Chapter 2

Fingerprints of Anthocyanins and Flavonols in Wild Grapes (*Vitis vinifera* L. ssp. *sylvestris* (Gmelin) Hegi)

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http://dx.doi.org/10.5772/intechopen.70861

Abstract

Phenolic compounds are a group of natural products that play an important role in the quality of wines. Most phenolic compounds present in wine are derived from those contained in grapes and extracted from skins, seeds, and pulp during the initial steps of winemaking. Among them, anthocyanins and flavonols are involved in the color of red wines as pigments or copigments and also as precursors of polymeric pigments after reaction with other phenols. Biosynthesis of those phenolics in grapes is regulated by different genes; thus, each grape genotype presents a characteristic phenolic fingerprint, which is modulated by different environmental conditions. In this chapter, the anthocyanins and flavonols composition of different genotypes of wild grapes preserved at El Encin Germplasm Bank has been examined in detail. Wild grapevines are a remarkable genetic resource that may be used in breeding programs to improve the phenolic composition of cultivated grapes and, hence, the quality of red wines.

Keywords: wild grapes, anthocyanins, flavonols, fingerprint, HPLC

1. Introduction

Anthocyanins and flavonols are two families of phenolic compounds that play important roles in Enology. Free anthocyanins are the pigments responsible for the coloration of young red wines and take part in the reactions leading to the formation of stable polymeric pigments responsible for the coloration of aged red wines [1]. On the other hand, flavonols are involved in copigmentation of the flavylium form of anthocyanins in young red wines [2]. Moreover, flavonols present antioxidant properties that pose positive effects on human health [3]. The pathways involved in the biosynthesis of these molecules are well-known, and the core structural genes of those pathways, leading to the formation of primitive anthocyanins (delphinidin-3-*O*-glucoside

and cyanidin-3-*O*-glucoside) and flavonol aglycones, like myricetin and quercetin, have been cloned and characterized [4, 5]. Moreover, several *O*-methyltransferases involved in the methylation of anthocyanins and flavonol glycosides have been identified [6, 7]; it has been demonstrated that the color exhibited by different grape cultivars may be associated with the *VvmybA1* and *VvmybA2* regulatory genes [8–10] that activate the expression of structural genes involved in the late steps of the anthocyanins biosynthetic pathway.

Anthocyanins are red pigments accumulated in skins during grape maturation (and also in pulp in teinturier cultivars), and their content has been related to several agroecological factors [11, 12], especially light and temperature, light being indispensable for anthocyanin biosynthesis and accumulation in the skins of berries and for phenylalanine ammonia lyase activity. Thus, their concentration is quite variable, even if the same cultivar or the same clone grown in a given location has been examined in several consecutive years [13, 14]. Nevertheless, the proportion of different anthocyanins, or anthocyanin fingerprint, is quite similar in the late stages of grape maturation of a cultivar grown in a given location from year to year [14]. On the other hand, the accumulation of flavonols (that are yellow pigments predominantly synthesized in grape skins [15]), is affected by shading treatments. The studies carried out in Shiraz grapes suggest that the branch of flavonoid biosynthetic pathway leading to flavonol biosynthesis is light-dependent, in contrast to anthocyanin and flavanol biosynthesis, which are little affected by shading treatments [16].

Cultivated grapevines are thought to be domesticated from genotypes of *Vitis vinifera* L. ssp. *sylvestris* (Gmelin) Hegi, which are present in small, isolated wild populations, located in riverbanks from the Western Himalayas to Western Europe [17, 18]. The sanitary status of those populations and their morphological and genetic characteristics have been recently studied [19–22]. Mature fruits of wild grapevines usually show high acidity, low pH, and a high intensity of color if compared with cultivated grapes [23, 24]; these features might be used to adapt Viticulture to the new climatic conditions, mitigating the potential effects of global warming on grape production.

The qualitative and quantitative anthocyanin composition of wild grape accessions preserved at El Encin Germplasm Bank has been examined by our research group after 2006 [25, 26], as well as their flavonol fingerprint [27–29]. The main objective of this study is to evaluate whether the anthocyanin and flavonol composition of wild grapes differs from that presented by cultivated grapes, and to determine whether some wild genotypes present some genetic characters of interest related to anthocyanins and flavonols accumulation during grape maturation. For this purpose, different female genotypes preserved at El Encin Germplasm Bank, that were collected in various natural populations located in different Spanish regions, were sampled in 2008 and have been fully examined for anthocyanins and flavonols content by HPLC.

2. Sampling of grapes

Samples of 25 genotypes of wild grapevines from different Spanish natural populations preserved at El Encin Grapevine Germplasm Bank (IMIDRA, Alcalá de Henares, Spain), grafted

Figure 1. Location of natural populations of wild grapes where genotypes under study were originally collected.

on 110R and trained to cordon Royat, were collected in October 2008 at optimum stage of maturation (between 200 and 240 g of glucose + fructose by kg of must). Each sample consisted of four clusters, as only two plants of each genotype were available. Those genotypes, grown in El Encin, were originally collected from natural populations located in different Spanish regions (see **Figure 1**); 10 of them came from Northern Spain (Asturias, Cantabria, Castilla-León, Basque Country, and Navarra), the other 15 from Southern Spain (Andalousie, Castilla-La Mancha, and Extremadura). Every natural population was identified by two letters and by one or two numbers, and each genotype was identified with the population code and an additional number, as well as the suffix bis in some cases. Once in the laboratory, samples were stored at −20°C until sample preparation.

