Anthocyanin profile of grape (*Vitis vinifera* L.) hybrids studies through high performance liquid chromatography

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ABSTRACT

The anthocyanin profile was analyzed by HPLC for assessing the genotypic variation in berry of eleven grape hybrids and two parental genotypes. Eleven peaks were detected in all the grape hybrids. In most cases, fifth and sixth peaks were found to be major. All the anthocyanins were monoglucoside derivatives of delphinidin, cyanidin, petunidin, peonidin and cyanidin. Derivatives include 3-monoglucoside and 3-monoglucoside acetate. The highest anthocyanins were found in hybrid 16/2A-R1P8. The peonidin derivatives were most abundant in most of the hybrids. The maximum content of malvidin and cyanidin derivatives was found in hybrid '16/2A-R_4P_8. The anthocyanin rich genotype could be further utilized in breeding programme to bred nutritionally superior grape varieties with potential health benefits.

Key Words: Grape, Anthocyanins, HPLC, Hybrids

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INTRODUCTION

Grape is one of the important and ancient fruit crops of the world and is produced 67.2 million tons annually from and an area of around 6.9 million ha (FAO, 2013). India is also have its significant contribution and ranks ninth in grape production and first in average productivity to 21.10 t/ha (NHB, 2014). Internationally, approximately 23 per cent of the total grapes produced are used for fresh consumption and remaining 86.6 per cent goes to processing, mainly for wine-making (Liu *et al*., 2006).

The quality of the grapes is greatly depends on the berry compositions and its colour (Liang et al., 2008). The berry colour is one of the important quality trait affects the market value of grapes and its byproducts like wine, juice etc. The skin colour greatly depends on the availability of anthocyanins in the grape berry skin and pulp (Copper-Driver, 2001). The composition of anthocyanin is primarily depends on the genetic constitution and relative factors. The relative content of the any one anthocyanin is stable in grape skin for a given genotype and which does not differ from one year to the next year (Pomar *et al*., 2005; Liang et al., 2008). Therefore the berry colour is the important factor presents among the Indian origin grape hybrids developed at IARI. The Objective of the present study was to explore the characterization of anthocyanin composition and content in eleven grape within grape genotypes. Previously, several studies has been reported about the profiling of grape genotypes for anthocyanins by paper chromatography (PC), thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Out of these three techniques, the HPLC method reported as a more precise, versatile and popular for analyzing anthocyanins, because of its rapid separation capacity and high sensitivity to detection (Pomar *et al*., 2005).

Anthocyanins in grape comprised mainly of cyanidin, malvidin, delphinidin, petunidin), peonidin, -3-monoglucosides (or 3, 5-diglucosides) along with corresponding acetyl. *P* coumaroyl, and caffeoyl derivatives in genotypes with red, blue and purple/violet skin (Liang et *al al*., 2008). In *Vitis vinifera*, majority of the anthocyanin derivatives are 3-monoglucoside (Goldy *et al*., 1989). Around the world, several studies have been reported about some selected cultivar anthocyanin profiles. However, there are very few reports about Indian originated grape hybrids or genotypes. In this direction, Indian Agricultural Research Institute (IARI), New Delhi, has evolved some grape hybrids for intended use like table and juice purpose. These hybrids have been evaluated for morpho-physical characteristics, but did studies for bioactive compounds like anthocyanins. Therefore, it would be valuable to profile the anthocyanins hybrids, in order to acquire information for future breeding efforts aimed at improvement of berry quality in grapes via effects on anthocyanins.

MATERIALS & METHODS

Present study was carried out at the Division of Fruits and Horticultural Technology and Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute (IARI), New Delhi, India. The vineyard of IARI is situated at an altitude of 228 m above mean sea level with 28^0 40' N latitude and 77^0 13' E longitudinal geographical coordinates. It has sub-tropical climate. The soils are alluvial with slightly alkaline pH with clay loam texture and generally low in organic matter.

Plant Material

Grape hybrids and parental cultivarsEleven grape hybrids and two parental cultivars were used in the present study (Table 1).

Harvesting and sampling

Fully matured bunches were randomly harvested from the vineyard. Five uniform bunches were selected from each replicated genotypes. Bunches were sorted out, packed in polythene bag and subsequently transported to laboratory for recording observation.

