Anthocyanin Pigments of Wild and Seibel No. 13053 Grapes Harvested in Tokachi*†

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Anthocyanin pigment contents in three strains (A, B and C) of wild grapes in Tokachi district and Seibel No. 13053 grapes were respectively 71.9, 37.4, 97.8 and 64.3 mg per 100 g of fresh weight in terms of delphinidin-3-monoglucoside.

From these grapes, 12 anthocyanins were isolated and identified as follows: 3-monoglucosides and 3,5-diglucosides of delphinidin, petunidin, malvidin and peonidin; delphinidin-3-monoglucoside and -3,5-diglucoside acylated with *p*-coumaric acid; peonidin-3,5-diglucoside and malvidin-3-monoglucoside acylated with *p*-coumaric acid.

The species of wild grapes are discussed from the viewpoint of these pigment patterns mentioned above.

Introduction

Tokachi wine is popular in Japan. The species of wild grapes from which this wine is produced had been reported to belong to *Vitis amurensis*¹⁾ that has only 3-monoglucoside pigments. Later on, IWANO²⁾ changed his opinion and claimed that this grapes belongs to *V. coignetiae* because of the presence of 3,5-diglucosides. As far as one can judge from his reports, he seems to have misidentified the anthocyanins and unknown RIBÉREAU-GAYON's papers.

RIBÉREAU-GAYON^{3), 4)} reported on the distribution of anthocyanins among different species of the genus *Vitis* and its application for chemotaxonomy. He proved the presence of 3-glucosides of malvidin, petunidin and peonidin, and 3,5-diglucosides of malvidin and peonidin, and the absence of acylated anthocyanins in *V. amurensis*. However, the pigment pattern of *V. coignetiae* was not referred to in his reports.

In this paper, the results of investigation of the anthocyanin pigments of wild grapes in Tokachi district and Seibel No. 13053

are reported, with a discussion of the species of wild grapes.

Materials and Methods

Grapes: Ripe grapes (3 kg) of three wild strains (A, B and C) and Seibel No. 13053 (S-13053) were obtained in autumn, 1975, from the vineyard of Tokachi-Ikeda Viticulture and Enology Experiment Station in Hokkaido.

Determination of soluble sugar and acid contents: Grape juice was prepared by cold pressing and then centrifuged at 10,000 rpm for 20 min at 0°C. The soluble sugar content in the grape juice (100 ml) was determined by a modified Somogyi method⁵⁾, calculated in terms of glucose. Acid was titrated with alkali (0.1N NaOH) up to pH 8.2 with Methorom Potentiography E436, calculated in terms of tartaric acid.

Preparation of pigment solution: Each 200 g of grapes was macerated in a Waring blender with 500 ml of 0.1% HCl-methanol solution for 5 min. The mixture was filtered in a Buchner funnel through filter paper. The process was repeated five more times with the same solvent until filtrates became

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colorless. After combining those extracts, crude anthocyanin solution was prepared by the basic lead acetate method⁶⁾.

Paper chromatography (PPC) and thin layer chromatography (TLC): The solvent systems for PPC and TLC were as follows. iBAW, isobutanol-acetic acid-water (8:2:3, v/v); BAW, *n*-butanol-acetic acid-water (4:1:5, v/v); 1% HCl, conc. HCl-water (3:97, v/v); Forestal, acetic acid-water-conc. HCl (30:10:3, v/v); FHW, formic acid-conc. HCl-water (5:2:3, v/v); BHW, *n*-butanol-conc. HCl-water (7:2:5, v/v); BPW, *n*-butanol-pyridine-water (6:3:1, v/v); 2% AcOH, acetic acid-water (2:98, v/v), and BzAW, benzene-acetic acid-water (2:2:1, v/v).

Toyo No. 50 filter paper (40×40 cm) was used for separation of pigments with iBAW solvent system. Purification of individual anthocyanins (IAcy) was carried out by the same method except for paper size (20×20 cm, ascending manner) with the following solvent system; AHW, BAW and 1% HCl. Two-dimensional chromatography was done on a thin layer (Avicel cellulose) plate (20×20 cm) with iBAW as the first solvent and AHW as the second.

Identification of IAcy: Identification of anthocyanin was carried out by usual methods (comparison of color and *R_f* values, absorption spectroscopy, partial acid hydrolysis^{7),8)}, alkaline degradation of aglycone¹³⁾ and other chromatographic techniques).

