Journal of Chromatography, 279 (1983) 365–372
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 159

QUALITATIVE AND QUANTITATIVE EVALUATION OF *VACCINIUM MYRTILLUS* ANTHOCYANINS BY HIGH-RESOLUTION GAS CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A. BAJ*, E. BOMBARDELLI, B. GABETTA and E.M. MARTINELLI
Inverni della Beffa S.p.A., Research Laboratories, Via Ripamonti 99, 20141 Milan (Italy)

SUMMARY

An analytical method for the separation and characterization of anthocyanins in Vaccinium myrtillus extracts has been developed based on capillary gas chromatography and mass spectrometry. In addition, a method involving reversed-phase high-performance liquid chromatography for the quantitative evaluation of anthocyanins in these pharmacologically important extracts is presented.

INTRODUCTION

The physiological effect of anthocyanins is related to the prevention of capillary fragility. For this reason, pharmaceutical preparations containing extracts of *Vaccinium myrtillus* fruits, a very rich source of this type of plant pigments, are today widely used for the treatment of various microcirculation diseases.

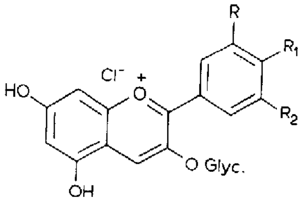
Paper and thin-layer chromatography, coupled with colorimetric measurements at fixed or different pH values before and after acidic hydrolysis, have been the traditional tools of investigators working with anthocyanins, but all these methods have drawbacks since they are time-consuming when large numbers of samples have to be analysed and require the use of reference compounds in most cases. Because of the complexity of their composition, the analytical problem posed by *V. myrtillus* extracts, which contain up to fifteen anthocyanins (*i.e.*, 3-O-arabinosides, 3-O-glucosides and 3-O-galactosides of cyanidin, delphinidin, peonidin, petunidin and malvidin, the structures of which are shown in Table I), is of considerable interest. Recent developments in both instrumentation and columns have opened up new possibilities for the separation of these plant products through the use of gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC).

We have demonstrated[1,2] that, in spite of the structural transformations induced in these pigments by pH variations[3,4], it is possible to obtain homogeneous derivates suitable for GLC analysis. In fact, treatment of anthocyanins with trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) yields nitrogen-containing compounds which, after injection into the gas chromatograph, are transformed into quinoline derivatives according to the scheme presented in Fig. 1. The quinoline derivatives thus obtained give rise to sharp chromatographic peaks and, when subjected to mass spec-

0021-9673/83/$03.00 © 1983 Elsevier Science Publishers B.V.

TABLE I

STRUCTURES OF *V. MYRTILLUS* ANTHOCYANINS



Glyc. = arabinoside, glucoside or galactoside

Compound	*R*	R_1	R_2
Delphinidin 3-O-glycoside	OH	OH	OH
Cyanidin 3-O-glycoside	OH	OH	H
Petunidin 3-O-glycoside	OH	OH	OCH_3
Peonidin 3-O-glycoside	OCH_3	OH	H
Malvidin 3-O-glycoside	OCH_3	OH	OCH_3

trometry (MS), they show fragmentation patterns useful for the identification of the starting anthocyanin.

A further possibility of separating complex anthocyanin mixtures, which has been reported almost simultaneously, is based on the use of HPLC[5,6]. In this method the use of reversed-phase column support materials enabled separation of the anthocyanin mixture from *Vitis viniferia*, which, however, yields a less complex extract in comparison to *Vaccinium myrtillus* and eluents of very low pH were required in order to avoid the broadening of the peaks arising from the interconversion of structural forms.

In the present paper we report the separation of the fifteen anthocyanins contained in Myrtocyan, a purified *V. myrtillus* extract employed in pharmaceutical preparations, by high-resolution gas chromatography (HRGC) and a method based on the (HPLC) technique, which can be utilized for the routine analysis of all types of *V. myrtillus* extracts.

For the attribution of HPLC peaks, authentic samples of anthocyanins and (MS) according to the results reported in ref. 2 were used.

