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Flavonol Profiles of *Vitis vinifera* Red Grapes and Their Single-Cultivar Wines

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The main flavonols found in seven widespread *Vitis vinifera* red grape cultivars include the 3-glucosides and 3-glucuronides of myricetin and quercetin and the 3-glucosides of kaempferol and isorhamnetin. In addition, the methoxylated trisubstituted flavonols, laricitrin and syringetin, were predominantly found as 3-glucosides. As minority flavonols, the results suggest the detection of the 3-galactosides of kaempferol and laricitrin, the 3-glucuronide of kaempferol, and the 3-(6″-acetyl)glucosides of quercetin and syringetin. The flavonol profiles based on the eight above-mentioned flavonols allowed the cultivar differentiation of the grape samples. With regard to flavonol biosynthesis in the berry skin, quercetin 3-glucuronide predominated at véraison, followed by quercetin 3-glucoside, and only trace amounts of trisubstituted flavonols were detected. The proportion of quercetin 3-glucoside remained almost constant during berry ripening, whereas the proportion of quercetin 3-glucuronide decreased and the other flavonols, especially myricetin 3-glucoside, increased their importance. In wines, flavonol 3-glycosides coexisted with their corresponding free aglycones released by hydrolysis. The presence of laricitrin, syringetin, and laricitrin 3-glucoside in red wines is reported here for the first time. The extent of hydrolysis was widely variable among wines made from the same grape cultivar, and the results suggest the influence of the type of aglycone and glycoside on the rate of hydrolysis. Due to hydrolysis, the differentiation of single-cultivar wines gave acceptable results only when aglycone-type flavonol profiles were used.

KEYWORDS: Flavonols; laricitrin; syringetin; grape; red wine; *Vitis vinifera*; cultivar authenticity; ripening

INTRODUCTION

Flavonols are a class of flavonoid compounds found in *Vitis vinifera* L. grape berry skins, where they are involved in UV screening, due to their strong absorbance in UV-A (325−400 nm) and UV-B (280−325 nm) wavelengths, and their accumulation in response to supplemental UV-A, UV-B, and sunlight radiation treatments (*1*, *2*). Moreover, the study of the effect of bunch shading on the flavonoid accumulation in Shiraz grapes (*3*) suggests that the branch of the flavonoid pathway leading to flavonol biosynthesis is light-dependent, in sharp contrast to anthocyanin and tannin synthesis, which was little affected by the shading treatments. Regarding the color of the wines, flavonols are yellow pigments that contribute directly to the color of white wines, but in red wines flavonols are masked by anthocyanins, the red pigments. However, flavonols are one of the best wine phenolics involved in the phenomenon of copigmentation in red wines (*4*). The formation of copigmentation complexes between anthocyanins and copigments, such as flavonols, causes an enhancement of the extraction of anthocyanins during winemaking, which is reflected in a more intense red color together with a bathochromic shift to purplish hues of the red color (*5*). These color effects can be easily observed in young red wines. In addition, flavonols have been identified as one of the best phenolics with antioxidant activity in wine, especially in white wines (*6*−*8*), although their antioxidant effects in red wines are usually exceeded by other more abundant phenolics, such as flavan-3-ols and anthocyanins (*9*−*11*).

Regarding the substitution pattern of the flavonoid structure of flavonols, the 4′-hydroxy, the 3′,4′-dihydroxy, and the 3′,4′,5′-trihydroxy flavonols, known as kaempferol (**1**), quercetin (**2**), and myricetin (**3**), respectively, have been found in *V. vinifera* red grapes and wines, together with isorhamnetin (**4**), the methoxylation product of the 3′-OH of quercetin (**Figure 1**). Flavonols in grapes exist only as 3-glycosides, whereas the corresponding free aglycones can be found in wines, together with the 3-glycosides, as a result of acid hydrolysis that occurs during winemaking and aging. Glucose is the common sugar attached to the C-3 position of kaempferol, quercetin, myricetin, and isorhamnetin, but glucuronic acid has been also found as

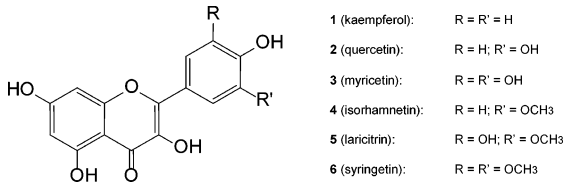

Figure 1. Flavonoid structure of flavonols found in *V. vinifera* grapes.

the glycosylation sugar of kaempferol, quercetin, and myricetin, the nonmethoxylated flavonols (*12*). Furthermore, quercetin has been found in grapes as 3-rhamnosylglucoside (the flavonol so-called rutin), 3-glucosylgalactose, and 3-glucosylxyloside, whereas other kaempferol 3-glycosides include 3-glucosylarabinoside and 3-galactoside, although many of the latter 3-glycosides have not been structurally confirmed and their assignations sometimes were based on only retention times and UV−vis spectra (*13*). For instance, it has been very common to assign rutin instead of quercetin 3-glucuronide (*14*), which has been confirmed by NMR (*15*). Kaempferol- and quercetin-type flavonols are present in both white and red grapes and wines, and it has been suggested that myricetin- and isorhamnetin-type flavonols account for only *V. vinifera* red grapes and wines. However, the presence of small amounts of isorhamnetin in white grape skins has been reported recently (*16*), and myricetin has been described in *Vitis rotundifolia* white grapes and wines (*17*, *18*).

In the same way as isorhamnetin is the methoxylation product of quercetin, the methoxylation product in C-3′ of myricetin is known as laricitrin (**5**), whereas the 3′,5′-dimethoxyl derivative of myricetin is called syringetin (**6**). The occurrence of these methoxylated trisubstituted flavonols, or their 3-glycosyl derivatives, in *V. vinifera* grapes or their corresponding products has been described only in a few examples in recent years. The presence of both syringetin 3-glucoside and syringetin 3-(6″-acetyl)glucoside was established for the first time in California Cabernet Sauvignon grape and wine (*19*). Additionally, laricitrin, syringetin, and laricitrin 3-glucoside were detected for the first time as minority flavonols in grape pomace extracts of the Sicilian red grape cultivar Nerello Mascalese (*20*). The latter workgroup further analyzed the grape pomaces of other cultivars, but this time only laricitrin was found in Cabernet Sauvignon samples (*21*). In a recent review dealing with the content of flavonols in grapes, grape products, and wines, no reference was given about the presence of laricitrin- and syringetin-type flavonols in such materials (*22*). Laricitrin and syringetin 3-glycosides have been found in the skins of 64 red-skinned grape varieties, and it has been suggested that the main sugar attached to these flavonols is galactose instead glucose, especially in the case of syringetin (*23*).

Phenolic compounds have been suggested as chemical markers for confirmation of cultivar authenticity in grapes and wines. In past years, the cultivar-characteristic profiles of monomeric anthocyanins have been widely used for the classification and differentiation of grape cultivars and single-cultivar wines (*24* and references cited therein). Recent studies have demonstrated that flavonol profiles also serve as a differentiation tool for table grape cultivars (*25*), and the characterization of some flavonols found in different red wine grape cultivars has also been made (*26*), although former studies did not find consistent differences among cultivars of red wine grapes (*27*). Recent researchers have suggested that flavonol profiles could be used as a general chemical indicator for the authenticity of both red and white *V. vinifera* grape cultivars and their corresponding single-cultivar wines (*16*, *23*, *28*−*30*). However, the usefulness of flavonol profiles for grape and wine cultivar authenticity needs to be based in the correct assignation of the main flavonols found. Nowadays, there are still important doubts about the correct assignation of the glycoside moiety of some flavonols found in *V. vinifera* grapes and wines, and the presence of new flavonols derived from aglycones different from the classic kaempferol, quercetin, myricetin and isorhamnetin, might need to be carefully checked. Many researchers have avoided this obstacle, having recourse to the hydrolysis of the flavonol glycosides, but this procedure has the disadvantage of the lack of the important information given by the glycosylation pattern.

