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On the absence of acylated anthocyanins in some wild grapevine accessions

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Summary

Our current research is focused on the anthocyanin composition of female grape accessions, mostly Spanish, preserved at El Encín Germplasm Bank. In 2008, berries of 126 accessions were taken at maturity. After the extraction from grape skins, total anthocyans were determined by spectrophotometry, and the anthocyanin fingerprint of grapes by HPLC, considering the relative amount of 15 anthocyanins. Among those 126 accessions, 23 genotypes (18.25 %) did not present acylated anthocyanins. Thus, those 23 genotypes presented a characteristic anthocyanin fingerprint, similar at a certain extent to that presented by some Rhine basin and Italian grape cultivars, e.g., 'Pinot Noir' and 'Gaglioppo'. Nevertheless, the absence of acylated anthocyanins has not been described in any Spanish grape cultivar. The examination of the anthocyanin fingerprint of wild grapes without acylated anthocyanins reveals that the regulation of the anthocyanin biosynthesis may differ in various wild grape accessions.

K e y w o r d s : Wild grapevine, anthocyanins, anthocyanin fingerprint, grapes, HPLC.

Introduction

Most of the red grape cultivars used for winemaking contain variable quantities of anthocyanins conjugated with acetic, *p*-coumaric and caffeic acids, known as acylated anthocyanins. Acylation of anthocyanins is caused by action of acyltranferases, which have been studied in some detail in the last several years (Nakayama *et al*. 2003). It is usually considered that these reactions increase anthocyanin stability (Mazza 1995). Moreover, acylation with aromatic acids (*p*-coumaric and caffeic acids) leads to an increase in the anthocyanins blue tint, as a consequence of the phenomena known as intramolecular copigmentation (Mazza 1995). On the other hand, acylation with aliphatic acids, like acetic acid, increases water solubility of anthocyanins, and can increase color intensity compared to the acylation with aromatic acids (Mazza 1995). For all these reasons, the presence of acylated anthocyanins in red grapes used for winemaking affects wine characteristics, as acylation may stabilize the colour of young red wines, and leads to wines with a slightly blue tint. Certainly, some grape cultivars used for making premium red wines, like 'Cabernet Sauvignon' and 'Tempranillo', contain remarkable quantities of acylated anthocyanins. Thus, 'Cabernet Sauvignon' grapes may content up to 40 % of acylated anthocyanins, and especially anthocyanins acylated with acetic acid (Ryan and Revilla 2003).

Nevertheless, the absence of acylated anthocyanins in grapes does not mean that colour and quality of red wines become diminished. For decades, it is well known that 'Pinot Noir' grapes, used for making premium red wines in Bourgogne, do not contain acylated anthocyanins (Ribéreau-Gayon 1964). This genetic trait appears in several 'Pinot Noir' coloured mutants (e.g. 'Pinot Gris', 'Pinot Tête de Negre') and in several cultivars genetically close to 'Pinot Noir', which are grown especially in the Rhine basin, like 'Schwarzriesling' ('Müllerrebe', 'Pinot Meunier'), 'Blauer Arbst' and 'Deckrot' (Wenzel *et al*. 1987, Mattivi *et al*. 2006). Nevertheless, that genetic trait is quite rare in red grapes from other areas. Only two red grape cultivars from Southern Italy, known as 'Gaglioppo' and 'Tintilia', do not contain acylated anthocyanins (Lovino *et al*. 2006, Mattivi *et al*. 2006), but their skins are slightly coloured, and probably should not be considered as red cultivars. Moreover, acylated anthocyanins are also absent in some grey and rosé cultivars (e.g., 'Muscat Rouge de Madère'), which are usually mutants of white cultivars (Mattivi *et al*. 2006). When studying in detail red cultivars used for winemaking studied in detail, none showed lack of acylated anthocyanins (García-Beneytez *et al*. 2002, Pomar *et al*. 2005, Gómez-Alonso *et al*. 2007). Thus, the absence of acylated anthocyanins in red grape cultivars used in winemaking appears to be a rare genetic character, which is nearly restricted to a group of cultivars known as Pinots and to other cultivars closely related to them from a genetic point of view (Regner *et al*. 2000). The lack of acylation in red grapes is not well understood, and two different hypotheses should be considered: the lack of genes related to the synthesis of acyltransferases, or the lack of regulation genes that module the expression of the first set of genes.