3. Sample preparation

Fifty berries were randomly selected and weighed once berries were separated from clusters, and grape skins were removed from pulps and seeds and stored at −20°C in methanol. Afterward, grape skins were grinded in a Kinematica PCU-2 blender for 1 minute. Then, they were sequentially extracted, using 25 mL of solvent for each extraction step: methanol for 16 hours at −25°C, 80% methanol for 4 hours at room temperature, 50% methanol for 4 hours at room temperature, deionized water for 16 hours at −25°C, and 75% acetone for 1 hour at

room temperature [30]. At the end of each extraction step, the liquid was centrifugated at 3500 rpm for 20 minutes in a Rotofix 32A centrifuge, and the residue was submitted to extraction again. The volume of the combined liquid extracts was raised between 125 and 200 mL with methanol. Then, the extracts were stored at −20°C prior to analysis.

Flavonols were isolated prior to HPLC analysis to avoid interferences caused by anthocyanins, using solid-phase extraction on Oasis MCX cartridges (6 mL capacity) filled with 500 mg of an adsorbent containing a mixture of reverse-phase and cationic-exchanger materials (Waters Corp., Milford, MA), following a procedure described previously [31]. For this purpose, 3 mL grape skins extract was dried in a rotary evaporator (40°C) and resolved in 0.1 M hydrochloric acid (3 mL). Then, it passed through the MCX cartridges, previously conditioned with methanol (5 mL) and water (5 mL). After washing with 0.1 M hydrochloric acid (5 mL) and water (5 mL), the flavonol fraction was eluted with methanol (3 × 5 mL). This fraction also contained other neutral or acidic polyphenols. Fixed anthocyanins were removed using 2% ammonia in 80% methanol (3 × 5 mL). Finally, the cationic-exchanger material was regenerated with 0.52 M hydrochloric acid in 80% methanol (3 × 5 mL). Subsequent conditioning of the cartridge with methanol and water allows its reuse at least four or five more times. The eluate containing flavonols was dried in a rotary evaporator (30°C) and resolved in 1 mL of methanol.

4. Analytical procedures

The anthocyanin and flavonol fingerprints of skin extracts were obtained with HPLC-DAD [27], using a Waters Corp. 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. The separations were performed 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. Water/acetonitrile (95:5) adjusted to pH 1.3 with trifluoroacetic acid (solvent A), and water/acetonitrile (50:50) adjusted to pH 1.3 with trifluoroacetic acid (solvent B) were used as mobile phases. Elution was performed at a 0.8 mL/min flow rate. For anthocyanins, a 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 was used. A linear gradient from 10% B to 35% B in 30 min, from 35% B to 50% B in 6 min, from 50% B to 100% B in 8 min, 100% B for 3 min, and from 100% B to 10% B in 1 min was used for flavonols. 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. The identity of the different anthocyanins and flavonols was elucidated by HPLC-MS, using an 1100 HPLC system (Agilent Technologies, Santa Clara, CA) with a PDA UV–Vis detector coupled to a QTOF mass spectrometer (AB SCiex, Framingham, MA). Chromatographic conditions were those used for the HPLC-DAD analysis. The MS analysis was carried out in the ESI+ mode, scanning from m/z 50 to 2000, with the following conditions: spray voltage, 5500 V; gas pressure, 80 psi; declustering potential, 50 V; focus potential, 210 V; CAD, 3 psi.

Total anthocyanins were determined in grape skins extracts, using the procedure described by Niketic-Aleksic and Hrzadina [32] using a BOECO S-22 UV–Vis spectrophotometer. Quantitative analysis of flavonols was carried out by HPLC, considering the surface of the different peaks, using standard solutions of quercetin-3-*O*-glucoside in the range of 20–100 mg/L.

5. Anthocyanin fingerprint of wild grapes

The HPLC analysis of anthocyanins extracted from wild grape skins permits the separation of 15 different anthocyanins. **Table 1** shows name, abbreviation, and number of peaks for each compound considered. The anthocyanin fingerprint of wild grapes revealed the presence of three groups of wild grapes genotypes, as it has been previously reported [25, 26]. **Figure 2** displays three typical chromatograms of those groups of genotypes.

The three groups of genotypes differ in different aspects linked to the pathways involved in anthocyanin biosynthesis [33] that are shown in **Figures 3** and **4**. First, the presence or absence of acylated anthocyanins, which implies important differences in the expression of genes involved in acyltransferase activity. Second, the prevalence of anthocyanins derived from delphinidin (Dp) or from cyanidin (Cy), which implies the differential expression of genes that control flavonoid-3′-hydroxylase and flavonoid-3′,5′-hydroxylase activities. Finally, the extent of methylation of Dp-3-gl and Cy-3-gl, due to the differential expression of genes controlling *O*-methyltransferase activity.

Anthocyanin	Abbreviation	Number of peaks
Delphinidin-3-*O*-glucoside	Dp-3-gl	1
Cyanidin-3-*O*-glucoside	Cy-3-gl	2
Petunidin-3-*O*-glucoside	Pt-3-gl	3
Peonidin-3-glucoside	Pn3-gl	4
Malvidin-3-glucoside	Mv-3-gl	5
Delphinidin-3-acetyl-glucoside	Dp-3-acgl	6
Cyanidin-3-acetylglucoside	Cy-3-acgl	7
Petunidin-3-acetylglucoside	Pt-3-acgl	8
Delphinidin-3-*p*-coumarylglucoside	Mv-3-cmgl	9
Peonidin-3-acetylglucoside	Pn-3-acgl	10
Malvidin-3-acetylglucoside	Mv-3-acgl	11
Petunidin-3-*p*-coumarylglucoside	Pt-3-cmgl	12
Malvidin-3-caffeoylglucoside	Mv-3-cfgl	13
Peonidin-3-*p*-coumarylglucoside	Pn-3-cmgl	14
Malvidin-3-*p*-coumarylglucoside	Mv-3-cmgl	15

Table 1. Name, abbreviation, and number of peaks for the different anthocyanins analyzed by HPLC.

