Kinetic Comparisons of Anthocyanin Reactivities towards 2,2′-Azobis(2-amidinopropane) (AAPH) Radicals, Hydrogen Peroxide and *tert*-Buthylhydroperoxide by Capillary Zone Electrophoresis

Takashi Ichiyanagi,[a] Yoshihiko Hatano,[b] Seiichi Matsugo,[c] and Tetsuya Konishi*,[b]

[a] *Faculty of Applied Life Sciences, Niigata University of Pharmacy and Applied Life Sciences;* [b] *Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences; Niigata 950–2081, Japan: and* [c] *Division of Biotechnology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi; 4–3–11 Takeda, Kofu 400–8511, Japan.* Received December 26, 2003; accepted January 22, 2004; published online January 29, 2004

Twelve major anthocyanins identified in bilberry extracts were studied *in vitro* using capillary zone electrophoresis (CZE) for their reactions towards 2,2′-azobis(2-amidinopropane) (AAPH) radicals, hydrogen peroxides (H_2O_2) and *tert*-buthylhydroperoxides (*t*-BuOOH). Reactivity towards AAPH radicals was primarily determined by the aglycon structure, not by the type of sugar moiety. Delphinidins carrying three-hydroxyl groups on the B ring were most reactive followed by cyanidins, with two-hydroxyl groups. Further, methylation of the hydroxyl groups reduced reactivity towards AAPH radicals. However, reactivity of anthocyanins towards H_2O_2 was not significantly affected by aglycon structure or by the type of sugar moiety; there being no marked difference in reaction rates among the anthocyanins. Reactivity towards *t*-BuOOH was essentially the same as towards H_2O_2, although the reaction rate was several times smaller. Also, the reaction rate of anthocyanin towards peroxide was relatively high compared to that of (+)-catechin (approximately 30 times larger) measured as a reference antioxidant, whereas the reactivities of anthocyanins and (+)-catechin towards AAPH radicals were similar.

Key words anthocyanin; capillary zone electrophoresis; bilberry; structure–reactivity relationship; reactive oxygen species; hydroperoxide

Antioxidant food ingredients have attracted attention as preventive factors against many diseases. Polyphenols, including flavonoids[1—4] for example, catechins from green tea, which have been reported to be one of the strongest radical scavengers of peroxynitrite and other radicals.[5—7] Their pharmacokinetic behavior has also been extensively examined.[8—10] It is therefore generally accepted that flavonoids are a major food sourced antioxidant.

Anthocyanins are a kind of flavonoid widely distributed in colored fruits and vegetables such as eggplants, red cabbages, grapes and blueberries. As with other flavonoids, anthocyanins have been discussed in relation to a wide range of physiological functions such as improvement of vision,[11,12] prevention of cancer[13—15] and generally[16—18] together with their antioxidant activity.[19—23]

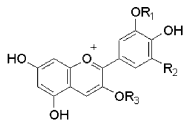
For example, Noda *et al.* studied the hydroxyl radical and superoxide radical scavenging activities of nasunin using ESR-spin trapping methods and revealed that delphinidin, the aglycon of nasunin, chelates iron ions to prevent hydroxyl radical generation during the Fenton reaction.[19,20] Whilst Tsuda *et al.* reported the antioxidant effects of cyanidin 3-*O*-*β*-D-glucopyranoside (Cy3G) both *in vivo* and *in vitro*.[21,22] Several other studies indicate the potential antioxidant properties of anthocyanins,[24—29] whilst the absorption and metabolic properties of anthocyanin have recently attracted much attention.[30—34] However, only a few systematic studies have investigated the structure–activity relationship of anthocyanins in terms of their reactivity towards reactive oxygen species (ROS), possibly because there is limited availability of anthocyanin standards.[35,36]

In the present study, we simultaneously determined the reactivities towards ROS of twelve major anthocyanins present in a wild berry extract by capillary zone electrophoresis (CZE),[37] and discussed the structure–reactivity relationship for anthocyanins towards 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) radicals, hydrogen peroxide (H_2O_2) and *tert*-buthylhydroperoxide (*t*-BuOOH).

