Complete Assignment of Bilberry (*Vaccinium myrtillus* L.) Anthocyanins Separated by Capillary Zone Electrophoresis

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Capillary zone electrophoresis (CZE) mobilities of fifteen anthocyanins in bilberry extract were completely characterized. Four minor anthocyanins in bilberry extract (malvidin 3-*O*-α-L-arabinopyranoside (Mv 3-ara), peonidin 3-*O*-β-D-galactopyranoside (Pn 3-gal), peonidin 3-*O*-α-L-arabinopyranoside (Pn 3-ara), and petunidin 3-*O*-α-L-arabinopyranoside (Pt 3-ara)) that remained unidentified in our previous CZE study were isolated from the bilberry extract, and the chemical structures were assigned by NMR and MS. Their CZE mobilities were then precisely examined together with those of other major anthocyanins in the extract. When the CZE mobilities of the fifteen anthocyanins assigned here were plotted against their molecular weight/numbers of free phenolic group, it was found that separation of anthocyanins by CZE is primarily determined by the type of conjugated sugar present, and secondly by the aglycon structure.

Key words anthocyanin; capillary zone electrophoresis; blueberry; anthocyanin 3-*O*-α-L-arabinopyranoside

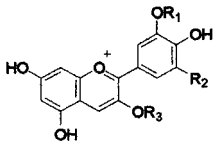
Flavonoids attract a lot of attention due to their variety of physiological functions, which include antioxidant activity.[1—3] To date, more than 5000 flavonoids have been identified in natural sources. Anthocyanin, a reddish plant pigment, is a kind of flavonoid distributed widely in colored fruits and vegetables, including eggplants,[4] black currants,[5] grapes[6] and blueberries.[7,8] As with other flavonoids, the phytoceutical significance of anthocyanins has been discussed in relation to a wide range of physiological functions such as vision improvement,[9,10] anticancer activity,[11,12] and antioxidant activity.[13—17]

Capillary electrophoresis (CE) has been successfully used in addition to HPLC to analyze flavonoids.[18—25] As we showed previously, capillary zone electrophoresis (CZE) is especially useful for the analysis of anthocyanins in extracts of fruits[26] and colored rice,[13] because the silica capillary tube used in CZE is applicable to samples like these that have not been purified.

We previously developed a CZE method for the analysis of anthocyanins in bilberry (a wild type blueberry) extract, and this method was used to evaluate the anthocyanin composition of different blueberry sources.[26] The method can also be used for the kinetic study of anthocyanin reactivity, for example the acid-mediated hydrolysis of anthocyanins.[27] In the previous studies, twelve major peaks were separated by the CZE method, and ten of them were identified by comparison with authentic reference samples[26] of fifteen anthocyanins contained in bilberry extract[7,8] (Fig. 1). In addition, one of the remaining peaks could be assigned as delphinidin 3-*O*-α-L-arabinopyranoside (III), based on a mobility comparison.[26,32] However, four other minor anthocyanins (malvidin 3-*O*-α-L-arabinopyranoside (Mv 3-ara, (XV)), peonidin 3-*O*-β-D-galactopyranoside (Pn 3-gal, (XI)), peonidin 3-*O*-α-L-arabinopyranoside (Pn 3-ara, (XII)) and petunidin 3-*O*-α-L-arabinopyranoside (Pt 3-ara, (IX))) in the bilberry extract (Bilberon 25) could not be identified in the previous CZE study, because of the lack of authentic samples to use as references, although we have discussed the possible migration time of the minor anthocyanins by electrophoretic rules in which both molecular weight and charges affect the mobility. In the present study, we purified these minor anthocyanins and determined their CZE mobility behaviors. When the mobility behavior of fifteen anthocyanins in bilberry was investigated by plotting the migration time against the molecular weight to numbers of free phenolic group in the molecule ratios, a correlation between these two variables was observed for a series of anthocyanins with the same conjugated sugar. From this correlation, the structures of unidentified anthocyanins could be predicted by analysis of their elution positions.