Methods

Sample Preparations

Grape berries of uniform size; shape and colour, free from injuries were sorted out and used for this experiment. Randomly selected 100 berries from each genotype were chosen for evaluating the anthocyanin content. Four replicates for each cultivar were used for analytical work.

High performance liquid chromatography for anthocyanin

Extraction of anthocyanins

Anthocyanin rich fruit skin and pulp were carefully removed (2-5 g) and taken in the amber colour flask and extracted with 500ml of acidified methanol (0.1% HCl). The content was sonicated in the dark for 15 min on an ultrasonicator (Misonix, NY, U.S.A). The combined extract was concentrated under vacuum (35±1°C) in a rotary evaporator (Heidolph, Germany) for complete removal of methanol.

Detection of anthocyanins

Purity of anthocyanin powder concentrate was checked by HPLC instrument (Alliance, Waters Corp., Milford, Mass., U.S.A.) equipped with e2695 quaternary pump, auto injector (20µL loop), a 2998 photodiode array detector and an "Empower 2" software programme. A C-18 column (Thermo, USA) 25 cm × 4.6 mm × 5µ was used for anthocyanin separation using a mobile phase comprising of a gradient mixer of solvent A: water (0.1% TFA) and solvent B: water: ACN: TFA (53:46:1 v/v) at a flow rate of 0.6 ml min^{-1}. The gradient mobile phase is as follows:

Time	Flow	%A	%B
1 min	0.60	80.0	20.0
26 min	0.60	40.0	60.0
30 min	0.60	80.0	20.0
40 min	0.60	80.0	20.0

Chromatogram was acquired at 520 nm and peak assignments were made based on MS fragmentation patterns and published literature (UV-Vis spectra and elution order).

Characterization of anthocyanins

Purity of anthocyanin powder concentrate was checked by HPLC instrument using a mobile phase comprising of a gradient mixer of solvent A: water (0.1% TFA) and solvent B: water: ACN: TFA (53:46:1 v/v) at a flow rate of 0.6 ml min^{-1}. The gradient mobile phase was A: 80% for 0 min, 40% in next 26 min, 80% for 14 min, total run time was 40 min. Chromatogram was acquired at 520 nm after injection of 20 µl. Standard Cyanidin-3-glucoside were also run according to the above mentioned flow rate. UV spectra also recorded for each peak found in HPLC analysis. Concentration of individual anthocyanins was calculated based on C_3G equivalent.

The data represented the mean of two years. The mean values of each grape genotype were from four replicates and were used for further analysis. The values of different parameters were expressed as the mean values (Duncan, 1955).

RESULTS & DISCUSSION

The anthocyanins were measured through HPLC. Its content in berries showed significant variation among genotypes and within a genotype. Anthocyanins belong to the parent group of flavonoids, which are very sensitive and degrade very quickly under heat in presence of oxygen. Therefore, the extraction tube was flushed with nitrogen to protect the sample from oxidation; and the samples were extracted in 80:20 (v/v) methanol–water solution containing 0.1 mL/L HCl at low temperature (30°C) to analyze the intact form of anthocyanins (Wu and Prior, 2005). Under such circumstances, the anthocyanins are stable and the glycosidic bonds never go to hydrolysis.

Identification of anthocyanins

Table 2 indicates the identification results based on the data including the peak number and the corresponding derivatives. There were eleven major peaks detected in all the genotypes (11- hybrids and 2- parental genotypes). All the anthocyanins were monoglucoside derivatives of 5 anthocyanins; delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn) and malvidin (Mv). Derivatives include 3- monoglucoside, and 3- monoglucoside acetate. Two unknown components (peak 4 and peal 11) have not been identified due to scarcity of the information. These findings are in agreement with Liang et al. (2008) reports wherein, they also reported only the monoglucoside derivatives in *Vitis vinifera* genotypes as in the present study.

