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Metabolite Profiling of Grape: Flavonols and Anthocyanins

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Flavonols are products of the flavonoid biosynthetic pathway, which also give rise to anthocyanins and condensed tannins in grapes. We investigated their presence in the berry skins of 91 grape varieties (*Vitis vinifera* L.), in order to produce a classification based on the flavonol profile. The presence of laricitrin 3-O-galactoside and syringetin 3-O-galactoside in red grapes is reported here for the first time. In red grapes, the main flavonol was quercetin (mean = 43.99%), followed by myricetin (36.81%), kaempferol (6.43%), laricitrin (5.65%), isorhamnetin (3.89%), and syringetin (3.22%). In white grapes, the main flavonol was quercetin (mean = 81.35%), followed by kaempferol (16.91%) and isorhamnetin (1.74%). The delphinidin-like flavonols myricetin, laricitrin, and syringetin were missing in all white varieties, indicating that the enzyme flavonoid 3′,5′-hydroxylase is not expressed in white grape varieties. The pattern of expression of flavonols and anthocyanins in red grapes was compared, in order to gain information on the substrate specificity of enzymes involved in flavonoid biosynthesis.

KEYWORDS: ***Vitis vinifera*; grape; flavonols; anthocyanins; flavonoid 3′-hydroxylase; flavonoid 3′,5′-hydroxylase; O-methyltransferase**

INTRODUCTION

Grapes and wine contain different classes of polyphenols. Flavonols are flavonoids that are found in most higher plants, usually in glycosidic forms. They are products of the flavonoid biosynthetic pathway, which also give rise to anthocyanins and to condensed tannins in grapevines (*1*, *2*). Flavonols are predominantly synthesized in the grape skin (*3*). During red wine making, flavonols are extracted from the grape skin, making their way into red wine, where they play a fundamental role in terms of quality. This is not the case with white wines, as the grape skins are separated earlier from the must, meaning that there is insuffcent time for significant extraction.

Their most widespread roles in plants appear to be as UV protectants localized in the upper epidermis (*4*, *5*) and as copigments with the anthocyanins in flowers and fruit (*6*, *7*). They can also participate in plant−pathogen interactions (*5*, *8*). The main flavonols reported in grape berries are quercetin-3-O-glucoside and quercetin-3-O-glucuronide (*3*, *9*, *10*).

Flavonols are involved in the stabilization of the flavilium form of anthocyanins in young red wines through copigmentation (*11*). Flavonols are also important from a nutritional point of view, being highly bioactive compounds widely distributed in dietary plants (*12*). Pharmacokinetics and the metabolism of quercetin and quercetin glycosides in humans have been extensively investigated (*12*, *13*).

Wines that contain higher concentrations of flavonols are produced from thick-skinned grapes such as Cabernet Sauvignon, which are characterized by a high skin:volume ratio, rather than thinner-skinned varieties such as Grenache with a low skin: volume ratio (*14*). There is also a trend toward higher flavonol levels in wines made from grapes grown in sunnier climates. Price et al. (*3*) reported that Pinot Noir wines made from sun-exposed grape clusters contain 10 times more quercetin glycosides than wines made from shaded berries. Similar relationships between sunlight exposure and an increase in quercetin 3-glucoside were reported by Spayd et al. (*15*). Downey et al. (*16*) reported that reduced biosynthesis rather than degradation is responsible for the low levels of flavonols present in shaded grapes and that all flavonol biosynthesis in Syrah berries was light induced.

The highest flavonol concentrations in grapes were found at flowering, followed by a decrease as the grapes increased in size. Subsequently, a significant level of flavonol biosynthesis was observed during berry development and the greatest increase in flavonols per berry can be observed 3−4 weeks postveraison (*10*).

Polyphenols are also important from the taxonomical point of view. It is known that the patterns of some classes of flavonoids, such as anthocyanins, are under strict genetic control and that their distribution varies considerably among different grape cultivars (*17*). The profiles of anthocyanins for each variety are relatively stable, while absolute concentrations can vary widely between different vintages, due to both environmental and agronomical factors. The anthocyanin profile can therefore be used as a chemotaxonomic parameter for the classification of red *Vitis vinifera* varieties (*18*−*20*).

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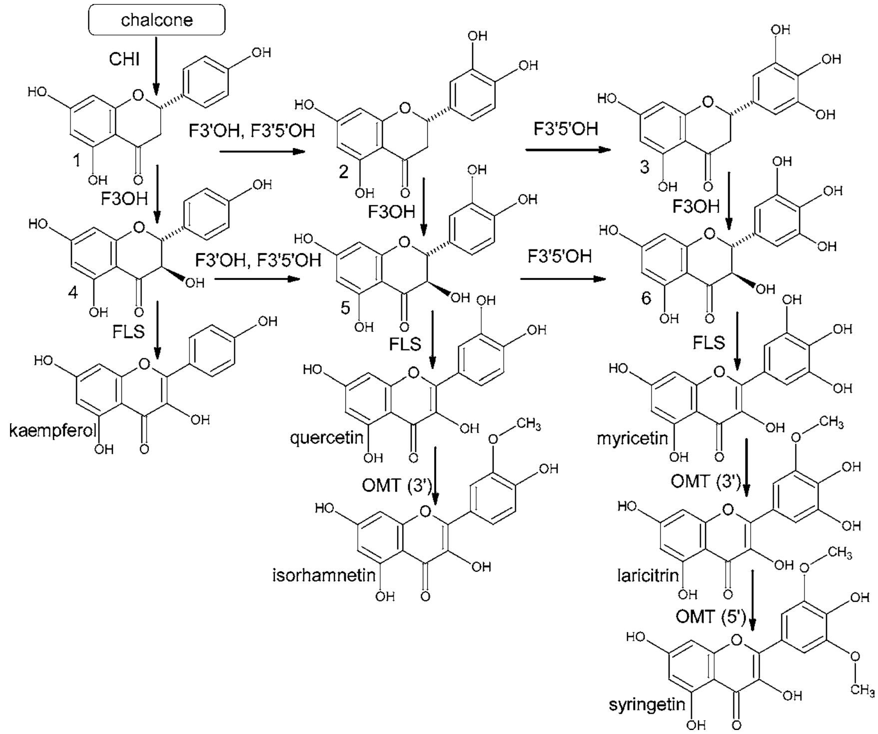

Figure 1. General pattern for flavonol biosynthesis. Precursors: 1, naringenin; 2, eriodictyol; 3, 3′,4′,5,5′,7-pentahydroxyflavanone; 4, dihydrokaempferol; 5, dihydroquercetin; and 6, dihydromyricetin. Enzymes: CHI, chalcone isomerase; F3OH, flavanone 3-hydroxylase; FLS, flavonol synthase; F3′OH, flavonoid 3′-hydroxylase; F3′5′OH, flavonoid 3′,5′-hydroxylase; and OMT, methyltransferases.

Downey et al. reported that the pattern of flavonol accumulation over three seasons also showed very little variation (*10*), and the quercetin/myricetin ratio has proved useful for discriminating Carmenere from Merlot wines (*21*). It is expected that in a similar way to anthocyanins (*22*, *23*), it will also be possible to use flavonols for taxonomical classification and metabolite profiling (*24*, *25*), thus providing new information on the metabolism of flavonoids in both red and white grape varieties. Unfortunately, only the three major grape flavonols (quercetin, myricetin, and kaempferol) have been considered in previous studies, whereas knowledge of the whole pattern would be necessary for a more complete understanding of the flavonol metabolism.

The molecular structure and the expression of the main enzymes involved in the biosynthesis of flavonoids have been extensively investigated (*10*, *23*−*30*). On the basis of these studies, a general diagram for the biosynthesis of grape flavonols can be established, and the presence of six different aglycons is expected (**Figure 1**).

The aim of this paper was to investigate the presence of all of these flavonols in the berry skins of 91 grape varieties picked at maturity. The flavonol data were analyzed using cluster and principal component analysis, in order to produce a classification based on the flavonol profile. Finally, the flavonol pattern was compared with the anthocyanin pattern in order to provide useful information about the relative importance of flavonoid 3′,5′-hydroxylase (F3′5′OH), 3′-O-methyltransferase (3′-OMT), and 5′-O-methyltransferase (5′-OMT) enzymatic activities in the metabolic pathways of these two classes of flavonoids.

MATERIALS AND METHODS

Standards and Reagents. All reagents and chromatographic solvents (acetonitrile, methanol, formic acid, and trifluoroacetic acid) were purchased from Carlo Erba (Rodano, Italy). Ethyl acetate, β-glucosidase from almonds (EC 3.2.1.21, catalogue #G-0395), and β-galactosidase from *Aspergillus oryzae* (EC 3.2.1.23, catalogue #G-5160) were purchased from Sigma (Steinheim, Germany); myricetin, kaempferol, quercetin, syringetin, syringetin-3-O-glucoside, and syringetin-3-O-galactoside standards were purchased from Extrasynthèse (Genay, France), and isorahmnetin was from Roth (Karlsruhe, Germany).