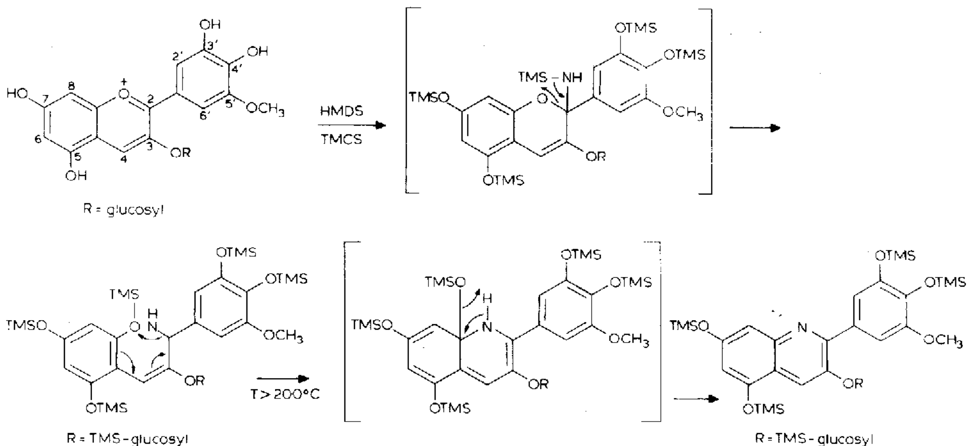


Fig. 1. Reaction scheme for anthocyanis (petunidin 3-O-glucoside) after treatment with TMCS and HDMS and thermal rearrangement, occuring during GLC.

EXPERIMENTAL

Reagents and chemicals

TMCS, HMDS and anhydrous pyridine (silylation grade) were purchased from Supelco (Bellefonte, PA, U.S.A.).

The HPLC eluents, methanol, acetonitrile (Lichrosolv Type) and formic acid were obtained from E-Merck (Darmstadt, F.R.G.).

The purified extract Myrtocyan was obtained from Inverni della Beffa (Milan, Italy). Authentic samples of cyanidin 3-O-glucoside, cyanidin 3-O-galactoside, cyanidin 3-O-arabinoside, delphinidin 3-O-glucoside, petunidin 3-O-glucoside, peonidin 3-O-glucoside, and malvidin 3-O-arabinoside were available in our laboratories.

Gas–liquid chromatography

Equipment. A Carlo Erba Model 2900 gas capillary chromatograph, equipped with a flame ionization detector and a Grob-type splitter injector were used. The chromatographic separations were accomplished on a 20 m x 0.3 mm I.D. glass capillary column, coated with SE-52 (film thickness 0.056 μm). The column temperature was increased at 1°C/min from 280°C to 310°C. The injection splitter (splitting ratio 35:1) and detector were heated to 350°C. The glass injection liner was filled with Chromosorb W HP (80–100 mesh). Helium was used as the carrier gas at a flow-rate of 2ml/min.

Analysis samples. About 50 g frozen *V. myrtillus* fruits were repetitively extracted (five times) at room temperature with stirring for 5 h with 100 ml of methanol. The combined extracts were concentrated under vacuum to low volume at 40°C and then lyophilized. The sample was diluted to 50 ml in methanol, 0.5 ml were transferred to a screw capped vial with PTFE cap-liner and the solvent was evaporated under a stream of nitrogen and dried under vacuum at 40°C. The residue was silanized with 0.2 ml of TMCS, 0.4 ml of HDMS, 0.03 ml of anhydrous dioxane and 0.015 ml of tetrahydrofuran by heating at 60°C for 30 min in a heating block. Aliquots of 1 μl were injected.

The purified extract (Myrtocyan) sample was prepared by dissolving 0.5 g in methanol, containing two drops of 10% (v/v) concentrated hydrochloric acid solution per 50 ml methanol. The analysis was carried out as described above for the fruits.