It has been mentioned that 'Pinot Noir' grapes present several morphological characters closed to those presented by wild grapevines (Bassermann-Jordan 1975). It has been postulated that 'Pinot Noir' is an ancient cultivar, and even that was introduced in Central Europe by Romans (Ambrosi *et al*. 1994). Nevertheless, Regner *et al*. (2000) have suggested that 'Pinot Noir' is a cross of 'Schwarzriesling' and 'Traminer' cultivars, and 'Schwarzriesling' is closer to wild grapes than 'Pinot Noir'.

Since 2006, our research group is studying the chemical composition of a number of wildgrape accessions pre-

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served at El Encín Germplasm Bank. A previous paper, focused on the study of 18 accessions over three years (Revilla *et al*. 2010), showed that the anthocyanin composition of wild grapevine accessions is quite similar to that shown by cultivated grapevines. In this paper, data on a set of 23 wild grapevine accessions which do not contain acylated anthocyanins are reported.

Material and Methods

Plant material: In this research 126 wild accessions preserved in El Encín's Germplasm Bank (IMIDRA, Alcalá de Henares, Madrid, Spain) have been considered. Genetic comparison of those accessions with cultivated genotypes from Spain showed that the majority of the accessions are different genotypes (Andrès *et al*. 2012). Photographs of the clusters of two wild genotypes are displayed in Fig. 1. Samples were collected in the first fifteen days of October 2008. 23 accessions did not present acylated anthocyanins, and they were the main objective of this work. 19 of the 23 accessions came from eight populations of the north of the Iberian Peninsula, and the other four came from three populations of Andalusia, which is located in the Southern part of Spain. Only two plants of each accession, grafted on 110R and trained to Royat cordon, were available, and all clusters were collected at the proper time.

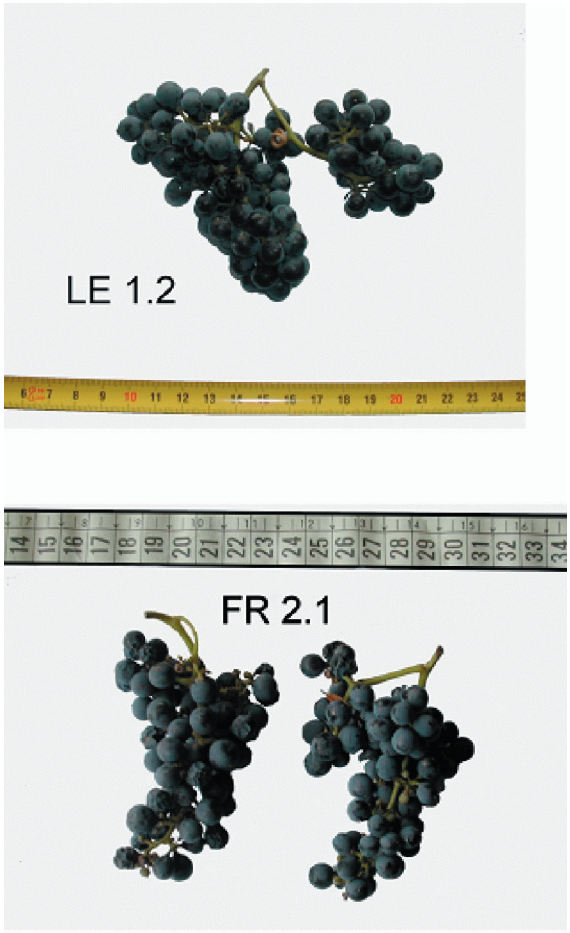

Fig. 1: Photographs of clusters of accessions LE-1.2 and FR-2.1.

