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Flavonol 3-*O*-Glycosides Series of *Vitis vinifera* Cv. Petit Verdot Red Wine Grapes

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To gain knowledge of the characteristic flavonol 3-*O*-glycosides of red grape *Vitis vinifera* varieties, we isolated the flavonol fraction from *V. vinifera* cv. Petit Verdot grape skins by solid-phase extraction using a combination of reverse-phase and ion-exchanging materials. This procedure allowed us to separate a fraction of anthocyanin-free flavonol 3-*O*-glycosides that was further split into neutral and acidic subfractions, thus facilitating flavonol identification. By means of semipreparative reverse-phase high-performance liquid chromatography, we isolated several of these flavonol 3-*O*-glycosides for structural elucidation. The identification of different flavonol 3-*O*-glycosides was based on liquid chromatography−diode array detection−electrospray ionization−tandem mass spectrometry and NMR data when available. The results suggest that red grape flavonol 3-*O*-glycosides comprise three different complete series, according to the nature of the sugar moiety linked to the C-3 position. The 3-*O*-glucosides were the main derivative of the six possible flavonol aglycones (kaempferol, quercetin, isorhamnetin, myricetin, laricitrin, and syringetin), whereas the 3-*O*-galactoside derivatives were found as minor compounds for all of the flavonol aglycones. The 3-*O*-glucuronides are the third kind of red grape flavonol derivatives and normally account as minor compounds for all of the flavonol aglycones, with the exception of quercetin 3-*O*-glucuronide, which was as abundant as quercetin 3-*O*-glucoside. In addition, the presence of quercetin 3-*O*-(6′′-rhamnosyl)-glucoside (rutin) was also detected as a trace compound in the skins of Petit Verdot grapes.

KEYWORDS: Flavonol; galactoside; glucoside; glucuronide; LC-MS; NMR; red grape; *Vitis vinifera*

INTRODUCTION

Flavonols are a class of flavonoid compounds located in *Vitis vinifera* grape berry skins, where they are involved in UV screening (*1*, *2*). Moreover, the branch of the flavonoid pathway leading to flavonol biosynthesis has been suggested to be light-dependent (*3*), in sharp contrast to anthocyanin and tannin synthesis, which is 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, like 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, like flavan-3-ols and anthocyanins (*9*−*11*).

Phenolic compounds biosynthesis in *V. vinifera* grapes is under genetic control, and the differences among grape varieties are sometimes enough so that the phenolic composition of the grape can be used as a tool for authenticity and varietal differentiation. In the case of red wine grape cultivars, the anthocyanin profiles have been widely used for varietal authenticity purposes, and also, flavonol profiles have demonstrated some ability for both red and white wine grape cultivars (*12*−*17*).