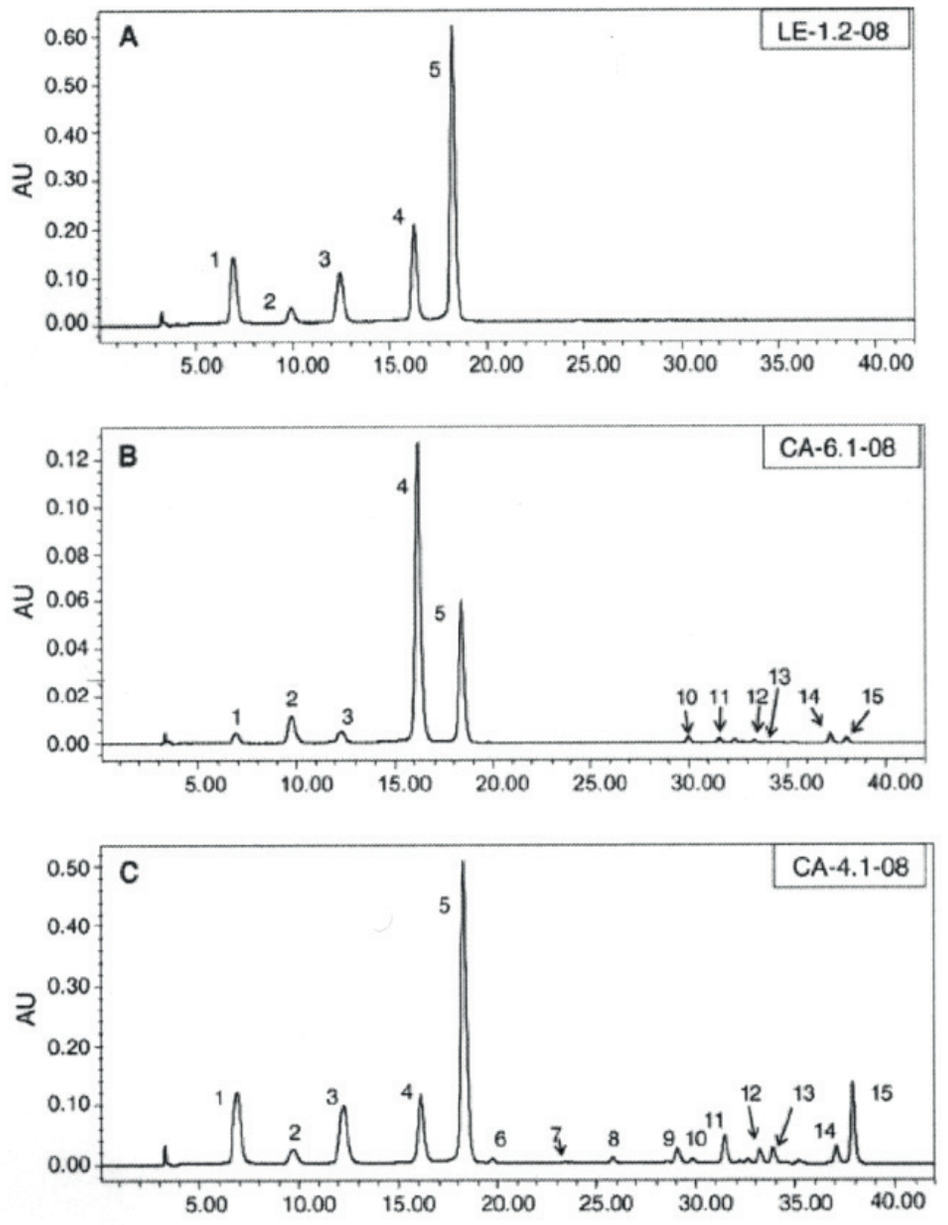

Figure 2. Chromatograms registered at 520 nm for three grape skins extracts representative of phenotypic groups A, B, and C. For key to peaks, see **Table 1**.

Genotypes of group A (tree samples) did not contain acylated anthocyanins (**Figure 2A**). This character is unusual in cultivated grapevines, occurring primarily in cv. Pinot Noir and its colored mutants [34, 35]. In these genotypes, genes encoding or regulating acyltransferase activity is neither presented nor expressed. To our knowledge, this type of anthocyanin fingerprint has not been described in grape cultivars usually considered of Spanish origin [35–37]. **Table 2** displays the percentages of several groups of anthocyanins presented in these genotypes. As can be observed, one genotype (BI-1.3bis) contained a remarkable amount of Cy-derived anthocyanins, over 50%, and the extent of methylation was very high (over 60%) in two genotypes (BI-1.3bis and LE-1.2). Similar trends were observed in several wild grapevine accessions that do not contain acylated anthocyanins in a previous report [26].