Gas–liquid chromatography–mass spectrometry

A Varian Model 3700 gas chromatograph equipped with a Grob type splitter injector and combined with a Finnigan MAT Model 8200 mass spectrometer through an open splitter system was used. The data system was a PDP 11-24. The GC conditions were the same as reported above. The mass spectra were registered at 70 eV (electron impact source). Aliquots of 1.0 μl of the sample were injected in the "splitless" mode.

High-performance liquid chromatography

The equipment used consisted of two Waters Associates Model 6000 A pumps, a Model 660 solvent programmer and a Model 7010 Reodyne injector. The detector was a Perkin-Elmer LC-55 variable-wavelength spectrophotometer set at 530 nm; peak areas were measured with a Hewlett-Packard Model 3385 A. A prepacked reversed-phase column (Aquapore RP-300, 10 μm, 22 cm x 4.6 mm; Brownlee Labs, Santa Clara, CA, U.S.A.) and guard column (Aquapore RP-300, 10 μm, 3 cm x 2.2 mm) were used. The solvents for gradient elution were: A, water–formic acid (90 : 10, v/v); B, methanol–acetonitrile–water–formic acid (22.5 : 22.5 : 45 : 10). Eluent B was increased from 0 to 30 % in 40 min by a concave gradient (curve 7 of Model 660 solvent programmer).

Fig. 2. GLC separation of the *V. myrtillus* anthocyanins after reaction with TMCS and HMDS.

Analysis samples. A 30-g sample of frozen *V. myrtillus* fruits was extracted as above; the extract was concentrated to a low volume and diluted to 100 ml in methanol containing 2 % (v/v) of concentrated hydrochloric acid solution. An aliquot of 5 ml was transferred to a 25-ml volumetric flask and diluted to volume in a solvent containing 10 % (v/v) of concentrated phosphoric acid in water. Aliquots of 20 μl were injected.

The purified extract (Myrtocyan) sample was prepared by dissolving 0.15 g in methanol containing 2 % (v/v) of concentrated hydrochloric acid, diluting to 50 ml in the same solvent and subsequently diluting as described for fruit analysis.

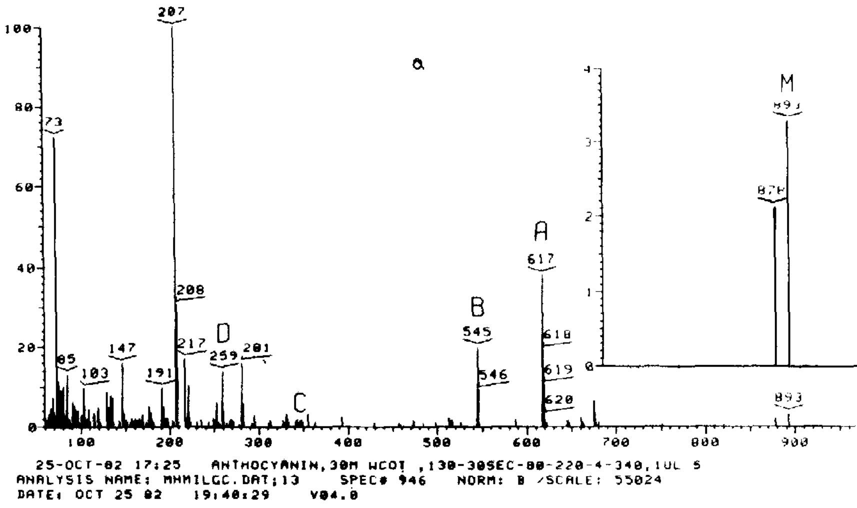
Standard solutions. A 20-mg sample of cyanidin 3-O-glucoside was dissolved and diluted to 50 ml in methanol containing 2 % (v/v) of concentrated hydrochloric acid, and 5 ml of this solution were diluted to 50 ml in water containing 10 % (v/v) of concentrated phosphoric acid. Aliquots of 20 μl were injected.

