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Characterization and structures of anthocyanin pigments generated in rosé cider during vinification

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Abstract

Anthocyanin pigments, which are not found in apple juice, were detected in rosé cider. We confirmed by HPLC/DAD and LC/ESI–MS analyses that some of these anthocyanin pigments generated in rosé cider during vinification corresponded to those formed in model cider containing anthocyanin and flavan-3-ol in the presence of acetaldehyde. To confirm their structures, two anthocyanin pigments formed in a model cider containing cyanidin-3-galactoside and (−)-epicatechin in the presence of acetaldehyde were isolated and purified, and their structures were elucidated by high resolution FAB–MS and ^{1}H and ^{13}C NMR analyses. These two pigments were found to consist of cyanidin-3-galactoside and (−)-epicatechin linked by a CH_3–CH bridge at the 8-position. They were diastereomers that differed in the configuration of the asymmetric methine carbon.

Keywords: Rosé cider; Model cider; Cyanidin-3-galactoside; (-)-Epicatechin; Acetaldehyde; LC/ESI–MS; ^{1}H and ^{13}C NMR

1. Introduction

Apple cider and wine contain many kinds of polyphenols such as phenol carboxylic acid derivatives, flavan-3-ols, proanthocyanidins, chalcones, anthocyanins, and flavonoids. These compounds are very important in terms of taste, color, flavor, and longevity (Cheynier et al., 1998). Among these properties, the color of rosé cider and red wine is associated with the kinds and contents of anthocyanins present. Furthermore, anthocyanins continuously change from monomeric anthocyanins to polymeric anthocyanins during the vinification process (fermentation, maturation, bottling, and aging) (Somers, 1971; Timberlake and Bridle, 1976; Shoji et al., 1999). However, the properties and structures of polymeric anthocyanins generated during vinification remain largely unexplored.

In our previous studies, we separated the anthocyanin pigments in apple juice and rosé cider by size-exclusion chromatography (SEC) and detected several peaks, that were not found in apple juice, in fractions from rosé cider by reversed phase HPLC analyses (Shoji et al., 1999; Yanagida et al., 1999). Since only the earlier fractions from rosé cider contained these peaks, we thought that they were polymeric anthocyanins with larger molecular size and were generated in rosé cider during vinification.

In this study, to confirm the formation of several anthocyanin pigments generated in rosé cider during vinification, we prepared a model cider containing cyanidin-3-galactoside (**1**), a main anthocyanin in rosé cider, and other apple polyphenols in the absence or presence of acetaldehyde. HPLC/DAD and LC/ESI–MS analyses revealed that several main anthocyanin pigments generated in rosé cider corresponded to those generated in a model cider containing (**1**) and (−)-epicatechin (**2**) in the presence of acetaldehyde. Therefore, we isolated two pigments from the model cider and investigated their structures by FAB/MS, and ^{1}H and ^{13}C NMR analyses.

2. Results and discussion

Anthocyanins from rosé cider were separated using SEC with a mixture of acetone and 8 M urea (pH 2.0) as

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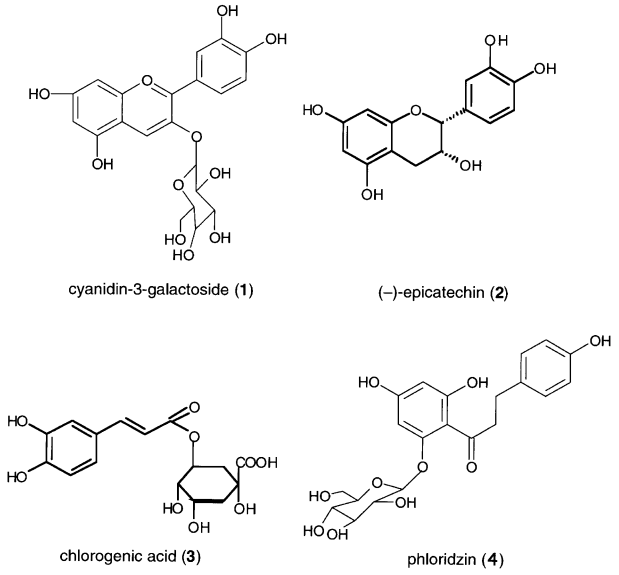