Samples. Ninety-one samples of *V. vinifera* from the ampelographic collection of the Istituto Agrario di San Michele all'Adige (IASMA, Trento, Italy) were included in the study. All varieties in this collection were of certain origin, checked, and named in agreement with existing literature (*31*) and cultivated using a standardized system, with Guyot trellising. All varieties were sampled in 2004 at technological maturity, defined as a content of soluble solids in the must corresponding to 18 °Brix, and the grapes were immediately stored in a freezer at −30 °C. A number of 64 red-skinned varieties (**Table 1**) and 27 white-skinned varieties (**Table 2**) were included in this study.

Extraction of Flavonols and Anthocyanins. This was done according to the literature (*32*). The skins of 20 frozen berries were peeled and subjected to extraction for 24 h in 100 mL of methanol. After the first extraction, the extract was separated and 50 mL of methanol was added to the skins, which were subjected to further extraction for 2 h. Both methanolic extracts were combined and stored in a freezer at −30 °C until analysis was carried out.

Acid Hydrolysis of Flavonol Glycosides. The total content of each aglycon was determined by high-performance liquid chromatography (HPLC) after acid hydrolytic cleavage of the flavonol conjugates, which released the aglycons (*33*). An aliquot of 10 mL of methanolic extract was evaporated to dryness in a 50 mL pear-shaped flask, using rotary evaporation under reduced pressure at 50−55 °C. The sample was brought back to 10 mL with 5 mL of trifluoroacetic acid (2 M in water) and 5 mL of methanol. The flask was placed in a boiling hot water bath with a condenser for 120 min. The mixture was cooled, dried in a rotating evaporator at 50−55 °C, dissolved in 40 mL of phosphate buffer at pH 7.00 and 80 mL of ethyl acetate, and transferred to a separatory funnel. Extraction was performed twice (shaking for 5 min) with a total of 160 mL of ethyl acetate. The combined extracts were anhydrified with anhydrous sodium sulfate, completely dried, and redissolved in 1 mL of methanol. The sample was filtered through 0.45 μm, 13 mm PTFE syringe-tip filters (Millipore, Bedford, MA) into LC vials for HPLC analysis.

Table 1. Flavonols in Red-Skinned Grape Varieties[a]

		%						total		factor		ratio		
no.	variety	myricetin	quercetin	laricitrin	kaempferol	isorhamnetin	syringetin	flavonols (mg/kg)	group	1	2	3′,5′-dihydroxy/ 3′- hydroxy derivatives	3′,5′-methoxy/ 3′,5′-hydroxy	3′-methoxy/ 3′-hydroxy
1	Aglianico	59.39	24.76	6.63	4.07	0.85	4.30	16.96	1	−2.07	1.44	2.75	0.07	0.03
2	Alicante Bouquette	48.49	25.52	9.25	4.98	5.40	6.36	40.53	1	−2.85	−0.59	2.07	0.13	0.21
3	Ancellotta	65.79	18.35	6.63	2.89	1.72	4.62	48.91	1	−2.50	1.26	3.84	0.07	0.09
4	Bovale Sardo	65.27	18.30	6.60	1.06	5.17	3.58	21.03	1	−2.64	0.12	3.21	0.05	0.28
5	Cabernet Sauvignon	45.68	34.06	5.65	5.31	5.05	4.26	34.31	1	−1.73	−0.34	1.42	0.09	0.15
6	Carmenere	47.95	33.30	5.13	7.12	3.17	3.33	78.40	1	−1.31	0.45	1.55	0.07	0.10
7	Casetta	57.58	30.94	3.85	5.09	1.34	1.20	80.37	1	−0.96	1.50	1.94	0.02	0.04
8	Croatina	56.22	24.10	6.94	3.28	5.38	4.08	38.70	1	−2.39	−0.21	2.28	0.07	0.22
9	Dolcetto	35.34	37.44	11.21	5.84	3.64	6.53	23.27	1	−2.49	−0.32	1.29	0.18	0.10
10	Lagrein	44.15	36.37	7.00	4.22	3.76	4.50	29.88	1	−1.85	0.04	1.39	0.10	0.10
11	Lambrusco Oliva	70.84	16.80	8.37	0.55	0.42	3.03	24.11	1	−2.60	1.93	4.78	0.04	0.03
12	Lambrusco Salamino	48.49	30.96	7.15	3.01	6.34	4.05	42.72	1	−2.22	−0.73	1.60	0.08	0.20
13	Malvasia Nera di Lecce	42.05	35.26	10.66	3.61	2.85	5.57	12.93	1	−2.48	0.19	1.53	0.13	0.08
14	Marzemino	62.58	18.97	8.24	3.47	1.76	4.99	20.10	1	−2.68	1.13	3.66	0.08	0.09
15	Nero	45.66	24.62	13.99	2.34	3.51	9.88	12.05	1	−4.10	−0.32	2.47	0.22	0.14
16	Nero d'Avola	53.58	29.98	5.25	6.64	2.28	2.26	41.01	1	−1.26	0.98	1.89	0.04	0.08
17	Pavana	50.62	22.56	11.01	2.23	4.37	9.20	16.08	1	−3.79	−0.42	2.63	0.18	0.19
18	Raboso del Piave	48.59	40.84	5.91	1.78	1.27	1.60	16.80	1	−1.13	1.25	1.33	0.03	0.03
19	Rebo	63.49	20.91	6.80	2.69	1.88	4.23	24.44	1	−2.38	1.18	3.27	0.07	0.09
20	Refosco	69.31	15.77	6.27	3.62	1.27	3.75	36.84	1	−2.33	1.57	4.66	0.05	0.08
21	Sagrantino	81.61	12.34	3.49	0.31	0.62	1.63	12.89	1	−2.01	2.28	6.69	0.02	0.05
22	Saint Laurent	41.81	33.32	8.03	5.15	5.64	6.04	48.68	1	−2.36	−0.80	1.43	0.14	0.17
23	Schioppettino	47.03	35.33	5.90	5.08	3.57	3.10	32.31	1	−1.45	0.30	1.44	0.07	0.10
24	Syrah	35.89	37.11	8.07	1.93	11.88	5.12	43.30	1	−2.63	−3.05	1.00	0.14	0.32
25	Tannat	60.74	31.68	2.51	0.00	1.41	3.66	8.37	1	−1.58	1.37	2.02	0.06	0.04
26	Tarrango	35.72	36.72	7.96	5.27	8.13	6.19	31.15	1	−2.37	−1.82	1.11	0.17	0.22
27	Tempranillo	48.47	32.02	4.63	11.72	1.52	1.64	54.46	1	−0.58	1.18	1.63	0.03	0.05
28	Teroldego	73.75	15.55	5.51	1.79	1.10	2.30	38.98	1	−2.14	1.86	4.90	0.03	0.07
29	Turca	43.19	33.42	8.25	3.38	6.72	5.04	59.37	1	−2.40	−1.07	1.41	0.12	0.20
30	Uva di Troia	56.86	33.59	4.06	1.14	2.06	2.30	8.17	1	−1.40	1.14	1.77	0.04	0.06
31	Zweigelt	54.29	17.23	11.33	2.93	4.70	9.52	31.03	1	−4.04	−0.48	3.43	0.18	0.27
32	Aleatico	18.54	54.68	5.73	13.96	3.91	3.18	16.42	2	−0.06	−0.49	0.47	0.17	0.07
33	Barbera	42.92	42.56	5.35	6.34	1.16	1.68	57.01	2	−0.66	1.16	1.14	0.04	0.03
34	Cabernet Franc	22.73	50.99	4.21	8.96	10.64	2.47	78.26	2	−0.64	−2.68	0.48	0.11	0.21
35	Calabrese	39.26	43.70	4.19	8.13	3.00	1.72	73.89	2	−0.46	0.44	0.97	0.04	0.07
36	Cannonau	28.32	42.02	9.17	8.64	6.21	5.64	9.41	2	−1.86	−1.29	0.89	0.20	0.15
37	Cesanese	34.71	48.71	9.44	1.81	2.34	2.99	7.05	2	−1.52	0.41	0.92	0.09	0.05
38	Cigliegiolo	27.32	53.27	6.52	6.54	3.43	2.92	18.22	2	−0.70	−0.11	0.65	0.11	0.06
39	Corvina	20.68	57.84	2.90	10.88	5.10	2.60	12.48	2	0.18	−0.78	0.42	0.13	0.09
40	Enantio	33.60	47.57	4.08	10.21	2.76	1.78	58.04	2	−0.14	0.39	0.78	0.05	0.06
41	Franconia	28.46	44.45	6.44	7.83	6.88	5.93	18.97	2	−1.61	−1.52	0.80	0.21	0.15
42	Merlot	25.25	53.96	4.78	8.13	5.25	2.64	52.89	2	−0.40	−0.75	0.55	0.10	0.10
43	Montepulciano	41.06	40.53	5.12	9.91	1.43	1.95	41.76	2	−0.50	1.01	1.15	0.05	0.04
44	Negro Amaro	36.92	43.77	5.81	9.98	0.98	2.54	25.66	2	−0.53	1.01	1.01	0.07	0.02
45	Nera dei Baisi	31.79	54.86	4.82	2.87	3.69	1.96	70.94	2	−0.58	−0.01	0.66	0.06	0.07
46	Pinot Noir	16.28	59.30	4.73	10.14	7.11	2.44	49.37	2	−0.11	−1.60	0.35	0.15	0.12
47	Pinotage	27.39	44.93	6.37	9.53	7.63	4.15	49.91	2	−1.19	−1.67	0.72	0.15	0.17
48	Primitivo	32.65	43.66	6.96	11.80	1.53	3.40	30.39	2	−0.70	0.64	0.95	0.10	0.04
49	Primitivo di Gioia	36.87	41.25	7.07	9.63	1.61	3.57	21.22	2	−1.00	0.70	1.11	0.10	0.04
50	Rondinella	19.10	58.28	2.83	13.87	4.47	1.44	19.12	2	0.65	−0.51	0.37	0.08	0.08
51	Sangiovese	22.69	67.13	2.79	6.13	0.68	0.57	24.56	2	0.74	0.96	0.38	0.02	0.01
52	Galioppo	7.63	79.81	1.83	4.98	4.91	0.83	11.72	3	0.98	−0.88	0.12	0.11	0.06
53	Gewuerztraminer	2.57	87.76	0.00	5.99	3.68	0.00	39.61	3	1.76	−0.48	0.03	0.00	0.04
54	Grignolino	9.57	78.36	0.00	9.54	2.52	0.00	3.81	3	1.74	0.09	0.12	0.00	0.03
55	Groppello Gentile	8.91	76.68	3.31	9.50	1.32	0.30	23.58	3	1.31	0.44	0.16	0.03	0.02
56	Molinara	9.33	74.55	5.17	8.31	2.64	0.00	9.48	3	0.91	−0.02	0.19	0.00	0.04
57	Moscato Rosa	7.31	69.47	2.59	14.47	5.13	1.04	29.66	3	1.18	−0.98	0.15	0.14	0.07
58	Muscat Rouge de Madere	2.35	77.12	0.00	17.52	3.01	0.00	28.85	3	2.27	−0.24	0.03	0.00	0.04
59	Nebbiolo	9.98	69.72	1.89	14.57	3.34	0.51	41.95	3	1.46	−0.24	0.17	0.05	0.05
60	Pinot Gris	11.87	67.21	3.86	8.23	6.37	2.46	28.58	3	0.18	−1.43	0.25	0.21	0.09
61	Pinot Tete de Negre	12.41	62.75	4.33	5.40	10.91	4.20	17.79	3	−0.78	−3.15	0.28	0.34	0.17
62	Schiava Grigia	13.89	71.15	3.02	5.06	5.64	1.24	21.54	3	0.42	−1.03	0.24	0.09	0.08
63	Schiava Grossa	7.18	75.12	1.96	10.12	5.13	0.49	36.68	3	1.22	−0.94	0.12	0.07	0.07
64	Schiava Lombarda	5.86	74.39	2.26	10.23	5.98	1.29	20.31	3	0.99	−1.33	0.12	0.22	0.08