Experimental

Materials All reagents including trifluoroacetic acid (TFA), AAPH and H_2O_2 were obtained from Wako Pure Chemical Industries Co., Ltd., Japan. The *t*-BuOOH (70% aqueous solution) and 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) were obtained from Sigma. Bilberon 25 was donated by Tokiwa Phytochemical Co., Ltd., Japan. Bilberon 25 is the powdered pure extract of bilberry (*Vaccinium myrtillus* L., bilberry) containing fifteen anthocyanins with five different aglycon structures and three different type of sugar moiety (Fig. 1). The anthocyanin content in Bilberon 25 is 33% as malvidin equivalent. (+)-catechin was donated by Dr. Y. Kashiwada of the Department of Pharmacognosy, Niigata University of Pharmacy and Applied Life Sciences.

Methods **Setting up of the Reaction Conditions for Kinetic Analysis of Anthocyanin Reactivity towards ROS** To determine the appropriate conditions under which to study the kinetic reactivity of anthocyanins using CZE, their reactivity as a whole in the bilberry extract (Bilberon 25) was determined at various pHs and temperatures with various concentrations of AAPH radicals, H_2O_2 and *t*-BuOOH. Using a Hitachi U-3000 spectrophotometer the bleaching of absorbance at 520 nm was measured and deter-



	R_1	R_2	R_3
Dephinidin 3-*O*-β-D-glucopyranoside	H	OH	glucoside
Dephinidin 3-*O*-β-D-galactopyranoside	H	OH	galactoside
Dephinidin 3-*O*-α-L-arabinopyranoside	H	OH	arabinoside
Cyanidin 3-*O*-β-D-glucopyranoside	H	H	glucoside
Cyanidin 3-*O*-β-D-galactopyranoside	H	H	galactoside
Cyanidin 3-*O*-α-L-arabinopyranoside	H	H	arabinoside
Petunidin 3-*O*-β-D-glucopyranoside	H	OCH_3	glucoside
Petunidin 3-*O*-β-D-galactopyranoside	H	OCH_3	galactoside
Petunidin 3-*O*-α-L-arabinopyranoside	H	OCH_3	arabinoside
Peonidin 3-*O*-β-D-glucopyranoside	CH_3	H	glucoside
Peonidin 3-*O*-β-D-galactopyranoside	CH_3	H	galactoside
Peonidin 3-*O*-α-L-arabinopyranoside	CH_3	H	arabinoside
Malvidin 3-*O*-β-D-glucopyranoside	CH_3	OCH_3	glucoside
Malvidin 3-*O*-β-D-galactopyranoside	CH_3	OCH_3	galactoside
Malvidin 3-*O*-α-L-arabinopyranoside	CH_3	OCH_3	arabinoside

Fig. 1. Chemical Structures in Bilberry Extract

* To whom correspondence should be addressed. e-mail: konishi@niigata-pharm.ac.jp

mined as an index of the anthocyanidin. Appropriate conditions were then determined as described in the following sections.

Conditions for Studying the Reactivity of Anthocyanin and ROS (a) AAPH Radicals: The reactions of an anthocyanin aqueous solution (1 mM as malvidin equivalent) and 100 mM of AAPH at pH 5.6 (0.1 M phosphate buffer) were examined for a defined period at 50 °C during which time AAPH radicals were produced through thermolysis. The reactions were observed under aerobic and anaerobic conditions. For the anaerobic reactions, the solvent was purged with argon for 20 min before the reaction and the reaction was carried out in sealed tube. The final concentration of AAPH radicals (Ri) was then calculated from the original AAPH concentrations used for the reaction according to Niki *et al.*[38] The reactions were stopped by first adding 3% TFA to the solution, then cooled with ice and centrifuged. The supernatant was analyzed by the CZE method. As a reference, the reaction of the antioxidant (+)-catechin was examined independently under the same conditions as the anthocyanin mixture.

(b) H_2O_2: The reactivity of anthocyanin aqueous solution (1 mM as malvidin equivalent) with H_2O_2 was examined at 50 °C for a defined period in the presence of 1 mM diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid monohydrate (DTPA) or ferrozine at pH 5.6 (0.1 M phosphate buffer). Since anthocyanin reacts rapidly with H_2O_2, reactivity was determined using 5 mM H_2O_2. The reaction was stopped with the addition of 1000 units of catalase followed by dilution with 3% TFA. After centrifugation the supernatant was subjected to CZE analysis.