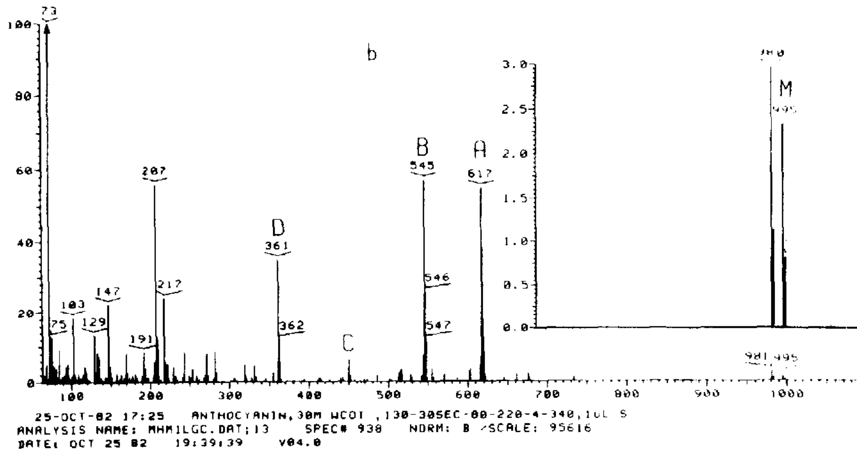
Fraction collection for high-resolution gas chromatography. Starting with a *V. myrtillus* extract solution (ten times more concentrated than the analysis solution) the fraction corresponding to each HPLC peak was collected, evaporated under vacuum at 40°C and derivatized for high-resolution gas chromatography with 100 μl of the silylating mixture as described for GLC.

RESULTS AND DISCUSSION

The GLC separation of *V. myrtillus* anthocyanins carried out on the fruit samples is shown in Fig. 2. The peaks of the quinoline derivatives obtained from the starting anthocyanins were assigned by comparison with authentic samples and interpretation of mass spectra. The MS fragmentation pattern of the anthocyanin derivatives is very characteristic, and glucosides, galactosides or arabinosides can easily be differentiated.

Fig. 3 shows the mass spectra corresponding to the three different 3-O-glycosides of malvidin.





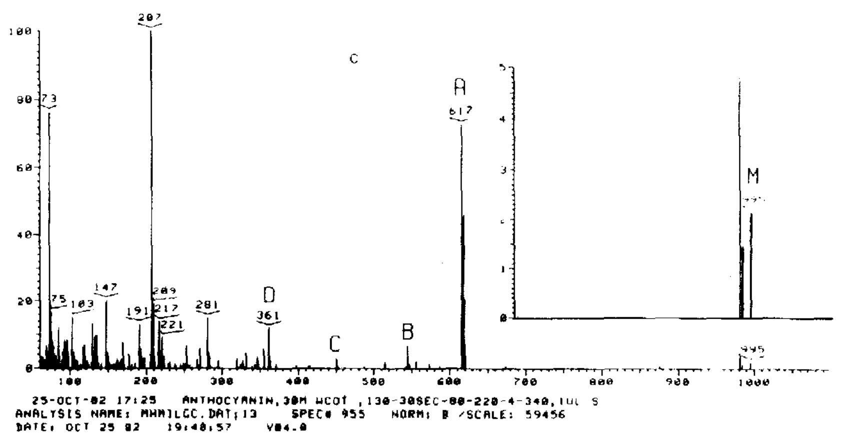


Fig. 3. HRGC–MS of malvidin 3-O-arabinoside (a), malvidin 3-O-glucoside (b) and malvidin 3-O-galactoside (c) after reaction with TMCS and HMDS.

TABLE II

MS FRAGMENTS OF ANTHOCYANIN DERIVATIVES OF *V. MYRTILLYS* OBTAINED BY HIGH-RESOLUTION GAS CHROMATOGRAPHY–MASS SPECTROMETRY

ara. = Arabinoside; gluc. = glucoside; gal. = galactoside.