the eluent and we obtained five fractions according to the elution volume. The HPLC profiles recorded at 520 nm for the SEC fractions separated from rosé cider are shown in Fig. 1. We named the main peaks RC-1–RC-3, respectively. Of these peaks, RC-1 was identified as cyanidin-3-galactoside (**1**), which is the main anthocyanin in apples, based on direct comparison with an authentic sample. The other peaks were considered to be novel pigments generated during vinification because they were detected in rosé cider but not in apple juice. The HPLC/DAD data of several peaks are shown in Table 1. The λ_{max} of RC-1 [cyanidin-3-galactoside (**1**)] was 518 nm, whereas those of RC-2 and RC-3 were shifted to a higher range. We previously considered that the change in the color of rosé cider from reddish orange to bluish pink was due to the formation of new anthocyanin pigments during vinification (Shoji et al., 1999).

Table 1
Retention time and spectra data of rosé cider and model cider measured by HPLC/DAD

Peak No.	Retention time (min)	λmax (nm)
Rosé Cider		
RC-1 (cyanidin-3-galactoside)	22.2	278, 518
RC-2	49.8	276, 449, 522
RC-3	64.9	280, 450, 530
Model Cider		
EC-C1 (5)	49.4	282, 450, 528
EC-C2 (6)	64.7	284, 450, 532

Rosé cider from apples contains cyanidin-3-galactoside (**1**), chlorogenic acid (**3**), phloridzin (**4**), flavan-3-ols [e.g. (+)-catechin and (−)-epicatechin (**2**)], and procyanidins [e.g. procyanidin B1 and procyanidin B2], as main polyphenols (Mazza and Velioglu, 1992; Suárez et al., 1996). Several authors have reported that in red wine, the polymerization reactions involve the condensation of polyphenols or anthocyanins with acetaldehyde, which is generated by yeast during fermentation (Timberlake and Bridle, 1976; Bakker et al., 1993; Rivas-Gonzalo et al., 1995; Dallas et al., 1996a,b; Francia-Aricha et al., 1997; Es-Safi et al., 1999), and they proposed that the polymeric pigments comprised anthocyanin and polyphenol condensed with acetaldehyde.

We performed LC/ESI–MS analyses with monitoring of ions at m/z 765, 829, and 912, which correspond to the $[M+H]^+$ of the products of the condensation of **1** and **2**, **3** and **4** linked by a CH_3–CH bridge from acetaldehyde, respectively. The results of LC/ESI–MS analyses of rosé cider with monitoring at m/z 765 are shown in Fig. 2. Four peaks were observed in the respective chromatograms. However, no peaks were observed with monitoring at m/z 829 and 912.

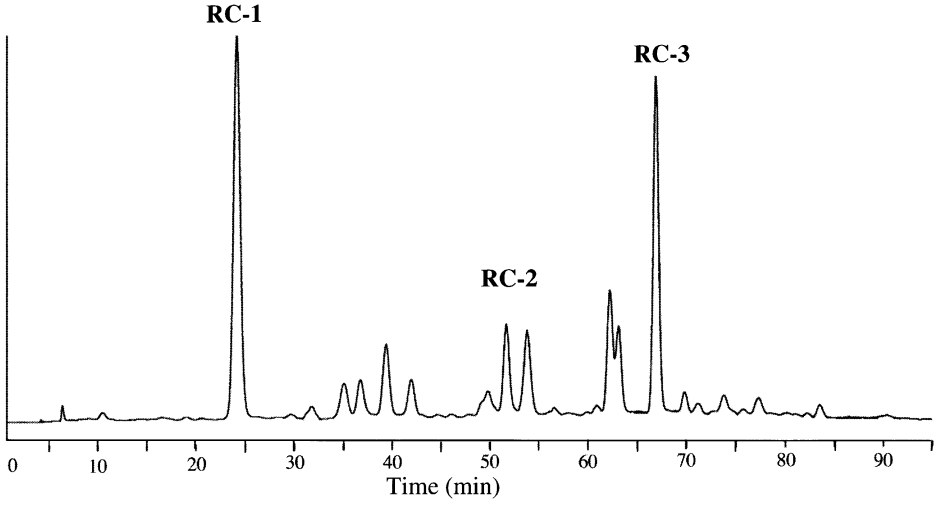

Fig. 1. HPLC profiles of SEC fractions obtained from rosé cider recorded at 520 nm. The peak (RC-1) with retention time of 22 min is **1**. RC-2 and RC-3 were generated during vinification.

