Structural Dependence of HPLC Separation Pattern of Anthocyanins from Bilberry (*Vaccinium myrtillus* L.)

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An HPLC method using isocratic elution was established for the analysis of fifteen anthocyanins contained in bilberry (*Vaccinium myrtillus* L.). Separation was attained by using an aqueous solution of 20% methanol containing 0.5% TFA as the mobile phase with a flow rate of 2 ml/min. The detection limit was 0.3 pmol for delphinidin 3-*O*-β-D-glucopyranoside, which is a major anthocyanin in bilberry extract. The reproducibility was 0.19—3.85% (S.E.M) for peak area and 0.64—0.77% (S.E.M) for relative mobility normalized by the elution position of the solvent peak. When the relative elution volumes of each anthocyanins were correlated to their corresponding anthocyanin structures, a characteristic pattern was observed. From this pattern, the structures of unknown anthocyanins could be predicted from their elution times. Therefore, the present method is useful for the study of anthocyanins from various biological sources.

Key words anthocyanin; bilberry; HPLC; separation condition; isocratic elution

Flavonoids are a subject of much interest due to the wide variety of physiological functions, including antioxidant activity.[1—3] Anthocyanin, a reddish plant pigment, is a widely distributed flavonoid found in colored fruits and vegetables such as 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 improvement of vision,[9,10] anticancer activity[11,12] and antioxidant activity.[13—15]

In bilberry (a wild type of blueberry) extract, fifteen anthocyanins are expected to be present[7,8] (Fig. 1). Baj *et al.* reviewed the chromatographic separation of anthocyanins, and showed that HPLC is a useful tool for the analysis of anthocyanins in plant materials.[7] However, their study used gradient elution for the separation, which involves time-consuming column conditioning and stabilization. Goiffon *et al.* studied various parameters affecting the retention time of blueberry and strawberry anthocyanins and showed that anthocyanins having the same aglycon group were separated by the type of sugar moiety under isocratic elution conditions.[8] However, under the conditions used, some anthocyanins such as cyanidin 3-*O*-β-D-galactopyranoside (Cy3-gal) and delphinidin 3-*O*-α-L-arabinopyranoside (Dp3-ara) were not completely separated.

In the present study, we investigated the separation of anthocyanins contained in bilberry using isocratic HPLC and each peak was assigned by the purified anthocyanins as standard. Results showed a characteristic separation pattern for the anthocyanins with different chemical structures in their relative elution volumes.

Experimental

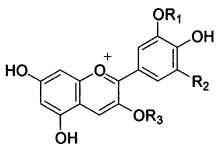
Materials All reagents, including trifluoroacetic acid (TFA), methanol (MeOH), acetonitrile (MeCN) and distilled water (HPLC grade) were purchased from Wako Pure Chemical Industries Co., Ltd., Japan. Bilberon 25, the powdered pure extract of bilberry (*Vaccinium myrtillus* L., bilberry), was donated by Tokiwa Phytochemical Co., Ltd., Japan. The anthocyanin content in Bilberon 25 is 33% as malvidin equivalent. Fifteen anthocyanins present in bilberry were isolated from Bilberon 25 using chromatographic techniques described elsewhere.[16,17]

Methods HPLC separation conditions were as follows: a HITACHI HPLC system (L-7100) equipped with a Develosil ODS-HG 5 column (4.6 mm×150 mm) was used. Anthocyanins were separated by isocratic elution with an aqueous solution of 20% MeOH containing 0.5% TFA, at a flow rate of 2 ml/min. The column temperature was maintained at 40 °C. Bilberon 25 (1 mg) was dissolved in 0.5% TFA (1 ml) and diluted 100 times by same solvent. Sample injection was performed by means of an autosampler (HITACHI L-7200, Japan), which loaded 100 μl of sample onto the column. Anthocyanin peaks were monitored by absorbance at 520 nm using HITACHI L-7420 UV-VIS detector.

Results and Discussion

We have recently isolated and purified fifteen anthocyanins present in bilberry, and assigned their chemical structures by NMR and MS.[16,17] Using these anthocyanins as standard, we set up a simple HPLC separation of anthocyanins in bilberry by isocratic elution mode.

First, the elution conditions required for good separation



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

Fig. 1. Chemical Structures of Bilberry Anthocyanins

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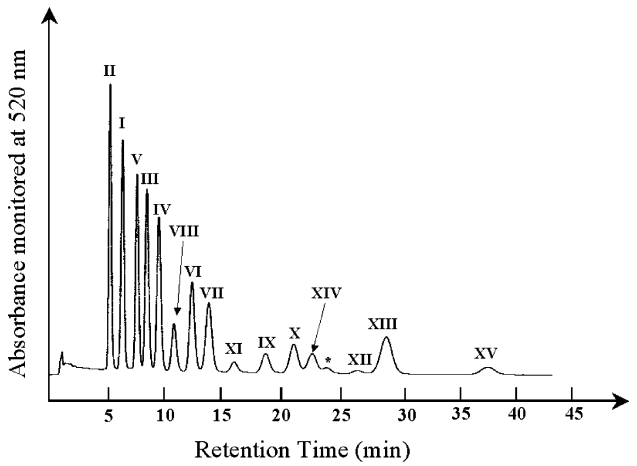