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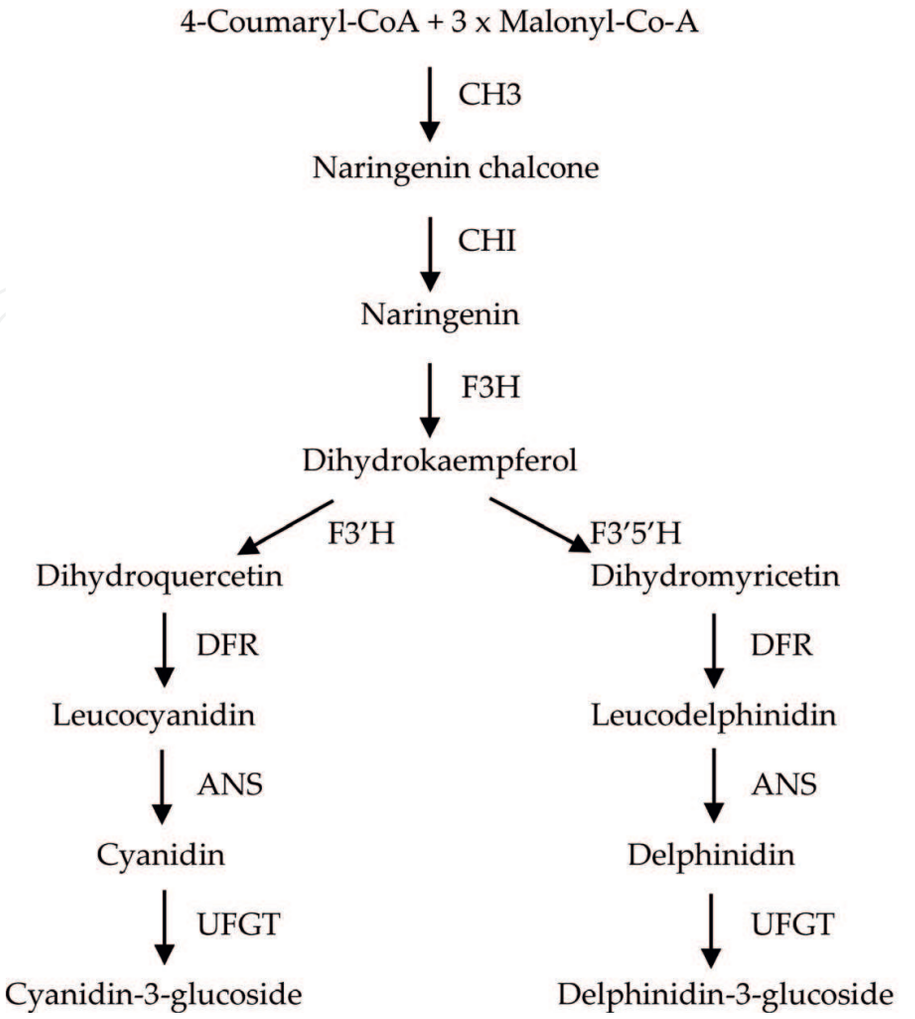

Figure 3. Biosynthesis of Dp-3-gl and Cy-3-gl. CH3, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase; F3′H, flavonoid-3′-hydroxylase; F3′5′H, flavonoid-3′,5′-hydroxylase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; UFGT, UDP-Glc-flavonoid 3-O-glucosyltransferase. See full compound names in **Table 1**.

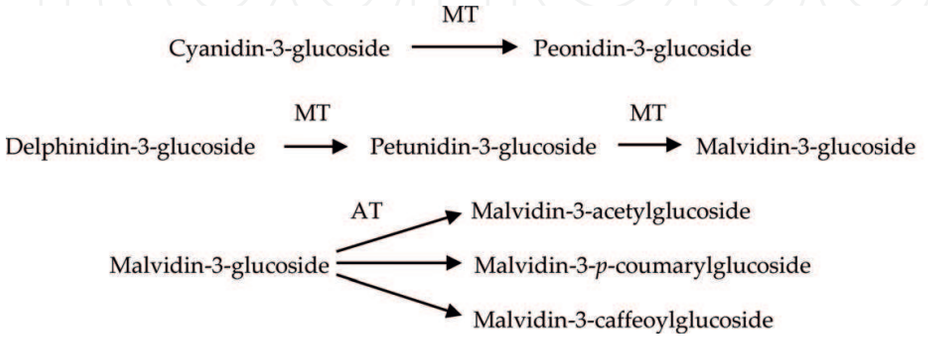

Figure 4. Biosynthesis of anthocyanins derived from Dp-3-gl and Cy-3-gl. MT, methyltransferase; AT, acyltransferase. See full compound names in **Table 1**.

Genotype	Dp-derived	Cy-derived	Methylated
BI-1.3bis	48.14	51.86	62.25
LE-1.2	80.61	19.39	82.14
SS-3.5bis	70.10	29.90	36.75

See full compound names in **Table 1**.

Table 2. Percentages of Dp-derived, Cy-derived, and methylated anthocyanins in genotypes of group A.

Genotypes of group B (six samples) contained acylated anthocyanins and a high proportion of Cy-derived anthocyanins (**Figure 2B**). This character is rare in cultivated grapevines, was observed only in 12 cultivars among 64 studied by Mattivi *et al*. [34], and it has been reported only in a cultivar considered of Spanish origin, cv. Brancellao [38]. Most cultivars of this type are gray or rosé cultivars, or even mutants of white cultivars. **Table 3** displays the percentages of several groups of anthocyanins presented in these genotypes. The percentage of Cy-derived anthocyanins ranged between 37 and 68%, and usually the percentage of methylated anthocyanins was up to 50%. Thus, Pn-3-gl usually was the major anthocyanin; the most remarkable exception was genotype SS-6.5bis. In this genotype, Cy-3-gl was the major anthocyanin (40.82%), as methylation was not very intense. Acylation was quite variable; it was too low in genotype CO-5.1, but considerably high in genotypes CA-13.3 and H-6.1. Moreover, most acylated anthocyanins were *p*-coumarylated derivatives; this character is quite common in red cultivars usually considered as Spanish, like Garnacha and Tempranillo [35].