Total content of anthocyanins

The total anthocyanin content among the hybrids ranged from 45.99 to 247.68 µg g^{-1} fresh weight (fw). The hybrid '16/2A-R_1P_8' had the maximum anthocyanins (247.68 247.68 µg g^{-1} fw). Hybrids 16/2A-R_1P_{19}, ER-R_1P_{19}, 16/2A-R_1P_2 and 16/2A-R_1P_7 also produced more than 100 µg g^{-1} fw of anthocyanins. However, it was found moderate in rest of the hybrids. Among the parental genotypes, 'Madeline Angevine' was found with highest

anthocyanin content. All the grape hybrids were identified with monoglucoside derivatives of anthocyanins. These were mainly composed of Pn, Dp, Pt, Mv and Cy derivatives. The peonidin and delphinidin derivatives, accounting for 38% and 16.45%, of the total anthocyanins. The maximum content of malvidin and cyanidin derivatives was found in hybrid 16/2A-R4P8 (49.26 247.68 and 34.73 µg g^{-1} fw) respectively. Each anthocyanin fraction gives a particular type of hue. Among anthocyanins, delphinidin derivatives give blueness, cyanidin derivatives are reddish colour, while malvidin and petunidin derivatives are associated with blue/dark blue, and with peonidin ones are purple.

HPLC chromatogram (Figure 1) indicates ten peaks separated base to base except one or two (Table 2 and 3). All the peaks belong to anthocyanin group that can be seen from the UV-Vis spectrum of each compound (Figure 1). Characteristic peak at around 517 nm shows the typical absorbance of anthocyanin. Table 2 represents the per cent area of individual peaks in terms of total peak area. Table 2 represents C_3G equivalent content of individual anthocyanins. In the HPLC analysis, individual content of different anthocyanin fractions corresponding to different peaks were estimated. It was seen that highest content of anthocyanin was identified at peak 5 in almost maximum genotypes under study. The anthocyanin content at peak 5 ranged from 41.35 µg g^{-1} C_3G eq. in 'Hy. 16/2A-R_1P_2' to 23.41 µg g^{-1} C_3G eq. in 'Hy. ER-R_1P_{19}', which were their corresponding maximum values among all other peaks identified. But in 'Hy. 16/2A R_1P_{14}', 'Hy. ER-R_2P_{36}', var. 'Madeleine Angevine', 'Hy. 16/2A-R_1P_{18}', var. 'Beauty Seedless', the maximum anthocyanin fraction i.e. 23.70, 26.25, 61.87 and 17.04, 14.63 µg g^{-1} C_3G eq. were recorded at peak 1, peak 6, peak 4, peak 9 and peak 4 respectively.

It was also observed that maximum area percentage was observed at peak 5 in most of the genotypes studied. 31.44%, 27.39%, 31.86%, 53.88,%, 60.72%, 40.42%, 53.56%, 37.86% were the corresponding maximum area percentage recorded at peak 4 in hybrids like '16/2A R1P2', '16/2A R1P7','16/2AR1P8', '16/2AR1P19', '16/2AR3P12', '16/2AR4P13', 'ER-R1P19', 'ER-R2P19' respectively. In terms of peak area, peak 5 and 6 were major in nature and the variations across the cultivar were presented in Table 2.

Conclusion

Anthocyanins profile largely varied with the genetic constitution as well as the environmental conditions under the vineyard grows. The content of anthocyanins is important in grapes for use. Eleven hybrids and two parental genotypes were characterized for anthocyanin profile through HPLC. Eleven peaks were detected in all the grape hybrids. All the anthocyanins were monoglucoside derivatives of Dp, Cy, Pt, Pn and Cy. Derivatives include 3-monoglucoside and 3-monoglucoside acetate. The highest anthocyanins were found in hybrid 16/2A-R1P8. The peonidin derivatives were most abundant in most of the hybrids. The maximum content of malvidin and cyanidin derivatives was found in hybrid 16/2A-R_4P_8.

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Table 1. List of grape hybrids and their parents

S. No.	Hybrid	Female parent ♀	Male parent ♂
1	16/2A R1P2	Madeleine Angevine	Ruby Red
2	16/2A R1P7	Madeleine Angevine	Ruby Red
3	16/2A R1P18	Banqui Abyad	Beauty seedless
4	16/2A R1P19	Banqui Abyad	Beauty seedless
5	16/2A R4P13	Banqui Abyad	Beauty seedless
6	16/2A R3P12	Black Muscat	Beauty seedless
7	ER-R1P19	Pearl of csaba	Beauty seedless
8	ER-R2P36	Pearl of csaba	Beauty seedless
9	ER-R2P19	Pearl of csaba	Beauty seedless
10	16/2A R1P14	Cardinal	Beauty seedless
11	16/2A-R1P8	Hur	A-5