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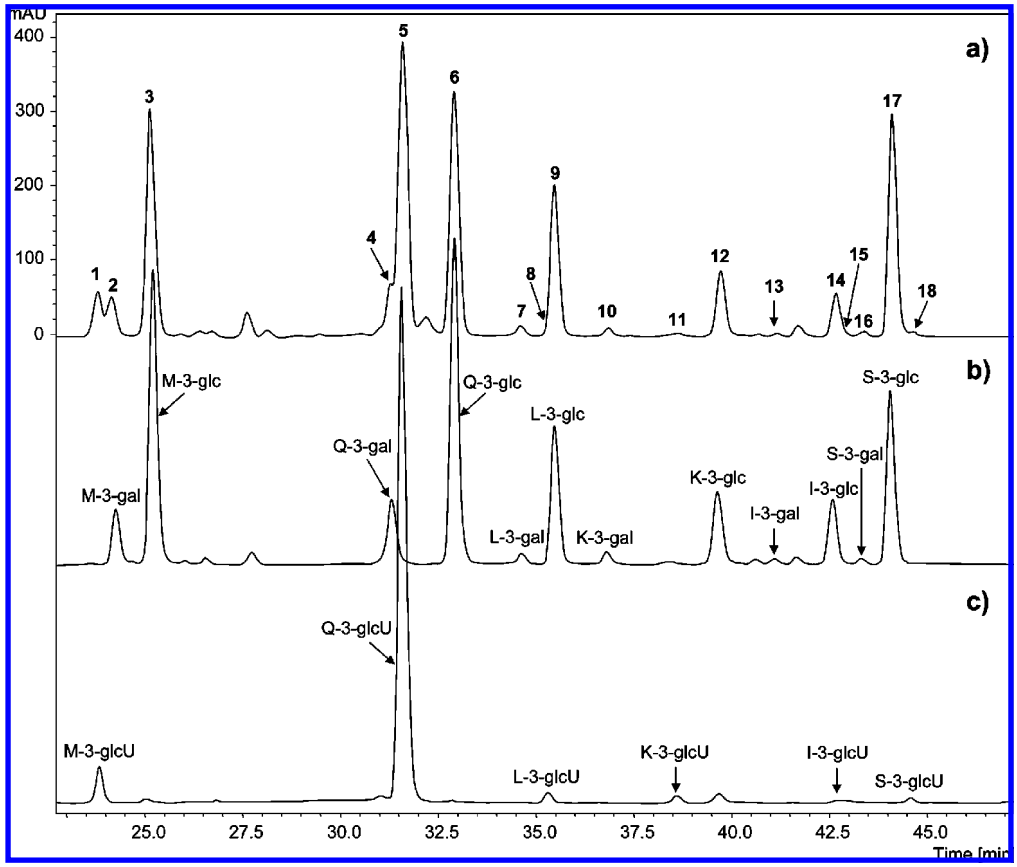

Figure 1. DAD chromatograms (detection at 360 nm) of flavonol 3-*O*-glycosides from Petit Verdot grape skins: (**a**) total flavonol extract, (**b**) fraction of neutral flavonol 3-*O*-glycosides, and (**c**) fraction of acidic flavonol 3-*O*-glycosides. The peak numbering is like in that in **Table 1**. Peak assignation: M, myricetin; Q, quercetin; L, laricitrin; K, kaempferol; I, isorhamnetin; S, syringetin; gal, galactoside; glc, glucoside; and glcU, glucuronide.

However, the results dealing with the usefulness of flavonol profiles as chemical markers for confirmation of cultivar authenticity in grapes have not always shown consistent results. Maybe one of the reasons for the inconsistency shown by grape flavonol profiles is the scarce knowledge of such a kind of grape phenolics in contrast to the very well-known grape anthocyanins. It is very common to find in the literature an incomplete description of the grape flavonols, with regard to both the substitution pattern of the B ring of the flavonoid structure and the type of sugar moiety linked to the 3-position. For many years, the only flavonoid structures considered were the 4′-hydroxy derivative named kaempferol, the 3′,4′-dihydroxy derivative named quercetin, the 4′-hydroxy-3′-methoxy derivative named isorhamnetin, and the 3′,4′,5′-trihydroxy derivative named myricetin, the two latter being considered as exclusive to red grape cultivars. Recently, the presence of two new types of flavonoid structures in the pool of red grape flavonols has been demonstrated in every red grape cultivar analyzed, the 3′,4′-dihydroxy-5′-methoxy derivative named laricitrin, and the 4′-hydroxy-3′,5′-dimethoxy derivative named syringetin (*17*). The aforementioned finding has afforded completion of the whole possible substitution patterns in the B -ring of the flavonol structure in the case of *V. vinifera* grape flavonols, in contrast to grape anthocyanins in which the monohydroxyl derivative (known as pelargonidin) is not present, although it exists in many other plants.

Flavonols in grapes only exist as 3-*O*-glycosides, while the corresponding free aglycones can be found in wines, together with the 3-*O*-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 all of the flavonol structures, but galactose and glucuronic acid have also been found as the glycosylation sugar of some of the flavonol structures (*17*). Furthermore, quercetin has been suggested to occur in grapes as 3-*O*-(6″-rhamnosyl)-glucoside (the flavonol so-called rutin), 3-*O*-glucosylgalactose, and 3-*O*-glucosylxyloside, while other kaempferol 3-*O*-glycosides include 3-*O*-glucosylarabinoside and 3-*O*-galactoside, although much of the latter 3-*O*-glycosides have not been structurally confirmed, and their assignations have sometimes only been based on retention times and UV−vis spectra (*18*). Very little is known about the glycosylation pathway of *V. vinifera* grape flavonols (*19*), and it has been suggested that the glucosylation of flavonols may be not catalyzed in vivo by the same UDP-glucose:flavonoid 3-*O*-glucosyltransferase responsible for the glucosylation of anthocyanins (*20*).