Experimental

Reagents All reagents including sodium borate ($NaBO_4$), trifluoroacetic acid (TFA) and methanol (HPLC grade) were purchased from Wako Pure



	R_1	R_2	R_3
Delphinidin 3-O-β-D-glucopyranoside (I)	H	OH	Glucose
Delphinidin 3-O-β-D-galactopyranoside (II)	H	OH	Galactose
Delphinidin 3-O-α-L-arabinopyranoside (III)	H	OH	Arabinose
Cyanidin 3-O-β-D-glucopyranoside (IV)	H	H	Glucose
Cyanidin 3-O-β-D-galactopyranoside (V)	H	H	Galactose
Cyanidin 3-O-α-L-arabinopyranoside (VI)	H	H	Arabinose
Petunidin 3-O-β-D-glucopyranoside (VII)	H	OCH_3	Glucose
Petunidin 3-O-β-D-galactopyranoside (VIII)	H	OCH_3	Galactose
Petunidin 3-O-α-L-arabinopyranoside (IX)	H	OCH_3	Arabinose
Peonidin 3-O-β-D-glucopyranoside (X)	CH_3	H	Glucose
Peonidin 3-O-β-D-galactopyranoside (XI)	CH_3	H	Galactose
Peonidin 3-O-α-L-arabinopyranoside (XII)	CH_3	H	Arabinose
Malvidin 3-O-β-D-glucopyranoside (XIII)	CH_3	OCH_3	Glucose
Malvidin 3-O-β-D-galactopyranoside (XIV)	CH_3	OCH_3	Galactose
Malvidin 3-O-α-L-arabinopyranoside (XV)	CH_3	OCH_3	Arabinose

Fig. 1. Chemical Structures of Bilberry Anthocyanins

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Chemical Industries, Co. Ltd., Japan. *trans*-1,2-Diaminocyclohexane *N*,*N*,*N*′,*N*′-tetra acetic acid monohydrate (CyDTA) was purchased from Dojin Chemical Industries, Co. Ltd., Japan. CLAN K200 was purchased from Clean Chemical Co. Ltd., Japan. MCI gel CHP 20P (70—150 μm) and Sephadex LH-20 (25—100 μm) were purchased from Mitubishi Chemical Industries Ltd., Japan and Amarsham Pharmacia Co. Ltd., U.S.A. respectively. Bilberon 25, the concentrated extract of bilberry (*Vaccinium myrtillus* L., Bilberry), was kindly donated by Tokiwa Phytochemicals Co. Ltd., Japan. The anthocyanin content in Bilberon 25 is 33% as malvidin equivalent.

Methods. 1) Analytical Conditions for CZE CZE was carried out as reported previously, in constant voltage mode at 25 °C using a CAPI-3100 capillary electrophoresis system (Otsuka Electronics Co. Ltd., Japan) equipped with a fused-silica capillary (50 μm i.d.×72.5 cm long: effective length=60.0 cm).[26] The sample solution was loaded onto the capillary in hydrodynamic mode (25 mm×30 s), and then the electrophoresis was carried out, typically with an applied voltage of +25 kV. The absorption spectrum of the eluent was recorded between 400 and 600 nm with a time constant of 0.12 s using a photodiode array detector, and the electrophoretogram was recorded with a monitoring wavelength of 580 nm.

2) Isolation and Identification of Minor Anthocyanins Bilberon 25 (10 g) was dissolved in 1% TFA aqueous solution (10 ml) and subjected to low-pressure liquid chromatography (Waters, Co. Ltd., U.S.A.). Anthocyanins were recovered in the fraction eluted with 30% MeOH containing 1% TFA (yield 3.3 g). The anthocyanin fraction was further chromatographed over MCI-gel CHP (4.5 cm×45 cm) with H_2O containing increasing amounts of MeOH (0 : 1→1 : 0) to give eleven fractions. Fractions 5 and 6, containing Mv 3-ara (XV), Pn 3-gal (XI), Pn 3-ara (XII), Pt 3-gal (VIII) and Pt 3-ara (IX), were further separated by Sephadex LH-20 chromatography (2.5 cm×26 cm). Each anthocyanin was successfully purified by HPLC using a HITACHI L-7120 HPLC pump, a Develosil ODS-HG5 column (4.6×250 mm for analytical use, 20.0×250 mm for preparative use, Nomura Chemical Co. Ltd., Japan), and a HITACHI L-7420 UV detector (520 nm), with an eluent of 20% MeOH containing 0.1% TFA.

The anthocyanin structures were assigned by extensive 1D and 2D NMR as well as by MS analysis. NMR spectra were recorded on a JEOL ECA500 spectrometer and chemical shifts are reported as δ (ppm) with tetramethylsilane (TMS) as the internal standard. For the analysis by MS the sample was dissolved in methanol, and 20 μl of this sample was subjected to LC-TOF MS (Micromass LCT).