[a] Flavonol profile (percentage of each aglycon out of the total), total concentration of flavonols in the berry (mg/kg), classification in groups according to cluster analysis, factor coordinates of the cases obtained using principal component analysis, and some ratios correlated to hydroxylase and O-methoxylase activities.

Enzymatic Hydrolysis of Flavonols Glycosides. Laricitrin and syringetin glycosides (3-glucoside and 3-galactoside) were identified with enzymatic hydrolysis following the protocol published by Vrhovsek et al. (*34*), in the skin extracts from the berries of four varieties, which contained high levels of flavonols: Cabernet Franc, Marzemino, Merlot, and Syrah. Each extract was analyzed using HPLC−diode array detection−mass spectrometry (DAD-MS) before and after hydrolysis (**Figure 2**).

HPLC-DAD Analysis of Flavonols. HPLC separation and quantification of flavonols were performed on a Hewlett-Packard Series 1090 instrument equipped with DAD, using a reversed-phase column Purospher RP18 250 mm × 4 mm (5 μm), with precolumn. Used were the following solvents: A = $HClO_4$ 0.3% in water; B = methanol. The linear gradient was as follows: from 40 to 90% B in 30 min, the flow rate was 0.45 mL/min. The time of equilibration for the column was 5 min, and the injection volume was 5 μL. The presence of flavonol aglycons was confirmed by coinjection with the corresponding standards. Each flavonol was quantified at 370 nm and expressed as mg/kg grapes by means of the external standard method, specific for each compound, with the exception of laricitrin, which—due to the lack of a commercial standard—was quantified as equivalent to myricetin. The results are given in **Tables 1** and **2**.

Table 2. Summary of the Data for White-Skinned Grape Varieties[a]

no.	variety	myricetin (%)	quercetin (%)	laricitrin (%)	kaempferol (%)	isorhamnetin (%)	syringetin (%)	total flavonols (mg/kg)	group	factor 1	factor 2
65	Catarratto	0.00	92.18	0.00	7.82	0.00	0.00	1.63	3	2.25	0.75
66	Chardonnay	0.00	72.73	0.00	25.99	1.28	0.00	19.88	3	2.84	0.31
67	Fiano	0.00	86.55	0.00	11.88	1.57	0.00	17.67	3	2.27	0.20
68	Garganega	0.00	74.75	0.00	23.12	2.13	0.00	15.20	3	2.65	0.01
69	Grechetto	0.00	79.12	0.00	20.88	0.00	0.00	12.12	3	2.76	0.76
70	Greco di Tufo	0.00	74.63	0.00	23.76	1.61	0.00	26.14	3	2.72	0.19
71	Inzolia	0.00	76.46	0.00	18.16	5.38	0.00	3.17	3	2.17	–1.13
72	Italia	0.00	75.65	0.00	22.98	1.37	0.00	3.34	3	2.72	0.28
73	Kozma Palne Muskotali	0.00	77.47	0.00	19.03	3.50	0.00	13.69	3	2.38	–0.47
74	Madeleine Angevine	0.00	77.54	0.00	22.46	0.00	0.00	1.36	3	2.82	0.76
75	Malvasia Bianca di Candia	0.00	83.94	0.00	13.58	2.48	0.00	17.90	3	2.26	–0.12
76	Malvasia Puntinata	0.00	83.51	0.00	13.42	3.07	0.00	4.49	3	2.20	–0.32
77	Marsanne	0.00	80.99	0.00	16.10	2.91	0.00	8.63	3	2.32	–0.27
78	Nosiola	0.00	80.31	0.00	16.76	2.92	0.00	9.78	3	2.34	–0.27
79	Ortrugo	0.00	72.46	0.00	26.34	1.20	0.00	14.53	3	2.86	0.34
80	Perla di Csaba	0.00	88.03	0.00	9.36	2.62	0.00	9.55	3	2.08	–0.17
81	Peverella	0.00	88.31	0.00	9.53	2.16	0.00	9.36	3	2.13	–0.01
82	Pignoletto	0.00	80.73	0.00	19.27	0.00	0.00	8.06	3	2.69	0.76
83	Prosecco	0.00	94.42	0.00	5.58	0.00	0.00	1.67	3	2.17	0.75
84	Ribolla Gialla	0.00	76.07	0.00	22.18	1.76	0.00	14.25	3	2.65	0.14
85	Riesling	0.00	84.88	0.00	12.46	2.66	0.00	5.40	3	2.20	–0.18
86	Rousanne	0.00	85.89	0.00	11.84	2.28	0.00	6.59	3	2.21	–0.05
87	Sauvignon Blanc	0.00	83.82	0.00	13.78	2.40	0.00	9.61	3	2.27	–0.09
88	Verdicchio Marche	0.00	73.22	0.00	25.49	1.28	0.00	13.14	3	2.82	0.31
89	Verduzzo Friuliano	0.00	80.57	0.00	19.43	0.00	0.00	5.29	3	2.70	0.76
90	Viogner	0.00	96.90	0.00	2.33	0.77	0.00	30.21	3	1.98	0.47
91	Xarello	0.00	75.40	0.00	22.96	1.65	0.00	9.71	3	2.69	0.18

[a] Flavonol profile (percentage of each aglycon out of the total), total concentration of flavonols in the berry (mg/kg), classification in groups according to cluster analysis and factor coordinates for the cases, obtained using principal component analysis.