(c) *t*-BuOOH: The reactivity of anthocyanin aqueous solution (1 mM) with 100 mM *t*-BuOOH was examined at 50 °C for a defined period. The cooling protocol was identical to that of the H_2O_2 reaction experiments. After centrifugation the supernatant was subjected to CZE analysis.

Analytical Conditions of the CZE Method CZE was carried out with a constant voltage 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).[37] The sample solutions were loaded onto the capillary with a hydrodynamic mode (25 mm×30 s). Electrophoresis was then allowed to run with an average applied voltage of +30 kV. The absorption spectra were recorded at a wavelength range of 400 to 600 nm with a time constant of 0.12 s using a photodiode array detector. Electrophoretograms were recorded at 580 nm by monitoring the wavelength. For the analysis of (+)-catechin, the detection wavelength and applied voltage were set at 240 nm and +25 kV, respectively.

Results

Reaction of Anthocyanin towards AAPH Radicals First, self-decompositions of anthocyanins at various pHs were studied. Anthocyanins were quite unstable, decomposing rapidly at neutral and alkaline pHs. Therefore, the reactions were carried out at pH 5.6. To examine the heat stability of the anthocyanins and (+)-catechin, they were kept in the reaction buffer at 50 °C without adding AAPH, and the peak-height changes were followed by CZE; but no significant decomposition was observed even after 120 min of the reaction. Heat decomposition of both the anthocyanins and (+)-catechin was, therefore, negligible under the present reaction conditions (data not shown).

The bleaching rate of the anthocyanin mixture toward AAPH radicals under anaerobic conditions was approximately half the magnitude of the rate seen under aerobic conditions (data not shown). It was thus suggested that oxygen derived secondary species such as peroxylradicals were involved in the accelerated decomposition of anthocyanin under aerobic conditions. To avoid this complexity, the following experiments were carried out under anaerobic conditions. Typical electrophoretograms of the anthocyanin mixture before and after the reactions with AAPH are shown in Figs. 2A and B. Changes in each anthocyanin peak were plotted against the reaction time in a logarithmic scale. The results showed that the decreasing rate of CZE peaks was followed, the pseudo-first order kinetics as shown in Fig. 3. When the reaction rates of anthocyanins with the same aglycon were compared, the reaction rates were similar among them (Fig. 3A). The reaction rates among anthocyanins carrying the same sugar moiety were also compared (Fig. 3B). In this case, the reaction rates were dependent on the aglycon structure; such that delphinidin glycosides disappeared faster than any other anthocyanins. Thus the aglycon structure is a major determinant for reactivity of anthocyanins towards AAPH radicals.

The rate constants for each anthocyanin towards AAPH radicals were calculated from the slopes in Fig. 3, and are summarized in Table 1. It is clearly shown that delphinidins were more reactive than cyanidins in any glycopyranosides ($p<0.01$). Furthermore, reactivity towards AAPH radicals decreased with the extent of methylation of the hydroxyl group on the B ring. The extent of the reactivities among the

