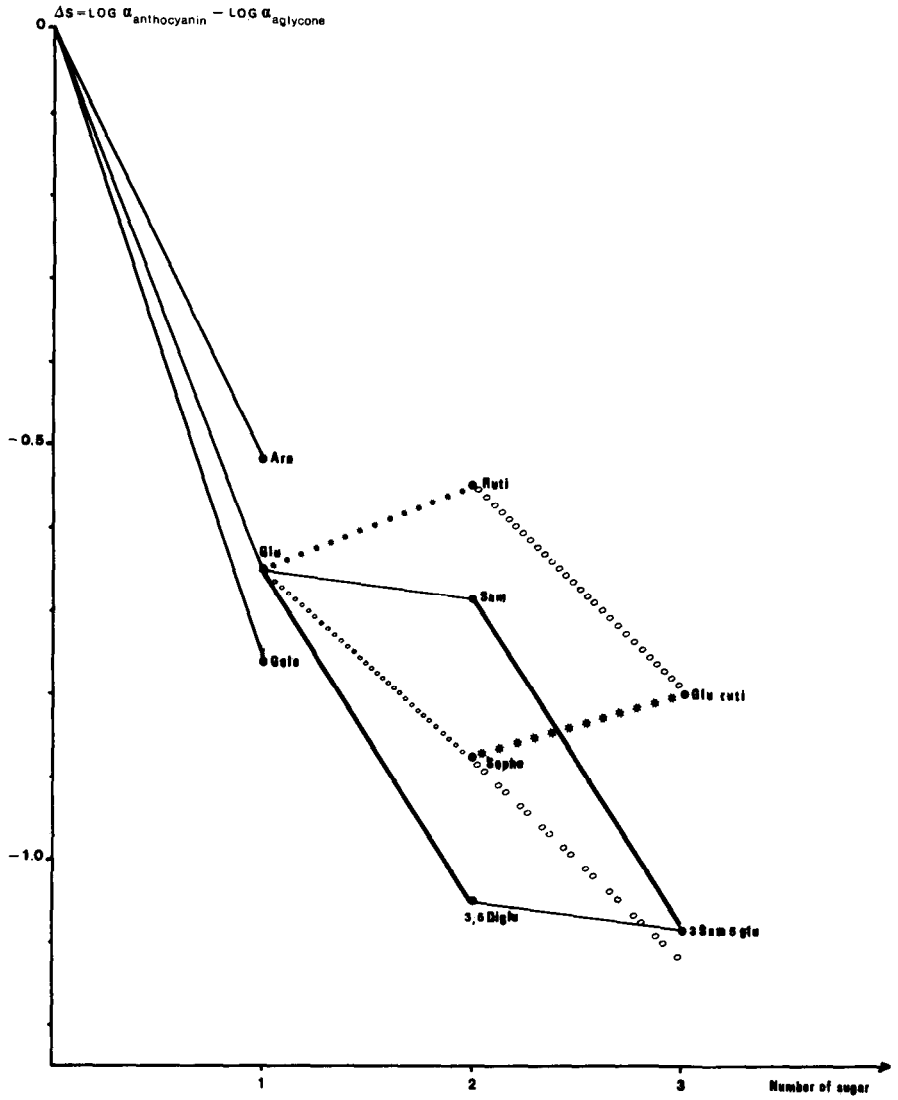


Fig. 8. Sugar contribution to anthocyanin retention. Mobile phase: water–acetonitrile–formic acid (81:9:10).

the mobile phase. She reported a linear variation of *a* with the molar volume of solutes. In other words the retention variations due to an increase in the acetonitrile content are higher for large than for small molecules. This allowed us to separate cyanidin 3-glucoside from cyanidin 3-sambubioside by using different acetonitrile contents. When the acetonitrile content is less than 6%, elution is very slow, and we used a slow gradient from 3 to 6% in 20 min, the 6% content then being maintained until the elution was completed. In this instance cyanidin 3-glucoside appears before cyanidin 3-sambubioside (*cf.*, Fig. $4C_2$). Experiments with an acetonitrile content higher than

6% were also performed and good results were obtained with 10% acetonitrile. The elution order is then reversed when compared with the previous results (*cf.*, Fig. $4C_3$).

Contribution of aglycone moiety to anthocyanin retention

In eqn. 9 this contribution is denoted as $\log \alpha_{Ag}$, and the experimental values are given in Table 4. The plot in Fig. 6 shows that the addition of a hydroxyl group to pelargonidin, cyanidin or peonidin leads to the same variation, Δ(OH), of $\log \alpha$. The corresponding segments are colinear or parallel. The same effect occurs with addition of a methoxy group to cyanidin or pelargonidin [$\Delta(OCH_3)$ variation of $\log \alpha$]. However, this does not account well for such an addition to peonidin.

This is also valid for the lines corresponding to anthocyanins and is of obvious practical interest. The possibilities of deducing the retention values of one anthocyanin from another are thus increased. With 3-glucosides, the values obtained are $\Delta(OH) = -0.23$ and $\Delta(OCH_3) = 0.22$.

With the same anthocyanins which were chromatographed with the 9% acetonitrile mobile phase (see Fig. 3), the Δ(OH) value is nearly the same (-0.24) whereas $\Delta(OCH_3)$ is slightly lower (0.18). This result is in agreement with Bitteur's data: an increasing acetonitrile concentration has more effect upon the largest mole which contains OCH_3 and then $\Delta(OCH_3)$ is lowered. As OH is small, this behaviour is not shown by Δ(OH).

CONCLUSION

From this study a better understanding of the retention of anthocyanins has been obtained. The experimental results explained by Rekker's theory established that the retention of these compounds is due to two independent factors, one specific to the anthocyanidine and the other to the sugar. These two factors were studied and rules governing the chromatography of anthocyanins have been established and it is now possible to predict the retention of any compound in this family, either graphically or by calculation.

Now, with the identification of the peaks in the redcurrant chromatogram, the anthocyanidine profiles of most common red fruits are known and HPLC analysis is therefore a suitable method for determining which red fruit has been used in the preparation of a particular food product.

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