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Antioxidant Activity of Black Currant Anthocyanin Aglycons and Their Glycosides Measured by Chemiluminescence in a Neutral pH Region and in Human Plasma

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The antioxidant activity of nine anthocyanin glycosides was measured in a neutral pH region using a chemiluminescence (CL) emission system in the presence of an H_2O_2−acetaldehyde system, and the intensities were found to be affected by three factors, pH value and both moieties of the aglycon and C-3 sugar. With an increase in pH from 4.0 to 9.0, the CL intensities increased from pH 5.0, reached their maxima at pH 6.0−7.0, and decreased at pH 9.0. Comparison of the intensities among the 3-glucosides with five different aglycons and the 3-glycosides with three different sugar moieties at C-3 showed that their strongest intensities were given by the delphinidin aglycon and 3-rutinosyl moiety, respectively. Monitoring of the CL intensity of human blood plasma for 8 h after oral administration of black currant anthocyanins (BCA) showed a rapid increase until 2 h, and a significant difference ($P < 0.05$) was recognized at 1−8 h.

KEYWORDS: Anthocyanin; antioxidative activity; black currant; delphinidin; rutinoside; chemiluminescence; photon emission; plasma

INTRODUCTION

Dietary anthocyanins have attracted a lot of attention due to their health-promoting benefits in terms of reducing the risk of coronary heart disease and preventing some chronic diseases (*1*, *2*). Recently, we reported that black currant anthocyanins (BCA), prepared from black currant fruits, have improving effects on dark adaptation and video display terminal (VDT) work-induced transient refractive alternation in a human study (*3*), and four anthocyanin components of BCA were absorbed into the blood after oral intake (*4*).

The beneficial effects are supposed to associate with their antioxidative properties, and the evaluation of the structure−activity relationship has been one of the main targets. However, there has been little comparative study on the structure−activity of anthocyanin glycosides because of the lack of purified samples, although almost all of their natural anthocyanins exist as glycosides and are used as dietary anthocyanins. There have been two interesting reports regarding extending the structure to flavonoid 3-glycosides that describe different results about the structure−antioxidant activity of quercetin 3-glycosides. In a comparison of the activity among quercetin (Qu), isoquercetin (Qu3Glc), and rutin (Qu3Rut), Hopia et al. (*5*) reported that the order was Qu > Qu3Glc > Qu3Rut by a method of oxidizing methyl linoleate, but an entire reverse order of Qu < Qu3Rut was obtained by our chemiluminescence (CL) system (*6*). The activity of anthocyanins is also supposed to be affected by the pH. With the change of pH, anthocyanins are well-known to change to five different equilibrium forms, the flavylium cation, carbinol pseudobase, chalcone, quinoidal base, and quinoidal anion, and the equilibrium distribution is reported to change according to the structural effect of the sugar moiety at C-3 (*7*). However, there has been little information of the pH influence on the antioxidant activity of anthocyanins.

Recently, we developed a preparative scale isolation of four components of BCA, delphinidin 3-rutinoside (Dp3Rut), delphinidin 3-glucoside (Dp3Glc), cyanidin 3-rutinoside (Cy3Rut), and cyanidin 3-glucoside (Cy3Glc) (*9*), and these purified components may be useful for the elucidation of their structure−activity relationship. Furthermore, we developed an imaging system for hydroperoxide and hydrogen donors by photon emission (*8*). This photon emission system was found to be applicable to solid samples regardless of the water solubility (*8*), and this result suggests the possibility of a direct application for the measurement of biomaterials such as blood and organs.

This paper presents an investigation into the antioxidant activity of nine anthocyanins including purified BCA by a CL system in a neutral pH region, and the relative activities were compared among the 3-glucosides with five different aglycons

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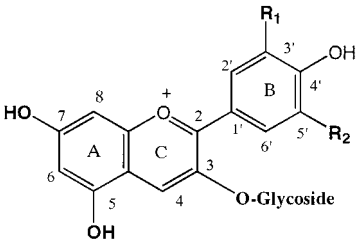

	R_1	R_2
Delphinidin	-OH,	-OH
Cyanidin	-OH,	-H
Pelargonidin	-H,	-H
Malvidin	$-OCH_3$,	$-OCH_3$
Peonidin	$-OCH_3$,	-H

Figure 1. Chemical structures of anthocyanin 3-glycosides.

and the 3-glycosides with three different sugar moieties of C-3 glycosylation. The antioxidant activity of human blood after oral intake of BCA was also determined as the photon emission.