Determination of anthocyanin content in grapes: Crude anthocyanin pigments from 20 g of grapes were treated with basic lead acetate reagent⁶⁾ and diluted to 250 ml with 0.1% HCl-methanol solution. Anthocyanin content was determined by measuring absorbance at 543 nm (λ_{max}), calculated in terms of delphinidin-3-monoglucoside; $\epsilon_{543nm}^{0.1\%HCl-MeOH} = 2.9 \times 10^4$.

IAcy contents were analyzed in accordance with the previous reports⁷⁾⁸⁾.

Results and Discussion

1. Soluble sugar and acid

Contents in juices A, B, C and S-13053 were respectively as follows: sugar in terms of glucose: 11.40, 10.60, 18.50 and 12.45 g/100

ml; acid in terms of tartaric acid: 2.94, 2.98, 2.35 and 1.44 g/100 ml; sugar/acid ratio: 3.88, 3.56, 7.87 and 8.65.

S-13053 had a high sugar/acid ratio suitable for grape juice. A and B had larger acid quantities than the others. C was the most suitable for winemaking because of its large content of sugar.

2. Two-dimensional TLC of anthocyanins

Two-dimensional TLC from three wild and S-13053 grapes indicated the presence of 12 anthocyanins shown in Fig. 1.

Fig. 1 Two-dimensional thin layer chromatograms of anthocyanins in grapes (A, B, C and Seibel No. 13053)

Pigments Nos. 1 and 3-1 were the most abundant pigments with bluish purple colors. Pigments Nos. 3-2, 4 and 7-B proved to be 3,5-diglucoside pigments, since they emitted fluorescence under ultra-violet radiation.

Pigments Nos. 1, 2, 3-1, 5, 7-A and 8-1 turned blue after being sprayed with molybdate reagent, which provides information on the presence of ortho hydroxylation in the B-ring.

3. Chromatographic properties of IAcy

The *R_f* values in four solvent systems, and colors under visible and u.v. light are given in Table 1.

Table 1 Comparison of R_f values and colors of individual anthocyanins in grapes

Pigment No.	R_f values ($\times 100$) at 23°C				Colors* in		
	iBAW	BAW	AHW	1% HCl	visible light	(H-V-C)	u.v. light
1	9	16	30	8	Pale purple	(21-18-3)	Bright purple
2	15	24	32	8	Pale purple	(22-18-3)	Bright purple
3-1	19	25	18	3	Pale purple	(20-18-3)	Dull purple
3-2	20	30	40	12	Light red purple	(23-16-6)	Fluor. purple
4	25	35	43	15	Dull red purple	(2-16-5)	Fluor. pink
5	28	34	22	4	Pale purple	(21-18-4)	Dull purple
6-1	35	38	28	6	Red purple	(23-15-6)	Dull purple
6-2	38	39	33	9	Light red	(1-17-6)	Dull purplish red
7-A	40	41	31	12	Pale purple	(21-18-4)	Bright purple
7-B	42	41	42	11	Light red	(1-17-6)	Fluor. pink
8-1	48	51	10	3	Pale purple	(20-18-4)	Dull purple
8-2	50	52	21	3	Light red purple	(22-17-4)	Light purple

Fluor.: Fluorescent

*: Classified in accordance with "Guide to Color Standard," (1951)¹².

The comparison of these data with previous reports^{7,8,10,11} indicated that pigments Nos. 1, 2, 3-1, 3-2, 4, 5, 6-1 and 6-2 were delphinidin-3,5-diglucoside (DI-3,5DG, abbreviation in Table 4), Pt-3,5DG, DI-3G, Mv-3,5DG, Pn-3,5DG, Pt-3G, Mv-3G and Pn-3G*, respectively. Pigments Nos. 7 and 8 seemed to be acylated anthocyanins, since they showed larger R_f values in organic solvent systems (iBAW and BAW) and smaller R_f values in aqueous solvent system (AHW).

4. Spectral properties of IAcY

The absorption maxima, the ratios of the absorbance at 440 nm to the maxima of IAcY and the adding effect of aluminium chloride

on these spectra are listed in Table 2.

The presence of shoulder peaks (about 308 nm) in the spectra of pigments Nos. 7-A, 7-B, 8-1 and 8-2 showed that these pigments were acylated anthocyanins.