Results

When bilberry extract was separated by CZE, eleven major peaks were identified, as reported previously, and each peak was assigned by comparison of its electromobility with those of anthocyanin standards.[26,27] However, peaks corresponding to Mv 3-ara (XV), Pn 3-gal (XI), Pn 3-ara (XII) and Pt 3-ara (IX) were not identified in the previous CZE study because appropriate standard samples were not available. To complete the assignment of all anthocyanin CZE peaks present in bilberry extract, minor anthocyanins Pn 3-gal (XI), Pn 3-ara (XII), Pt 3-ara (IX) and Mv 3-ara (XV) together with Pt 3-gal (VIII) were isolated from the bilberry extract (Bilberon 25) by repeated column chromatography and preparative HPLC. Their chemical structures were assigned by MS and NMR spectroscopies. The *m*/*z* values determined by MS for the purified minor anthocyanins were 463 for Mv 3-ara (XV), 463 for Pn 3-gal (XI), 433 for Pn 3-ara (XII), 449 for Pt 3-ara (IX) and 479 for Pt 3-gal (VIII), respectively, and these values matched their molecular masses. ^{1}H- and ^{13}C-NMR data are summarized in Tables 1 and 2. Mv 3-ara (XV), Pn 3-gal (XI), Pn 3-ara (XII), Pt 3-ara (IX) and Pt 3-gal (VIII) were assigned to CZE peaks 5, 5, 7, 9 and 8, respectively (Fig. 2). By the present CZE method, the analysis of anthocyanins present in bilberry extract were completed within 20 min including capillary conditioning step (Fig. 2).

When the relative CZE mobilities of anthocyanins assigned here were plotted against the molecular weight to the numbers of free phenolic group in the molecule ratios, a characteristic correlation was obtained among the anthocyanins carrying the same sugar moiety, as shown in Fig. 3.

Table 2. ^{13}C-NMR Data for Compounds VIII, IX, XI, XII and XV (δ-Values)

	VIII	IX	XI	XII	XV
2	165.09	165.18	165.00	165.34	164.25
3	146.07	146.56	146.43	146.35	145.77
4	137.89	137.62	138.22	138.02	137.33
5	158.70	158.64	158.75	158.77	158.06
6	104.39	104.43	104.37	104.47	103.72
7	171.55	171.52	171.72	171.73	171.1
8	96.12	96.17	96.14	96.24	95.56
4a	114.43	144.15	114.53	114.46	113.77
8a	160.19	160.03	180.27	*a)*	*a)*
1′	120.92	120.88	122.05	122.07	120.01
2′	114.69	110.36	118.57	118.54	110.97
3′	150.76	150.81	150.41	150.57	149.97
4′	146.70	146.20	160.27	157.54	146.48
5′	148.33	148.34	116.21	116.27	149.97
6′	110.38	114.88	129.61	130.08	110.97
OCH_3	58.20	58.25	57.84	57.96	57.57 (2C)
Gal					
1	105.46		105.32		
2	73.16		73.12		
3	75.95		75.94		
4	71.02		71.00		
5	78.72		78.69		
6	63.30		63.28		
Ara					
1		105.44		105.28	104.85
2		74.92		74.8	74.28
3		73.12		73.11	72.42
4		69.99		69.75	69.31
5		68.22		67.93	67.62

Measured at 125 MHz in CD_3OD+TFA (9 : 1). *a*) Overlapped with TFA signals.

Table 1. ^{1}H-NMR Data (δ, *J* in Hz) for Compounds VIII, IX, XI, XII, and XV

	VIII	IX	XI	XII	XV
4	9.02 (s)	8.95 (s)	8.98 (s)	9.01 (s)	8.88 (s)
6	6.92 (d, 2)	6.91 (d, 2)	6.85 (d, 2)	6.95 (d, 2)	6.84 (d, 2)
8	6.68 (d, 2)	6.68 (d, 2)	6.61 (d, 2)	6.70 (d, 2)	6.58 (d, 2)
2′	8.00 (d, 2)	7.97 (d, 2)	8.19 (d, 2)	8.25 (d, 2)	7.89 (s)
5′	—	—	0.99 (dd, 8, 2)	7.08 (d, 8, 2)	—
6′	7.81 (d, 2)	7.83 (d, 2)	8.16 (d, 8)	8.33 (d, 8)	7.89 (s)
OCH_3	4.02 (s)	4.02 (s)	3.95 (s)	4.05 (s)	3.92 (s) (2C)
1″	5.29 (d, 8)	5.28 (d, 8)	5.20 (d, 8)	5.29 (d, 8)	5.16 (d, 8)

Measured at 500 MHz in CD_3OD+TFA (9 : 1).