MATERIALS AND METHODS

Materials. Gallic acid was purchased from Nakarai Tesque Co. Ltd. (Kyoto, Japan). Dp3Rut, Dp3Glc, Cy3Rut, and Cy3Glc were prepared from commercial black currant juice according to the methods described in a previous report (*9*). Cyanidin 3-galactoside (ideanin chloride, Cy3Gal), pelargonidin 3-glucoside (callistephin chloride, Pg3Glc), malvidin 3-glucoside (oenin chloride, Mv3Glc), malvidin 3-galactoside (Mv3Gal), and peonidin 3-glucoside (Pn3Glc) were purchased from Extrasynthese S.A. (Lyon, France). A powdered BCA concentrate was analyzed and found to contain Dp3Rut (5.09%), Dp3Glc (1.48%), Cy3Rut (3.76%), and Cy3Glc (0.50%) (*9*). The anthocyanins used are flavylium chlorides unless otherwise stated, and their chemical structures are summarized in **Figure 1**. Buffer solutions were prepared as follows: 1 M HCl−1 M CH_3COONa buffer (pH 4.0 and 5.0), 50 mM Na_2HPO_4−50 mM KH_2PO_4 buffer (pH 6.0 and 7.0), and 50 mM $Na_2B_4O_7$−100 mM HCl (pH 8.0 and 9.0).

CL Measurement. The CL intensity was measured using a chemiluminescence detector model CLD-110 (Tohoku Electronic Industrial Co., Ltd., Sedndai, Japan), based on a single photon counting method, connected to a Waters model 510 pump and U6K injector (Waters Co., Milford, MA). A sample solution of 50% methanol diluted with each pH buffer containing 1.5% H_2O_2 and 5% acetaldehyde was used as the mobile phase, its flow rate and temperature being set to 1 mL/min and 23 °C, respectively. Five microliters of each sample solution (1 μM) in 50% methanol solution was injected. The dispersed light at the grating was simultaneously detected on the photocathode with the image sensor set at wavelengths from 300 to 650 nm. The photons counted at the respective wavelengths were computed totally as spectral intensities in counts per second (cps).

Plasma Samples of Human Blood. Three healthy male volunteers participated in this study, which was performed according to the Helsinki Declaration. On the day before the experiment, the subjects did not consume any food rich in anthocyanin (vegetables, fruits, juice, etc.). They did not ingest any food or beverages except for water in the 12 h period prior to the experiment. On the day of the experiment, each subject orally took a BCA concentrate (33.0 mg/kg body weight) in water (150 mL), corresponding to 2.75 mmol (1.68 mg) as Dp3Rut, 2.08 mmol (1.24 mg) as Cy3Rut, 1.04 mmol (0.488 mg) as Dp3Glc, and 0.37 mmol (0.165 mg) as Cy3Glc per kilogram body weight. Blood samples were intravenously collected at 0.5, 1.0, 2.0, 4.0, and 8.0 h postadministration, and the plasma samples were prepared as whole blood following a method described in a previous report (*4*).

Photon Emission Intensity and Anthocyanin Measurement of Plasma. Photon emission (PE) intensity was detected using charge-coupled device (CCD) camera (Hamamatsu Photonics, Hamamatsu, Japan) in the same manner as described in a previous report (*8*). The reaction mixture contained hydroperoxide (0.5 mL), plasma samples (0.5 mL), and saturated $KHCO_3$ in 356 mmol/L of MeCHO (0.5 mL). The reaction mixtures were added into a 12-hole multiplate (i.d. 12 mm; total volume 2.0 mL) in the following order: hydroperoxide, $KHCO_3$ in 356 mmol/L of MeCHO, and the sample solution. Photon intensity, imaging with pseudocolor, is converted into luminance (cd/m^2) according to the standard bar. The identification and quantification of the four anthocyanins were performed using an HP 1100 series HPLC system (Hewlett-Packerd Co., Wilmington, DE) by the method described in our previous study (*4*). Significant difference was assessed by one-way ANOVA with Turkey's test to compare the individual means. P-values <0.05 were regarded as significant.