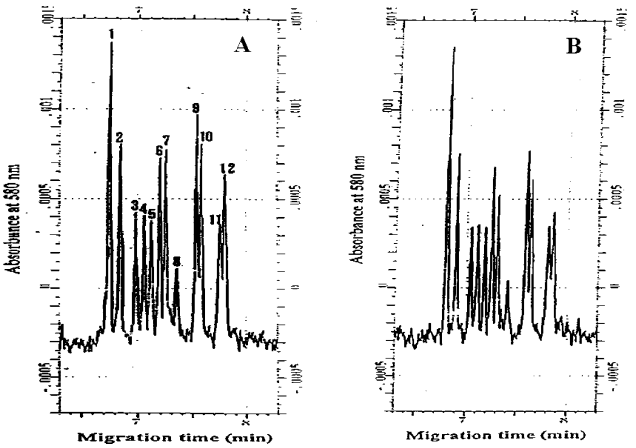


Fig. 2. Change of Electrophoretogram of Anthocyanins

(A) before the reaction, (B) after 60 min reaction with AAPH radicals. 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. Electrophoretogram was monitored at 580 nm. Peaks identified are the followings; 1 malvidin 3-*O*-β-D-glucopyranoside, 2 peonidin 3-*O*-β-D-glucopyranoside, 3 malvidin 3-*O*-β-D-galactopyranoside, 4 petunidin 3-*O*-β-D-glucopyranoside, 5 peonidin 3-*O*-β-D-galactopyranoside, 6 cyanidin 3-*O*-β-D-glucopyranoside, 7 delphinidin 3-*O*-β-D-glucopyranoside, 8 petunidin 3-*O*-β-D-galactopyranoside, 9 cyanidin 3-*O*-β-D-galactopyranoside, 10 delphinidin 3-*O*-β-D-galactopyranoside, 11 cyanidin 3-*O*-α-L-arabinopyranoside, 12 delphinidin 3-*O*-α-L-arabinopyranoside.

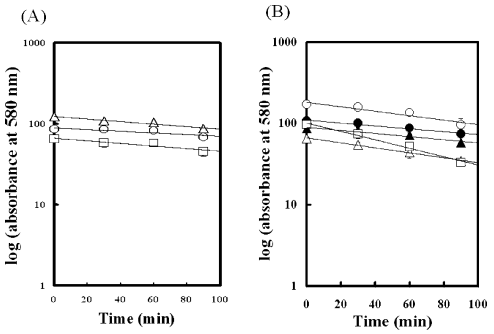


Fig. 3. Kinetic Comparison of Anthocyanidin 3-*O*-β-D-Glucopyranoside Reactivity toward AAPH Radicals

Bilberry extract (1 mM for malvidin equivalent) was reacted with AAPH for a defined period. After the reaction, the supernatant was subjected to CZE. The peak height was plotted in logarithmic scale to calculate the rate constant of each anthocyanin. (A) Sugar based comparison (in the case of cyanidin). Symbols: ○ glucoside, △ galactoside, □ arabinoside. Values are means±S.D. for three independent reactions. (B) Aglycon based comparison (in the case of glucoside). Symbols: ○ malvidin, ● peonidin, △ petundin, ▲ cyanidin, □ delphinidin. Values are means±S.D. for three independent reactions.

Table 1. Pseudo-First Order Rate Constants for the Reaction of Anthocyanins toward AAPH Radicals

($min^{-1} \times 10^3$)

Anthocyanidin	Glucoside	Galactoside	Arabinoside	(+)-Catechin
Cyanidin	1.05±0.19	1.55±0.23	1.6±0.49	3.78±0.53
Peonidin	0.85±0.31	1.17±0.44	—	
Delphinidin	2.77±0.43	3.04±0.41	3.22±0.23	
Petunidin	1.61±0.32	2.35±0.49	—	
Malvidin	1.21±0.22	1.52±0.32	—	

Bilberry extract (1 mM for malvidin equivalent) or 1 mM of (+)-catechin was reacted with AAPH for a defined period. After the reaction, the supernatant was subjected to CZE. The rate constants were obtained from the slopes of each anthocyanin as shown in Fig. 3. Values are means±S.D. for three reactions. Difference between delphinidin and other anthocyanins (cyanidin, peonidin and malvidin) was significant in all of the glycosides ($p<0.01$) and also petunidin-glucoside ($p<0.05$). Difference between (+)-catechin and anthocyanin (cyanidin, peonidin and malvidin) was significant in all of the glycosides ($p<0.01$) and also petundin glycoside ($p<0.05$). Statistical comparisons were made by Student *t*-test.

Table 2. Pseudo-First Order Rate Constants for the Reaction of Anthocyanins toward H_2O_2

($min^{-1} \times 10^3$)

Anthocyanidin	Glucoside	Galactoside	Arabinoside	(+)-Catechin
Cyanidin	5.86±0.79	6.53±0.73	5.80±0.61	0.187±0.02
Peonidin	5.74±0.39	4.8±0.54	—	
Delphinidin	5.2±0.88	5.99±0.6	5.37±0.51	
Petunidin	4.36±0.73	4.74±0.66	—	
Malvidin	4.61±0.26	4.76±0.55	—	