Fig. 2. HPLC Chromatogram of Bilberry Anthocyanins Using an Aqueous 20% Methanol Solution Containing 0.5% TFA as the Mobile Phase with a Flow Rate of 2 ml/min

Biberon 25 was dissolved in 0.5% TFA solution and aliquot of this solution (1 μg as Bilberon 25) was injected into HPLC system. Anthocyanins were detected absorbance at 520 nm. Peak labels correspond to numbers given in Fig. 1, * is delphinidin.

of fifteen anthocyanins in bilberry extact (Bilberon 25) were studied using MeCN or MeOH containing TFA as mobile phases with flow rate of 1 ml/min. Some anthocyanin peaks were not separated perfectly by MeCN, thus MeOH was used subsequently to separate the anthocyanins. The best separation of all fifteen anthocyanins was attained with an aqueous solution of 20% MeOH containing 0.5% TFA. However, with this solvent, malvidin 3-*O*-α-L-arabinopyranoside, which is the most hydrophobic anthocyanin, took longer than 90 min to elute. When the HPLC flow rate was increased to 2 ml/min, however, separation was completed within 50 min, and the fifteen anthocyanins in bilberry were completely separated (Fig. 2). Each peak was assigned by the authentic anthocyanins obtained by ourselves[16,17] and also those provided by Dr. K. Igarashi that were used for the peak identification of our previous capillary zone electrophoresis (CZE) study.[18] Under the conditions used, the backpressure of the column remained below the limit (about 180 kg·f/kg).

The detection limit of this method was 0.3 pmol for delphinidin 3-*O*-β-D-glucopyranoside (Dp3-glc), which is a major anthocyanin in bilberry extract. The reproducibility of peak area and retention time of the present HPLC method was also studied for each anthocyanin using the purified anthocyanins from Bilberon 25. From the results summarized in Tables 1 and 2, this method was found to have high reproducibility and low detection limit and thus is applicable even for the analysis of anthocyanins in biological samples, such as blood plasma. Indeed, we recently applied this method to measure plasma concentration of Dp3-glc in rats after oral administration.[19] Moreover, in the present method, large volume of sample can be injected directly into the system, because the sample is dissolved in aqueous 0.5% TFA solution which does not contain MeOH. Hence, the anthocyanins are first absorbed and concentrated on the top of the column, and then separated by the elution solution.

When the retention times of anthocyanins carrying the same sugar moiety were compared, it was seen that the sequence of their retention time was determined by the aglycon structure, therefore the retention times were in the following order: delphinidin<cyanidin<petunidin<peonidin<malvidin. On the other hand, when anthocyanins carrying the same aglycon structure were compared, the sequence of retention times was as follows: galactoside<glucoside<arabinoside.

Table 1. Reproducibility of Relative Mobility of Anthocyanins with Respect to Solvent Peak

	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin
Galactoside	0.77	0.65	0.71	0.64	0.7
Glucoside	0.64	0.67	0.73	0.66	0.72
Arabinoside	0.69	0.66	0.7	0.75	0.69

The HPLC conditions were described in the text. Values are S.E.M (%) for n=20.

Table 2. Reproducibility of Peak Area of Each Anthocyanin

	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin
Galactoside	0.19	0.28	0.51	1.77	1.77
Glucoside	0.19	0.24	0.38	1.59	1.77
Arabinoside	0.26	0.24	1.36	2.17	3.85

The HPLC conditions were described in the text. Values are S.E.M (%) for n=20.

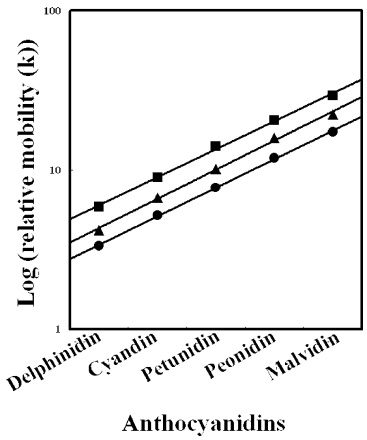

Fig. 3. Correlation between Anthocyanidin Structures and Their Relative Retention Volumes

Sugar-based comparison. Symbols: ●: galactoside, ▲: glucoside, ■: arabinoside.

Previously, Goiffon *et al.* analyzed anthocyanins present in bilberry and strawberry, and observed a similar relationship in anthocyanins with the same sugar moiety.[8] However, petunidin-glycopyranosides did not fit this relation when the mobilities were plotted as $\log(k_n+1/k_n)$ ($k_n=(t_n-t_0)/t_0$ where t_n is the retention time of compound n ($n=1, 2, \ldots, 15$) and t_0 is the dead time of the column). Moreover, in their study, some anthocyanins such as Cy3-gal and Dp3-ara were not separated clearly.

In order to examine the mobility–structure relationship of anthocyanins more precisely, we calculated relative elution volume (k_n) of each anthocyanin. When the values were plotted against anthocyanin structure, a characteristic pattern was obtained both in terms of sugar moiety and aglycon group (Figs. 3, 4).