The absorbance ratio ($E_{440}/E_{\max}$) can be used for differentiating 3-monoglucoside (3G) from 3,5-diglucoside (3,5DG). Therefore, pigments Nos. 1, 2, 3-2, 4, 7-A and 7-B were found to be 3,5DG pigments because of their smaller values than 18. The other pigments proved to be 3G pigments.

The absorption peak (pigments Nos. 1, 2, 3-1, 5, 7-A and 8-1) shifted when aluminium chloride was added as a chelating agent.

Table 2 The absorption spectra of individual anthocyanins in grapes

Pigment No.	λ max in 0.01% HCl-MeOH (nm)		$E_{440}/E_{\text{vis. max}}(\%)$ Shift with AlCl ₃	
1	280	535	13	+
2	279	533	11	+
3-1	275	536	20	+
3-2	280	533	12	-
4	280	523	13	-
5	278	534	20	+
6-1	280	535	27	-
6-2	280	525	20	-
7-A	283	308 sh 534	11	+
7-B	280	308 sh 526	11	-
8-1	280	308 sh 538	25	+
8-2	280	308 sh 538	16	-

sh: Shoulder

*: Abbreviations are referred to in Table 4.

This indicates the presence of ortho hydroxy groups in the B-ring.

5. Partial acid hydrolysis

The chromatograms of the hydrolysis products developed with BHW and AHW solvent systems indicated that pigments Nos. 3-1, 5, 6-1 and 6-2 were 3-glucoside pigments which yielded two spots and that Nos. 1, 2, 3-2 and 4 were 3,5-diglucoside pigments which yielded four spots. Pigments Nos. 7-A and 7-B gave five spots on each chromatogram, and Nos. 8-1 and 8-2 gave three spots.

6. Aglycones, sugar moieties and acyl moieties

The aglycones were identified by their *R_f* values and colors on the paper as follows: pigments Nos. 1, 3-1, 7-A and 8-1 were delphinidin (*R_f*; 0.33 in forestal, 0.15 in FHW, dull purple), Nos. 2 and 5 were petunidin (*R_f*; 0.47 in forestal, 0.22 in FHW, purple), 0.59 in forestal, 0.26 in FHW, red purple), and Nos. 4, 6-2 and 7-B were peonidin (*R_f*; 0.65 in forestal, 0.33 in FHW, pink).

The sugars in IAcy were determined by PPC of acid hydrolysis products. Glucose (*R_f*; 0.28 and 0.34 in BAW and BPW, respectively) was the only sugar isolated from IAcy.

Acid hydrolysis^{(8), (14)} can remove acyl groups

from the anthocyanin. The acyl moieties in pigments Nos. 7-A, 7-B, 8-1 and 8-2 were identified to be *p*-coumaric acid (*R_f*; 0.92 in BAW, 0.67 in BzAW and 0.44 in 2% AcOH, orange color after spraying with diazotized *p*-nitro-aniline reagent).

7. Alkaline degradation of the aglycone

The results of alkaline degradation of aglycones (except Nos. 7-A, 7-B, 8-1 and 8-2) are given in Table 3, which shows that aglycones were delphinidin (Nos. 1 and 3-1), petunidin (Nos. 2 and 5), malvidin (Nos. 3-2 and 6-1) and peonidin (Nos. 4 and 6-2).

8. Conclusion

On the basis of the above observation, the anthocyanins in three wild and S-13053 grapes were identified as listed in Table 4. The amounts of IAcy are given in Table 5. Besides, total anthocyanin contents were calculated in terms of D1-3G. It was concluded that C grape was the most suitable fruit for winemaking because of its high contents of sugar and pigments.

These grapes were characterized as follows; (1) the presence of acylated delphinidin glucosides was first found in grapes harvested in Japan and (2) delphinidin and petunidin contents were greater than malvidin and peonidin contents that were detected as the major pigments in previous reports^{(7), (8), (10), (11)}.