Values are means±S.D. for three reactions. Bilberry extract (1 mM for malvidin equivalent) or 1 mM of (+)-catechin was reacted with 5 mM H_2O_2 at 50 °C for a defined period. After the reaction, the supernatant was subjected to CZE. The rate constants were obtained from the slopes of each anthocyanin. Values are means±S.D. for three reactions. Difference between (+)-catechin and all anthocyanin was significantly different ($p<0.01$). Statistical comparisons were made with Student *t*-test.

anthocyanins carrying three hydroxyl substituents on the B ring was in the following order: delphinidin>petunidin>malvidin ($p<0.01$ in the case of delphinidin *versus* petunidin and delphinidin *versus* malvidin, $p<0.05$ in the case of petunidin *versus* malvidin). The same trend was observed for anthocyanins carrying two hydroxyl substituents on the B ring, for example, cyanidin>peonidin, although this difference was not statistically significant. It was further revealed that delphinidins showed a nearly equal reactivity towards AAPH as (+)-catechin, the reference antioxidant.

Reaction of Anthocyanins towards H_2O_2 The reaction of anthocyanins towards H_2O_2 was then examined. As shown in Table 2, all the anthocyanins showed remarkably high reactivity towards H_2O_2 compared to that of (+)-catechin ($p<0.005$ in the case of cyanidin, delphinidin, peonidin and malvidin glycopyranosides *versus* catechin, $p<0.01$ in the case of petunidin glycopyranosides *versus* (+)-catechin). Moreover, all the anthocyanins showed rather similar reactivity towards H_2O_2. This is in contrast to the reactions towards AAPH radicals. Reactivity of anthocyanins with the same sugar moiety towards H_2O_2 was in the following order: cyanidin>delphinidin>peonidin>petunidin>malvidin ($p<0.05$ in the case of delphinidin *versus* malvidin, and no significant difference between the other anthocyanins). H_2O_2 may generate hydroxyl radicals if free iron exists in the reaction mixture (Fenton reaction). Although the Bilberon sample was proven to contain only a trace amount of Fe (<10 ppm), the reactions were carried out in the presence of excess amounts of DTPA or ferrozine to ensure the reactions observed here were not due to secondarily generated hydroxyl radicals. Both the changing patterns of the CZE phoretograms and the bleaching rates of aglycon absorption at 520 nm were completely identical in the reactions with and without ion chelating reagents. It can be concluded therefore, that there is not a significant production of hydroxyl radicals during the H_2O_2 mediated-decomposition of the anthocyanins studied.

Table 3. Pseudo-First Order Rate Constants for the Reaction of Anthocyanins toward *t*-BuOOH

($min^{-1} \times 10^3$)

Anthocyanidin	Glucoside	Galactoside	Arabinoside	(+)-Catechin
Cyanidin	5.19±0.95	5.44±0.53	5.45±0.48	0.124±0.072
Peonidin	4.9±0.46	4.18±1.14	—	
Delphinidin	4.44±0.35	5.11±0.5	5.13±0.55	
Petunidin	4.54±0.92	4.53±0.6	—	
Malvidin	3.77±0.34	3.82±0.85	—	

Bilberry extract (1 mM for malvidin equivalent), or 1 mM of (+)-catechin reacted with *t*-BuOOH for a defined period. After the reaction, the supernatant was subjected to CZE. The rate constants were obtained from the slopes of each anthocyanin. Values are means±S.D. for three reactions. Difference between (+)-catechin and all anthocyanin was significantly different ($p<0.01$). Statistical comparisons were made by Student *t*-test.

Reaction of Anthocyanins towards *t*-BuOOH The reaction of anthocyanins towards *t*-BuOOH was also examined. The results are summarized in Table 3. The trend of anthocyanin reaction towards *t*-BuOOH was essentially the same as that towards H_2O_2. That is, the reactivity was not significantly affected by either aglycon structure or the type of sugar moiety. Furthermore, the anthocyanins reacted several times faster to *t*-BuOOH and H_2O_2 than (+)-catechin.