Intact Effect of Four BCA Components on the PE Intensity in Human Plasma. After a 2 week BCA administration, a washout plasma of one subject was prepared in a manner similar to that for a postadministration plasma. The intact effect was measured by a direct addition of four purified components of BCA into the plasma. Each washout plasma sample shown after 0.5, 1.0, 2.0, 4.0, and 8.0 h was prepared by addition of a respective amount of four BCA components, which had been quantified by HPLC analysis in a postadministration study. PE intensity was measured in a similar manner as described above.

Table 1. Chemiluminescence Intensity of Anthocyanins (5 pmol) in the Presence of Hydrogen Peroxide at the Neutral pH Range (cps)

	pH					
sample	4.0	5.0	6.0	7.0	8.0	9.0
GA	0.709	0.766	1.814	4.616	1.389	0.567
Dp3Glc	0.583	0.518	2.271	2.937	0.301	1.002
Dp3Rut	0.818	1.381	2.028	5.994	2.158	1.465
Cy3Glc	0.000	0.145	1.098	0.744	0.225	0.128
Cy3Gal	0.485	0.211	0.938	0.550	0.225	0.516
Cy3Rut	0.000	0.420	1.262	1.726	0.776	0.631
Pg3Glc	0.298	0.172	0.940	1.158	0.423	0.157
Mv3Glc	0.718	0.223	0.546	0.649	0.239	0.172
Mv3Gal	0.890	0.564	0.195	1.162	0.480	0.172
Pn3Glc	0.599	0.217	0.631	1.529	0.167	0.000

RESULTS

CL Measurement of Anthocyanins. The CL intensity of five commercial anthocyanins and four purified components of BCA was measured in an H_2O_2−acetaldehyde system at each of six points in a neutral pH region from 4.0 to 9.0. The result is summarized in **Table 1** as the CL intensity of each 5 pmol, showing that the intensity was influenced by the pH value. All nine anthocyanins were found to have maximal intensity at pH 6.0−7.0. Using the data of five anthocyanin 3-glucosides (Dp3Glc, Cy3Glc, Pg3Glc, Mv3Glc, and Pn3Glc), the structural effect of the aglycon moiety on the CL intensity is compared in **Figure 2A**, showing different intensities depending on the aglycons. Dp3Glc showed the highest intensity among five 3-glucosides. The other structural effect of the sugar moiety at C-3 on the CL intensity is compared in **Figure 2B,C** using the data of three cyanidin 3-glycosides (Cy3Glc, Cy3Gal, and Cy3Rut) and two delphinidins (Dp3Glc and Dp3Rut), respectively. Their highest intensities appeared at pH 6.0−7.0, and both 3-rutinosides showed a higher intensity than the 3-glucosides of the corresponding aglycon.

PE Intensity and Anthocyanin Measurement of Human Plasma. The PE intensity of human plasma was measured in an H_2O_2−acetaldehyde system. **Figure 3A** shows the time course of change in the PE intensity of the plasma of three subjects after oral administration of the BCA concentrate. The PE intensity increased during 0−2 h postadministration, reaching maxima at 2−4 h, and maintaining a higher level at 8 h than those at 0 h. Statistical analysis of their average intensities (mean ± SD) compared with that at 0 h (2.010 ± 0.381) showed that