Table 2. Anthocyanin measured at various peaks through HPLC

Hybrid/	Anthocyanin (μg g^{-1} C_3G eq.) at various peaks											
Varieties	Peak1	Peak2	Peak3	Peak4	Peak5	Peak6	Peak7	Peak8	Peak9	Peak10	Min.	Max.
Hy.16/2A R1P2	5.19	11.42	7.22	32.82	41.35	7.05	19.79	1.28	5.41	-	1.28	41.35
Hy.16/2A R1P7	18.25	3.78	8.63	6.57	29.14	0.25	18.41	21.39	-	-	0.25	29.14
Hy.16/2AR1P8	15.14	34.73	15.15	1.29	78.91	49.26	1.52	3.14	4.61	43.93	1.52	78.91
Hy.16/2AR1P14	23.70	8.47	7.67	7.06	18.71	11.78	0.50	0.39	19.21	-	0.39	23.70
Hy.16/2AR1P18	1.50	0.94	0.23	1.69	1.20	10.80	2.74	9.85	17.04	-	0.23	17.04
Hy.16/2AR1P19	4.40	26.53	4.04	1.85	78.74	17.65	1.31	1.61	4.20	5.80	1.31	78.74
Hy.16/2AR3P12	2.46	1.68	4.84	4.08	40.56	5.39	7.79	-	-	-	1.68	40.56
Hy.16/2AR4P13	0.37	9.70	1.55	17.44	24.29	0.13	0.18	0.41	6.01	-	0.13	24.29
Hy.ER-R1P19	6.52	2.02	10.45	17.12	72.03	4.14	5.00	1.17	16.03	-	1.17	72.03
Hy.ER-R2P19	1.97	1.50	2.98	8.16	23.41	2.36	15.31	1.27	7.23	-	1.27	23.41
Hy.ER-R2P36	2.94	0.73	3.60	1.09	13.19	26.25	0.86	1.28	-	-	0.73	26.25
Madeline Angevine	20.24	33.46	19.46	61.87	60.09	2.67	4.76	5.02	16.02	-	2.67	61.87
Beauty Seedless	0.98	1.71	0.63	14.63	5.42	10.56	3.75	-	-	-	0.63	14.63
Min.	0.37	0.73	0.23	1.09	1.20	0.13	0.18	-	-	-	0.13	-
Max.	23.7	34.73	19.46	61.87	78.91	49.26	19.79	21.39	19.21	43.93	19.46	-

Table 3. Percentage area measure by HPLC at various peaks

Hybrid/Varieties	Peak1	Peak2	Peak3	Peak4	Peak5	Peak6	Peak7	Peak8	Peak9	Peak10
Hy.16/2A R1P2	3.95	8.68	5.49	24.95	31.44	5.36	15.05	0.97	4.11	-
Hy.16/2A R1P7	17.15	3.55	8.11	6.17	27.39	0.24	17.3	20.1	-	-
Hy.16/2AR1P8	6.11	14.02	6.12	0.52	31.86	19.89	0.61	1.27	1.86	17.74
Hy.16/2AR1P14	24.31	8.69	7.87	7.25	19.19	12.08	0.51	0.4	19.7	-
Hy.16/2AR1P18	3.25	2.03	0.5	3.68	2.62	23.5	5.95	21.42	37.05	-
Hy.16/2AR1P19	3.01	18.15	2.77	1.26	53.88	12.08	0.9	1.1	2.88	3.97
Hy.16/2AR3P12	3.68	2.51	7.25	6.1	60.72	8.07	11.66	-	-	-
Hy.16/2AR4P13	0.62	16.15	2.58	29.03	40.42	0.22	0.3	0.68	9.99	-
Hy.ER-R1P19	4.85	1.5	7.77	12.73	53.56	3.08	3.72	0.87	11.92	-
Hy.ER-R2P19	0.58	15.12	2.42	27.19	37.86	0.21	0.28	0.64	9.36	-
Hy.ER-R2P36	5.88	1.46	7.2	2.18	26.41	52.58	1.73	2.56	-	-
Madeline Angevine	9.05	14.97	8.7	27.67	26.87	1.19	2.13	2.25	7.16	2.6
Beauty Seedless	2.6	4.53	1.67	38.84	14.39	28.03	9.95	-	-	-