Discussion

The antioxidant properties of flavonoids, including some anthocyanins, have been studied extensively,[3,5,7,19—29] but no systematic study has been undertaken in terms of the structure–reactivity relationship of anthocyanin reactions towards ROS. As the authors previously showed, the CZE method is useful not only to profile anthocyanin distribution in plant extracts but also to follow the kinetic changes of the contents under certain reaction conditions.[39] In the present experiments, the reactions of twelve major anthocyanins present in a bilberry extract (Bilberon 25) towards ROS were quantitatively evaluated by CZE. The reactions with ROS were carried out at pH 5.6, not at the physiological pH, because anthocyanins are quite unstable at alkaline and neutral pHs. The temperature was kept at 50 °C, also not at the physiological temperature, to promote the reactions for an accurate determination of reaction rates. Temperature, however, did not affect the relative reactivity of anthocyanins with ROS.

Although the contents of each anthocyanin in Bilberon 25 samples are not identical, for example, malvidin 3-*O*-β-D-glucopyranoside presents in a quantity more than 5 times excess to petunidin 3-*O*-β-D-galactopyranoside, all the anthocyanin peaks decreased linearly in a log plot (See Fig. 3) during the reaction period up to 120 min; indicating that all the anthocyanins present in the sample followed pseudo-first order kinetics under the present reaction condition. Therefore, the relative reactivities of the twelve different anthocyanins were reliably compared using Bilberon 25 as the an-

thocyanin sample by CZE.

When the reactivity towards AAPH radicals was compared among the bilberry anthocyanins, it was discovered that the number of hydroxyl substituents on the anthocyanin B ring primarily determined the radical scavenging ability of the anthocyanins. The methylation of these hydroxyl groups led to a significant decrease in reactivity (see Table 1). Structure–activity relationships of anthocyanins for superoxide quenching reactions have been reported.[23] In those studies, the numbers of free hydroxyl groups on the anthocyanin B ring related directly to the scavenging activities. In this study, the reactivity of anthocyanins towards AAPH radicals showed a similar trend as that towards superoxide anion radicals. Since the methylation of these hydroxyl substituents reduced reactivity towards AAPH radicals, it is suggested that the AAPH radicals reacted to anthocyanins *via* hydrogen atom abstraction from the hydroxyl group(s) on the B ring as in other flavonoids.

On the other hand, the type of sugar moiety did not really affect the reactivity towards AAPH radicals. This trend is different from previous observations where acid mediated hydrolysis of the anthocyanins was shown to be governed mainly by the type of sugar moiety, not the aglycon structure.[39]

The reactivity of anthocyanins towards H_2O_2, however, was completely different from that towards AAPH radicals. Anthocyanins again showed remarkably higher reactivity than (+)-catechin, which is known to be a strong radical scavenger. The reactivity of cyanidins was a little more than that of delphinidins, but the difference was not statistically significant. Therefore it seems the B ring structure is not critical in determining the reactivity of anthocyanins towards H_2O_2.

Although the reaction rate of (+)-catechin was not determined in the presence of Bilberon 25 because the CZE peak overlapped with other ingredients in the mixture, the reaction rate was comparable to that towards AAPH radicals, or several times smaller than those for anthocyanins towards peroxides. Since the presence of competitive reactions might effect the reaction rate of (+)-catechin towards ROS, reducing rather than increasing, it was concluded that the reactivity of anthocyanins towards AAPH radical and peroxides was at least stronger than (+)-catechin.

Some chemical studies have been done on the bleaching reaction of anthocyanin to sodium sulfite[40,41] and H_2O_2.[42,43] In these studies, malvin (malvidin 3,5-*O*-β-D-diglucopyranoside) was reacted with H_2O_2 to cleave the C ring leading to malvon formation.[42,43] It is suggested that the same reactions occurred here between anthocyanidins 3-*O*-β-D-monoglycoside, and H_2O_2 in this study, in that H_2O_2 attacks the 2,3-double bond of the anthocyanidin C ring. Since (+)-catechin does not have a double bond in the 2,3-position of the C ring, the low reactivity of (+)-catechin towards peroxides can be substantiated. Another factor, which may accelerate bleaching of the anthocyanins, might be hydroxyl radicals, which must be produced by the Fenton reaction if iron is present in the bilberry extract.[44] The iron content in Bilberon 25 is approximately 1 mg/100 g (assay data by Tokiwa Phytochemical Co., Ltd.,). From this data, it was calculated that the iron concentration in the reaction mixture was less than 60 nM. Therefore, the anthocyanin decomposition reactions in the presence of excess amounts of ferrozine or DTPA (0.5 mM for ferrozine and 0.1 mM for DTPA respectively) were studied to eliminate the possible involvement of the Fenton reaction. The results clearly showed that the electrophoretogram of the reaction mixture was almost identical to that without ferrozine. It was concluded therefore, that hydroxyl radical mediated reactions were negligible in these experiments.