To gain knowledge of the characteristic flavonol 3-*O*-glycosides of red grape *V. vinifera* varieties, the aim of this work was the separation, isolation, and identification of the flavonol 3-*O*-glycosides present in *V. vinifera* cv. Petit Verdot. We first isolated the flavonol fraction from grape skins, and then, we split it into neutral and acidic flavonols, by means of solid-phase extraction using a combination of reverse-phase and ion-exchanging materials. The analytical high-performance liquid chromatography (HPLC) method was improved for a better resolution of the different flavonol peaks, and the isolation of individual compounds was performed by repetitive analytical HPLC separation helped by the use of a fraction collector, due to the complexity of the HPLC flavonol profile. Only flavonol 3-*O*-glycosides were isolated, and the assignment of the different identified compounds was based on liquid chromatography−diode

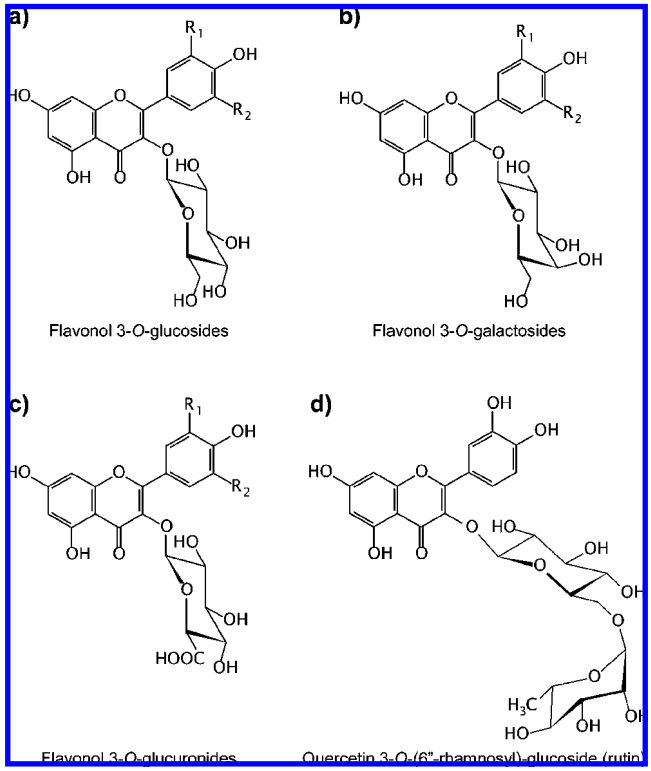

Figure 2. Flavonol 3-*O*-glycosides found in *V. vinifera* cv. Petit Verdot grapes: (**a**) flavonol 3-*O*-glucosides, (**b**) flavonol 3-*O*-galactosides, (**c**) flavonol 3-*O*-glucuronides, and (**d**) quercetin 3-*O*-(6″-rhamnosyl)-glucoside (also known as rutin). Flavonoid structures (flavonol aglycone): $R_1 = R_2 = H$, kaempferol; $R_1 = OH$ and $R_2 = H$, quercetin; $R_1 = OCH_3$ and $R_2 = H$, isorhamnetin; $R_1 = R_2 = OH$, myricetin; $R_1 = OCH_3$ and $R_2 = OH$, laricitrin; and $R_1 = R_2 = OCH_3$, syringetin.

array detection−electrospray ionization−tandem mass spectrometry (LC-DAD-ESI-MS/MS) and also by NMR data when possible.

MATERIALS AND METHODS

Chemicals and Grape Samples. All solvents were HPLC quality, and all chemicals were analytical grade (>99%). Water was Milli-Q quality. Commercial standards of flavonol glycosides 3-*O*-glucosides of quercetin, kaempferol, isorhamnetin, and syringetin and 3-*O*-galactosides of quercetin and syringetin (Extrasynthese, Genay, France) were used. Other noncommercial flavonol standards (myricetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, and kaempferol 3-*O*-glucuronide) were kindly supplied by Dr. Ullrich Engelhardt (Institute of Food Chemistry, Technical University of Braunschweig, Germany).