The aim of this work was to test the use of flavonol profiles as a marker for the authentication of grape cultivar for *V. vinifera* red grapes and wines, including the recently discovered methoxylated trisubstituted flavonols, that is, laricitrin- and syringetin-type flavonols. For this purpose, the flavonols of several red grape cultivars and single-cultivar red wines were isolated by solid-phase extraction and analyzed by LC-DAD-ESI-MS^n. To establish the stage of development of the grape berry in which the characteristic flavonol profile is setting, the concentration of flavonols in the berry during ripening was monitored for six red grape cultivars. The *V. vinifera* cultivars assayed comprised the well-known Spanish Tempranillo and the worldwide-grown Cabernet Sauvignon, together with other representative, widespread Spanish and French cultivars.

MATERIALS AND METHODS

Chemicals and Samples. All solvents were of HPLC quality and all chemicals of analytical grade (>99%). Water was of Milli-Q quality. Commercial standards of flavonol glycosides, 3-glucosides of quercetin, kaempferol, isorhamnetin, and syringetin and 3-galactosides of quercetin and syringetin, were obtained from Extrasynthese (Genay, France). Commercial standards of flavonol aglycones were obtained as indicated: myricetin, quercetin, kaempferol, and syringetin (Extrasynthese, Genay, France); isorhamnetin (Sigma, St. Louis, MO). Other noncommercial flavonol standards (myricetin 3-glucoside and quercetin 3-glucuronide) were kindly supplied by Dr. Ullrich Engelhardt (Institute of Food Chemistry, Technical University of Braunschweig, Germany).

Healthy red wine grapes grown in five vineyards located in the region of La Mancha (central southern Spain) were collected at optimum ripeness for harvesting (estimated alcoholic strength of 13−14%, v/v). The sampling was randomly made by picking berries from the top, central, and bottom parts of the cluster, following a zigzag path between two marked rows of 10 vines. We tried to sample berries from both exposed and shaded clusters by picking berries of four to five clusters per vine. The size of the sample was around 200 berries, which were bulked and separated in two subsamples of approximately 100 berries. The study of flavonol evolution during ripening was conducted in two different vineyards of the La Mancha region: Tempranillo, Cabernet Sauvignon, Syrah, and Petit Verdot samples were collected from 6-year-old vines in a vineyard located in Miguelturra (Ciudad Real, Spain); Garnacha and Garnacha Tintorera samples were collected from 15-year-old (Garnacha) and 6-year-old (Garnacha Tintorera) vines in a vineyard located in Orgaz (Toledo, Spain); all of the vines were drip-irrigated and grown using a bilateral Royat cordon trellis. The grape samples were picked starting at the moment of véraison and finishing at harvest, with a periodicity of 1 week for the cultivars Tempranillo, Cabernet Sauvignon, Syrah, and Petit Verdot and of 9 days for the cultivars Garnacha and Garnacha Tintorera. The sampling procedure was the same as described above for ripe grape samples. The wine samples were obtained directly from cellars or purchased in wine shops. The criteria for choosing wine samples comprised both the cultivar employed in their elaboration and different aging times when available. We tried to get wine samples of the most widespread red grape cultivars (Tempranillo, Garnacha, Cabernet Sauvignon, Merlot, and Syrah), together with some minor cultivars (Bobal, Petit Verdot, Dornfelder, Carmenère, and Sangiovese). All of the wine samples were single-

cultivar wines, having in mind that the cultivar origin was that declared from the producers. All of the determinations were performed in duplicate.

Grape Skin Extraction. An amount of 100 g of healthy grapes was finger pressed to remove the pulp and the seeds. The remaining skins were washed three times in water (Milli-Q) and softly dried twice by patting them between sheets of filter paper. The dried skins were extracted with 100 mL of a mixture 50:48.5:1.5 (v/v) of CH_3OH/H_2O/HCOOH (*31*), using a homogenizer (Heidolph DIAX 900) for 2 min and then centrifuged at 2500*g* at 5 °C for 15 min. A second extraction of the skin pellets yielded nearly 99% of the grape skin phenolic content, as confirmed by HPLC of successive extractions (up to five). The combined supernatants were stored at −18 °C until use.

Isolation of Grape and Wine Flavonols. Solid-phase extraction on Oasis MCX cartridges (Waters Corp., Milford, MA; cartridges of 6 cm^3 capacity filled with 500 mg of adsorbent) containing a mixture of reverse-phase and cationic-exchanger materials allowed the isolation of grape and wine flavonols. The samples need some preparation prior to separation. For grape skin extracts, 3 mL was dried in a rotary evaporator (40 °C) and re-solved in 3 mL of 0.1 M hydrochloric acid. In the case of wine samples, 3 mL of wine was diluted with 3 mL of 0.1 M hydrochloric acid. The separation procedure was adapted from that of González-Manzano et al. (*32*) to allow the reuse of the cartridges. The prepared samples were passed through the MCX cartridges previously conditioned with 5 mL of methanol and 5 mL of water. After washing with 5 mL of 0.1 M hydrochloric acid and 5 mL of water, the flavonol fraction was eluted with 3 × 5 mL of methanol. This fraction also contained other neutral or acidic polyphenols (flavan-3-ols or tannins and hydroxycinnamic acid derivatives, respectively). Fixed anthocyanins were removed using 3 × 5 mL of 2% ammonia in 80% methanol, and the cationic-exchanger material was regenerated with 3 × 5 mL of 2% hydrochloric acid in 80% methanol. 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 (40 °C) and re-solved in 3 mL of the solvent A used in the HPLC separation.

HPLC-DAD-ESI-MS^n Analysis of Flavonols. HPLC separation, identification, and quantification of flavonols were performed on an Agilent 1100 series system (Agilent, Waldbronn, Germany), equipped with a DAD (G1315B) and LC/MSD Trap VL (G2445C VL) electrospray ionization mass spectrometry (ESI-MS^n) system and coupled to an Agilent Chem Station (version B.01.03) data-processing station. The mass spectral data were processed with the Agilent LC/MS Trap software (version 5.3). The samples, after filtration (0.20 μm, polyester membrane, Chromafil PET 20/25, Machery-Nagel, Düren, Germany), were injected (50 μL) on a reversed-phase column Zorbax Eclipse XDB-C18 (4.6 × 250 mm; 5 μm particle; Agilent), thermostated at 40 °C. The chromatographic conditions were adapted from the OIV method for analyzing anthocyanins (*33*). The solvents were water/acetonitrile/formic acid (87:3:10, v/v/v, solvent A; 40:50:10, v/v/v, solvent B), and the flow rate was 0.63 mL/min. The linear gradient for solvent B was as follows: 0 min, 6%; 15 min, 30%; 30 min, 50%; 35 min, 60%; 38 min, 60%; 46 min, 6%. Quantification was made using the DAD chromatograms obtained at 360 nm, by means of external standard calibration curves (due to the lack of certain standards, myricetin 3-glucuronide and laricitrin 3-glucoside were quantified as myricetin 3-glucoside, and laricitrin was quantified as myricetin). For identification, ESI-MS^n was used, setting the following parameters: positive ion mode; dry gas, N_2, 11 mL/min; drying temperature, 350 °C; nebulizer, 65 psi; capillary, −2500 V; capillary exit offset, 70 V; skimmer 1, 20 V; skimmer 2, 6 V; scan range, *m*/*z* 50−1200.

Statistical Analysis. The data obtained from the grape and wine samples were subjected to an analysis of variance (Student−Newman−Keuls test) to identify the existence of statistically significant differences between samples grouped by cultivars. For grapes we used data corresponding to peaks 1−8 (see **Table 2** and **Figure 2**), whereas for wines the data used corresponded to peaks 1−14 (see **Table 3** and **Figure 6**). The same data were also subjected to principal component analysis, but no previous cultivar classification was made. All of these statistical tests were performed using SPSS software, version 12.0.