Genotypes of group C (16 samples) contained acylated anthocyanins and a high proportion of delphinidin-derived anthocyanins (**Figure 2C**), as do most grapevine cultivars [33–37]; this implies that the expression of genes controlling flavonoid-3′,5′-hydroxylase is too high if compared with that of genes controlling flavonoid-3′-hydroxylase. The percentages of several groups of anthocyanins presented in these genotypes are displayed in **Table 4**. As can be noted, these genotypes also presented a high extent of methylation; the percentage of methylated anthocyanins was higher than 60%, except in four genotypes (BA-1.1, NA-1.4bis, SE-3.4,

Genotype	Dp-derived	Cy-derived	Methylated	Acylated	Acetylated	*p*-Coumarylated
CA-6.1	32.98	67.02	91.26	4.84	1.58	2.83
CA-13.3	54.35	45.65	80.84	14.77	4.42	9.97
CO-5.1	48.04	51.96	55.07	1.78	0.63	1.06
H-6.1	62.83	37.17	54.62	17.41	9.05	8.29
SS-6.5bis	42.70	57.30	31.17	4.46	2.09	2.38
VI-2.1bis	58.34	41.66	52.55	6.63	2.96	3.58

See full compound names in **Table 1**.

Table 3. Percentages of Dp-derived, Cy-derived, methylated, acylated, acetylated, and *p*-coumarylated anthocyanins in genotypes of group B.

Genotype	Dp-derived	Cy-derived	Methylated	Acylated	Acetylated	*p*-Coumarylated
BA-1.1	85.75	14.25	59.97	3.56	1.85	1.61
CA-4.1	82.69	17.31	71.97	14.70	4.76	9.01
CA-9.7	87.90	12.10	93.26	6.33	2.62	1.90
CA-11.3	83.92	16.08	65.99	34.26	27.74	6.06
CO-2.2	87.13	12.87	68.84	15.40	7.60	6.55
CO-3.7	81.95	18.05	73.27	16.32	8.00	7.08
CR-1.6	91.87	8.13	84.54	10.73	5.12	4.16
H-1.1	79.67	20.33	72.15	3.65	1.97	1.21
J-2.4	88.23	11.77	90.76	9.01	4.08	3.90
NA-1.4bis	73.82	26.18	43.96	2.82	1.26	1.56
O-1.5bis	80.48	19.52	62.43	6.64	3.26	3.15
S-1.3bis	88.95	11.05	66.03	16.43	7.42	8.10
S-1.9	90.97	9.03	65.99	8.84	4.37	4.11
SE-1.5	92.97	7.03	86.35	25.39	11.12	12.96
SE-3.4	84.65	15.35	58.70	16.42	6.52	9.59
SS-3.5	78.68	21.32	54.09	21.27	17.41	3.58

See full compound names in **Table 1**.

Table 4. Percentages of Dp-derived, Cy-derived, methylated, acylated, acetylated, and *p*-coumarylated anthocyanins in genotypes of group C.

and SS-3.5). In these late genotypes, Dp-3-gl was the major anthocyanin, but in the other 11 genotypes, the major genotype was Mv-3-gl. Sometimes, its content was higher than 90%.

The extent of acylation among genotypes included in group C was quite variable and not related to the extent of methylation. The percentage of acylated anthocyanins ranged from less than 3% (NA-1.4bis, 2.82%) to nearly 35% (CA-11.3, 34.26%). In two genotypes with a high extent of acylation (CA-11.3 and SS-3.5), acetylated anthocyanins were much more abundant than *p*-coumarylated anthocyanins. This character is well-documented in several French cultivars (e.g., Cabernet Sauvignon and Merlot), but is rare in Spanish cultivars. Most genotypes present less than 15% acylated anthocyanins, and percentages of acetylated and *p*-coumarylated anthocyanins were quite similar, as it has been observed in many grape cultivars considered of Spanish origin [35].

Data reported in **Table 5** point out that the total content of anthocyanins was quite variable, ranging from 273 to 3534 mg/kg, but, there is a remarkable difference among genotypes collected in populations located in Northern Spain and those from populations located in Southern Spain. As can be noted, genotypes from Northern Spain contained a higher amount of anthocyanins than those originated in Southern Spain ($p < 0.05$). As it is well-known, the accumulation of anthocyanins in grapes, that take place after veraison, is affected, at a great extent, by day-night thermal contrast [12], which can be considered neutral in our study, as all

	Range	Mean value	Standard deviation
Northern Spain	956–3078	2088	693
Southern Spain	273–3534	1697	797

Table 5. Range, mean value, and standard deviation for the content of total anthocyanins (mg/kg of grapes) in genotypes originated in northern Spain and southern Spain.

genotypes grew in the same environment. Thus, differences observed in anthocyanin content can be considered of genetic nature. The most probable explanation is that genotypes from Northern Spain have evolved in oceanic climate environments, where veraison takes place at the end of summer, and day-night thermal contrast is smaller than that in the Mediterranean climate environments in which evolved those genotypes collected from Southern Spain. Thus, it is probable that wild grapes in Northern Spain have evolved to accumulate enough anthocyanins capable of attracting birds and other animals to facilitate the dispersion of seeds, despite the limiting weather conditions for anthocyanin accumulation. Thus, when genotypes from Northern Spain grow in a warmer environment, like that of El Encin Germplasm Bank, the accumulation of anthocyanins may be very high.

6. Flavonol fingerprint of wild grapes

Six different flavonols were fully identified by HPLC-MS: a myricetin derivative (3-*O*-glucoside, My-3-gl), three quercetin derivatives (3-*O*-glucoside, Qu-3-gl; 3-*O*-glucuronide, Qu-3-gr; 3-*O*-rhamnoside, Qu-3-rh), a laricitrin derivative (3-*O*-glucoside, La-3-gl), and a syringetin derivative (3-*O*-glucoside, Sy-3-gl). All these flavonols have been identified in berries of several red grapevine cultivars [31, 38], and their presence in wild grapes should be expected. The flavonols tentatively identified were 3-*O*-galactosides of myricetin (My-3-gal) and quercetin (Qu-3-gal), which have been previously detected in red grape skins [38].