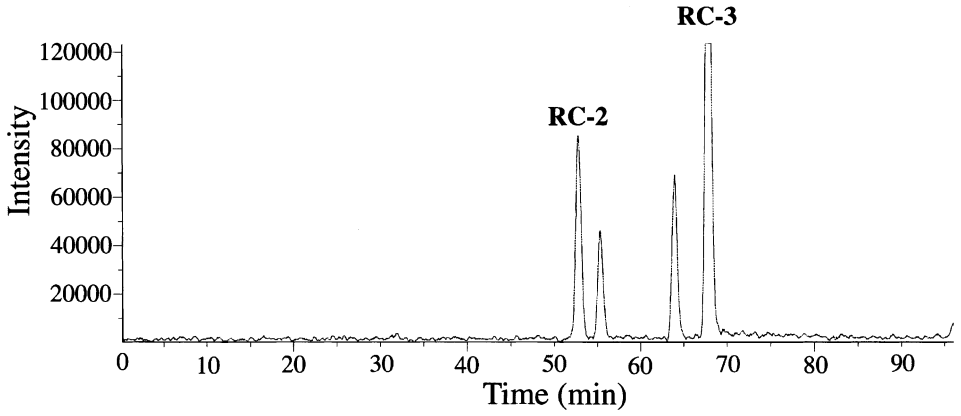

Fig. 2. LC/ESI–MS analysis of SEC fraction obtained from rosé cider. The chromatograms show m/z values (765 m/z).

Since rosé cider contains many kinds of polyphenols or anthocyanins found in apples as well as other non polyphenolic compounds (e.g. acetaldehyde) found in juice and/or cider, the polymerization reactions in rosé cider are complicated. Therefore, we prepared model cider solutions containing cyanidin-3-galactoside (**1**) and one of the other main apple polyphenols (**2**, **3** and **4**) in the absence or presence of acetaldehyde, and the resulting reaction products were compared to the polymeric anthocyanins generated in rosé cider (Shoji et al., 2002).

Regardless of the presence of acetaldehyde, in the reactions between **1** and **3** or **4**, or with **1** alone, while compound **1** slowly was decomposed during storage, new pigments were not detected. Similarly, in the reaction between **1** and **2** in the absence of acetaldehyde, the concentration of **1** slowly decreased but new pigments were not detected. However, in reaction between **1** and **2** in the presence of acetaldehyde, we detected several major pigments, EC-C1 (**5**) and EC-C2 (**6**), as shown in Fig. 3. Timberlake and Bridle (1976) postulated that polymerization starts with the protonation of acetaldehyde, followed by electrophilic substitution of polyphenol. Since the electron density is highest at the 8 position of **2**, the carbocation preferentially attacks the 8-position. However, since **3** and **4** show an electron density with low stability, polymerization does not start.

EC-C1 (**5**) and EC-C2 (**6**) showed retention times of 49.4, and 64.7 min, respectively, and their LC/ESI–MS analyses showed a molecular ion peak at m/z 765. The λ_{max} of **5** and **6** were shifted to a higher value than that of **1** (518 nm), and a shoulder at around 450 nm indicated a CH_3–CH bridge, as reported by Francia-Aricha et al. (1997). Two corresponding peaks (RC-2 and RC-3), with the same retention times, spectral data (Table 1), and molecular ions, were present in the chromatogram of rosé cider. Since these data were identical to those of **5** and **6** in the model cider, RC-2 and RC-3 were considered to be the same as **5** and **6**, respectively.