RESULTS AND DISCUSSION

Flavonol Composition of Ripe *V. vinifera* Red Grapes. The anthocyanins present in red grape skin extract usually cause a great interference in the chromatographic separation and identification of flavonols, in spite of the use of a specific detection wavelength for the latter compounds (i.e., 360 nm). The almost complete removal of the anthocyanins present in the grape skin extracts, by means of solid-phase extraction using a combination of reversed-phase and cationic-exchanger material, allowed a better chromatographic separation of the flavonols found in these extracts. Representative chromatograms of the isolated flavonol fraction (with and without separation of anthocyanins) are shown in **Figure 2** for a sample of Petit Verdot red grape cultivar. In these chromatograms at least 13 peaks can be assigned as flavonol glycosides, and no flavonol aglycones were found. Peaks 1−8 were considered to be the main flavonol glycosides in *V. vinifera* grapes, because they were assigned to the 3-glucosides (peaks 2 and 4−8) of all the flavonol aglycones found in grape (**Figure 1**) and to the well-known 3-glucuronides (peaks 1 and 3) of myricetin and quercetin. Peaks A−E corresponded to minority flavonols having different glycosylation patterns.

The assignations of myricetin 3-glucoside (peak 2), quercetin 3-glucuronide (peak 3), quercetin 3-glucoside (peak 4), kaempferol 3-glucoside (peak 6), isorhamnetin 3-glucoside (peak 7), and syringetin 3-glucoside (peak 8) were confirmed by comparison of the retention times as well as the UV−vis and MS^n data of their corresponding standards run under the same chromatographic conditions (**Table 1**). With the exception of syringetin 3-glucoside, all of the latter flavonols had been detected in the skins of many *V. vinifera* red grape cultivars (*22*). However, to our knowledge, the occurrence of syringetin 3-glucoside had been reported in only one sample of Cabernet Sauvignon grape skin (*19*) and as a minority flavonol in Syrah and Marzemino grapes, being absent in Merlot and Cabernet Franc grapes (*23*). In the latter study, syringetin 3-glycosides were found in amounts ranging from 0 to 9.88% (mean value = 3.22%), and the authors suggested the single chromatographic peak they observed at 13.2−13.6 min was in fact a mixture of both the 3-glucoside and the 3-galactoside of syringetin, syringetin 3-galactoside being the main isomer (92.5−100%) on the basis of enzymatic hydrolysis results. Under the chromatographic conditions used in the present research, syringetin 3-galactoside and syringetin 3-glucoside do not coelute (**Figure 3b**). We also found this behavior for the isomers quercetin 3-galactoside and quercetin 3-glucoside, the 3-galactoside isomer eluting 0.8 min before the 3-glucoside isomer (data not shown). As **Figure 3b** shows, the syringetin 3-galactoside added to a sample of isolated grape skin flavonols can be easily detected in the extracted ion chromatogram (EIC) at *m*/*z* 347. This compound seems to be present only as trace amounts in the original sample of isolated grape skin flavonols (**Figure 3a**, very small peak in the EIC at *m*/*z* 347). Besides, syringetin 3-galactoside partially overlapped with isorhamnetin 3-glucoside, but it is possible to distinguish them using the respective EICs at *m*/*z* 347 and 317. The above-mentioned results indicate that peak 8 corresponds to only syringetin 3-glucoside and allow us to suggest that 3-glucoside is the main form of syringetin-type flavonols in red grape skins. These results are in agreement with previous data reporting 3-glucoside to be the main type of glycoside found in grape for the flavonol aglycones myricetin, quercetin, kaempferol, and isorhamnetin (*22*). Very little is known about the glycosylation pathway of *V. vinifera* grape flavonols (*34*), and it has been suggested that the glucosylation of flavonols may be not

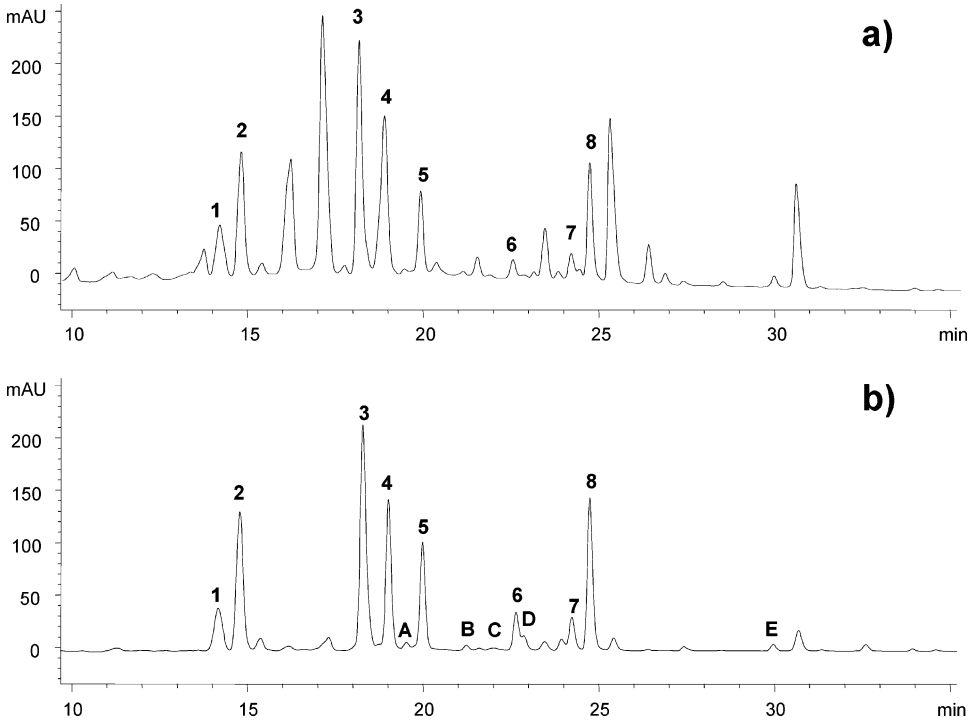

Figure 2. Chromatographic flavonol pattern (detection at 360 nm) of Petit Verdot grape skins: (**a**) crude solid–liquid extract of grape skins; (**b**) flavonol fraction isolated by solid-phase extraction of the crude extract on MCX cartridges (combination of reverse phase and cationic exchanger materials).

Table 1. Retention Times and Mass Spectral and UV–Vis Data of Flavonols Identified in *V. vinifera* Red Grape Skins and Single-Cultivar Red Wines

peak	flavonol assignation	HPLC t_R (min)	UV–vis maxima (nm)	molecular and product ions (*m*/*z*)
1	myricetin 3-glucuronide	13.93	257 (sh), 261, 301 (sh), 353	495, 319
2	myricetin 3-glucoside	14.55	257 (sh), 262, 298 (sh), 355	481, 319
3	quercetin 3-glucuronide	18.05	257, 265 (sh), 299 (sh), 354	479, 303
4	quercetin 3-glucoside	18.82	256, 265 (sh), 295 (sh), 354	465, 303
5	laricitrin 3-glucoside	19.87	256, 265 (sh), 301 (sh), 357	495, 333
6	kaempferol 3-glucoside	22.63	265, 298 (sh), 320 (sh), 348	449, 287
7	isorhamnetin 3-glucoside	24.35	255, 265 (sh), 297 (sh), 354	479, 317
8	syringetin 3-glucoside	24.91	255, 265 (sh), 300 (sh), 357	509, 347
A	laricitrin 3-galactoside	19.41	256, 265 (sh), 302 (sh), 357	495, 333
B	kaempferol 3-glalactoside	21.14	266, 292 (sh), 320 (sh), 348	449, 287
C	kaempferol 3-glucuronide	21.94	265, 290 (sh), 320 (sh), 348	463, 287
D	quercetin 3-(6″-acetyl)glucoside	22.89	257, 265 (sh), 295 (sh), 352	517, 303
E	syringetin 3-(6″-acetyl)glucoside	30.41	255, 265 (sh), 298 (sh), 358	551, 347
9	myricetin	22.02	253, 265 (sh), 303 (sh), 372	319
10	quercetin	30.12	255, 265 (sh), 300 (sh), 370	303
11	laricitrin	32.10	253, 265 (sh), 305 (sh), 372	333
12	kaempferol	37.90	264, 295 (sh), 320 (sh), 363	287
13	isorhamnetin	40.82	254, 265 (sh), 305 (sh), 371	317
14	syringetin	41.10	253, 265 (sh), 304 (sh), 372	347

catalyzed in vivo by the same UDP-glucose:flavonoid 3-*O*-glucosyltransferase responsible for the glucosylation of anthocyanidins (*35*). However, it is difficult to suppose a different behavior of syringetin toward glucosylation in comparison to the rest of flavonols, especially that of its precursor, myricetin.