A question might arise that present observation is only the chemical reactivity of anthocyanins, and not their scavenging potential against ROS. However, the high reactivity of anthocyanins compared to (+) catechin as a reference strongly suggests the high potential of their scavenging ability against ROS. Moreover, Yamasaki *et al.* reported that anthocyanin bleaching was accelerated when cyanidin 3-*O*-β-D-sophoroside scavenges superoxide anion radical.[45] Clarification of the quantitative correlation between chromophore bleaching and antioxidant activity, however, has to be waited until each anthocyanin is isolated and purified.

In conclusion, the present experiments revealed that anthocyanins readily reacted, to bleach, with AAPH radicals and hydroperoxides (H_2O_2 and *t*-BuOOH), but their reactivity towards peroxides was more remarkable compared to the reactions towards AAPH radicals. The reactivity of anthocyanins was mainly governed by the aglycon structure, and not by the type of sugar moiety in both reactions; for example, delphinidins were the most reactive anthocyanin towards both AAPH radicals and peroxides. These observations will be valuable for the elucidation of the antioxidant role of anthocyanins in certain food sources.

Acknowledgements The authors would like to thank Tokiwa Phytochemical Co. Ltd. for providing Bilberon 25 and Dr. Y. Kashiwada of the Department of Pharmacognosy, Niigata University of Pharmacy and Applied Life Sciences, for providing (+)-catechin. This study was supported by a grant from the Promotion and Mutual Aid Corporation for Private schools in JAPAN.

References

1) Ficarra R., Ficarra P., Tommasini S., Calabro M. L., Ragusa S., Barbera R., Rapisarda A., *Il Farmaco.*, **50**, 245—256 (1995).
2) Sorata Y., Takahama U., Kimura M., *Biochem. Biophys. Acta*, **799**, 313—317 (1984).
3) Lamson D. W., Brignall M. S., *Altern. Med. Rev.*, **5**, 196—208 (2000).
4) Shih H., Pickwell G. V., Quattrochi L. C., *Arch. Biochem. Biophys.*, **373**, 287—294 (2000).
5) Pannala A. S., Singh S., Rice-Evans C., *Method. Enzymol.*, **299**, 207—235 (1999).
6) Zhu N., Huang T., Yu Y., LaVoie E. J., Yang C. S., Ho C., *J. Agric. Food Chem.*, **48**, 979—981 (2000).
7) Miyazawa T., Nakagawa K., *Biosci. Biotech. Biochem.*, **62**, 829—832 (1998).
8) Nakagawa K., Miyazawa T., *Anal. Biochem.*, **248**, 41—49 (1997).
9) Nakagawa K., Miyazawa T., *J. Nutr. Sci. Vitaminol.*, **43**, 679—684 (1997).
10) Nakagawa K., Okuda S., Miyazawa T., *Biosci. Biotech. Biochem.*, **61**, 1981—1985 (1997).
11) Mercier A., Perdriel G., Rozier J., Cheraleraud J., *Bull. Soc. Ophtalmol. Fr.*, **65**, 1049—1053 (1995).
12) Matsumoto H., Nakamura Y., Tachibanaki S., Kawamura S., Hirayama M., *J. Agric. Food Chem.*, **51**, 3560—3563 (2003).
13) Bomser J., Madhavi D. L., Singletary K., Smith M. A. L., *Planta Med.*, **41**, 212—216 (1996).
14) Katsube N., Iwashita K., Tsushida T., Yamaki K., Kobori M., *J. Agric. Food Chem.*, **51**, 68—75 (2003).
15) Hou DX., *Curr. Mol. Med.*, **3**, 149—159 (2003).
16) Matsui T., Ueda T., Oki T., Sugita K., Terahara N., Matsumoto K., *J. Agric. Food Chem.*, **49**, 1948—1951 (2001).
17) Matsui T., Ueda T., Oki T., Sugita K., Terahara N., Matsumoto K., *J.*