Healthy red wine grapes grown in the experimental vineyard of the Instituto de la Vid y el Vino de Castilla-La Mancha (middle-southern Spain) were collected at optimum ripeness for harvesting (estimated alcoholic strength of around 13%, 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 4−5 clusters per vine. The size of the sample was around 200 berries, which were bulked and separated in two subsamples of approximately 100 berries. All of the vines were drip-irrigated and grown using a bilateral Royat cordon trellis.

Grape Skin Extraction and Sample Preparation. 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 $CH_3OH/H_2O/HCOOH$, using a homogenizer (Heidolph DIAX 900) for 2 min and then centrifuging 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 using.

Anthocyanin-free flavonol fractions from grape skin extracts were obtained following the procedure previously described (*17*) using SPE cartridges (Oasis MCX cartridges, Waters Corp., Mildford, MA; cartridges of 6 mL capacity filled with 500 mg of adsorbent). The eluate containing flavonols was dried in a rotary evaporator (40 °C) and resolved in 3 mL of 25% methanol.

Separation of Neutral and Acidic Flavonol 3-*O*-Glycosides. Solid-phase extraction on Oasis MAX cartridges (Waters Corp.; cartridges of 6 mL of capacity filled with 500 mg of adsorbent) containing a mixture of reverse-phase and anionic-exchanger (dimethyl butylamine groups) materials allowed the separation of grape flavonols into two groups: the neutral and the acidic flavonols, according to the nature of the glycosyl moiety. The procedure was inspired in the previous work of Cheynier and Rigaud, which separated grape flavonol 3-*O*-glycosides using a polyamide column (*21*). An amount of 3 mL of anthocyanin-free flavonol fraction isolated from grape skins was dried under vacuum and resolved in 3 mL of water. Furthermore, this solution was passed through the SPE cartridges previously conditioned with 5 mL of methanol and 5 mL of water. After the columns were washed with 2 × 5 mL of water, the neutral flavonol fraction was eluted with 3 × 5 mL of 2% formic acid in methanol. Acidic flavonols were recovered using 3 × 5 mL of 2% hydrochloric acid in methanol. The eluates containing neutral and acidic flavonols were separately dried in a rotary evaporator (40 °C) and resolved in 3 mL of 25% methanol.

HPLC-DAD-ESI-MS^n Analysis of Flavonols. HPLC separation, identification, and quantification of flavonols were performed on a Agilent 1100 Series system (Agilent, Germany), equipped with DAD (G1315B) and LC/MSD Trap VL (G2445C VL) ESI-MS^n system, and coupled to an Agilent Chem Station (version B.01.03) data-processing station. The mass spectra 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 mm × 250 mm; 5 μm particle; Agilent), thermostatized at 40 °C. The chromatographic conditions were modified from a previous method (*17*). The solvents were as follows: solvent A (acetonitrile/water/formic acid, 3:88.5:8.5, v/v/v), solvent B (acetonitrile/water/formic acid, 50:41.5:8.5, v/v/v), and solvent C (methanol/water/formic acid, 90:1.5:8.5, v/v/v). The flow rate was 0.63 mL/min. The linear solvents gradient was as follows: zero min, 96% A and 4% B; 7 min, 96% A and 4% B; 38 min, 70% A, 17% B, and 13% C; 52 min, 50% A, 30% B, and 20% C; 52.5 min, 30% A, 40% B, and 30% C; 57 min, 50% B and 50% C; 58 min, 50% B and 50% C; and 65 min, 96% A and 4% B. For identification, ESI-MS^n was used in both positive and negative modes, setting the following parameters: dry gas, N_2, 11 mL/min; drying temperature, 350 °C; nebulizer, 65 psi; capillary, −2500 V (positive ionization mode) and +2500 V (negative ionization mode); target mass, 600 *m*/*z*; compound stability, 40% (negative ionization mode) and 100% (positive ionization mode); trap drive level, 100%; and scan range, 50−1200 *m*/*z*.