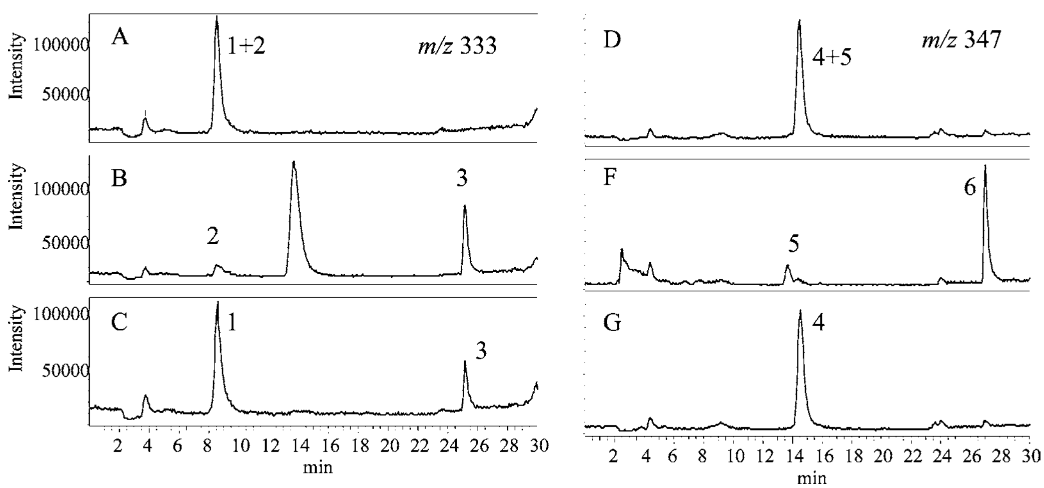

Figure 2. Enzymatic hydrolysis of flavonol glycosides in a Marzemino grape skin extract. HPLC-ESI-MS chromatographic signals of laricitrin (*m/z* 333) and syringetin (*m/z* 347) before (**A** and **D**) and after enzymatic hydrolysis with β-galactosidase (**B** and **F**) or β-glucosidase (**C** and **G**). Key to compounds: 1, laricitrin 3-O-glucoside (RT = 8.4 min; *m/z* 495.3); 2, laricitrin 3-O-galactoside (RT = 8.3 min; *m/z* 495.3); 3, laricitrin; 4, syringetin-3-O-glucoside (RT = 13.6 min; *m/z* 509.2); 5, syringetin-3-O-galactoside (RT = 13.2 min; *m/z* 509.2); and 6, syringetin.

HPLC-DAD-MS Analysis of Flavonols. Identification of laricitrin and syringetin conjugates was carried out on a Waters 2690 HPLC system equipped with Waters 996 DAD (Waters, Milford, MA), Micromass ZQ electrospray ionization mass spectrometry (ESI-MS) system (Micromass, Manchester, United Kingdom), and Empower software (Waters), using the method of Vrhovsek et al. (*34*). An example of a chromatogram is given in **Figure 2**. Syringetin and its conjugates were identified by comparison of their retention times, UV spectra, and MS spectra registered in the positive mode with those of the corresponding standards: syringetin, syringetin-3-O-glucoside, and syringetin-3-O-galactoside. Molecular ions $[M + H]^+$ for syringetin (*m/z* 347.23, CV 30V) and syringetin glycosides (*m/z* 509.23, CV 50V) were used for quantification. Laricitrin-glycosides were identified on the basis of their relative position in the chromatogram and by their UV and MS spectra. Molecular ions $[M + H]^+$ for laricitrin (*m/z* 333.3, CV 30 V) and laricitrin glycosides (*m/z* 495.2, CV 50 V) were monitored in the grape extracts. The results are given in **Table 3**.

HPLC-DAD Analysis of Anthocyanins. According to the literature (*35*), the samples were filtered through 0.22 mm, 13 mm PTFE syringe-tip filters (Millipore) into LC vials and immediately injected into the same system, column, and eluents used for HPLC-DAD analysis of flavonols. Separation of the 16 main free anthocyanins was obtained at 40 °C, with a flow of 0.45 mL/min. The binary gradient was applied as follows: from 27 to 44.5% of B in 32 min, then to 67.5% of B in 13 min, to 100% B in 2 min, isocratic 100% B for 3 min; total analysis time, 50 min. Delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside, malvidin 3-glucoside, and their relevant acetic acid and *p*-coumaric acid esters and malvidin 3-gluco-

Table 3. Enzymatic Hydrolysis of Laricitrin and Syringetin Glycosides

	flavonols released after treatment with β-galactosidase				flavonols released after treatment with β-glucosidase			
variety	laricitrin (peak area)	% laricitrin galactoside	syringetin (peak area)	% syringetin galactoside	laricitrin (peak area)	% laricitrin glucoside	syringetin (peak area)	% syringetin glucoside
Syrah	2611727	54.7	4446408	97.1	2166526	45.3	134115	2.9
Marzemino	2368753	76.7	3709489	92.5	720164	23.3	302198	7.5
Merlot	1505245	57.2	1989919	100.0	1127008	42.8	0	0.0
Cabernet Franc	1505245	57.2	2499588	100.0	1127647	42.8	0	0.0

side-caffeoate were identified according to Castia et al. (*32*) and quantified at 520 nm with a calibration curve with malvidin 3-glucoside chloride. The results are given in **Table 4**.

Statistical Analysis. Statistical analysis was carried out using STATISTICA s/w (data analysis software system), version 6 (StatSoft, Tulsa, OK). Cluster analysis of the flavonol profiles (the percentage of each of the six aglycons out of the total) was carried out using the tree clustering method, computing the Euclidean distances between raw data, with the amalgamation rule unweighted pair−group method arithmetic averages. Principal component analysis of the flavonol profiles was based on the correlations. The percentage of total variance highlighted was as follows: factor 1, 69.19%; factor 2, 17.61%; and factor 3, 7.72%. The results are summarized in **Figures 3** and **4**.

RESULTS AND DISCUSSION

Identification of Grape Flavonols. After acid hydrolysis, in addition to free forms of quercetin, myricetin, kaempferol, and isorhamnetin, which are often reported to be present in grapes and wines and whose conjugated forms have already been noted in grapes and wine (*9*, *10*, *14*, *36*), a further two free flavonols were detected, syringetin and laricitrin. The six flavonols characterized in our study include all of the aglycons expected on the basis of the biosynthetic pathway (**Figure 1**).

The presence of two syringetin conjugates (assigned to syringetin 3-O-glucoside and syringetin 3-O-acetylglucoside) in Cabernet Sauvignon wines was reported for the first time by Wang et al. (*37*), and the presence of laricitrin 3-O-glucoside has to date only been reported in the Nerello Mascalese grape pomace by Amico et al. (*38*). In another recent paper, two unknown flavonols were also reported in Spanish red wines (numbered as peaks 45 and 50 in ref *39*), whose molecular ions $[M - H]^-$ correspond to the theoretical values expected, respectively, for laricitrin and syringetin and hexoside residue.

In this paper, the study of syringetin and laricitrin was included since they were required for the classification of red and white grape varieties on the base of their flavonol profiles.

After acid hydrolysis, it was possible to detect and identify five free flavonols (quercetin, myricetin, kaempferol, isorhamnetin, and syringetin) using HPLC-DAD, by comparison of their retention times and DAD spectra with standards. The free form of laricitrin was identified by HPLC-DAD-MS and its relative retention time in the chromatogram.

In order to find out the nature of syringetin and laricitrin conjugates, identification with LC-DAD-MS before and after enzymatic treatments with β-galactosidase and β-glucosidase was performed. Laricitrin and syringetin glycosides were identified with enzymatic hydrolysis in four varieties, which contained high levels of flavonols: Cabernet Franc, Marzemino, Merlot, and Syrah. The disappearance of two peaks in the chromatogram and the formation of the corresponding aglycons was observed using HPLC after 20 h of incubation at 40 °C with β-glucosidase and β-galactosidase, respectively (**Figure 2**). In the samples before hydrolysis, syringetin-3-O-glucoside and syringetin-3-O-galactoside and laricitrin-3-O-glucoside and laricitrin-3-O-galactoside were identified. The problem of their correct quantification was resolved through the enzymatic hydrolysis experiment, since in both cases the galactoside coeluted with the relevant glucoside (**Figure 2**). The chromatographic area of laricitrin 3-O-galactoside was higher than that of laricitrin 3-O-glucoside, accounting for from 54.7 to 76.7% of the total laricitrin released by enzymatic hydrolysis (**Table 3**). Syringetin 3-O-galactoside accounted for from 92.5 to 100% of the total syringetin (**Table 3**). The galactosides of some flavonols such as quercetin (*39*) and kaempferol (*9*, *40*) have already been reported, but to our knowledge, this is the first time that the presence of laricitrin 3-O-galactoside and syringetin 3-O-galactoside has been reported in grapes. On the basis of our findings, previous identification of laricitrin 3-O-glucoside (*37*) and syringetin 3-O-glucoside (*38*) in grapes can be reasonably reassigned, respectively, as a mixture of laricitrin 3-O-galactoside and laricitrin 3-O-glucoside and as syringetin 3-O-galactoside with traces of syringetin 3-O-glucoside.