Among those flavonols, the most abundant were My-3-gl (trihydroxysusbtituted in B-ring, analogous to Dp-3-gl) and two quercetin derivatives (Qu-3-gl and Qu-3-gr, analogous to Cy-3-gl because they are dihydroxysubstituted in B-ring). Other myricetin derivatives, like laricitrin and syringetin derivatives (La-3-gl and Sy-3-gl), were minor components, and some of them were absent in several samples. **Figure 5** displays the chromatogram registered at 350 nm for an extract of genotype H-6.1, with three major peaks, corresponding to My-3-gl, Qu-3-gr, and Qu-3-gl.

Three phenotypic groups of wild grapes have been considered, taking into account the amounts of My-3-gl, Qu-3-gl, and Qu-3-gr. Group 1 includes eight genotypes, which did not contain My-3-gl (**Table 6**). This fact implies that, in these genotypes, dihydroxylation of dihydrokaempferol by flavonoid-3′,5′-dihydroxylase is blocked (**Figure 6**). In these genotypes, the major flavonol was Qu-3-gl or Qu-3-gr, and in some cases, contained very small amounts of other flavonol; anyway, La-3-gl and Sy-3-gl were absent (**Table 6**). Group 2 is formed by nine genotypes, which contain My-3-gl, but major flavonol was Qu-3-gl or Qu-3-gr (**Table 7**). These genotypes usually contained several minor flavonoids, including La-3-gl and Sy-3-gl,

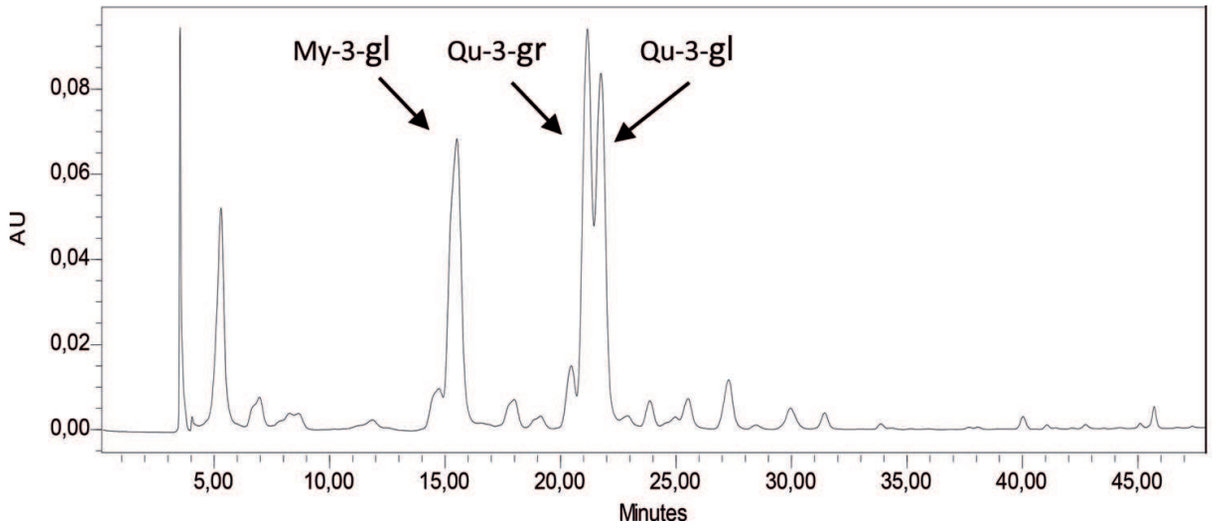

Figure 5. Chromatogram registered at 350 nm for a grape skin extract. My-3-gl, myricetin-3-*O*-glucoside; Qu-3-gr, quercetin-3-*O*-glucuronide; Qu-3-gl, quercetin-3-*O*-glucoside.

Genotype	My-3-gl	Qu-3-gr	Qu-3-gl	Other flavonols	Total
CA-6.1	nd	27.0	60.8	14.0	101.9
CA-13.3	nd	11.5	47.7	9.0	68.2
H-6.1	nd	54.2	73.6	17.3	145.1
NA-1.4bis	nd	18.0	10.7	0.0	28.7
S-1.3bis	nd	10.0	6.8	12.5	29.4
SS-3.5bis	nd	21.1	26.5	nd	47.6
SS-6.5bis	nd	26.1	61.5	nd	87.5
VI-2.1bis	nd	22.3	44.9	nd	67.2

nd: not detected. See full compound names in **Figure 5**.

Table 6. Content of flavonols (mg/kg of grapes) in genotypes that did not contain My-3-gl.

the exceptions being genotypes CA-11.3 and SE-3.4. In these two genotypes, methylation of My-3-gl by action of a *O*-methyltransferase is blocked. Finally, group 3 includes eight genotypes presenting My-3-gl as a major flavonol (**Table 8**). In most cases, these genotypes contained several minor flavonoids, including La-3-gl and Sy-3-gl, the exceptions being genotypes O-1.5bis and SS-3.5. Like in genotypes CA-11.3 and SE-3.4, methylation of My-3-gl by action of a methyltransferase is blocked.