HPLC Semipreparative Separation of Flavonols. For semipreparative HPLC, we used the same equipment and the same chromatographic conditions as for analytical HPLC due to the complexity of the mixture to be separated. With the help of an analytical fraction collector (Agilent G1364C), we made repeated injections (ca. 100 injections) of the neutral and the acidic flavonol isolates. The collected fractions were dried in a rotary evaporator (40 °C) and cleaned-up from residual anthocyanins using Oasis MCX cartridges. We collected up to 20 fractions, but only seven of them corresponded to enough chromatographically pure compounds, and only four of the latter contained sufficient amounts of flavonol 3-*O*-glycoside for the recording of NMR spectra.

NMR Analysis of Flavonol 3-*O*-Glycosides. The assignment of the proton (^{1}H) and carbon (^{13}C) peaks was done by ^{1}H NMR, ^{1}H−^{1}H correlation spectroscopy (COSY), ^{1}H−^{13}C heteronuclear multiple-quantum coherence (HMQC), and ^{1}H−^{13}C heteronuclear multiple-bond correlation (HMBC) experiments in CD_3OD. The NMR experiments were carried out using a Bruker Avance II NMR spectrometer operating

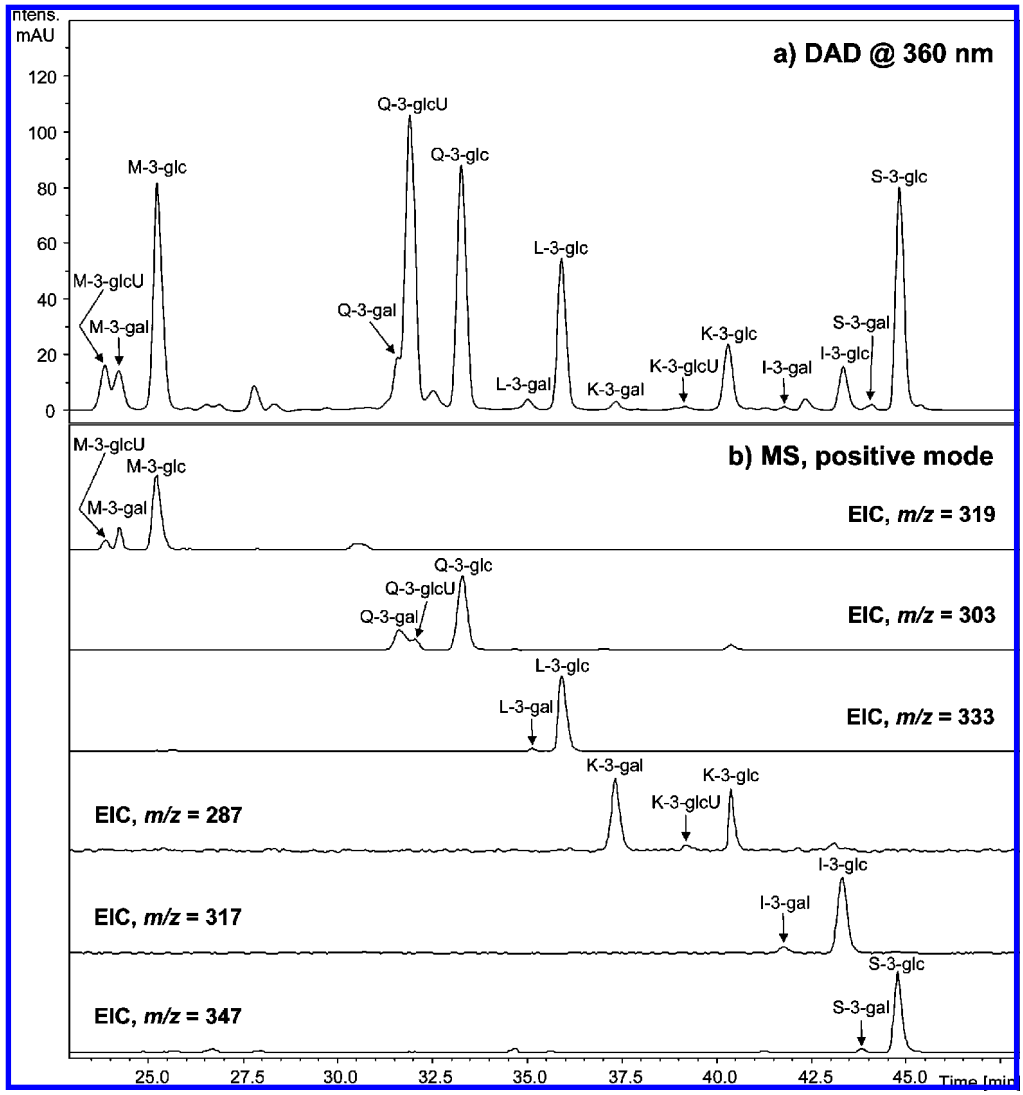

Figure 3. HPLC chromatograms corresponding to the fraction of total flavonol 3-*O*-glycosides isolated from Petit Verdot grape skins: (**a**) DAD chromatogram (360 nm) and (**b**) EIC chromatograms in positive ionization mode at the *m*/*z* values of the flavonol aglycones, resulting from the loss by fragmentation of the sugar moiety of flavonol 3-*O*-glycosides, $[(M\text{-gly}) + H]^+$. Peak assignation: M, myricetin; Q, quercetin; L, laricitrin; K, kaempferol; I, isorhamnetin; S, syringetin; gal, galactoside; glc, glucoside; and glcU, glucuronide.

at 600.13 MHz for ^{1}H and at 150.03 MHz for ^{13}C (14.1 T). The spectrometer was equipped with a Great 3/10 gradient amplifier and a triple-nucleus CP-TXI cryoprobe with *z*-gradient. Cryogenic NMR probe technology significantly increased the signal-to-noise ratio (S/N) of NMR spectroscopy (*22*), making the study of samples at very low concentrations possible (*23*). All one- and two-dimensional experiments (COSY, HMQC, and HMBC) were performed at 300 K using standard pulse sequences from the Bruker library.