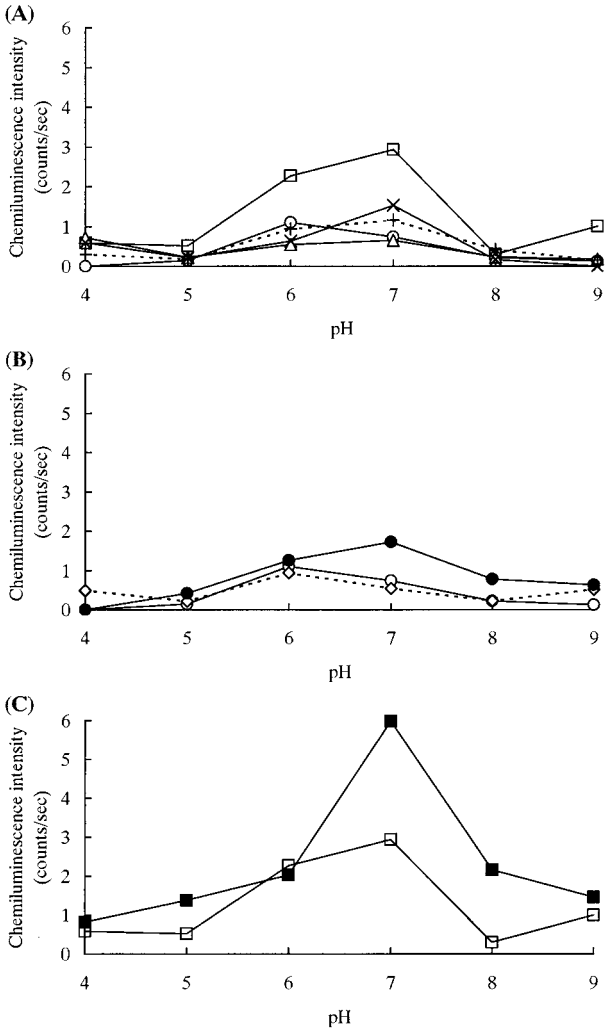

Figure 2. Chemiluminescence intensities of anthocyanin 3-glycosides in a neutral pH region: (A) anthocyanin 3-glucosides with five different aglycons; (B) cyanidin 3-glycoside with three different C-3 sugars; (C) delphinidin 3-glycoside with two different C-3 sugars. Symbols: Dp3Glc (□); Dp3Rut (■); Cy3Glc (○); Cy3Gal (◇); Cy3Rut (●); Pg3Glc (+); Mv3Glc(△); Pn3Glc (×).

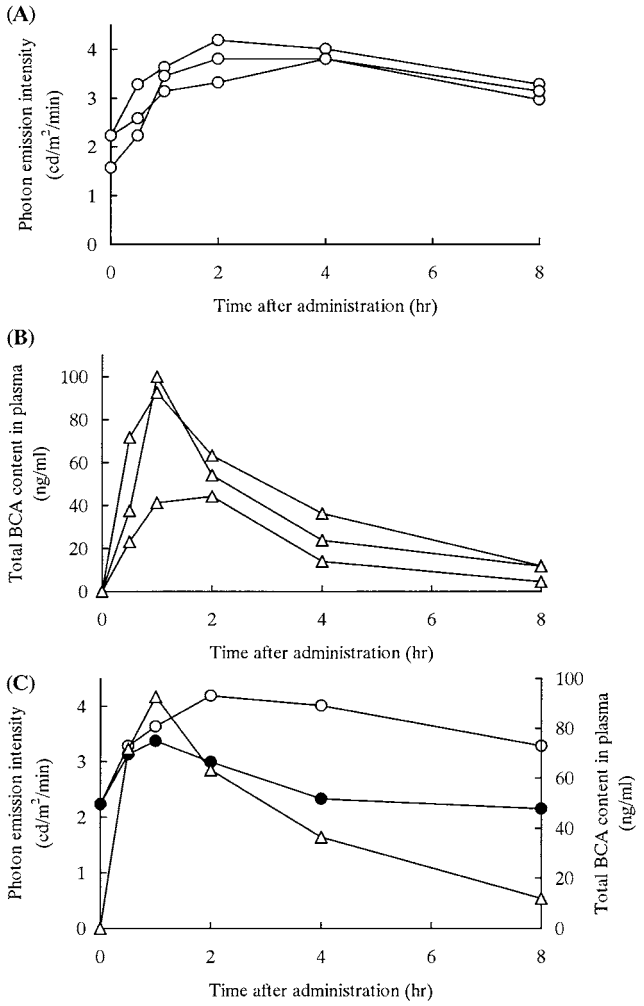

Figure 3. Time course of change in CL intensity and amount of BCA in plasma: (A) CL intensity of postadministration plasma (○, $n = 3$); (B) amount of BCA in postadministration plasma (△, $n = 3$); (C) comparison of CL intensity of postadministration plasma (○) with amount of BCA in postadministration plasma (△) and CL intensity of washout plasma (●) of one subject.

significance was recognized at the later time points of 1−8 h [$P < 0.01$ after 1 h (3.401 ± 0.248), $P < 0.01$ after 2 h (3.770 ± 0.436), $P < 0.01$ after 4 h (3.870 ± 0.121), and $P < 0.05$ after 8 h (3.130 ± 0.155)]. Amounts of four BCA components (Dp3Rut, Cy3Rut, Dp3Glc, and Cy3Glc) in plasma were monitored by HPLC after administration, and the result is shown in **Figure 3B**. Four components of BCA in plasma were observed to have their maximal concentrations at 1−2 h after oral administration of BCA concentrate and then rapidly decreased to about one-fifth of the maximum amounts at 8 h, in a pattern similar to that of our previous findings (*4*).