Data reported in **Tables 6**–**8** point out that the total content of flavonols was quite variable, ranging from 29 to 324 mg/kg. Nevertheless, there is a remarkable difference among genotypes from populations located in Northern Spain and those from populations located in Southern Spain (**Table 9**). As can be noted, genotypes from Northern Spain contained a lower amount of flavonols than those originated in Southern Spain. The accumulation of flavonols in grapes

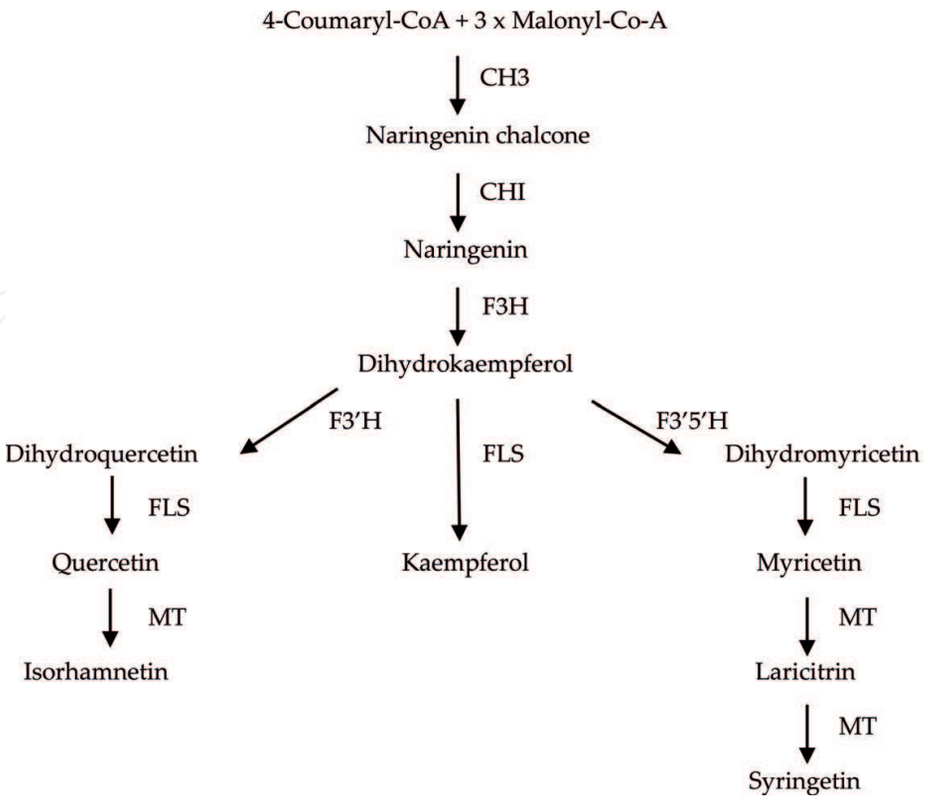

Figure 6. Biosynthesis of flavonol aglicones. CH3, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3β-hydroxylase; FLS, flavonol synthase; F3′H, flavonoid-3′-hydroxylase; F3′5′H, flavonoid-3′,5′-hydroxylase; MT, methyltransferase.

Genotype	My-3-gl	Qu-3-gr	Qu-3-gl	Other flavonols	Total
BI-1.3bis	1.4	14.0	14.3	3.1	32.8
CA-9.7	2.9	13.8	9.4	13.7	39.8
CA-11.3	10.3	25.8	27.4	nd	63.4
CO-5.1	4.8	37.8	74.1	6.7	123.4
CR-1.6	6.3	31.4	20.3	25.2	83.2
J-2.4	8.1	14.4	7.8	14.2	44.5
LE-1.2	3.2	16.2	11.5	24.0	54.8
SE-1.5	80.8	106.1	71.4	46.2	304.4
SE-3.4	98.3	113.4	112.5	nd	324.1

nd: not detected. See full compound names in **Figure 5**.

Table 7. Content of flavonols (mg/kg of grapes) in genotypes that contained My-3-gl, being Qu-3-gl or Qu-3-gr the major flavonol.

increases with sun exposure, as demonstrated by other authors [16]. This factor can be considered as neutral in our study because all genotypes grown in the same environment; thus, differences observed in flavonols content can be considered of genetic nature. The most probable explanation is that genotypes originally collected in Northern Spain have evolved in

Genotype	My-3-gl	Qu-3-gr	Qu-3-gl	Other flavonols	Total
BA-1.1	84.8	47.0	42.0	9.5	183.3
CA-4.1	22.7	15.2	15.2	1.3	54.4
CO-2.2	48.7	14.6	22.8	14.0	100.1
CO-3.7	26.8	24.9	9.8	23.5	84.9
H-1.1	40.4	11.1	18.0	25.2	94.7
O-1.5bis	77.7	35.1	59.2	nd	172.1
S-1.9	58.0	30.1	28.3	12.2	128.6
SS-3.5	22.9	11.2	14.0	nd	48.1

nd: not detected. See full compound names in **Figure 5**.

Table 8. Content of flavonols (mg/kg of grapes) in genotypes in which My-3-gl was the major flavonol.

	Range	Mean value	Standard deviation
Northern Spain	28.7–172.1	69.7	47.3
Southern Spain	39.8–324.1	121.0	87.3

Table 9. Range, mean value, and standard deviation for the content of total flavonols (mg/kg of grapes) in genotypes originated in northern Spain and southern Spain.

oceanic climate environments, where sunlight exposure is lower than in the Mediterranean climate environments in which evolved genotypes from Southern Spain.

7. Comparison between anthocyanin fingerprint and flavonol fingerprint

The anthocyanin and flavonol fingerprints of wild grape genotypes are quite different, taking into account the pathways involved in their biosynthesis. Thus, in most genotypes B-ring trisubstituted anthocyanins (Dp-derived) predominate, but B-ring disubstituted flavonols (Qu derivatives) are more abundant than My derivatives (B-ring trisubstituted). Moreover, some genotypes do not present B-ring trisubstituted flavonols (**Table 7**), but they always present Dp-derived anthocyanins, sometimes in a high proportion (e.g., genotype S-1.3bis, see **Table 4**). On the other hand, some genotypes presenting a very low amount of Cy-derived anthocyanins (e.g., CR-1.6 and SE-1.5, see **Table 4**) contain a remarkable amount of Qu derivatives (**Table 7**). These data suggest that flavonol synthase activities linked to the formation of Qu and My are regulated in a different way than enzymatic activities linked to the formation of Dp-3-gl and Cy-3-gl from the corresponding dihydroflavonols. Other relevant biosynthetic difference among flavonols and anthocyanins is B-ring *O*-methylation. This reaction seems to be more intense for anthocyanins than for flavonols; thus, in most genotypes, methylated anthocyanins predominate. This fact suggests that primitive anthocyanins (Cy-3-gl and Dp-3-gl) are better substrates for *O*-methyltransferases (OMT) than quercetin and myricietin, as pointed out previously [34].