Among several peaks in the chromatogram of rosé cider, RC-1, RC-2, and RC-3 were identified to be **1**, EC-C1 (**5**), and EC-C2 (**6**), respectively in the model cider, and this suggested that these new anthocyanin pigments generated in rosé cider were composed of **1** and **2**, and are linked by a CH_3–CH bridge derived from acetaldehyde. Es-Safi et al. (1999) reported the condensation products generated in a model red wine solution containing malvidin-3-glucoside and (+)-catechin in the presence of acetaldehyde, and their structures

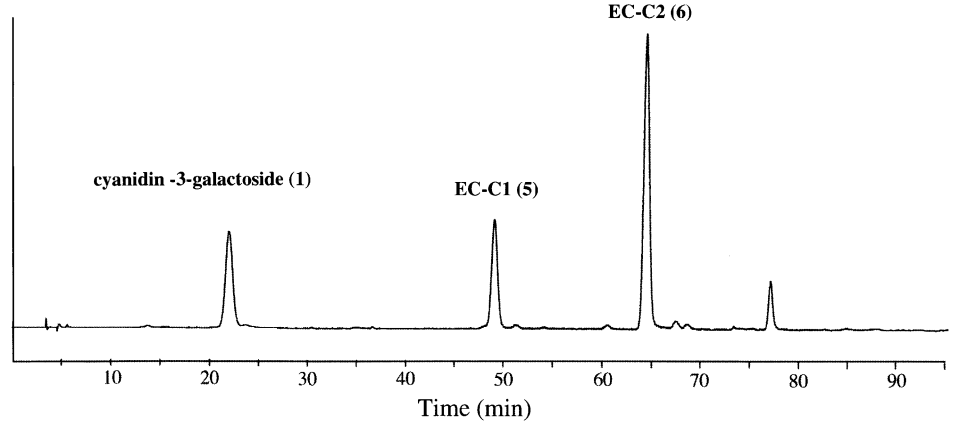

Fig. 3. HPLC profiles of model cider containing compounds **1** and **2** with acetaldehyde recorded at 520 nm. The peak with retention time of 22 min is **1**, and other peaks are new pigments EC-C1 (**5**) and EC-C2 (**6**), which are produced during storage.

were confirmed by thiolysis and LC/ESI–MS analyses. However, the condensation products were not detected in red wine. Our findings are the first unambiguous confirmation of the condensation products of anthocyanins and flavan-3-ols with acetaldehyde in rosé cider.

EC-C1 (**5**) and EC-C2 (**6**) generated in the model cider were purified by preparative HPLC, and the structures of these new pigments were studied. High resolution FAB–MS analyses of both new pigments showed a molecular ion peak $[M+H]^+$ at m/z 765, and the molecular formula $C_{38}H_{37}O_{17}$ (EC-C1, observed: 765.1989; calculated: 765.2031; EC-C2, observed: 765.1996; calculated: 765.2031) was confirmed by results of elemental analysis. These results agreed well with the molecular ion peak obtained by LC/ESI–MS. The ^1H and ^{13}C NMR spectroscopic data of both new pigments were obtained in a solvent mixture of CD_3OH and 20% HCl (9:1), and were almost identical (Tables 2 and 3). The typical coupling constants of the B rings of **1** and **2**, the C-ring of **2** and the galactosyl unit of **1**, and the deshielded sharp signal assignable to the 4-position of **1** are retained in the ^1H NMR spectra of both **5** and **6**. In addition, CH_3–CH signals and two singlets derived from the A ring of **1** and **2** were observed. These data suggested that **5** and **6** were composed of cyanidin-3-galactoside and (−)-epicatechin units linked through a CH_3–CH bridge, and that each unit was connected to the CH_3–CH bridge at either the 6- or 8-position of the A-ring. The ^{13}C NMR spectra were also consistent with this structure.

To determine the positions linked by the CH_3–CH bridge, 1D difference NOE and HMBC experiments were performed. When the methyl signal was irradiated, NOE was observed at the signals of the corresponding 2′- and 6′-positions of the B ring of the cyanidin-3-galactoside unit in both **5** and **6**. However, NOE was not observed with any signal of the (−)-epicatechin unit. Therefore, an HMBC experiment was performed to determine the position at which the CH_3–CH bridge was linked to the (−)-epicatechin unit.