Concentration and Pattern of Flavonols in Grape. The absolute concentrations of the free forms of flavonols after acid hydrolysis are shown in **Tables 1** and **2** and, in accordance with the literature (*40*), are on the average three times higher in the red-skinned varieties than in the white-skinned varieties. The total amount of flavonols found after the hydrolysis of the grape extracts ranged from 3.81 to 80.37 mg/kg, with mean = 32.46 mg/kg in the red varieties (**Table 1**) and from 1.36 to 30.21 mg/kg, with mean = 10.83 mg/kg, in the white varieties (**Table 2**).

Looking at the pattern of the flavonols, the main compound in the red varieties was quercetin (mean = 43.99%; range, 12.34−87.76%), followed by myricetin, which is present in a similar percentage (mean = 36.81%; range, 2.35−81.61%), then, in descending order: kaempferol (mean = 6.43%; range, 0−17.52%), laricitrin (mean = 5.65%; range, 0−13.99%), isorhamnetin (mean = 3.89%; range, 0.42−11.88%), and syringetin (mean = 3.22%; range, 0−9.88%). Quercetin, myricetin, and isorhamnetin were always present in all red-skinned varieties. Myricetin was found only in traces in two pink varieties, Gewuerztraminer and Muscat Rouge de Madere, whose flavonol pattern is very close to that of white varieties. For the purposes of this study, these samples were included with the red varieties, in spite of the fact that the wines produced using these varieties are white, because their grapes contain a small amount of anthocyanins (25−35 mg/kg), consisting of over 95% cyanidin 3-glucoside (**Table 4**). Laricitrin and syringetin were undetectable in the samples of Gewuerztraminer, Muscat Rouge de Madere, and Grignolino, and kaempferol was absent in the sample of Tannat (**Table 1**).

In the white-skinned varieties (**Table 2**), the main flavonol by far was always quercetin (mean = 81.35%; range, 72.46−96.90%), followed by kaempferol (mean = 16.91%; range, 2.33−26.34%) and then isorhamnetin (mean = 1.74%; range, 0−5.38%). The delphinidin-like flavonols (myricetin, laricitrin,

Table 4. Anthocyanins in Red-Skinned Grape Varieties[a]

		%									ratio		
no.	variety	delphinidin 3-glucoside	cyanidin 3-glucoside	petunidin 3-glucoside	peonidin 3-glucoside	malvidin 3-glucoside	sum of acetates	sum of *p*-coumarates	malvidin 3-glucoside caffeoate	total anthocyanins (mg/kg)	3′,5′-dihydroxy/3′-hydroxy derivatives	3′,5′-methoxy/3′,5′-hydroxy	3′-methoxy/3′-hydroxy
1	Aglianico	9.79	0.57	10.05	3.77	58.38	3.50	13.73	0.20	1558.30	18.01	5.96	6.59
2	Alicante Bouquette	5.77	1.93	5.70	23.67	44.22	3.27	15.29	0.15	4932.54	2.18	7.67	12.24
3	Ancellotta	17.49	2.35	11.48	5.21	34.37	15.45	13.46	0.18	4885.43	8.38	1.97	2.21
4	Bovale Sardo	11.30	3.65	13.74	5.43	40.84	3.52	21.52	0.00	2697.57	7.25	3.62	1.49
5	Cabernet Sauvignon	12.44	1.77	7.16	5.86	37.62	27.08	7.62	0.45	2150.24	7.50	3.02	3.31
6	Carmenere	11.88	1.37	8.98	4.69	39.22	15.42	18.36	0.07	3030.58	9.92	3.30	3.42
7	Casetta	26.24	3.06	18.92	5.37	32.11	3.32	10.74	0.23	6279.37	9.16	1.22	1.75
8	Croatina	16.57	2.95	12.95	11.88	35.82	10.54	9.25	0.05	2325.34	4.41	2.16	4.03
9	Dolcetto	4.47	0.68	4.85	7.81	50.62	8.78	22.58	0.20	1260.00	7.06	11.33	11.46
10	Lagrein	9.28	1.86	8.60	10.33	39.73	15.34	14.72	0.13	1911.82	4.73	4.28	5.55
11	Lambrusco Oliva	10.55	2.90	13.23	4.27	41.14	3.38	23.81	0.73	1002.28	9.06	3.90	1.47
12	Lambrusco Salamino	13.79	5.04	10.09	18.13	30.92	11.59	10.25	0.19	3705.33	2.37	2.24	3.60
13	Malvasia Nera di Lecce	5.88	0.97	6.85	10.61	50.66	2.83	21.41	0.77	1140.36	5.47	8.61	10.91
14	Marzemino	11.31	0.92	7.42	2.08	35.07	27.56	15.16	0.46	2038.57	17.89	3.10	2.26
15	Nero	4.14	2.39	7.10	9.11	47.44	10.76	18.90	0.16	1076.97	5.10	11.47	3.81
16	Nero d'Avola	12.72	1.31	9.67	4.62	47.69	11.34	12.42	0.22	1463.42	11.82	3.75	3.53
17	Pavana	3.96	0.69	4.29	6.97	48.50	12.70	22.62	0.27	1053.61	7.41	12.23	10.11
18	Raboso del Piave	15.75	17.89	13.52	10.97	26.08	9.95	5.83	0.00	2340.21	1.92	1.66	0.61
19	Rebo	12.89	1.76	7.79	3.00	30.28	29.60	14.49	0.20	1802.35	10.72	2.35	1.71
20	Refosco	17.76	2.81	11.71	6.19	28.57	24.25	8.61	0.10	2865.38	6.45	1.61	2.21
21	Sagrantino	18.54	5.00	14.10	8.96	35.48	2.33	15.41	0.18	1758.84	4.88	1.91	1.79
22	Saint Laurent	7.13	1.17	7.60	7.89	41.17	16.72	18.26	0.07	1838.37	6.17	5.78	6.71
23	Schioppettino	16.17	1.47	11.73	4.30	35.78	15.93	14.50	0.12	1947.80	11.04	2.21	2.93
24	Syrah	6.35	0.75	6.41	8.22	33.46	17.57	26.95	0.29	2336.73	5.15	5.27	10.93
25	Tannat	11.13	1.60	11.67	3.88	36.81	10.47	24.32	0.13	1138.83	10.87	3.31	2.43
26	Tarrango	6.11	0.68	5.56	5.94	49.87	4.84	26.53	0.48	1338.98	9.30	8.16	8.68
27	Tempranillo	19.92	2.84	13.60	5.57	41.04	2.85	14.01	0.16	1776.58	8.87	2.06	1.97
28	Teroldego	15.83	3.46	10.55	6.80	29.99	21.34	11.86	0.17	4853.56	5.50	1.89	1.97
29	Turca	7.52	0.62	8.23	3.92	37.73	6.84	34.88	0.26	2821.22	11.79	5.02	6.35
30	Uva di Troia	9.32	2.55	6.45	5.58	31.28	20.73	23.67	0.41	698.93	5.78	3.36	2.19
31	Zweigelt	10.31	1.68	9.50	9.76	43.44	10.52	14.70	0.09	2806.83	5.53	4.21	5.80
32	Aleatico	4.47	1.83	5.08	5.59	52.25	9.67	20.67	0.43	255.21	8.33	11.68	3.05
33	Barbera	16.10	5.04	13.64	5.46	36.66	14.53	8.28	0.30	2231.48	6.32	2.28	1.08
34	Cabernet Franc	7.61	1.34	6.14	8.88	36.61	25.11	14.06	0.24	1786.61	4.93	4.81	6.63
35	Calabrese	14.14	1.89	10.85	5.79	46.36	9.40	11.57	0.00	1456.47	9.28	3.28	3.06
36	Cannonau	1.90	0.42	2.87	5.56	65.55	2.83	20.31	0.57	356.27	11.76	34.51	13.29
37	Cesanese	13.05	9.46	11.29	19.66	37.98	3.78	4.79	0.00	415.03	2.14	2.91	2.08
38	Cigliegiolo	4.86	3.96	6.79	15.40	65.08	1.03	2.68	0.19	721.97	3.96	13.40	3.89
39	Corvina	7.15	4.94	6.36	29.46	41.38	0.70	9.82	0.18	1111.58	1.60	5.78	5.97
40	Enantio	13.95	5.87	10.63	23.45	34.32	5.62	6.09	0.08	3004.99	2.01	2.46	3.99
41	Franconia	3.89	1.34	4.71	19.16	61.01	1.86	7.90	0.13	1545.02	3.40	15.69	14.30
42	Merlot	9.22	2.99	6.89	7.90	35.13	22.22	15.30	0.36	1145.25	4.71	3.81	2.65
43	Montepulciano	14.10	2.98	13.00	5.14	33.69	16.97	13.90	0.21	2233.09	7.48	2.39	1.73
44	Negro Amaro	15.28	4.86	15.46	5.02	44.30	2.12	12.96	0.00	3149.00	7.60	2.90	1.03
45	Nera dei Baisi	9.35	2.26	9.95	6.25	63.34	2.89	5.97	0.00	1893.61	9.71	6.77	2.77
46	Pinot Noir	4.95	4.44	5.68	34.65	50.27	0.00	0.00	0.00	985.86	1.56	10.15	7.80
47	Pinotage	4.49	0.66	5.33	5.53	49.71	15.97	17.97	0.34	1254.12	9.61	11.08	8.37
48	Primitivo	6.23	2.24	7.59	8.27	52.02	3.36	20.05	0.23	1313.14	6.26	8.35	3.69
49	Primitivo di Gioia	4.29	1.10	5.53	6.60	50.97	3.77	27.61	0.14	947.88	7.90	11.88	6.01