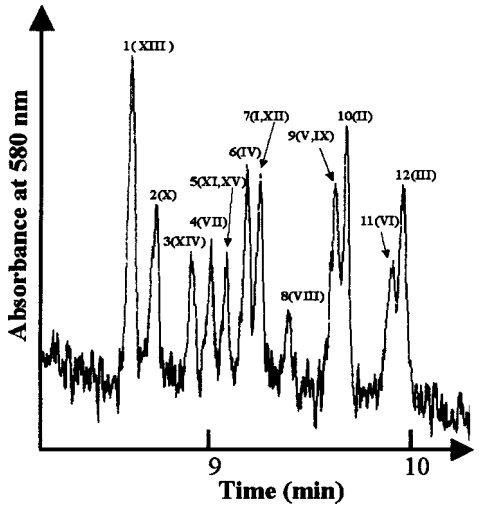

Fig. 2. CZE Peak Assignment of Bilberry Anthocyanins

The electrophoresis carried out using 30 mM Na-borate containing 7.5 mM *trans*-1,2-diaminocyclohexane *N*,*N*,*N*′,*N*′-tetra acetic acid monohydrate as a carrier buffer. After each run, the capillary was washed with CLEAN99K200, distilled water, 0.5 M NaOH and distilled water every 2 min, then finally with running buffer. Numbers (I—XV) correspond to those in Fig. 1.

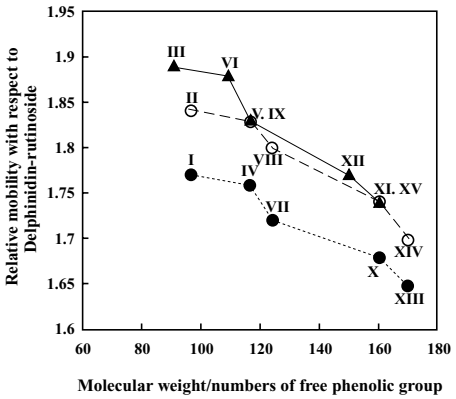

Fig. 3. Relationship between the Relative CZE Mobility and Molecular Weight/Numbers of Free Phenolic Group of Anthocyanins

Symbols: ●: glucoside, ○: galactoside, ▲: arabinoside.

Disucussion

Separation of anthocyanins by CZE has been reported for fruit extracts such as black currant, which contains only four major anthocyanins.[28—31] We developed a method for the analysis of bilberry anthocyanins by CZE, and showed that this method is useful for the analysis of anthocyanins from different sources[13,26] and also for studies of their chemical reactivity.[27] However, in the previous study, minor anthocyanins with galactose and arabinose as the conjugated sugar remained unidentified due to several reasons, including the lack of authentic reference samples, insufficient CZE detection limits and relatively smaller amounts of these anthocyanins in the bilberry extract. In the present study, we assigned all the anthocyanins present in bilberry to the CZE peaks by using authentic anthocyanins, including minor anthocyanins purified here as reference samples.

The electrophoretic mobility of anthocyanins is governed mainly by molecular mass and electrical charge, according to the electrophoresis rule. To analyze the CZE mobility behavior of anthocyanins further, the mobilities of the fifteen anthocyanins determined in bilberry extract were plotted against the molecular weight/numbers of free phenolic group in the molecule, as shown in Fig. 3. The plot clearly showed that anthocyanins are separated primarily according to their conjugated sugar type, and this separation is further modified by the aglycon structure, as suggested in our previous report.[26] That is, glucosides move faster than galactosides, followed by arabinosides, when the mobilities of anthocyanins carrying the same aglycon structure are compared (Fig. 3).

In our previous study, peak 5 in Fig. 3 was assigned to Pt 3-gal (VIII) by comparison with the mobility of the authentic reference, even though this meant that the CZE behavior of Pt 3-gal (VIII) did not fit to the electrophoretic rule mentioned above.[26] In the present study, we evaluated the CZE behavior of Pt 3-gal (VIII) using the purified Pt 3-gal (VIII) isolated here, and found that Pt 3-gal (VIII) migrated with peak 8, not with peak 5, as reported previously. It was concluded that this discrepancy was due to the authentic Pt 3-gal (VIII) sample used previously as a reference being incorrect. Consequently, it was shown that all the anthocyanins fit the CZE mobility rule worked out in the present study well (Fig. 3).

In conclusion, when the relative CZE mobilities of anthocyanins were plotted against the molecular weight to the numbers of free phenolic group ratios, a characteristic correlation was observed among the anthocyanins carrying the same sugar moiety, as shown in Fig. 3. Patterns for this correlation were similar among the anthocyanins with same conjugated sugar (glucose, galactose and arabinose). Hence, anthocyanins were primary separated by the type of conjugated sugar and then secondary, further separation was attained the structure of aglycon B ring. Based on these characteristic features of anthocyanin mobility in CZE, we can deduce the chemical structures of certain anthocyanins isolated from different sources.

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