Anthocyanins extraction and general analysis of musts: Immediately after sampling, the berries were separated from the clusters. Then, 50 berries were randomly selected and weighed. After that, grape skins were separated from pulps and seeds, and conserved in methanol at -20 °C. Then, grape skins were grinded in a Kinematica PCU-2 blender for 1 min, and submitted to sequential extraction with different solvents (methanol for 16 h at -25 °C, 80 % methanol for 4 h at room temperature, 50 % methanol for 4 h at room temperature, deionized water for 16 h at -25 °C, and 75 % acetone for 1 h at room temperature) using 25 mL of solvent for each extraction step, as described elsewhere (Revilla *et al*. 1998, based on Bourzeix *et al*. 1986). When each extraction step was finished, the liquid extract was separated by centrifugation at 3500 rpm for 20 min in a Rotofix 32A centrifuge, and the residue was submitted to extraction again. For each sample, all the liquid extracts were combined, and their volume was raised between 125 and 200 mL with methanol. Then, the extracts were stored at 4 °C prior to their analysis. Additionally, ~ 50 g berries were crushed for determining sugar concentration of must by refractometry and total acidity by potentiometry, following standard procedures (International Organisation of Vine and Wine 2008).

Reagents and standards: Deionized water was purified with a Milli-Q water system (Millipore, Bedford, MA) before use. Acetonitrile of HPLC grade was purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid of analytical reagent grade was obtained from Sigma-Aldrich (Tres Cantos, Spain). All other chemicals (analytical-reagent grade) were obtained from Panreac (Mollet del Vallès, Spain). Standards of several anthocyanins were prepared from fresh red grape skins as described elsewhere (Bakker and Timberlake 1985). The identity of standards was determined by HPLC-MS, as described elsewhere (García-Beneytez *et al*. 2003). Briefly, HPLC-MS analyses were carried out using an HP 1100 system (Hewlett-Packard, Palo Alto, CA) with a PDA UV-vis coupled to a mass spectrometer system equipped with an ESI interface. Stationary phase, mobile phase, oven temperature, and injection volume were the same used for HPLC analysis of anthocyanins (see below). MS parameters were: capillary voltage, 4000 V; fragmenter ramped from 90 to 120 V; drying gas temperature, 325°C; and gas flow (N_2), 12 $mL \cdot min^{-1}$. The instrument was operated in positive ion mode scanning from *m/z* 50 to 2000 at a scan rate of 1.47 s/cycle.

Spectrophotometric analysis: Total anthocyanins in grape extracts were determined by visible spectrophotmetry (Niketic-Aleksic and Hrazdina 1972), using a Boeco S-22 UV-vis spectrophotometer (Boeckel, Hamburg, Germany).

Chromatographic analysis: The anthocyanic profile of the skin extracts was obtained with HPLC considering the relative content of 15 anthocyanins, following the procedure of García-Beneytez *et al.* (2003) with small changes (Revilla *et al*. 2010). HPLC analysis of anthocyanins was performed with a liquid chromatograph consisting of a 600 quaternary pump, a 717 automatic injector, a TC2 controller for a column oven, a 996

photodiode array detector, and a Millennium 32 workstation (Waters, Milford, MA). The separation was carried out using a Waters Nova-Pak C18 steel cartridge (3.9 × 250 mm), filled with 5-µm particles, and furnished with a Waters Sentry Nova-Pack C18 guard cartridge (20 × 3.9 mm), both thermostated at 55 °C. Two mobile phases were used: water/acetonitrile (95:5) adjusted to pH 1.3 with trifluoraectic acid (solvent A), and water/acetonitrile (50:50) adjusted to pH 1.3 with trifluoracetic acid (solvent B). Gradient elution was applied at a 0.8 mL·min^{-1} flow rate according to the following program: linear gradient from 15 % B to 35 % B in 20 min, from 35 % B to 50 % B in 10 min, 50 % B for 6 min, from 50 % B to 100 % B in 5 min, 100 % B for 5 min, 100 % B to 15 % B in 1 min. Samples (20 µL) were injected in triplicate. Spectra were recorded every second between 250 and 600 nm, with a bandwidth of 1.2 nm. Samples, standard solutions, and mobile phases were filtered before analysis through a 0.45-µm pore size membrane. HPLC analyses were carried out by duplicate.