Table 4. Retention times & spectral characteristics of the chromatographic peaks identified

Peak number	Retention time (tR)	λ_{max}		Tentative identification
		Literature	Observed value	
1	19.022	277, 346, 524	276, 347, 526	Delphinidin-3-monoglucoside
2	21.089	279, 330, 515	279, 375, 518	Cyaniding-3-monoglucoside
3	22.198	277, 347, 526	276, 347, 526	Petunidin-3-monoglucoside
4	23.289	279, 515	265, 431.4, 505.4	Unidentified
5	24.360	277, 348, 526	279, 328, 517	Peonidin-3-monoglucoside
6	25.176	280, 523	277.2, 347.5, 527.4	Malvidin-3-monoglucoside
7	26.777	281, 514	276, 346, 524.9	Delphinidin-3-monoglucoside-acetate
8	29.196	278, 528	281.9, 362.9, 522.5	Delphinidin-3-monoglucoside-acetate
9	30.109	282, 529	279, 527.4	Petunidin-3-monoglucoside-acetate
10	32.834	280, 518	279, 333, 516	Peonidin-3-monoglucoside-acetate
11	33.712	278, 348, 529	279.5, 535.9	Unidentified

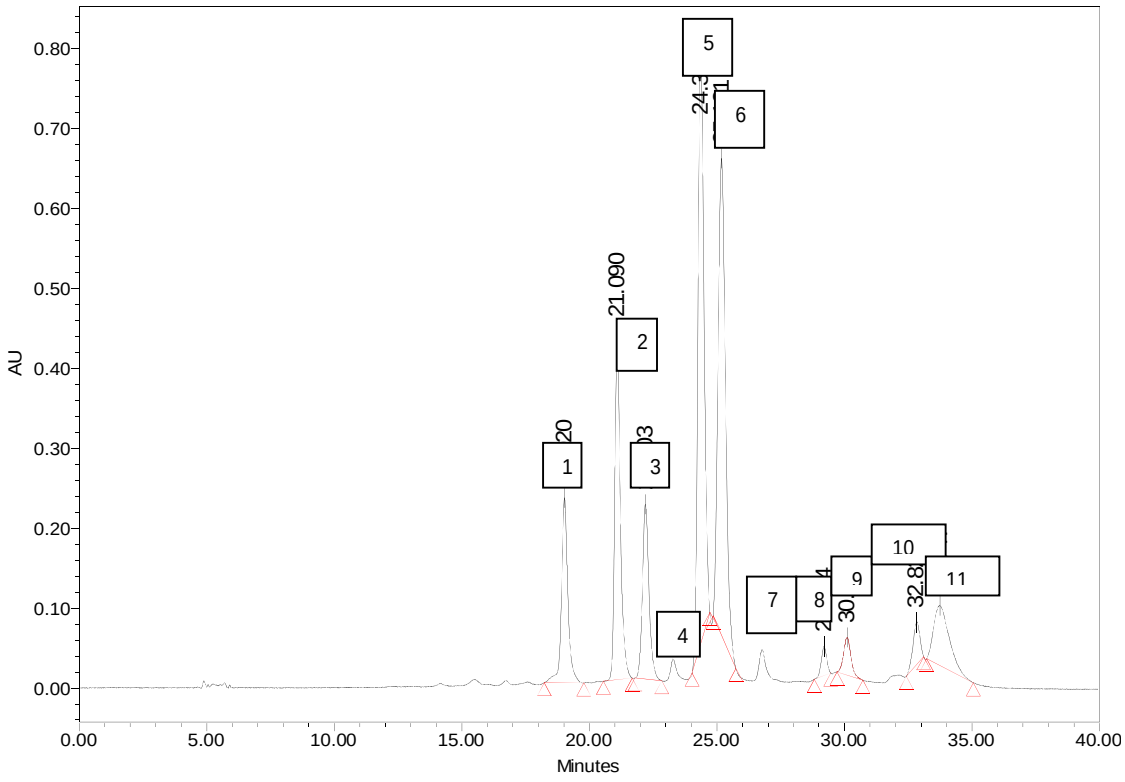

Figure 1. Typical HPLC chromatogram of anthocyanin extracts captured at 517 nm. Peak identification is shown in table 4.

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