The assignation of myricetin 3-glucuronide (peak 1) was mainly supported by its MS^n data. This peak had a typical UV−vis spectrum of flavonol 3-glycoside (**Table 1**) and showed a molecular ion ($[M + H]^+$) at 495 *m*/*z* units that underwent a fragmentation giving rise to a product ion at 319 *m*/*z* units. This behavior was similar to that showed by myricetin 3-glucoside (peak 2), but the difference now is that the loss of a fragment of 176 *m*/*z* units corresponds to a D-glucuronic acid molecule attached to the aglycone myricetin, instead of a D-glucose molecule (loss of a fragment of 162 *m*/*z* units) in the case of myricetin 3-glucoside. The same fragmentation patterns were shown by the analogous pair formed by quercetin 3-glucuronide (peak 3) and quercetin 3-glucoside (peak 4), but in this case the common product ion after losing the sugar molecule (D-glucuronic acid and D-glucose, respectively) was at 303 *m*/*z* units (**Table 1**).

The shape and the absorption maxima of the UV−vis spectrum belonging to peak 5 suggested the similarity to the typical UV−vis spectra of flavonol 3-glycosides (**Table 1**). In a previous work (*29*) we observed such a peak eluting after quercetin 3-glucoside, and we wrongly assigned it to quercetin 3-glucosylxyloside on the basis of only literature data (*12*). Now, on the basis of the MS^n data, this peak has been assigned to laricitrin 3-glucoside, a grape flavonol previously found as a minor compound only in the grape pomace of the cultivar Nerello Mascalese (*20*) and recently reported to be also present

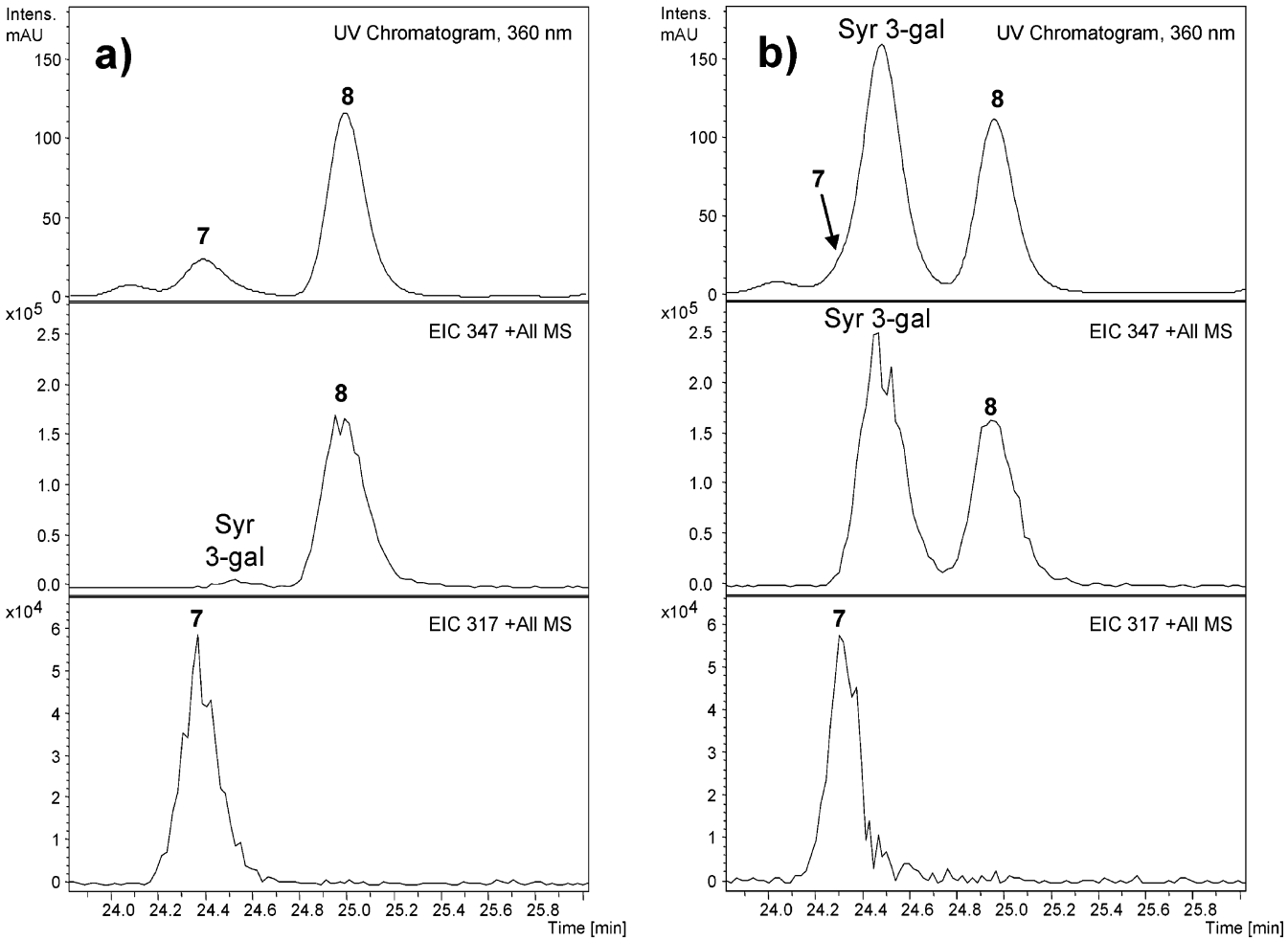

Figure 3. Expanded UV chromatogram (360 nm) and extracted ion chromatograms (EIC) at *m*/*z* values of 347 and 317, corresponding to the isolated flavonol fraction of Petit Verdot grape skins: (**a**) original sample; (**b**) sample with added syringetin 3-galactoside (Syr 3-gal). Peaks 7 and 8 are the same as in **Table 1**.

in many red-skinned grape cultivars (*23*). This peak showed in the MS^n experiments a molecular ion ($[M + H]^+$) at 495 *m*/*z* units that further fragmented, giving rise to a product ion at 333 *m*/*z* units. The loss of a fragment at 162 *m*/*z* units can be interpreted as a hexose molecule attached to a flavonol aglycone, whereas the ion at 333 *m*/*z* units is consistent with the structure of the aglycone laricitrin. As for syringetin-type flavonols, Mattivi et al. (*23*) suggested that laricitrin 3-glucoside and laricitrin 3-galactoside coeluted at 8.3−8.4 min under the chromatographic conditions they used, the 3-galactoside being the main isomer (54.7−76.7%) found after specific enzymatic hydrolysis. However, when an extracted ion chromatogram of our Petit Verdot flavonol fraction was obtained at 333 *m*/*z* units (**Figure 4**), a second minor peak appears (peak A) together with the expected peak 5. The UV−vis data of peak A were similar to those of peak 5 (**Table 1**), and the corresponding mass spectrum also showed signals at both 495 and 333 *m*/*z* units. Although no MS^n experiments were available for peak A due to the weak signal of the suspected molecular ion (495 *m*/*z* units), the results suggest that peaks 5 and A correspond to isomers of the same flavonol aglycone (laricitrin) but having different hexoses attached to the 3-position. The similarity of these results with those discussed for the glucoside/galactoside isomers of both syringetin and quercetin allowed us to suggest that peak 5 may be tentatively assigned to laricitrin 3-glucoside, whereas peak A may be tentatively assigned to laricitrin 3-galactoside. As for the case of syringetin-type flavonols, the main 3-glycoside of laricitrin found in grapes seems to be the 3-glucoside.