Statistical analysis: Correlation analyses were performed using the Statgraphics 5.0 Plus statistical package (Statistical Graphics Corp.).

Results and Discussion

Every accession presented an average berry weight between 200 and 600 mg, which means that the average weight of 50 berries is lower than 50 g. These values are smaller than in cultivated red grapes, which usually weigh more than 1000 mg. The consequence of this, joined to a high number of seeds, is that the grape juice volume is very small, and in some cases, like for SS-3.5', it was impossible to determine any ripening index. Ripening index shows that, in spite of the high sugar content in the grape juice, usually over 20 °Brix, total acidity is high, exceeding 15 g·L^{-1} in most samples. When looking the whole of the data, a strong, negative correlation (r = -0.54) among the weight of 50 berries and total acidity of the must, which is statistically significant ($p < 0.01$), was observed. In other words, the smaller is the berry size the higher uses to be the acidity of the grape juice, regardless of the amount of sugars accumulated.

In the wild accessions under study, the content of total anthocyans was too high when it was expressed in mg·kg^{-1} of grapes, with values exceeding sometimes 2000 mg·kg^{-1} grapes. When comparing these values with data obtained by the authors in the same Germplasm Bank, the total content of anthocyans in wild grapes, in mg·kg^{-1} grapes basis, was higher than in many cultivated grapes. This effect is due to the average size of the grapes, too small in wild accessions.

Fig. 2 show the chromatograms registered at 520 nm for an accession without acylated anthocyanins (LE-1.2) and another with acylated anthocyanins (CA-10.3). As can be noted, accession CA-10.3 shows a chromatogram with 15 different peaks, which is quite similar to that presented by most grapevine cultivars, and also by most of the wild grapevine accessions preserved at El Encín Germplasm Bank (Revilla *et al*. 2011). On the other hand, the chromatogram of accession LE-1.2 only present five peaks, corresponding to five non acylated anthocyanins: delphinidin-3-glucoside (DpGl), cyanidin-3-glucoside (CyGl), petunidin-3-glucoside (PnGl), peonidin-3-glucoside (PtGl)

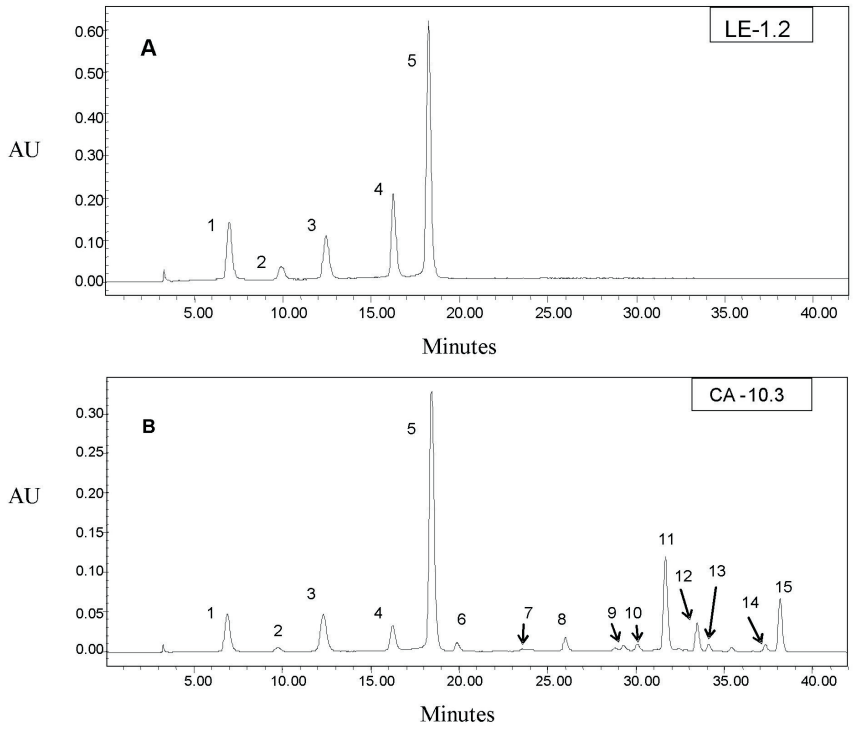

Fig. 2: Chromatograms corresponding to accession LE-1.2, without acylated anthocyanins (**A**), and to another accession (CA-10.3) which present acylated anthocyanins (**B**). Key for peaks: 1, DpGl; 2, CyGl; 3, PtGl; 4, PnGl; 5, MvGl; 6 to 15, acylated anthocyanins.

and malvidin-3-glucoside (MvGl). The other ten peaks in accession CA-10.3 chromatogram belong to ten different acylated anthocyanins.