The intact effect of four purified BCA components on the PE intensity in human plasma was measured in vitro by a direct addition of them into washout plasma after 2 week BCA administration, and the result (closed circle) is shown in **Figure 3C**, together with an in vivo study of postadministration plasma showing the PE intensity (open circle) and the amount of BCA quantified by HPLC analysis (triangle). Comparison among three shapes of their time-course changes revealed two characteristics as follows. The PE intensity of postadministration plasma increased until 2 h postadministration, had a maximum at 2 h, then decreased gradually, and still maintained at higher level at 8 h compared with that at 0 h. On the other hand, the PE intensity of washout plasma and amount of BCA increased until 1 h, had maxima at 1 h, and then decreased rapidly to return to an almost similar level of 0 h at 8 h.

DISCUSSION

For the evaluation of foods and biomaterials, it is important to measure their biomarkers in a neutral pH region. The antioxidant activity of nine anthocyanin glycosides in a neutral pH region was successfully measured by a CL emission system in the presence of an H_2O_2−acetaldehyde system, and the intensities were found to be affected by three factors, pH value and both moieties of the aglycon and C-3 sugar.

With the increase of pH value from 4.0 to 9.0, the CL intensities increased from pH 5.0, had their maxima during pH 6.0−7.0, and decreased at pH 9.0, as shown in **Table 1**. This result suggests that the CL intensities might correlate with the equilibrium forms of the anthocyanins. Regarding the influence of the equilibrium forms on the properties of anthocyanins, Cabrita et al. (*10*) made interesting observations using six anthocyanin 3-glucosides as follows: their colors, absorption intensities, and stability changed significantly in the pH 1−12

range, and their absorption intensities increased above pH 5.0 in a neutral pH region and reached their maxima at pH 9.0−9.5 in alkaline solutions. They also reported that the increase of the absorption intensities corresponded mainly to their quinoidal base and quinoidal anion form. In the present study, the CL intensities were observed to have their individual maxima only at pH 6.0−7.0, increasing from pH 5.0 and decreasing at pH 9.0, as shown in **Figure 2A**. This result suggests that the increase of the CL intensity might be associated with only the increase of the quinoidal base form in the equilibrium distribution of the anthocyanins.

The structural effect of aglycon moieties on the CL intensity was compared among five anthocyanins having the same glucose moiety at C-3 as shown in **Figure 2A**, which seems to show that the delphinidin aglycon apparently yields a higher intensity than the other four. Another structural effect of the sugar moiety at C-3 was also compared using three cyanidin and two delphinidin 3-glycosides, indicating that both of the 3-rutinosides had higher intensities at pH 7.0 than the corresponding 3-glucosides (see **Figure 2B,C**). Compared with previous studies on the antioxidant activity of quercetin 3-glycosides, the present result is in reverse order as measured by a method of oxidizing methyl linoleate (*5*) but is in good agreement with our previous findings using a CL system, showing that the 3-rutinoside had a higher CL intensity than the 3-glucoside (*6*). These results suggest that the presence of the rutinosyl moiety at C-3 may be associated with the strong CL of flavonoid 3-glycosides.

This photon emission system successfully measured the reactive oxygen scavenging activity of human blood plasma at pH 7.4 (*11*), and the PE intensity after oral administration of BCA concentrate was monitored for 8 h as shown in **Figure 3A**. The intensities increased rapidly until 2 h postadministration and then decreased gradually. The statistical significance of their average intensities compared with that at 0 h was recognized at the later time points of 1−8 h. Plotting the amount of four BCA components in the plasma (see **Figure 3B**), measured by HPLC analysis, showed a rapid increase until 1 h postadministration and then decreased rapidly at the later time points of 2−8 h. Comparison of the shapes between the PE intensities and amount of BCA showed an apparent difference at the latter time points of 2−8 h, at which the PE intensities were still maintained at higher levels.