Other minority peaks present in the chromatogram shown in **Figure 2** had properties that suggested they were flavonol 3-glycosides, too, but the assignation proposed is only tentative (**Table 1**). Peaks B and C had UV−vis spectra with shapes and absorption maxima similar to those of kaempferol 3-glucoside. Peak C had the same retention time as a standard of kaempferol 3-glucuronide, and its mass spectrum resembles the expected signals at 463 *m*/*z* units ($[M + H]^+$) and 287 *m*/*z* units (loss of a D-glucuronic acid molecule). However, the mass spectrum also contained other residual signals, and no MS^n experiments were available due to the weak signal of the suspected molecular ion. Peak B showed a mass spectrum containing signals at 449 and 287 *m*/*z* units. These two signals are the same as shown by kaempferol D-glucoside (peak 6), suggesting that peak B corresponds to a kaempferol aglycone attached to a hexose different from D-glucose. As the 3-galactosides of quercetin and syringetin elute before their 3-glucoside isomers under the chromatographic conditions used, and the same may be applicable to laricitrin isomers, peak B was tentatively assigned as kaempferol 3-galactoside. Peaks D and E were tentatively assigned as quercetin 3-(6″-acetyl)glucoside and syringetin 3-(6″-acetyl)glucoside, respectively, on the basis of their UV−vis and MS data. Syringetin 3-(6″-acetyl)glucoside was already found in Cabernet Sauvignon skins (*19*). The UV−vis spectra

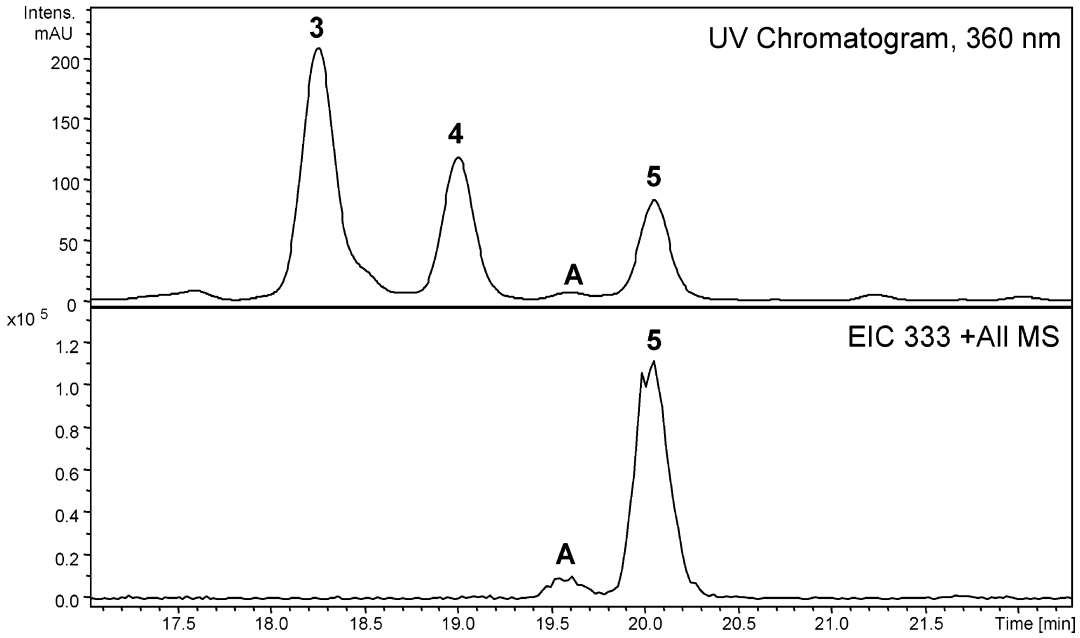

Figure 4. Expanded UV chromatogram (360 nm) and EIC at 333 *m*/*z* units, corresponding to the isolated flavonol fraction of Petit Verdot grape skins. Peak assignations are as in **Table 1**.

of these two peaks were similar to those of their corresponding nonacetylated compounds (peaks 4 and 8, respectively). In the mass spectrum of peak D a signal at *m*/*z* 303 was found, thus indicating a quercetin-type flavonol, together with a weak signal at 517 *m*/*z* units that can be associated with the molecular ion ($[M + H]^+$). In the same way, the mass spectrum of peak E showed the expected signals at 551 *m*/*z* units ($[M + H]^+$) and 347 *m*/*z* units (syringetin-type flavonol). However, the mass espectra of peaks D and E were not clean and, as in the case of peaks B and C, the assignation proposed must be confirmed.

Other *V. vinifera* red grape cultivars were analyzed for their content of flavonols, paying special attention to the occurrence of the main flavonol glycosides found in Petit Verdot grapes (peaks 1−8), which include the methoxylated trisubstituted flavonols laricitrin 3-glucoside and syringetin 3-glucoside. In total, three Spanish red grape cultivars (Tempranillo, Garnacha, and Garnacha Tintorera) and four French red grape cultivars (Cabernet Sauvignon, Merlot, Syrah, and Petit Verdot) were considered. All of the samples analyzed had a chromatographic pattern similar to that shown in **Figure 2b**, allowing the detection of the eight flavonols above-mentioned (**Table 2**). As a general rule, the main flavonols were myricetin 3-glucoside, quercetin 3-glucuronide, and quercetin 3-glucoside, their individual molar percentages being in the range of 13−33%. The myricetin 3-glucuronide had a remarkable importance in Tempranillo and Petit Verdot cultivars, contributing with molar percentages of >10%. The isorhamnetin 3-glucoside was an important flavonol for Garnacha Tintorera and Syrah cultivars (molar percentages of >10%), and kaempferol 3-glucoside always was a minority flavonol (molar percentages usually of <5%). The methoxylated trisubstituted flavonols, laricitrin 3-glucoside and syringetin 3-glucoside, accounted at least for a molar percentage of 5% for all of the cultivars studied. Laricitrin 3-glucoside contributed at higher molar percentages (8−9%) to the flavonol profiles of Syrah and Petit Verdot cultivars, whereas syringetin 3-glucoside accounted for as high as 9−10% in the cases of Garnacha Tintorera, Syrah, Petit Verdot, and Cabernet Sauvignon cultivars. The above molar percentages were similar to those previously reported for red-skinned grapes (*23*), after acid hydrolysis, in the case of total laricitrin-type flavonols (mean, 5.65%; range, 0−13.99%), but they were slightly higher for total syringetin-type flavonols (mean, 3.22%; range 0−9.88%). These results suggest that laricitrin 3-glucoside and syringetin 3-glucoside are important contributors to the flavonol pool of *V. vinifera* red grapes (up to a total of 15−20%, on a molar basis), and they might be considered, together with other important flavonols (myricetin- and quercetin-type flavonols), in studies dealing with the antioxidant capacity of grapes or their contribution to the stabilization of red wine color.

The latter results suggested that flavonols could be used as chemical markers for confirmation of cultivar authenticity of *V. vinifera* grapes. The principal component analysis of the flavonol profiles data (**Table 2**), performed with no previous cultivar assignation, provided the grouping of the grape samples of the same cultivar with remarkable success. The analysis explained 81.7% of the cumulative variance with three principal components (PCs), which had associated the following flavonol glycosides (loadings ≥ 0.8): isorhamnetin 3-glucoside and quercetin 3-glucuronide (PC 1); 3-glucosides of both myricetin and quercetin (PC 2); and laricitrin 3-glucoside (PC 3). The scores obtained with these three PCs for the grape samples allowed the separation of the groups formed by the samples of the Spanish cultivars (Tempranillo, Garnacha, and Garnacha Tintorera) and three of the four French cultivars (Cabernet Sauvignon, Merlot, and Petit Verdot). In contrast, the samples of the French cultivar Syrah showed some overlapping with the samples of Cabernet Sauvignon and Merlot cultivars. These preliminary results need to be confirmed by analyzing a higher number of grape samples, including samples from different vintages, and also need to be extended to other grape cultivars.