The Table shows data on the anthocyanins profile of the 23 accessions which were studied, and it can be seen that there were important differences related to the relative importance of each one of the pathways involved in anthocyanin biosynthesis. This also happens among cultivars of the same species (García-Beneytez *et al*. 2002, Pomar *et al.* 2005, Mattivi *et al*. 2006, Gómez-Alonso *et al*. 2007). Analyzing the whole set of samples, it should be pointed out that in 20 wild accessions B-ring trisubstituted anthocyanins (DpGl, PtGl and MvGl) predominate. These three anthocyanins are derived from delphidin, after *O*-glycosilation and, eventually, methylation (He *et al.* 2010). On the other hand, in three accessions (BI-1.3', BI-1.4'and VI-3.5') the proportion of B-ring disubstituted anthocyanins (CyGl and PnGl), derived from cyanidin after *O*-glycosilation and, eventually, methylation (He *et al.* 2010), is higher than 50 %. Moreover, in other five accessions (BI-1.4, H-1.3, O-1.9, VI-3.6' and VI-3.6'-bis), the proportion of B-ring disubstituted anthocyanins is higher than 40 %. Thus, it should be hypothesized that the expression of *F3'h* genes at the transcriptional level in grape skin seems to be very intense if it is compared with the expression of *F3'5'h* gene in several wild accessions. In accordance with literature data on cultivars without acylated anthocyanins, 'Gaglioppo' is the only cultivar in which B-ring disubstituted anthocyanins predominate (Lovino *et al.* 2006, Mattivi *et al.* 2006). However, total content of anthocyans in 'Gaglioppo' (427 mg·kg^{-1} after Lovino *et al.* 2006; 211 mg·kg^{-1} of grapes after Mattivi *et al.* 2006) is lower than in the wild accessions presenting this trait, as it is shown in the Table. Nevertheless, B-ring trisubstituted anthocyanins predominate in all the other cultivated grapes belonging to the Pinot family (Wenzel *et al.* 1986, Mattivi *et al.* 2006); thus, the regulation of the anthocyanin biosynthesis, in their earlier steps, should be similar in most of the wild accessions under study to that shown by cultivars of Pinot family.

Moreover, the extent of methylation is quite variable among wild accessions. In some of them (LE-1.1, LE-1.2, LE-1.3, LE-1.6 and O-1.7'), methylation of anthocyanins is quite intense, and only a small proportion of anthocyanins (less than 25 %) remain unmethylated. On the other hand, in six wild accessions (BI-1.4, SS-3.5, SS-3.5', VI-3.5', VI-3.6' and VI-3.6'-bis), methylation is relatively low, and more than 50 % anthocyanins remain unmethylated. In these accessions, the major anthocyanin is not methylated (DpGl for accessions BI-4, SS-3.5 and SS-3.5', CyGl for

Table

Anthocyanic profile of each accession, expressed as percentage of five anthocyanins, percentages of the different groups of anthocyanins in each accession, and total content of anthocyanins in each accession