Anthocyanin	*Mass fragment of the derivative, m/z (I%)*				
	M	*A*	*B*	*C*	*D*
Delphinidin 3-O-ara.	1009(18.2)	733(100)	661(69.4)	348(7.6)	259(58.7)
Delphinidin 3-O-gluc.	1111(2.5)	733(100)	661(97.5)	450(2.5)	361(28.4)
Delphinidin 3-O-gal.	1111(6.7)	733(100)	661(15.4)	450(5.1)	361(42.7)
Cyanidin 3-O-ara.	921(3.3)	645(100)	573(30.0)	348(14.4)	259(50.0)
Cyanidin 3-O-gluc.	1023(1.4)	645(91.2)	573(100)	450(14.2)	361(42.6)
Cyanidin 3-O-gal.	1023(3.9)	645(100)	573(15.5)	450(3.9)	361(29.1)
Peonidin 3-O-ara.	863(3.4)	587(100)	515(95.8)	348(8.5)	259(33.9)
Peonidin 3-O-gluc.	965(3.2)	587(82.8)	515(100)	450(10.7)	361(28.0)
Peonidin 3-O-gal.	965(3.0)	578(100)	515(19.4)	450(5.0)	361(34.9)
Petunidin 3-O-ara.	951(10.1)	675(100)	603(54.12)	348(3.7)	259(54.1)
Petunidin 3-O-gluc.	1053(2.3)	675(100)	603(59.8)	450(3.1)	361(13.0)
Petunidin 3-O-gal.	1053(4.1)	675(100)	603(13.2)	450(4.0)	361(29.1)
Malvidin 3-O-ara.	893(8.6)	617(100)	545(52.7)	348(5.4)	259(37.6)
Malvidin 3-O-gluc.	995(4.2)	617(96.4)	545(100)	450(11.3)	361(61.0)
Malvidin 3-O-gal.	995(2.8)	617(100)	545(8.9)	450(4.5)	361(16.7)

The molecular ions (M) are evident in all the registered spectra. In addition, ions A and B result from the loss of the sugar unit and migration from the latter of H and TMS radicals to the phenolic oxigen at C-3. 3-O-Galactosides can be distinguished from 3-O-glucosides by the fact that fragment B is less abundant in the former. The fragments due to the sugar units occur at *m/z* 450 (C) and *m/z* 361 (D) for glucosides and galactosides and, at *m/z* 348 (C) and *m/z* 259 for arabinosides.

The peaks at *m/z* 217, 207, 191, 103 are due to sugar fragments. The principal ions of all the anthocyanin derivatives are listed in Table II.

The gas chromatogram of the purified extract, Myrtocyan, is the same as that obtained for the fruits.

The GLC technique, in combination with MS and the employement of some pure anthocyanin samples, was also used to identify anthocyanin peaks resulting from

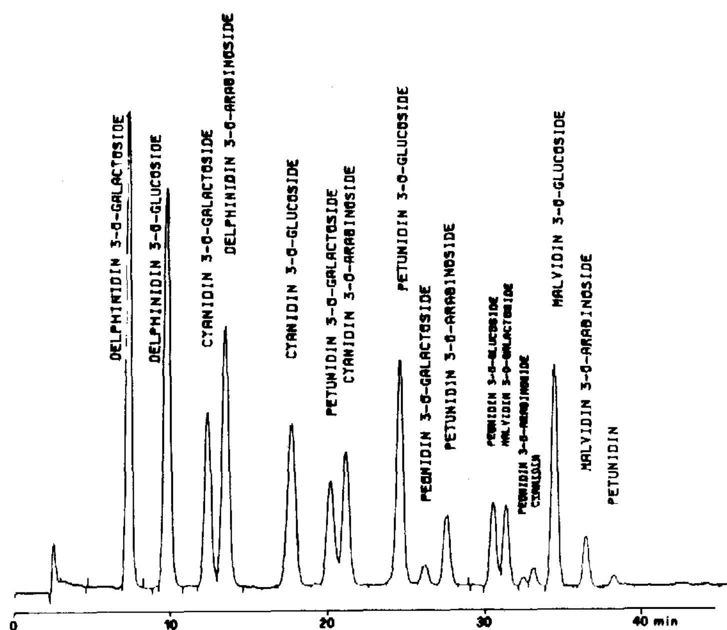


Fig. 4. HPLC separation of *V. myrtillus* anthocyanins.