In the HMBC spectra of both **5** and **6**, the methine proton signal (**5**: δ 5.30; **6**: δ 5.49) at the CH_3–CH bridge position was correlated to the carbon signal (**5**: δ 154.16; **6**

Table 2
^1H-NMR spectral data for EC-C1 and EC-C2 (dH in CD_3OH/HCl = 9:1)

Position	5, δ (ppm)	6, δ (ppm)
Cyanidin		
4	8.81 *s*	8.87 *s*
6	6.65 *s*	6.63 *s*
2′	7.83 *d* (2)	7.85 *d* (2)
5′	6.92 *d* (8)	6.91 *d* (8)
6′	8.06 *dd* (2,8)	8.08 *dd* (2,8)
Galactose		
G1	5.26 *d* (8)	5.28 *d*
G2	4.04 *dd* (8,9)	4.04 *dd* (8,9)
G3	3.78 *dd* (9,3)	3.78 *dd* (3,9)
G4	4.01 *d* (3)	4.02 *br. d* (3)
G5	3.90 *br. dd* (5,7)	3.92 (*m*)
G6a	3.87 *d* (7,11)	3.88 (*m*)
G6b	3.82 *dd* (11,5)	3.85 (*m*)
CH_3CH bridge		
CH	5.30 *d* (8)	5.49 *d* (8)
CH3	1.69 *d* (8)	1.73 *d* (8)
(-)-Epicatechin		
2	4.36 *s*	4.63 *s*
3	4.09 *br.dd* (3,4)	4.05 *dd* (3,4)
4a	2.65 *dd* (17,3)	2.81 *dd* (17,4)
4b	2.78 *dd* (4,17)	2.69 *dd* (17,3)
6	6.14 *s*	6.15 *s*
2′	6.10 *s*	6.09 *s*
5′	6.23 *d* (8)	6.28 *d* (8)
6′	6.34 *d* (8)	6.05 *d* (8)

Table 3
^{13}H-NMR spectral data for EC-C1 and EC-C2 in CD_3OH/HCl and CD_3OD/DCl)

Position	EC-C1 in CD_3OH/HCl δ (ppm)	EC-C2 in CD_3OH/HCl δ (ppm)	EC-C2 in CD_3OD/DCl δ (ppm)
Cyanidin			
2	162.93	163.06	163.04
3	144.97	145.38	145.20
4a	134.67	134.84	134.75
5	156.27	156.34	156.65
6	103.94	104.27	104.08
7	167.54	167.14	166.87
8	112.85	112.77	112.67
8a	154.16	153.40	152.62
1′	121.33	121.31	121.30
2′	118.21	118.36	118.24
3′	146.67	146.58	146.35
4′	154.79	154.97	154.67
5′	116.89	116.82	116.72
6′	128.10	128.40	128.40
Galactose			
G1	103.28	103.34	103.21
G2	72.17	72.21	72.01
G3	74.75	74.87	74.64
G4	70.04	70.09	69.91
G5	77.32	77.44	77.36
G6	62.24	62.27	62.10
CH_3CH bridge			
CH	26.96	26.48	26.43
CH_3	19.60	19.33	19.31
2	79.88	80.55	80.49
3	65.98	66.60	66.42
4	30.08	28.85	28.82
4a	100.89	100.70	100.57
5	155.97	156.28	156.11
6	96.74	96.83	
7	154.70	154.83	–
8	109.51	108.48	–
8a	155.57	155.30	155.28
1′	131.10	131.52	131.51
2′	115.86	115.00	114.91
3′	145.37	145.20	114.91
4′	145.61	145.20	145.16
5′	116.14	116.20	116.10
6	120.26	119.53	119.54

δ 153.40) at the 8a-position of the cyanidin-3-galactoside unit, which was assigned by the correlation of the proton signal (**5**: δ 8.81; **6**: δ 8.87) at the 4-position. These results strongly indicated that the CH_3–CH bridge was linked to the cyanidin-3-galactoside unit of both pigments at the 8-position, not the 6-position, on the A-ring.