Accession	DpGl	CyGl	PtGl	PnGl	MvGl	Mono-glucosides derived from DpGl	Mono-glucosides derived from CyGl	Methylated mono-glucosides	Non-methylated mono-glucosides	Total anthocyans (mg·kg^{-1} grapes)
BI-1.3‘	18,89	18,86	11,25	33,01	18,00	48,14	51,86	62,25	37,75	956
BI-1.4	31,38	21,60	13,34	20,82	12,87	57,59	42,41	47,02	52,98	830
BI-1.4‘	19,47	18,96	11,35	33,74	16,48	47,29	52,71	61,57	38,43	884
CO-7.1	35,27	6,37	21,14	8,53	28,70	85,10	14,90	58,37	41,63	1525
FR-2.1	28,17	16,19	19,09	12,88	23,68	70,94	29,06	55,65	44,35	2237
FR-2.2	26,82	21,95	17,50	13,69	20,04	64,36	35,64	51,23	48,77	2222
H-1.3	16,31	14,37	11,08	33,14	25,06	52,45	47,55	69,29	30,71	1366
J-2.1	32,09	11,53	16,95	14,80	24,64	73,67	26,33	56,38	43,62	1350
J-2.2	28,24	5,36	18,04	10,67	37,69	83,97	16,03	66,40	33,60	1855
LE-1.1	13,54	3,25	11,64	13,55	58,02	83,20	16,80	83,21	16,79	1538
LE-1.2	13,71	3,77	11,96	18,66	51,89	77,57	22,43	82,51	17,49	1742
LE-1.3	14,78	3,08	11,35	16,31	54,48	80,62	19,38	82,14	17,86	1716
LE-1.6	14,38	2,79	11,58	18,98	52,26	78,23	21,77	82,83	17,17	1426
NA-3.2‘	25,99	6,97	21,24	9,83	35,97	83,20	16,80	67,04	32,96	2507
O-1.7‘	21,10	3,81	20,19	9,10	45,80	87,10	12,90	75,09	24,91	1704
O-1.9	18,80	17,76	14,58	22,34	26,53	59,91	40,09	63,45	36,55	1617
SS-3.5‘	43,17	20,08	15,83	9,82	11,10	70,10	29,90	36,75	63,25	2050
SS-3.5“	44,94	18,16	16,19	10,76	9,94	71,08	28,92	36,90	63,10	2236
SS-5.5‘	33,12	5,26	19,55	10,44	31,63	84,30	15,70	61,62	38,38	2949
SS-5.6	36,20	4,21	20,89	7,09	31,61	88,70	11,30	59,59	40,41	2805
VI-3.5‘	27,31	34,93	13,79	15,27	8,71	49,80	50,20	37,76	62,24	2966
VI-3.6‘	27,93	29,38	15,52	15,41	11,77	55,21	44,79	42,70	57,30	2468
VI-3.6‘bis	27,03	32,73	14,31	15,37	10,56	51,90	48,10	40,24	59,76	3829

DpGl: delphinidin-3-glucoside; CyGl: cyanidin-3-glucoside; PtGl: petunidin-3-glucoside; PnGl: peonidin-3-glucoside; MvGl: malvidin-3-glucoside.

the others). In other accessions, methylation only slightly predominates and, as a consequence, the major anthocyanin is not methylated: DpGl in CO-7.1, FR-2.1, FR-2.2 and J-2.1; CyGl in SS-5.5' and SS-5.6. In most cultivated grapes, methylation of anthocyanins is quite intense, and usually MvGl or PnGl predominate (García-Beneytez *et al*. 2002, Pomar *et al*. 2005, Mattivi *et al*. 2006, Gómez-Alonso *et al*. 2007). This fact has been observed in 'Pinot Noir' and related cultivars (Wenzel *et al.* 1986, Mattivi *et al.* 2006). Thus, our results suggest that the regulation of genes involved in the methylation of anthocyanins is quite diverse in the wild accessions studied.

Conclusions

An important number (18.5 %) of female wild grape accessions preserved at El Encin Germplasm Bank do not contain acylated anthocyanins in berry skins. That proportion of individuals without acylated anthocyanins is higher than in grown cultivars, according with current literature. All those 23 accessions accumulate large quantities of anthocyanins, up to 1000 $mg \cdot kg^{-1}$ grapes. The anthocyanins profile show important differences among accessions. In most of them, B-ring trisubstituted anthocyanins predominate, resembling the cultivars of the 'Pinot Noir' family; nevertheless, the extent of methylation is quite variable. In the other accessions, B-ring disubstituted anthocyanins predominate, suggesting that they may be close, to a certain extent, to the Italian cultivar 'Gaglioppo'.

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