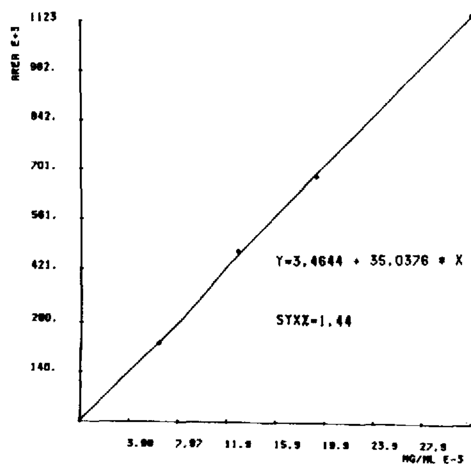


Fig. 5. HPLC calibration graph for cyanidin 3-O-glucoside.

HPLC separation. The HPLC conditions described in the Experimental allow good separation of all the anthocyanins, as shown in Fig. 4.

The alkylsilane-bonded large-pore-size (30 nm) stationary phase used offers specific advantages with the mostly aqueous eluents employed. Better-shaped peaks and enhanced resolution were observed in comparison with the results obtained by utilizing a conventional-pore-size (6–10 nm) stationary phase. At wavelength employed the profile of the raw extract is practically the same as for purified extracts. Two free aglycones (anthocyanidins), cyanidin and petunidin, are also visible as small peaks in the chromatogram.

To get quantitative information on the extracts, cyanidin 3-O-glucoside was chosen as reference compound. The use of different reference samples for the quantitative analysis was not justified due to the rather similar chromatographic responses of different anthocyanins at 530 nm. The linearity of the response for the cyanidin 3-O-glucoside is shown in Fig. 5. As an example, the results of the analysis of Myrtocyan are reported in Table III.

TABLE III

DETERMINATION OF ANTHOCYANINS IN MYRTOCYAN, A STANDARD COMMERCIALLY PURIFIED EXTRACT OF *V. MYRTILLUS* FRUITS.

Anthocyanin	*Mean per cent content*
Delphinidin 3-O-arabinoside	4.32
Delphinidin 3-O-glucoside	5.81
Delphinidin 3-O-galactoside	5.04
Cyanidin 3-O-arabinoside	2.19
Cyanidin 3-O-glucoside	3.42
Cyanidin 3-O-galactoside	2.75
Peonidin 3-O-arabinoside	0.22
Peonidin 3-O-glucoside	1.31
Peonidin 3-O-galactoside	0.34
Petunidin 3-O-arabinoside	1.08
Petunidin 3-O-glucoside	3.67
Petunidin 3-O-galactoside	1.89
Malvidin 3-O-arabinoside	0.81
Malvidin 3-O-glucoside	3.35
Malvidin 3-O-galactoside	1.27

ACKNOWLEDGEMENTS

We thank Mr. G. Chen for HPLC analysis, Mr. V. Cattani and Mr. E. Crippa for GLC separations and Mr. B. Porelli for the mass spectra.

REFERENCES

1 E.Bombardelli, A. Bonati, B.Gabetta, E.M.Martinelli, G.Mustich and B.Danieli, *J.Chromatogr.*, 120 (1976) 115–122.

2 E.Bombardelli, A.Bonati, B.Gabetta, E.M.Martinelli and G.Mustich, *J.Chromatogr.*, 139 (1977) 111–120.

3 C.F.Timberlake and P.Bridle, in J.B.Harbone, T.J.Nabry and J.Mabry (Editors), *The Flavonoids*, Chapman and Hall, London, 1975, pp. 214–266.

4 G.Hrazdina, in J.B.Harborne and T.J.Mabry (Editors), *The Flavonoids, Advances in Research*, Chapman and Hall, London, 1982, pp. 135–188.

5 L.W.Wulf and W.Nagel, *Amer. J. Enol. Vitic.*, 29 (1978) 42–49.

6 M.Williams, G.Hrazdina, M.M.Wilkinson, J.G.Sweeny and G.A.Iacobucci, *J.Chromatogr.*, 155 (1978) 389.