Table 3 *R_f* values and color characteristics of alkaline degradation products of individual anthocyanidins in grapes (except phloroglucinol)

Pigment No.	<i>R_f</i> values (×100)		Color with DPNA*+NH ₃
	BAW	2%AcOH	
1	67	42	Gray brown
2	77	50	Red purple
3-1	67	42	Gray brown
3-2	87	57	Blue
4	80	61	Purple
5	78	50	Red purple
6-1	87	57	Blue
6-2	80	60	Purple
Gallic acid	67	42	Gray brown
Vanillic acid	80	61	Purple
Syringic acid	87	57	Blue
Protocatechuic acids	81	54	Dull purple
<i>p</i> -Coumaric acid	90	43	Gray brown

*: Sprayed with diazotized *p*-nitroaniline reagent

Table 4 Identification of the anthocyanins in grapes (A, B, C and Seibel No. 13053)

Pigment No.	Identification	Abbreviation
1	Delphinidin-3,5-diglucoside	(Dl-3,5DG)
2	Petunidin-3,5-diglucoside	(Pt-3,5DG)
3-1	Delphinidin-3-monoglucoside	(Dl-3G)
3-2	Malvidin-3,5-diglucoside	(Mv-3,5DG)
4	Peonidin-3,5-diglucoside	(Pn-3,5DG)
5	Petunidin-3-monoglucoside	(Pt-3G)
6-1	Malvidin-3-monoglucoside	(Mv-3F)
6-2	Peonidin-3-monoglucoside	(Pn-3G)
7-A	Delphinidin-3,5-diglucoside acylated with <i>p</i> -coumaric acid	(Dl-3,5DG-Cm)
7-B	Peonidin-3,5-diglucoside acylated with <i>p</i> -coumaric acid	(Pn-3,5DG-Cm)
8-1	Delphinidin-3-monoglucoside acylated with <i>p</i> -coumaric acid	(Dl-3DG-Cm)
8-2	Malvidin-3-monoglucoside acylated with <i>p</i> -coumaric acid	(Mv-3G-Cm)

Table 5 Anthocyanin content of fresh grapes (A, B, C and Seibel No. 13053)

Acy*	mg/100 g berry	Cy**		Pn**		Dl**		Pt**		Mv**		T. acylated**	
		3	3,5	3	3,5	3	3,5	3	3,5	3	3,5	3	3,5
A	71.9	—	—	8	14	22	12	14	8	2	—	20	trace
B	37.4	—	—	10	14	29	14	13	10	2	3	3	2
C	97.8	—	—	7	13	22	12	12	11	8	10	3	2
S-13053	64.3	—	—	11	12	21	15	11	10	6	12	2	trace
<i>V. amurensis</i> ³⁾		—	—	13	15	—	—	5	—	27	40	—	—
<i>V. coignetiae</i> ²⁾		—	+	+	+	+	—	+	—	+	+	—	—

* : Anthocyanin content, **: Cy: cyanidin, Pn: peonidin, Dl: delphinidin, Pt: petunidin, Mv: malvidin, 3:3-monoglucoside, 3,5:3,5-diglucoside, T. acylated: total acylated pigments.

RIBÉREAU-GAYON^{3),4)} represented that *V. amurensis* was characterized by the absence of acylated anthocyanin, and cyanidin- and delphinidin-glucosides. IWANO^{1),2)} seems to have not identified to acylated anthocyanin and there were many interrogation points on his methods and results. Our results were not in accord with either the pigment patterns of *V. amurensis*^{3),4)} or of *V. coignetiae*²⁾. Consequently, we presume that this grapes belong to a variety of *V. amurensis*, probably *V. amurensis* Rupr. var. *glabrescens* NAKAI.¹⁵⁾

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十勝産・野生ブドウおよび Seibel No. 13053 ブドウのアントシアニン色素

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十勝地方の野生ブドウ3系統(A, B および C)とSeibel No. 13053 ブドウ中のアントシアニン色素含量は、デルフィニジン-3-モノグルコシドとして、各々、71.9, 37.4, 97.8 および 64.3 mg/100 g 新鮮重であった。

12コのアントシアニン色素が単離され、以下のように同定された。デルフィニジン、ペチュニジン、マルビジン、ペオニジンの3-モノグルコシドおよび3,5-ジグル

コシド、カクマル酸によってアシル化されたデルフィニジン-3-モノグルコシドと-3,5-ジグルコシド、そしてカクマル酸によってアシル化されたペオニジン-3,5-ジグルコシドとマルビジン-3-モノグルコシド。

上述の色素パターンから、本野生ブドウの種属する種について論議された。

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