To measure the intact effect of BCA components on the PE intensity in the plasma, the same amount of four purified BCA components, which were quantified by HPLC analysis, was directly added to washout plasma, and the PE intensities were plotted to give the closed circles in **Figure 3C**. Compared with the PE intensity of postadministration plasma (open circle) and the amount of BCA (triangle), the curve shape was found to be similar to the latter but to be different from the former. Similarity with the latter can be understood as the result that the intact effect of four BCA components on the PE intensity appeared in proportion to their amount in human plasma. Regarding the difference from the former, we are interested in the maintenance of the PE intensities of postadministration plasma in higher levels at 2−8 h, but we have no clear experimental evidence yet to explain the reason. However, we assume one possibility that BCA components are converted into some metabolites having strong PE intensities, because Tsuda et al. (*12*) had already reported that Cy3Glc was easily metabolized after oral ingestion in rats to afford protocatechuic acid in the plasma.

ABBREVIATIONS USED

BCA, black currant anthocyanins; CL, chemiluminescence; VDT, video display terminals; Qu, quercetin; Qu3Glc, quercetin 3-*O*-*β*-glucoside; Qu3Rut, quercetin 3-*O*-*β*-rutinoside; Dp3Rut, delphinidin 3-*O*-*β*-rutinoside; Dp3Glc, delphinidin 3-*O*-*β*-glucoside; Cy3Rut, cyanidin 3-*O*-*β*-rutinoside; Cy3Glc, cyanidin 3-*O*-*β*-glucoside; Cy3Gal, cyanidin 3-*O*-*β*-galactoside; Pg3Glc, pelargonidin 3-*O*-*β*-glucoside; Mv3Glc, malvidin 3-*O*-*β*-glucoside; Mv3Gal, malvidin 3-*O*-*β*-galactoside; Pn3Glc, peonidin 3-*O*-*β*-glucoside; PE, photon emission; CCD, charge-coupled device; HPLC, high-performance liquid chromatography.

LITERATURE CITED

(1) Renaud, S.; de Logeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.

(2) Morazzoni, P.; Bombardelli, E. *Vaccinium myrtillus* L. *Fitoterapia* **1996**, *67*, 3−29.

(3) Nakaishi, H.; Matsumoto, H.; Tominaga, S.; Hirayama, M. Effects of blackcurrant anthocyanoside intake on dark adaptation and VDT work-induced transient refractive alternation in healthy humans. *Altern. Med. Rev.* **2000**, *5*, 553−562.

(4) Matsumoto, H.; Inaba, H.; Kishi, M.; Tominaga, S.; Hirayama, M.; Tsuda, T. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. *J. Agric. Food Chem.* **2001**, *49*, 1546−1551.

(5) Hopia, A.; Heinonen, M. Antioxidant activity of flavonol aglycones and their glycosides in methyl linoleate. *J. Am. Oil Chem. Soc.* **1999**, *76*, 139−144.

(6) Yoshiki, Y.; Okubo, K.; Onuma, M.; Igarashi, K. Chemiluminescence of benzoic and cinnamic acids, and flavonoids in the presence of aldehyde and hydrogen peroxide or hydroxyl radical by fenton reaction. *Phytochemistry* **1995**, *39*, 225−229.

(7) Mazza, G.; Brouillard, R. Recent developments in stabilization of anthocyanins in food products. *Food Chem.* **1987**, *25*, 207−225.

(8) Yoshiki, Y.; Iida, T.; Akiyama, Y.; Okubo, K.; Matsumoto, H.; Sato, M. Imaging of hydroperoxide and hydrogen peroxide-scavenging substances by photon emission. *Luminescence* **2001**, *16*, 327−335.

(9) Matsumoto, H.; Hanamura, S.; Kishi, M.; Kawakami, T.; Sato, Y.; Hirayama, M. Preparative-scale isolation of four anthocyanin components of black currant (*Ribes nigrum* L.) fruits. *J. Agric. Food Chem.* **2001**, *49*, 1541−1545.

(10) Cabrita, L.; Fossen, T.; Andersen, Ø. M. Colour and stability of the six common anthocyanidin 3-glucosides in aqueous solutions. *Food Chem.* **2000**, *68*, 101−107.

(11) Rees, W. D.; Jenkins, H. S. T. pH profile of gut as measured by radiotelemetry capsule, *Br. Med. J.* **1972**, *2*, 104−106.

(12) Tsuda, T.; Horio, F.; Osawa, T. Absorption and metabolism of cyanidin 3-*O*-*β*-D-glucoside in rats. *FEBS Lett.* **1999**, *449*, 179−182.

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