In the HMBC spectrum of **5**, the methine signal (δ 5.30) of the CH_3–CH bridge was correlated to the carbon signal (δ 155.57) at the 8a-position of the (−)-epicatechin unit, which was assigned by the correlation of the proton signal (δ 4.36) at the 2 position of the (−)-epicatechin unit. This indicated that the connecting position of **5**, which was linked by the CH_3–CH bridge, was the 8-position on the A-ring of the (−)-epicatechin unit.

In the case of **6**, however, a correlation between the proton signal (δ 4.63) at the 2-position of the (−)-epicatechin unit and the corresponding quaternary carbon signal at the 8a-position of the (−)-epicatechin unit was not observed in the HMBC spectrum. Therefore, we measured the ^{13}C NMR spectra of **6** in a solvent system of CD_3OD/DCl to observe an isotopic shift and to determine the carbon signal at the 8a-position of the (−)-epicatechin unit (Table 3). Among the three quaternary carbons assignable to the 5-, 7-, or 8a-positions of the (−)-epicatechin unit, only the carbon signals at δ 155.30 (CD_3OH/HCl) and δ 155.28 (CD_3OD/DCl) did not show a significant isotopic shift. This indicated that the carbon signal must be assigned the 8a-position. The HMBC spectrum obtained in CD_3OH/HCl showed that the methine proton signal (δ 5.49) of the CH_3–CH bridge was correlated to the carbon signal at the 8a-position of EC-C2 (**6**). These results showed that the connecting position that was linked by the CH_3–CH bridge to the (−)-epicatechin unit was the 8-position on the A-ring of the (−)-epicatechin unit as well as the cyanidin-3-galactoside unit.

Consequently, the results of NOE and HMBC experiments showed that both the cyanidin-3-galactoside and (−)-epicatechin moieties of compounds **5** and **6** were linked by a CH_3–CH bridge at the 8-position on the A-ring, and that **5** and **6** were diastereomers that differed in the configuration of the asymmetric carbon at the position of the CH_3–CH bridge (Fig. 4). Furthermore, the circular dichroism (CD) spectra of cyanidin-3-galactoside (**1**), (−)-epicatechin (**2**) and compounds **5** and **6** were measured in 0.01% HCl–MeOH. The spectrum of compound **1** scarcely gave any sign of a CD band, whereas that of compound **2** displayed a weakly positive band at about 240 nm and a negative band at about 280 nm, as reported by Korver and Wilkins (1971) and Rensburg et al. (1999). However, compounds **5** and **6** exhibited strong absorption bands at 280, 450 and 520 nm. The CD bands of compound **5** were all positive, but those of compound **6** were all negative. These results indicated that compounds **5** and **6** are diastereomers with ethylidene stereocenter derived from acetaldehyde skeleton.

Polymeric anthocyanins in red wine have been proposed to be generated by direct condensation reactions between anthocyanins and various other wine components (Liao et al., 1992; Santos-Buelga et al., 1995; Fulcrand et al., 1996, 1998; Bakker et al., 1997), and indirect reactions between anthocyanins and polyphenols which involve acetaldehyde (Timberlake and Bridle, 1976; Baranowski and Nagel, 1983; Bakker et al., 1993; Garcia-Viguera et al., 1994; Rivas-Gonzalo et al., 1995; Dallas et al., 1996a,b; Francia-Aricha et al., 1997; Es-Safi et al., 1999). Numerous products formed by direct condensation reactions during vinification have recently been characterized and identified by HPLC/DAD, LC/MS, and NMR spectroscopic analyses (Bakker et al., 1997; Bakker and Timberlake, 1997; Fulcrand et al., 1996, 1998). In the case of indirect condensation reactions, purple violet pigments were detected in studies using a model solution. However, the structures of the pigments in red wine have not been confirmed; the positions in anthocyanins and flavan-3-ols or procyanidins that are linked by the CH_3–