Table 4. (Continued)

		%									ratio		
no.	variety	delphinidin 3-glucoside	cyanidin 3-glucoside	petunidin 3-glucoside	peonidin 3-glucoside	malvidin 3-glucoside	sum of acetates	sum of *p*-coumarates	malvidin 3-glucoside caffeoate	total anthocyanins (mg/kg)	3′,5′-dihydroxy/ 3′-hydroxy derivatives	3′,5′-methoxy/ 3′,5′-hydroxy	3′-methoxy/ 3′-hydroxy
50	Rondinella	6.93	5.67	6.23	32.45	39.01	0.57	8.92	0.22	1050.92	1.37	5.63	5.72
51	Sangiovese	11.12	18.48	13.11	15.58	40.20	0.24	1.27	0.00	765.11	1.89	3.61	0.84
52	Galioppo	4.32	30.78	3.62	49.44	11.84	0.00	0.00	0.00	211.21	0.25	2.74	1.61
53	Gewuerztraminer	1.96	95.73	0.53	0.80	0.83	0.00	0.15	0.00	36.28	0.03	0.42	0.01
54	Grignolino	7.48	17.51	4.02	35.96	26.55	1.34	7.15	0.00	106.71	0.71	3.55	2.05
55	Groppello Gentile	5.07	31.64	3.81	37.26	12.98	3.21	6.03	0.00	593.63	0.32	2.56	1.18
56	Molinara	16.82	21.06	8.20	23.62	19.20	4.50	6.61	0.00	409.39	0.99	1.14	1.12
57	Moscato Rosa	1.69	5.33	2.49	50.33	34.76	0.86	4.55	0.00	934.75	0.70	20.60	9.44
58	Muscat Rouge de Madere	0.00	95.38	0.00	0.00	4.62	0.00	0.00	0.00	25.58	0.05		0.00
59	Nebbiolo	6.73	13.29	4.94	45.55	21.09	3.27	5.12	0.00	1391.96	0.56	3.13	3.43
60	Pinot Gris	4.36	3.06	5.37	29.23	57.98	0.00	0.00	0.00	776.17	2.10	13.28	9.55
61	Pinot Tete de Negre	2.82	3.80	3.71	41.58	48.09	0.00	0.00	0.00	737.47	1.20	17.05	10.94
62	Schiava Grigia	3.82	9.79	4.35	45.16	32.21	0.67	3.98	0.03	569.95	0.73	8.43	4.61
63	Schiava Grossa	2.68	13.97	3.39	52.85	16.84	2.67	7.59	0.00	1175.49	0.34	6.29	3.78
64	Schiava Lombarda	1.14	4.99	1.34	71.56	17.48	0.61	2.88	0.00	421.55	0.26	15.27	14.34

[a] Anthocyanin profile (percentage), total concentration of anthocyanins in the berry (mg/kg) and some ratios correlated to hydroxylase and O-methoxylase activities.

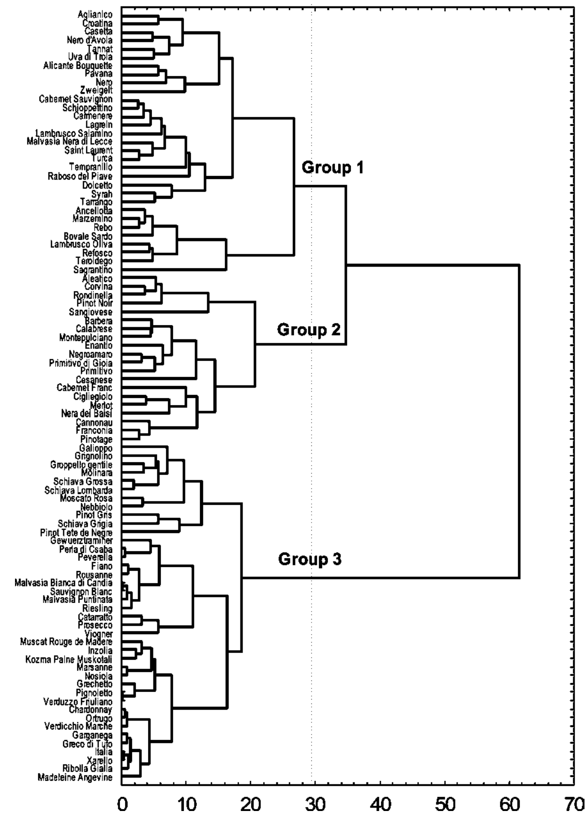

Figure 3. Hierarchical tree plot showing the classification produced using cluster analysis of the flavonol profile (the percentage of each aglycon out of the total). The linkage distance is given on the *X*-axis.

and syringetin) were missing in all 27 white-skinned varieties investigated (**Table 2**), thus indicating that the gene coding for the enzyme F3′5′OH is not expressed in white grape varieties. This finding was in agreement with the very low mRNA levels of F3′5′H reported in the berry skin of white varieties (*24*).

Origin of the Pink Varieties. Nowadays, in many cases, it is impossible to decide whether a white variety is the sport of a red one or vice versa. The observed lack of expression of F3′5′OH in all white varieties could be very useful for formulating a hypothesis as regards the origin of pink varieties. We know that white varieties lack expression of the gene encoding UFGT (UDP-glucose:flavonol 3-O-D-glucosyltransferase) (*41*), due to the role of MybA genes, playing a critical role in the regulation of anthocyanins biosynthesis via control of UFGT gene expression (*42*). A retrotransposon insertion in VvmybA1 has been suggested as the molecular basis of white coloring in a number of white grape varieties (*43*). All other structural genes, not including F3′5′OH, were expressed in both red and colored varieties (*41*, *42*).