In general, phenylpropanoid biosynthesis and subsequent flavonoid production are tightly linked to primary metabolism through phenylalanine as a precursor of flavonoids. Catalyzing the first committed step into the flavonoid biosynthetic pathway, chalcone synthase (CHS) plays a pivotal role to provide a common chalcone precursor for the production of all intermediates and final products of the flavonoid biosynthetic pathway which are therefore biogenetically and structurally related (**Figures 3** and **6**). In most plants, including grapevine, flavanones are preferentially used as substrates for flavanone-3β-hydroxylase (F3H), which produces dihydroflavonols as an important branch point flavonoid and an essential substrate for all classes of downstream compounds (**Figures 3** and **6**). The biosynthesis of flavonol aglycones through flavonol synthase 1, FLS4 [39, 40]; as well as the biosynthesis of proanthocyanidins and anthocyanin precursors through dihydroflavonol 4-reductase (DFR) employs dihydroflavonols as substrates thereby directly competing for the same substrate (**Figures 3** and **6**). DFR reshuffles substrates away from flavonol biosynthesis and converts dihydroflavonols to leucoanthocyanidins, which are precursors for proanthocyanidin and anthocyanin biosynthesis [41]. While DFR is specific for the anthocyanin/proanthocyanidin pathway, flavonoid-3′-hydroxylase (F3′H) and flavonoid-3′,5′-hydroxylase (F3′5′H) gene products are necessary for the production of all subclasses, namely flavonols, anthocyanins, and proanthocyanidins. In general, hydroxylation of the B-ring of dihydroflavonols, flavanones, and flavones changes the color of the resulting anthocyanin-derived pigment and increases dramatically the chemodiversity of flavonols, proanthocyanidins, and anthocyanins [42].

The known biosynthetic pathway of flavonoids shares common enzymatic steps, whereas the activities of enzymes specific for anthocyanins or flavonols lead exclusively to the biosynthesis of the respective flavonoid by competing for common substrates (**Figures 3** and **6**). The accumulation of flavonol compounds in the berry is mediated by an increase of transcripts encoding FLS (*VvFLS4* or *VvFLS5*) under the regulation of the transcriptional factor *VvMYF* [40]. Later during veraison, the anthocyanins are synthesized through the flavonoid pathway in grapevine cultivars that harbor the wild-type VvmybA1 transcription factor for the expression of UFGT [43]. The encoded enzyme UFGT catalyzes the glycosylation of unstable anthocyanidin aglycones into pigmented anthocyanins (**Figure 3**). Two primitive anthocyanins (Cy-3-gl and Dp-3-gl) are synthesized in the cytosol of berry epidermal cells. The B-ring of Cy-3-gl is dihydroxylated at the 3′ and 4′ positions, whereas Dp-3-gl has a tri-hydroxylated B-ring because of an additional hydroxyl group at the 5′ position. The 3′ position of Cy-3-gl and Dp-3-gl and sequentially the 5′ position of Dp-3-gl can be methoxylated by *O*-methyltransferase (*VvOMT*), generating Pn-3-gl, Pt-3-gl, and Mv-3-gl, respectively [44]. Anthocyanins can be further modified by acyltransferases, which produce 3-*O*-acetyl-, 3-*O*-coumaroyl-, and 3-*O*-caffeoyl-monoglucosides by attaching acyl groups to the C6″ position of the glucose moiety [45].

Taking into consideration the regulations and biosynthesis pathway, we can suggest that the differences in the anthocyanin and flavonol fingerprints of wild grapes are putative due to the different expression level of the structural genes *F3′H* and *F3′5′H*, which are essential in the breach point for the final anthocyanin and flavonol compounds and the transcriptional factor involved in the pathway. Finally, the different level of methylation of Cy-3-gl and Dp-3-gl could be due to different expression of *OMT* genes and the gene encoding an anthocyanin

acyltransferase, anthocyanin-3-*O*-glucoside-6″-*O*-acyltransferase (*3*), which is capable of producing the common acylated anthocyanins found in grape berries [46].

8. Conclusions

The anthocyanin fingerprint of wild grapes skins, considering the relative amount of 15 anthocyanins, showed a considerable variability, being possible to distinguish three phenotypic groups. Differences into those groups are related with the predominance of delphinidin- or cyanidin-derived anthocyanins and the expression of genes involved in acyltransferase activities. Moreover, it has been possible to separate 12 flavonol glycosides, eight of them were successfully identified. Major flavonols were Qu-3-gl, Qu-3-gr, and My-3-gl. The diversity and number of flavonols differed for each genotype. In most genotypes, Qu-3-gl or Qu-3-gr was the major flavonol, and My-3-gl was absent in some genotypes. Quantitative analysis of anthocyanins and flavonols revealed that genotypes collected in wild grapevine populations located in Northern Spain were richer in anthocyanins and poorer in flavonols than those collected in populations located in Southern Spain. This difference may be explained by the different expression level of the structural genes and transcriptional factors in the biosynthesis pathway in relation with the impact of climatic conditions on the evolution of wild grapes in different environments.

Acknowledgements

The authors acknowledge the financial support of Spain's Ministry of Economy and Competitiveness (Project RTA2014-0016-C003-01, MINECO-INIA-CCAA). AB holds an INIA fellowship for this project.

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