Evolution of the Flavonol Profile during the Ripening of the Berry. At the moment of véraison, the total content of the eight main flavonol 3-glycosides (peaks 1−8 in **Table 1**) detected in the analyzed berries was in the range of 60−120 μmol/kg, with the exception of Garnacha Tintorera cultivar, which had only 35 μmol/kg. The flavonol content, expressed both per kilogram and per berry, was increasing during the ripening period for all of the cultivars considered, following the same trend as previously found for Australian Shiraz and Chardonnay grapes (*3*, *36*). In the warm climate conditions of

Table 2. Flavonol Profile (Molar Percentage of Each Individual Flavonol) and Flavonol Content (Value Range) of Different *V. vinifera* Red Grape Cultivars[a]

	molar % of flavonol in						
flavonol	Tempranillo ($n = 4$)	Garnacha ($n = 4$)	Garnacha Tintorera ($n = 2$)	Syrah ($n = 3$)	Petit Verdot ($n = 2$)	Cabernet Sauvignon ($n = 4$)	Merlot ($n = 4$)
myricetin 3-glucuronide	10.70 a	2.17 b	1.43 b	3.49 b	10.82 a	6.02 c	3.12 b
myricetin 3-glucoside	25.92 a,c	15.98 b	29.63 c	16.20 b	20.85 a, b	20.88 a,b	18.98 a,b
quercetin 3-glucuronide	22.99 a,b	25.93 b	12.98 c	17.41 a,c	23.69 a,b	23.34 a,b	29.94 b
quercetin 3-glucoside	22.00 a	31.18 b,c	24.58 a,b	30.07 b,c	20.95 a	26.71 a,b,c	32.93 c
laricitrin 3-glucoside	5.92 a	5.43 a	5.28 a	8.57 b	8.28 b	6.48 a,b	4.54 a
kaempferol 3-glucoside	5.03 a	6.34 b	3.83 c	2.71 c,d	1.80 d	3.17 c	2.84 c,d
isorhamnetin 3-glucoside	2.67 a	6.70 a	11.74 b	11.13 b	3.16 a	4.51 a	3.22 a
syringetin 3-glucoside	4.78 a	6.26 a,b	10.53 b	10.42 b	10.44 b	8.89 a,b	4.42 a
flavonol content (μmol/kg)	187–223	129–177	140–158	300–346	178–246	194–242	241–285

[a] Different letters in the same row, for wines of the same cultivar, indicate significant differences according to the Student–Newman–Keuls test ($\alpha = 0.05$).

the Spanish winemaking region of La Mancha, the French red grape cultivars reached higher flavonol contents than the Spanish red grape cultivars. The highest flavonol content was reached by the Syrah berries (400 μmol/kg), followed by Cabernet Sauvignon and Petit Verdot cultivars (around 280 μmol/kg each). The flavonol content of Spanish red grape cultivars was within the range of 145−235 μmol/kg.

The quercetin-type flavonols were predominant over the ripening period, followed by the myricetin-type flavonols (**Figure 5**). The molar percentage of quercetin 3-glucoside was the most constant during the ripening for all of the cultivars studied, and it oscillated in the following ranges: Tempranillo, 20.5−25.8%; Garnacha, 28.7−35.9%; Garnacha Tintorera, 22.0−25.9%; Cabernet Sauvignon, 20.6−31.4%; Syrah, 26.6−34.0%; and Petit Verdot, 20.0−23.5%. At the moment of véraison, the main flavonol was always quercetin 3-glucuronide, its molar percentage being in the range of 51.3−74.3%. The importance of quercetin 3-glucuronide strongly decreased in the first steps of the ripening period, and two trends of evolution were found. On the one hand, the molar proportion of quercetin 3-glucuronide finally reached levels similar to those shown by quercetin 3-glucoside: this was the case of the cultivars Tempranillo (22.9 versus 20.6%), Cabernet Sauvignon (26.1 versus 26.5%), and Petit Verdot (23.8 versus 20.1%). On the other hand, the decrease in the molar percentage of quercetin 3-glucuronide continued until it was lower than those of quercetin 3-glucoside: Syrah, 16.7 versus 26.6%; Garnacha, 23.8 versus 34.1%; and Garnacha Tintorera, 12.4 versus 23.7%.

Myricetin 3-glucoside was a minor flavonol at the moment of véraison for all of the considered cultivars (0.6−4.1%) with the exception of Garnacha Tintorera (11.4%). Its contribution to the flavonol profile increased during ripening until it reached molar percentages similar to those found for quercetin-type flavonols. This result is in agreement with the observed higher levels of mRNA of flavonoid 3′,5′-hydroxylase in berry skin at harvest (*37*). In some cases myricetin 3-glucoside finally was the main flavonol at the end of ripening (27.7% in Tempranillo and 30.4% in Garnacha Tintorera). Cabernet Sauvignon was the only sample in which the final molar percentage of myricetin 3-glucoside did not reach the value of 15%, whereas the other cultivars had 17.5−19.7% of this flavonol.

Contrary to quercetin 3-glucuronide, the initial molar percentage of myricetin 3-glucuronide was very low, and its importance slightly increased over the ripening period. The final values were lower than 5% for Garnacha, Garnacha Tintorera, and Merlot. Tempranillo and Petit Verdot reached molar percentages slightly higher than 10%, and Cabernet Sauvignon had an intermediate behavior (7−8%). Kaempferol 3-glucoside was another minor flavonol that never reached molar percentage values higher than 5%, with the only exception of Garnacha, which reached 6−7% at the end of ripening.

The methoxylated flavonols were minor flavonols at véraison. Their molar percentages increased during ripening, when their corresponding precursors were accumulating. These precursors were disubstituted and trisubstituted flavonols and, as above-described, they also were minor flavonols at the beginning of ripening. The methoxylation of quercetin led to the formation of isorhamnetin 3-glucoside, its molar percentage in the ripe grapes being lower than 5% in three cultivars (Tempranillo, Garnacha, and Petit Verdot); Cabernet Sauvignon slightly exceeded 5% of this flavonol, but Garnacha Tintorera and Syrah cultivars reached 10% and more.

Gradual methoxylation of myricetin led to laricitrin 3-glucoside and then syringetin 3-glucoside. The molar percentage values of laricitrin 3-glucoside in the ripe grapes were >5% for all of the studied cultivars, the highest values being those corresponding to the French cultivars: Cabernet Sauvignon, 7.0%; Petit Verdot, 8.9%; and Syrah, 10.7%. Finally, the contribution of syringetin 3-glucoside to the flavonol profile of ripe grapes was rather important in four of the considered cultivars: Cabernet Sauvignon, 8.5%; Garnacha Tintorera, 10.9%; Syrah, 11.8%; and Petit Verdot, 12.1%.

Occurrence of Flavonols in *V. vinifera* Single-Cultivar Wines. The flavonol fraction of a total of 32 wine samples, made from 10 *V. vinifera* grape cultivars and produced in five different countries, was analyzed (**Table 3**). The main flavonol glycosides identified in red grape skins (peaks 1−8) were also found in all of the wine samples as expected (**Figure 6**). However, many samples showed only trace amounts of quercetin 3-glucoside (**4**), as well as small amounts of the 3-glucosides of both kaempferol (**6**) and isorhamnetin (**7**). Moreover, new flavonol-like peaks were detected in wine chromatograms (peaks 9−14). The mass spectral and UV−vis data (**Table 1**) suggested these new peaks corresponded to the flavonol aglycones produced by acid hydrolysis of the flavonol glycosides over the winemaking process and/or the wine aging. The assignation of the new peaks was possible by comparison with commercial available standards, with the exception of peak 11. This peak was assigned as laricitrin, because its UV−vis data resembled those of the other 3-substituted flavonol aglycones (myricetin and syringetin), and its mass spectrum showed a signal at 333 *m*/*z* units, which is compatible with the molecular ion ($[M + H]^+$) of laricitrin.