Agric. Food Chem., **49**, 1952—1956 (2001).
18) Satue-Gracia M. T., Heinomen M., Frankel E. K., *J. Agric. Food Chem.*, **45**, 3362—2267 (1997).
19) Noda Y., Kaneyuki T., Igarashi K., Mori A., Packer L., *Res. Commus. Molecul. Pathol. Pharmacol.*, **102**, 175—187 (1998).
20) Noda Y., Kneyuki T., Igarashi K., Mori A., Packer L., *Toxicology*, **148**, 119—123 (2000).
21) Tsuda T., Horio F., Osawa, T., *Lipids*, **33**, 583—588 (1998).
22) Tsuda T., Horio F., Kitoh J., Osawa T., *Arch. Biochem. Biophys.*, **368**, 361—366 (1999).
23) Tsuda T., Shiga K., Ohshima K., Kawakishi S., Osawa T., *Biochem. Pharmacol.*, **52**, 1033—1039 (1996).
24) Zheng W., Wang S. Y., *J. Agric. Food Chem.*, **51**, 502—509 (2003).
25) De Beer D., Joubert E., Gelderblom W. C., Manley M., *J. Agric. Food Chem.*, **51**, 902—909 (2003).
26) Lazze M., Pizzala R., Savio L. A., Prosperi E., Bianchi L., *Mutat. Res.*, **535**, 103—115 (2003).
27) Kahkonen M. P., Heinonen M., *J. Agric. Food Chem.*, **51**, 628—633 (2003).
28) Stintzing F. C., Stintzing A. S., Carle R., Frei B., Wrolstad R. E., *J. Agric. Food Chem.*, **50**, 6172—6181 (2002).
29) Noda Y., Kaneyuki T., Mori A., Packer L., *J. Agric. Food Chem.*, **5**, 166—171 (2002).
30) Mazza G., Kay C. D., Cottrell T., Holub B. J., *J. Agric. Food Chem.*, **50**, 7731—7737 (2002).
31) Wu X., Cao G., Prior R. L., *J. Nutr.*, **132**, 1865—1871 (2002).
32) Felgines C., Texier O., Besson C., Fraisse D., Lamaison J. L., Remesy C., *J. Nutr.*, **132**, 1249—1253 (2002).
33) Bub A., Watzl B., Heeb D., Rechkemmer G., Briviba K., *J. Nutr.*, **132**, 1249—1253 (2002).
34) Matsumoto H., Inaba H., Kishi M., Tominaga S., Hirayama M., Tsuda T., *J. Agric. Food Chem.*, **49**, 1546—1551 (2001).
35) Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., Rice-Evans C., *Free Radic. Biol. Med.*, **26**, 1231—1237 (1999).
36) Pannala A., Chan T., Brien P., Rice-Evans C., *Biochem. Biophys. Res. Commun.*, **282**, 1161—1168 (2001).
37) Ichiyanagi T., Tateyama C., Oikawa K., Konishi T., *Biol. Pharm. Bull.*, **23**, 492—497 (2000).
38) Niki E., *Methods Enzymol.*, **186**, 100—108 (1990).
39) Ichiyanagi T., Oikawa K., Tateyama C., Konishi T., *Chem. Pharm. Bull.*, **49**, 114—117 (2001).
40) Leonard J., *J. Food Sci.*, **29**, 16—19 (1967).
41) Swain T., E. Hillis., *J. Sci. Food Agric.*, **10**, 63—68 (1959).
42) Karrer P., Widmer R., Helfenstein A., Hurliman W., Nievergelt O., Monsarrat-Thoms P., *Helv. Chim. Acta*, **10**, 729—752 (1927).
43) Karrer P., Meuron G., *Helv. Chim. Acta*, **15**, 507—512 (1932).
44) Ali M. A., Ph. D. Thesis (1999).
45) Yamasaki H., Uefuji H., Sakihama Y., *Arch. Biochem. Biophys.*, **332**, 183—186 (1996).