The similar correlation between CZE mobility and anthocyanin structure has been observed in our previous CZE study.[16] When the k_n value obtained in the present HPLC was plotted against the relative mobility in CZE described in our previous report for anthocyanins, negative correlation was observed between them (data not shown). Therefore,

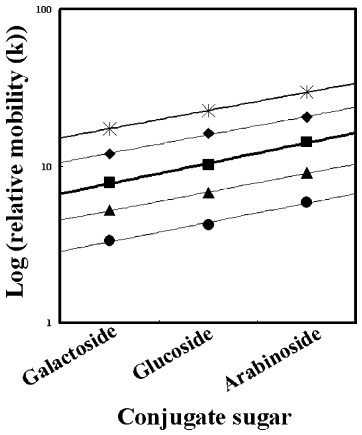

Fig. 4. Correlation between Anthocyanin Structures and Their Relative Retention Volumes

Aglycon-based comparison. Symbols: ●: delphinidin, ▲: cyanidin, ■: petunidin, ◆: peonidin, ∗: malvidin.

proper selection of these methods which have different separation principle will help predicting the structures of anthocyanins due to their sample conditions, although additional spectroscopic methods are necessary to identify the complete chemical structure.

Recently, we found that orally administered Dp3-glc was metabolized to 4′-*O*-methyl-Dp3-glc in rats.[19] In contrast, cyanidin 3-*O*-*β*-D-glucopyranoside was reported elsewhere to be metabolized to peonidin 3-*O*-*β*-D-glucopyranoside.[20,21] Hence, biotransformation studies of orally administered anthocyanis are important in order to determine the *in vivo* effects of anthocyanins. The present method will provide useful tool information not only for predicting the metabolites of anthocyanins but also anthocyanins from various fruits and vegetables in biological samples.

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References

1) Farombi E. O., Ogundipe O., Moody J. O., *J. Med. Sci.*, **30**, 213—215 (2001).
2) Horvathova K., Novotny L., Vachalkova A., *Neoplasma*, **50**, 291—295 (2003).
3) Vessal M., Hemmati M., Vasei M., *Comp. Biochem. Physiol. C*, **135C**, 357—364 (2003).
4) Noda Y., Kaneyuki T., Igarashi K., Mori A., Packer L., *Res. Commun. Mol. Pathol. Pharmacol.*, **102**, 175—185 (1998).
5) Matsumoto H., Hanamura S., Kawakami T., Sato Y., Hirayama M., *J. Agric. Food Chem.*, **49**, 1541—1545 (2001).
6) Igarashi K., Takanashi K., Makino M., Yasui T., *Nippon Shokuhin Kogyo Gakkaishi*, **36**, 852—856 (1989).
7) Baj A., Bombardelli E., Gabetta B., Martinelli E. M., *J. Chromatogr.*, **279**, 365—372 (1983).
8) Goiffon J. P., Brun M., Bourrier M. J., *J. Chromatogr.*, **537**, 101—121 (1991).
9) Mercier A., Perdriel G., Rozier J., Cheraleraud J., *Bull. Soc. Ophtalmol. Fr.*, **65**, 1049—1053 (1965).
10) Matsumoto H., Nakamura Y., Tachibanaki S., Kawamura S., Hirayama M., *J. Agric. Food Chem.*, **51**, 3560—3563 (2003).
11) Katsube N., Iwashita K., Tsushida T., Yamaki K., Kobori M., *J. Agric. Food Chem.*, **51**, 68—75 (2003).
12) Hou D. X., *Curr. Mol. Med.*, **3**, 149—159 (2003).
13) Ichiyanagi T., Hatano Y., Matsugo S., Konishi T., *ITE Lett.*, **4**, 788—793 (2003).
14) Ichiyanagi T., Hatano Y., Matsugo S., Konishi T., *Chem. Pharm. Bull.*, **52**, 434—438 (2004).
15) Ichikawa H., Ichiyanagi T., Xu B., Yoshii Y., Nakajima M., Konishi T., *J. Med. Food*, **4**, 211—218 (2001).
16) Ichiyanagi T., Kashiwada Y., Ikeshiro Y., Hatano Y., Shida Y., Horie M., Matsugo S., Konishi T., *Chem. Pharm. Bull.*, **52**, 226—229 (2004).
17) Ichiyanagi T., Kashiwada Y., Ikeshiro Y., Hatano Y., Konishi T., (in preparation).
18) Ichiyanagi T., Tateyama C., Oikawa K., Konishi T., *Biol. Pharm. Bull.*, **23**, 492—497 (2000).
19) Ichiyanagi T., Rahman M. M., Kashiwada Y., Ikeshiro Y., Shida Y., Hatano Y., Matsumoto H., Hirayama M., Tsuda T., Konisihi T., *Free Radic. Biol. Med.*, **36**, 930—937 (2004).
20) Miyazawa T., Nakagawa K., Kudo M., Muraishi K., Somcya K., *J. Agric. Food Chem.*, **47**, 1083—1091 (1999).
21) Tuda T., Horio F., Osawa T., *FEBS Lett.*, **449**, 179—182 (1999).