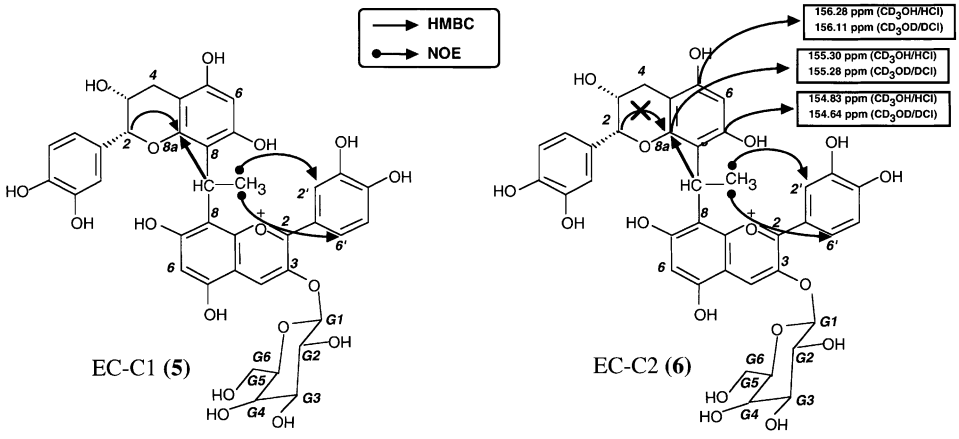

Fig. 4. Structures of anthocyanin pigments **5** and **6** in model cider.

CH bridge have not been resolved by spectroscopic analyses (^{1}H and ^{13}C NMR). Timberlake and Bridle (1976) proposed that in the reaction of anthocyanin and (+)-catechin with acetaldehyde, the 6- or 8-position on the A-ring of anthocyanin or (+)-catechin is probably involved in connection of the CH_3–CH bridge.

Anthocyanins and flavan-3-ols contain two reactive centers, the 6- and 8-positions, and therefore it is reasonable to expect that four new pigments would be generated in the model solution. Recently, Es-Safi et al. (1999) using thiolysis and LC/MS showed that since no pigments that contained more than two anthocyanin units were detected in the model solution, only one position on the A-ring of anthocyanins is involved in the connection of the CH_3–CH bridge. Our results with NMR spectral analyses and those of Es-Safi et al. (1999) reveal that only the 8-position of cyanidin-3-galactoside is involved in the connection of the CH_3–CH bridge. In this report, we have unambiguously demonstrated that two new anthocyanin pigments in rosé cider are linked at the 8-position of (−)-epicatechin by a CH_3–CH bridge, and are diastereomers that differ in the configuration of the asymmetric methine carbon.

3. Experimental

^{1}H (600 MHz); ^{13}C NMR (150 MHz); 1D–NOE and HMBC: UV–visible and CD spectra in 0.01% HCl–MeOH.

3.1. Materials

Cyanidin-3-galactoside (**1**) was isolated from apple juice using a Diaion HP-20 (Mitsubishi Kasei Co., Ltd., Japan) column and a preparative ODS column (FS 1830 FT, Organo Co., Ltd., Japan). The purity of **1** was confirmed to be greater than 95% by reversed-phase HPLC. Compound **2** was a commercial sample obtained from Sigma Chemical Co., Ltd. (USA), compound **3** was purchased from Wako Pure Chemical Industries Ltd. (Japan), and compound **4** was obtained from Funakoshi Co., Ltd. (Japan).

Unless otherwise stated, all other reagents and chemicals used were obtained commercially extra pure grade.

3.2. Preparation of polyphenol fractions from rosé cider

Rosé cider was obtained as previously described (Shoji et al., 1999). After fermentation, the cider was centrifuged, and allowed to stand at 4 °C in a CO_2 atmosphere for 6 months. To obtain polyphenol fractions containing anthocyanins, rosé cider was passed through a preparative ODS packed column. After washing the column with distilled water, polyphenols were eluted with 60% MeOH. The eluate was concentrated by rotary evaporation at 45 °C and lyophilized as polyphenol fractions. Furthermore, SEC of the lyophilized polyphenol fractions (300 mg) was performed according to the methods of Shoji et al. (1999) using a Toyopearl HW 40 (F) (Tosoh Co., Ltd., Japan) column with a 6:4 mixture of acetone and 8 M urea (adjusted to pH 2.0 with HCl) as the eluent. To remove urea, anthocyanins and other polyphenols were recovered from the fractions using a C_{18} Sep-Pak cartridge (Waters Associates, Japan). Finally, the adsorbed compounds were eluted with MeOH. The eluate was concentrated by rotary evaporation at 45 °C and lyophilized.