The results suggested that the rates of hydrolysis of the flavonol glycosides in wine were different according to the type of flavonol aglycone and also with respect to the nature of the

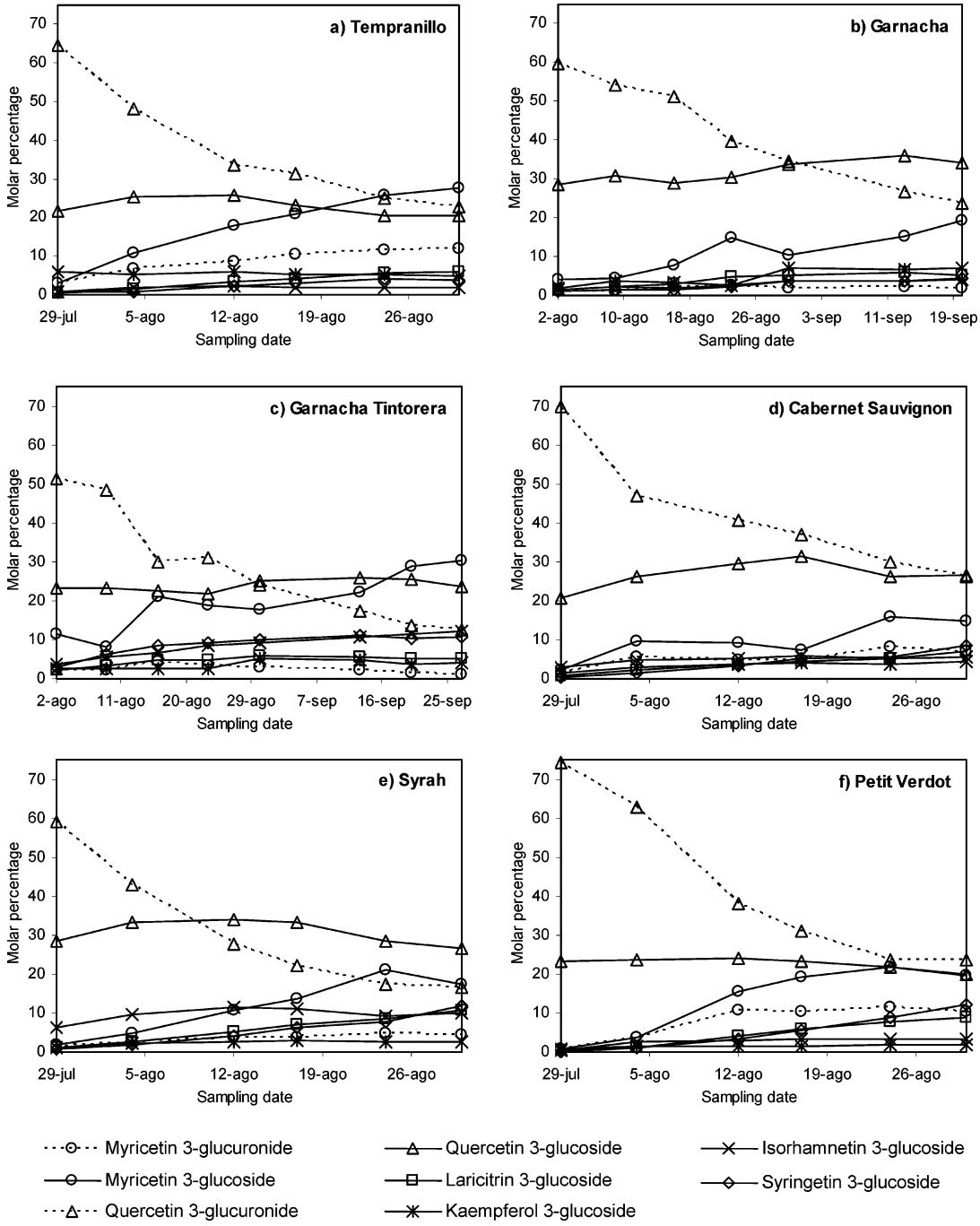

Figure 5. Evolution during ripening (from véraison until harvest) of the flavonol composition of different *V. vinifera* cultivars: (**a**) Tempranillo; (**b**) Garnacha; (**c**) Garnacha Tintorera; (**d**) Cabernet Sauvignon; (**e**) Syrah; (**f**) Petit Verdot.

glycoside moiety. The 3-glucosides of mono- and disubstituted flavonols (kaempferol, quercetin, and isorhamnetin) look to be easily hydrolyzed, because they usually accounted for very small amounts in many of the analyzed wine samples. As quercetin 3-glucoside is one of the main flavonol glycosides found in grapes, it is usual to observe an important peak of free quercetin in wines due to its hydrolysis. In contrast, the high remaining amounts of quercetin 3-glucuronide in wines suggested its resistance to acid hydrolysis, and the same can be said for the analogous myricetin 3-glucuronide. Although many wine samples had an important peak corresponding to free myricetin, the amounts of myricetin 3-glucoside never were a trace; thus, this flavonol seems to be not as easily hydrolyzed as quercetin 3-glucoside. Finally, the amounts of the 3-glucosides of both laricitrin and syringetin usually were much higher than those of their corresponding free aglycones.

With regard to the total content of flavonols, some suggestions can be made bearing in mind the low number of samples analyzed. First of all, the results showed a great variability that can be attributable to several factors, including some aspects of the raw material (the grape cultivar, the degree of grape ripening, or the initial flavonol content of grapes), the wine-making process, and the aging time. For instance, the wines made from the Spanish and Italian cultivars (Tempranillo, Garnacha, Bobal, and Sangiovese) have total contents usually around 100 μmol/L, whereas some Syrah, Cabernet Sauvignon, Merlot, and Dornfelder wines showed total contents as high as 250−300 μmol/L and more. A decrease in the flavonol content

Table 3. Flavonol Profile (Molar Percentage of Each Individual Flavonol) and Flavonol Content of Different *V. vinifera* Single-Cultivar Red Wines