We also know that some pink sport of red varieties, such as Cabernet Sauvignon and Syrah, have anthocyanin profiles very similar to those of the plant from which they derive (*41*). In some pink sport of white varieties, such as the Sultana and Chardonnay sport in Boss et al. (*41*) and in a Chardonnay sport in Mattivi (*44*), cyanidin 3-glucoside and peonidin 3-glucoside, located in the dihydroxylated branch of the anthocyanin pathway, were by far the main pigments (*41*, *44*). We suggest that the varieties having mostly cyanidin derivatives, such as, for example, the Muscat Rouge de Madere and the Gewuerztraminer in this study (**Table 1**) or the pink Muscats and the pink Chardonnay sport described by Mattivi (*44*), are expected to derive from white varieties. Those having a higher amount of delphinidin-derived anthocyanins, such as Pinot Gris (**Table**

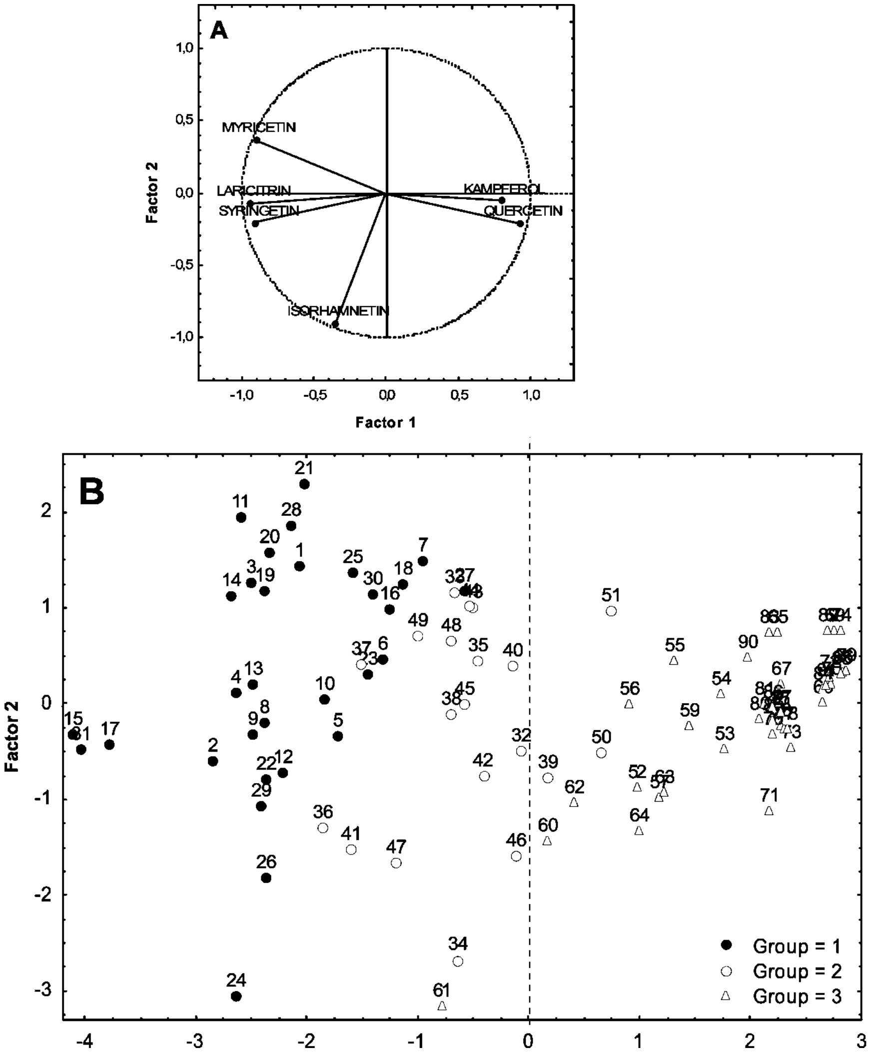

Figure 4. Principal component analysis of the flavonol profile. (**A**) Projection of the variables on the factor plane (1 × 2). (**B**) Scatterplot of 91 cases in the factor plane (1 × 2). The varieties are grouped according to the groups defined by cluster analysis and labeled in accordance with **Tables 1** and **2**.

1), are expected to derive from red varieties, in this case from Pinot Noir. A theory formulated by Kobayashi et al. (*43*), sustaining that every cultivated red variety is derived by a corresponding white variety, has been recently corrected by the same authors (*45*), according to Boss et al. (*41*), with both Pinot Blanc and Pinot Gris being derived by mutation on the gene VvmybA1c of Pinot Noir (*41*).

Classification of the Grape Varieties Based on the Pattern of Flavonols. For the classification of grape varieties, the concentrations of the six flavonols were expressed as the percentage of their total concentration for each variety. In this way, it was expected to diminish variability due to environment, which strongly influences the values of absolute concentrations of polyphenols in *V. vinifera* (*20*).

As a first exploratory phase of our research, as we did not have any pre-established hypotheses, we used cluster analysis (**Figure 3**) to find the most significant solution for the classification of the varieties based on the flavonol profiles. The distribution of the cases is continuous, which means that the groups identified by cluster analysis depend strongly on the data set and on the conditions in which the analysis was performed. Choosing a relatively large and safe cutting value at the linkage distance of 30 (**Figure 3**), the samples could be divided into three main groups. The group to which each sample was assigned is given in **Tables 1** and **2**.

A principal component analysis, based on the same variables, was performed in order to obtain further information on how composition affects classification. The cumulative percentage of the total variance explained by the first two factors was 86.62%. **Figure 4** shows the scatterplots of both the variables and the cases in the plane defined by the factors 1 and 2. A first large group (group = 1) contained 31 out of 64 red grape varieties, which were characterized by a relatively substantial presence of myricetin. The myricetin/quercetin ratio among these varieties varied, lying in the range 0.9−6.6. The samples with the highest values of this ratio were displayed in the negative part of the *X*-axis, while the samples with the highest percentages of isorhamnetin were in the lower part of the scatterplot (**Figure 4**). All of the 27 white-skinned varieties were clustered very close together in the classification tree (group 3 in **Figure 3**) and in the right part of the PCA scatterplot (**Figure 4**), due to the complete lack of myricetin, laricitrin, and syringetin (**Table 2**). The pale-colored varieties Gewuerztraminer and Muscat Rouge de Madere were assigned to the same branch of the cluster (**Table 1**) since they contained over 30 times more quercetin than myricetin (**Table 1**). A further 11 red or pale-red varieties (Galioppo, Grignolino, Groppello Gentile, Molinara, Moscato Rosa, Nebbiolo, Pinot Gris, Pinot Tete de Negre, Schiava Grigia, Schiava Grossa, and Schiava Lombarda) were assigned by cluster analysis to the same group 3, as they had

6−10 times more quercetin than myricetin. A further 20 red-skinned varieties were clustered in group 2 (**Figure 3**) because they had a higher percentage of quercetin than group 1 (quercetin ≥ 40% of the total flavonols) with similar or lower values of myricetin and above average values of kaempferol.

Flavonols vs Anthocyanins in Grape. The biosynthesis of flavonols is closely related to that of anthocyanins (*25*). The main difference is the presence of kaempferol among flavonols, while the corresponding 4′-hydroxylated anthocyanin (pelargonidin) is not present in grapes. It is interesting to observe the analogies between the hydroxylation of position 5′, which leads to the formation of the anthocyanidin delphinidin from cyanidin and the flavonol myricetin from quercetin. In addition to this, O-methylation of position 3′ of anthocyanidin cyanidin, which leads to the formation of peonidin, corresponds to the O-methylation, which converts flavonol quercetin into isorhamnetin. Finally, O-methylation of positions 3′ and 5′ of anthocyanidin delphinidin, which leads to the formation of petunidin and malvidin, corresponds to O-methylation, which converts flavonol myricetin into laricitrin and syringetin (**Figure 1**). These analogies depend on the same or parallel enzymatic activities for both classes of flavonoids. Even if there is no evidence that the enzymes involved in the O-methylation of the flavonols and the anthocyanins are the same, the chemical reaction is the same. This means that flavonols can be used in the taxonomical classification and metabolite profiling of *V. vinifera* varieties, also for profiling white varieties, which do not contain anthocyanins. To date, this possibility has been limited by the low number of flavonols identified in grapes, as in grapes the compounds considered were usually quercetin and myricetin and less frequently kaempferol. Available data on grape flavonols are scarce and limited to a few varieties.

In order to compare the global balance of biosynthesis generating both flavonols and anthocyanins, we computed the following three ratios, which are related to three key enzymatic activities, linked to known structural genes involved in the synthesis of anthocyanins (A) and flavonols (F).

F3′5′OH Activity.

$$\text{A ratio } \frac{3',5'\text{-dihydroxy}}{3'\text{-hydroxy}} = \frac{\text{sum of delphinidin, petunidin, and malvidin 3-glucosides}}{\text{sum of cyanidin and peonidin 3-glucosides}}$$

$$\text{F ratio } \frac{3',5'\text{-dihydroxy}}{3'\text{-hydroxy}} = \frac{(\text{myricetin} + \text{laricitrin} + \text{syringetin})}{(\text{quercetin} + \text{isorhamnetin})}$$

3′ OMT Activity.

$$\text{A ratio } \frac{3'\text{-methoxy}}{3'\text{-hydroxy}} = \frac{\text{peonidin 3-glucoside}}{\text{cyanidin 3-glucoside}}$$

$$\text{F ratio } \frac{3'\text{-methoxy}}{3'\text{-hydroxy}} = \frac{\text{isorhamnetin}}{\text{quercetin}}$$

5′ OMT Activity.

$$\text{A ratio } \frac{3',5'\text{-methoxy}}{3'\text{-}5'\text{-hydroxy}} = \frac{\text{malvidin 3-glucoside}}{\text{delphinidin 3-glucoside}}$$

$$\text{F ratio } \frac{3',5'\text{-methoxy}}{3',5'\text{-hydroxy}} = \frac{\text{syringetin}}{\text{myricetin}}$$

The ratios computed on flavonols in the 64 red grape varieties are given in **Table 1**. The relevant ratios computed on the anthocyanins are reported in **Table 4**.