3.3. HPLC/DAD analysis

Anthocyanins were analyzed using an HPLC/DAD equipped with an L-6200 intelligent pump (Hitachi Ltd., Japan), an AS-2000 autosampler (Hitachi), and a 991J diode array detector (Waters). An Inertsil ODS-3 (GL Sciences Inc., Japan) reversed-phase column (250×4.6 mm i.d.) was used at 30 °C. A mixture of 5% formic acid solution and MeOH was used as the mobile phase, and the flow rate was 1.0 ml/min. The eluent used was 15% MeOH for the first 15 min, followed by a linear gradient from 15 to 38% MeOH for 90 min, and then increasing to 50% in 5 min. The concentration was held at 50% MeOH for 10 min and then returned to the initial conditions to re-equilibrate for 10 min. UV–visible spectra were recorded from 250 to 650 nm using a DAD, and detection was performed using an L-4200 UV–vis detector (Hitachi) at 520 nm.

3.4. LC/ESI–MS

Mass measurements were performed on a Jeol LC-Mate. The mass spectrometer was operated in positive ion mode. The ion spray voltage was set at +50. HPLC separation was performed as described above for HPLC/DAD analysis.

3.5. Model rosé cider

The model rosé cider basal solution was a mixture of 10% (v/v) EtOH–4.5 g/l malic acid adjusted to pH 3.5. Compound **1**, other apple polyphenols (**2**, **3** and **4**), and acetaldehyde were dissolved in the model cider basal solution at a final concentration of 0.2, 1.0, and 35.8 mM, respectively. The preparations were placed in reaction vials and stored in the dark at 4 °C.

3.6. Purification of new anthocyanin pigments from model rosé cider

The model cider containing **1** and **2** in the presence of acetaldehyde was maintained at 4 °C. After 60 days of

storage, the chromatographic area of the peaks corresponding to the novel pigments **5** and **6** by reversed phase HPLC reached a maximum, and the model cider was treated to prevent degradation of the new pigments and to stop the polymerization reaction. The model cider was applied to a preparative ODS packed column and eluted with 30% MeOH–0.01 N HCl. Crude new anthocyanin pigments were obtained in highly purified form using an Inertsil PREP-ODS (GL Sciences) packed column. The purity of the new pigments was confirmed to be greater than 95% by reversed-phase HPLC.

3.7. NMR analysis

^{1}H and ^{13}C NMR spectra were measured at 30 °C with a Jeol A-600 spectrometer using 3 mm tubes with CD_3OH/HCl or CD_3OD/DCl (9/1) as the solvent. ^{1}H and ^{13}C chemical shifts are given in ppm relative to TMS as an internal standard. HMBC analyses confirmed the assignments of the proton and carbon signals of the new anthocyanin pigments from the model cider.

3.8. EC-C1 (**5**)

Bluish red powder. UV–visible λ_{max} (0.01% HCl–MeOH) nm: 286 (3.33), 534 (3.55), 445 (3.02). ^{1}H NMR see Table 1; ^{13}C NMR see Table 2. CD: $[\phi]_{285}$ 3757, $[\phi]_{450}$ 4452, $[\phi]_{534}$ 4427.

3.9. EC-C2 (**6**)

Bluish red powder. UV–visible λ_{max} (0.01% HCl–MeOH) nm: 287 (3.24), 450 (2.96), 536 (3.33). ^{1}H NMR see Table 1; ^{13}C NMR see Table 2. CD: $[\phi]_{287}$ −42490, $[\phi]_{450}$ −12070, $[\phi]_{538}$ −21670.

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