wine sample				flavonol content	molar percentage of individual flavonols[a]													
no.	cultivar	vintage	country	(μmol/L)	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Tempranillo	1999	Spain	98	2.77	45.69	18.26	14.03	6.76	0.80	1.67	5.17	2.77	1.69	ND	0.39	ND	ND
2	Tempranillo	1999	Spain	138	3.79	43.19	15.01	11.60	5.61	0.39	1.13	3.24	6.67	6.73	0.68	1.19	0.48	0.27
3	Tempranillo	2000	Spain	99	2.70	47.25	13.56	7.44	7.07	0.19	1.34	5.60	5.05	7.38	0.81	0.88	0.54	0.18
4	Tempranillo	2003	Spain	93	4.14	27.07	20.15	ND	8.68	0.32	0.66	8.14	11.43	14.20	1.51	1.82	1.43	0.45
5	Tempranillo	2003	Spain	49	6.09	15.78	17.01	1.18	8.97	0.22	ND	8.55	22.83	13.71	3.17	1.01	1.11	0.40
6	Tempranillo	2003	Spain	106	4.28	14.95	17.49	ND	7.59	0.55	0.38	7.87	23.32	17.80	2.69	1.05	1.54	0.50
7	Tempranillo	2006	Spain	282	4.91	38.90	16.87	16.17	5.62	1.45	1.59	2.84	7.12	3.93	0.10	0.26	0.10	0.14
8	Garnacha	2005	Spain	28	3.59	16.20	31.71	2.12	6.62	ND	0.87	7.63	6.97	17.61	3.60	1.29	1.78	ND
9	Garnacha	2005	Spain	45	3.86	13.70	27.98	1.61	5.82	ND	0.89	7.16	12.09	20.28	3.06	0.68	2.39	0.48
10	Garnacha	2005	Spain	99	4.53	16.10	41.58	2.81	7.95	ND	2.37	9.61	1.29	9.74	1.55	0.45	1.78	0.24
11	Garnacha	2005	Spain	71	4.22	19.58	41.95	3.66	7.59	ND	3.05	8.93	1.22	7.27	0.96	0.18	1.19	0.20
12	Garnacha	2005	Spain	92	3.95	21.50	36.13	4.52	7.22	ND	2.63	7.67	1.55	11.79	1.02	0.62	1.23	0.16
13	Bobal	2003	Spain	70	2.54	2.60	28.11	ND	5.65	0.57	0.38	11.15	10.35	30.28	3.02	1.48	3.23	0.65
14	Bobal	2003	Spain	127	4.26	1.29	20.06	ND	4.77	0.68	0.20	11.02	19.72	29.08	4.72	0.73	2.94	0.54
15	Bobal	2003	Spain	88	3.95	2.01	26.26	ND	5.20	0.44	0.51	11.69	11.19	29.78	3.73	1.45	3.17	0.61
16	Cabernet Sauvignon	2002	Spain	58	4.13	10.57	36.85	ND	5.99	1.81	0.47	16.06	8.18	10.24	3.69	ND	1.35	0.65
17	Cabernet Sauvignon	2002	Spain	126	4.16	15.36	25.17	ND	7.67	0.29	1.00	12.08	8.08	19.86	2.08	0.98	2.58	0.68
18	Cabernet Sauvignon	2004	Spain	293	4.04	25.16	20.85	11.08	4.48	0.38	2.09	5.46	6.31	15.67	1.22	1.58	1.33	0.35
19	Cabernet Sauvignon	2005	Spain	311	6.00	26.71	21.01	15.63	5.45	1.25	2.70	5.57	4.25	8.69	1.15	0.71	0.61	0.26
20	Cabernet Sauvignon	2005	Chile	236	2.01	12.13	12.01	1.79	4.37	0.21	1.34	7.85	17.83	28.31	7.10	1.06	3.46	0.53
21	Cabernet Sauvignon	2006	Chile	229	4.06	4.72	8.98	ND	3.34	ND	ND	9.38	32.09	25.42	6.45	0.54	4.29	0.73
22	Petit Verdot	2004	France	173	2.56	8.81	11.71	4.04	3.09	ND	0.53	5.50	24.38	31.09	3.49	1.64	2.45	0.70
23	Petit Verdot	2004	Spain	191	2.51	12.79	22.26	1.15	5.45	0.68	ND	9.34	14.71	25.56	1.99	1.65	1.25	0.66
24	Petit Verdot	2005	Spain	155	3.24	15.63	24.99	2.08	6.79	ND	2.06	14.10	21.95	2.59	3.91	0.40	1.63	0.61
25	Syrah	2003	Spain	159	3.62	5.61	25.03	0.68	10.00	0.44	0.43	15.59	16.29	11.92	4.17	ND	5.71	0.50
26	Syrah	2004	Spain	377	3.48	24.81	17.59	9.65	5.47	0.62	3.17	5.53	6.53	17.89	1.32	1.73	1.76	0.45
27	Merlot	2005	Spain	267	3.52	17.63	31.44	19.86	2.93	1.38	1.74	2.19	4.07	12.67	0.89	0.89	0.59	0.19
28	Merlot	2006	Spain	221	4.00	18.55	36.55	18.40	3.33	0.81	1.64	2.96	4.17	8.60	0.17	0.48	0.24	0.11
29	Dornfelder	2004	Germany	279	2.31	10.93	7.93	0.68	4.53	ND	0.48	6.17	31.84	23.67	4.70	1.62	4.14	1.01
30	Dornfelder	2004	Germany	270	2.61	25.43	14.98	0.98	3.08	0.13	2.62	2.83	13.23	27.34	1.36	2.47	2.36	0.58
31	Carmenère	2005	Chile	175	2.77	3.72	9.31	1.74	2.41	ND	0.22	5.83	26.67	37.09	4.18	1.70	3.60	0.74
32	Sangiovese	2004	Italy	82	1.28	5.39	13.23	1.41	2.94	0.94	0.51	8.35	21.41	36.05	4.64	1.11	2.00	0.75

[a] Flavonol assignation as in **Table 1**. ND, not detected.

during aging could be expected, due to the involvement of flavonols in oxidation processes and/or by insolubility, but the preliminary results obtained for commercial Tempranillo and Cabernet Sauvignon wines with different aging times did not suggest a clear trend. Determination of the role of aging on the evolution of the flavonol content of wine needs the evaluation of controlled wines over time.

Finally, the flavonol profiles of red wines could be also used for confirmation of cultivar authenticity. However, the attempts made using the molar percentages of the 14 flavonols (glycosides and free aglycones) present in wines were not successful (Student−Newman−Keuls test and principal component analysis; data not shown). The reason must be found in the interferences introduced by the acid hydrolysis of flavonol glycosides. However, when the flavonol profiles were redefined on the basis of the type of aglycone (by the summation of every flavonol glycoside and the free aglycone of the same type), some degree of differentiation was found. In this case, the principal component analysis explained 73.1% of the cumulative variance with two PCs, which had associated the following types of

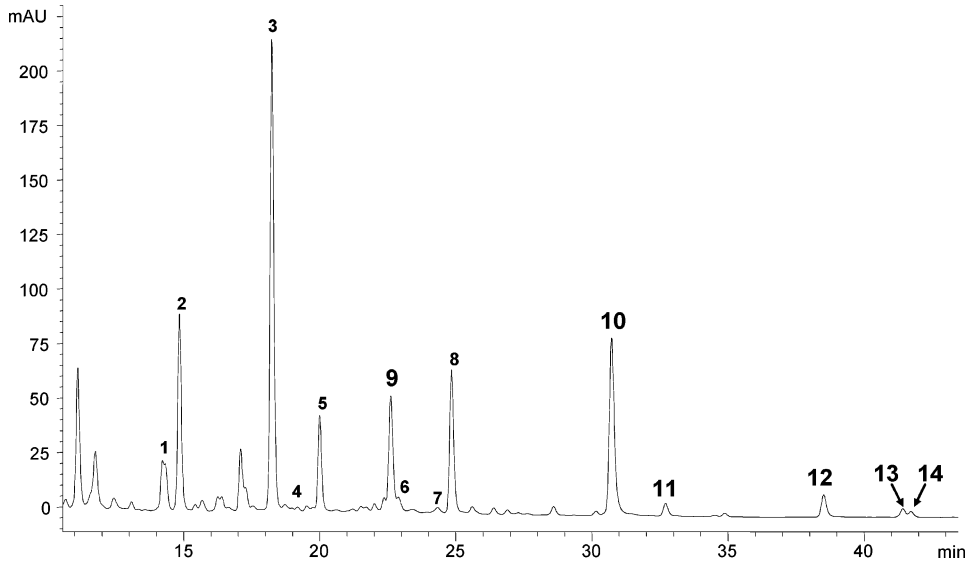

Figure 6. Chromatographic pattern (detection at 360 nm) of the flavonol fraction of a Petit Verdot wine. Peak assignations are as in **Table 1**.

aglycone (loadings ≥ 0.8): myricetin and quercetin (PC 1); laricitrin and syringetin (PC 2). The grouping of the samples only was acceptable in the case of Tempranillo, Garnacha, and Merlot wines, whereas some trend in grouping was shown by Cabernet Sauvignon and Bobal wines. These results are only preliminary and need to be carefully evaluated with a higher number of wine samples, paying special attention to the expected heavy effects of some technological factors, such as the use of exogenous enzymes, on the flavonol profiles of the wines.

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