The enzymatic activities that lead to flavonols largely overlap, from the qualitative point of view, with those leading to anthocyanins. A highly significant correlation ($p < 0.01$) was found between the ratios of F3′5′OH ($r = 0.45$), 3′-OMT ($r = 0.74$), and 5′-OMT ($r = 0.49$) activities in the two classes of flavonoids (**Figure 5**). Moreover, other significant correlations were found between 3′-OMT vs 5′-OMT activities in both anthocyanins ($r = 0.75$) and flavonols ($r = 0.51$) and between 3′-OMT activity in anthocyanins and 5′-OMT activity in flavonols ($r = 0.80$).

The significant correlation existing between the two metabolic pathways implies that any attempt to optimize the pattern of the anthocyanins might also be expected to affect the pattern of other metabolites, such as flavonols and flavanols. As an example, an increase in the expression of F3′5′OH activity would modify the anthocyanin profile toward a higher amount of delphinidin-like anthocyanins, a darker purple color and more stable than the cyanidin-like anthocyanins, but is also expected, to a variable extent, to convert the highly bioactive compound quercetin into myricetin, whose healthy properties are less well-characterized.

In order to further explore the commonality of the patterns between anthocyanins and flavonols, a PCA on red varieties was carried on both flavonols and anthocyanins (data not shown), giving rise to similar grouping as the PCA on flavonols (**Figure 4**).

While there is a certain degree of similarity between the absolute level of hydroxylation in the two classes of flavonoids (**Figure 5A**), differences of 1 order of magnitude were highlighted in O-methyltransferase activities, for which anthocyanins are much better substrates than flavonols (**Figure 5B**,**C**). In the case of anthocyanins, the majority of the varieties had very strong 3′-OMT and 5′-OMT activities and, thus, synthesized large amounts of malvidin and peonidin 3-glucosides, which were the major pigments in 62 out of 64 of the red grape varieties (**Table 4**). In agreement with the literature (*20*), the 3′- and 5′-OMT ratios (**Table 4**) assumed values >1 in all but three of the varieties classified (Gewuerztraminer, Muscat Rouge de Madere, and Sangiovese), with average values for the 3′-OMT and 5′-OMT ratios of 4.75 and 6.29, respectively, and maximal values as high as 14.34 (3′-OMT, Schiava Lombarda) and 34.51 (5′-OMT, Cannonau).

On the contrary, the 3′-OMT and 5′-OMT activities on flavonols were very weak, so that the free hydroxylated forms of the flavonols quercetin and myricetin (**Tables 1** and **2**) were always dominant in comparison to the corresponding O-methoxylated forms. For flavonols, the 3′-OMT and 5′-OMT ratios were always equal or below 0.34, with mean values equal to 0.10.

In general, a substantial presence of myricetin was found in the majority of red varieties characterized by their purple color, due to the large amount of delphinidin derivatives, such as Marzemino, Teroldego, and Lambrusco Oliva (**Table 4**). The red varieties characterized by a dominant amount of cyanidin derivatives in the anthocyanins, such as Nebbiolo, Moscato Rosa, Schiava Grossa, etc., were also characterized by the substantial prevalence of quercetin among flavonols (**Table 1**). The higher values of F3′5′OH activity for anthocyanins as compared to flavonols (**Figure 5A**) support the hypothesis that grape flavonol synthase has much higher specificity to dihydroquercetin than to dihydrokaempferol and dihydromyricetin

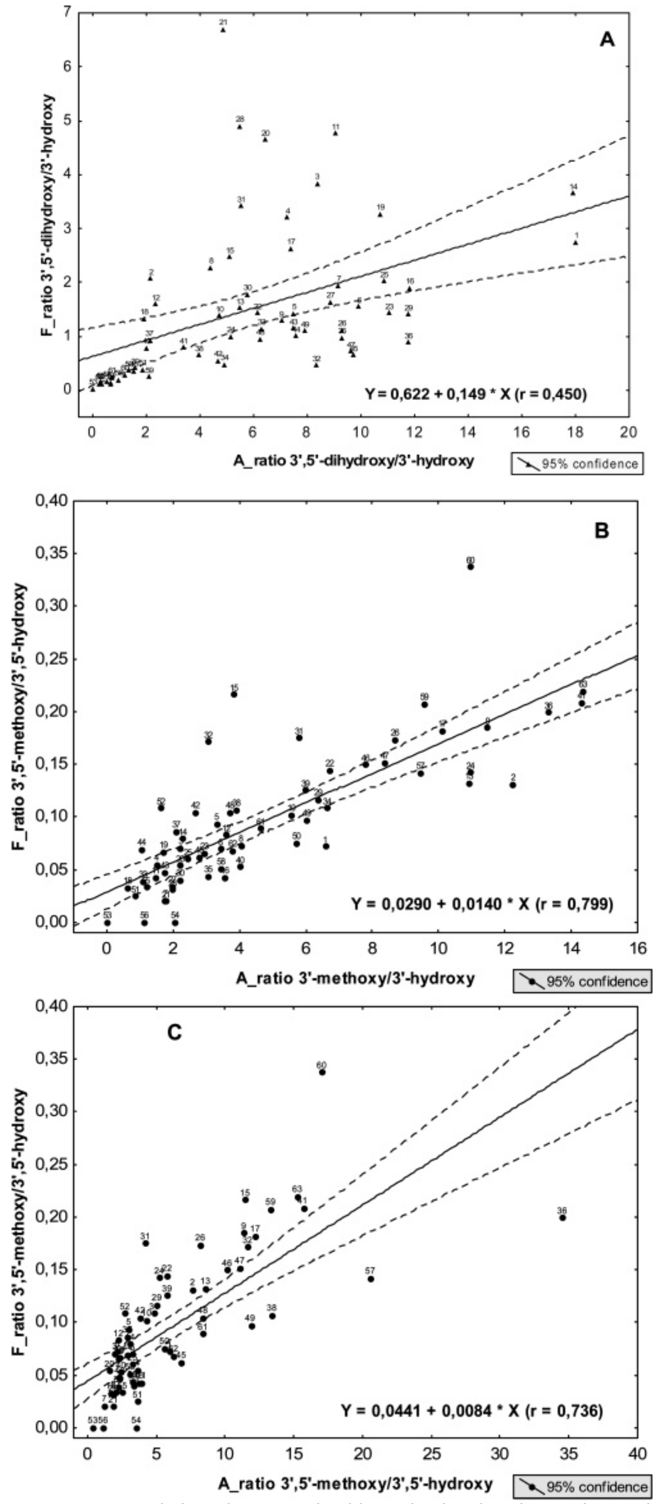

Figure 5. Correlations between the biosynthesis of anthocyanins and flavonols in 64 red grape varieties. Key: **A**, correlation between the ratios of hydroxylation (F3′5′OH activity); **B**, correlation between the ratios of 3′-OMT (3′-OMT activity) in anthocyanins and of 5′-OMT (5′-OMT activity) in flavonols; and **C**, correlation between the ratios of 5′-OMT (5′-OMT activity).

(**Figure 1**), leading to accumulation of quercetin even in varieties having high mRNA levels of F3′5′H (*25*).

Figure 5 shows that there was relatively important scattering of the samples in the scatterplots, since many varieties showed a significant difference in the relative importance of enzymatic activities in these two classes of flavonoids. The percent of variance of the ratios of F3′5′OH in the two classes of flavonoids explained by the model ($100 \times r^2$), which was limited to about 20%. The varieties plotted in the upper left part of **Figure 5A** (Alicante Bouquette, Lambrusco Oliva, Refosco, Sagrantino, Teroldego, etc.) had very similar absolute levels of F3′5′OH in the two classes of flavonoids, while the varieties located in the lower central part of the graph (Aleatico, Cabernet Franc, Cannonau, Merlot, Nera dei Baisi, Pinotage, etc.) displayed higher values of F3′5′OH in anthocyanins than in flavonols. This could be partially due to the differences in the relative expression of the genes involved in the common metabolic pathway, which is not unexpected, especially considering that the timing of the synthesis of these compounds in grapes is rather different (*10*, *25*, *46*).

On the other hand, the 64% of variance explained from the model 3′-OMT activity in anthocyanins vs 5′-OMT in flavonols (**Figure 5B**) could be explained if the enzymes involved in these O-methylation reactions are the same.

The comparison of the degrees of hydroxylation and methoxylation of flavonol and those of anthocyanin provided a unique and interesting approach to discuss the substrate specificity of the enzymes in flavonoid biosynthesis.

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