RESULTS AND DISCUSSION

The first task of our work was the improvement of the HPLC method for analyzing grape flavonol 3-*O*-glycosides. In a previous work (*17*), we reported the finding of the 3-*O*-glucoside derivatives of the six possible flavonol aglycones in red grapes, together with three 3-*O*-galactoside and three 3-*O*-glucuronide derivatives for only some of the flavonol structures. In that work, we also found some evidence of coelution of more than one flavonol 3-*O*-glycoside under the same chromatographic peak, and the presence of some of the very minor flavonol 3-*O*-glycosides, like the 3-*O*-(6″-acetyl)-glucoside derivatives of quercetin and syringetin, was only tentatively suggested. In the present work, we modified our previous chromatographic method combining the extension of the analysis time together with the partial substitution of acetonitrile by methanol as the modifier solvent. The aforementioned modifications allowed us to get a better separation of the resulting chromatographic peaks for flavonol 3-*O*-glycosides (**Figure 1a**). The newly developed HPLC method has been designed to be applied not only to grape flavonol analysis but also to wine flavonol analysis (including free flavonol aglycones), as well as to hydroxycinnamic acid derivatives analysis (the elution ranges are within 35−60 min for free flavonol aglycones and within 5−30 min for hydroxycinnamic acid derivatives; data not shown). Using these new chromatographic conditions, it was possible to assign a total of 18 peaks as flavonol 3-*O*-glycosides that appeared as single chromatographic peaks, by means of a combination of spectral data (UV−vis and MS^n spectra) and comparison to standards when available (**Table 1**). However, peak overlapping was observed involving some couples of a 3-*O*-glucuronide derivative and their respective 3-*O*-galactoside or 3-*O*-glucoside derivatives (peaks 4 and 5, 8 and 9, and 14 and 15, respectively). The results showed that for every kind of the six possible grape flavonol structures (the 4′-hydroxylated, kaempferol; the 3′,4′-dihydroxylated, quercetin; the 3′-methoxylated-4′-hydroxylated, isorhamnetin; the 3′,4′,5′-trihydroxylated, myricetin; the 3′-

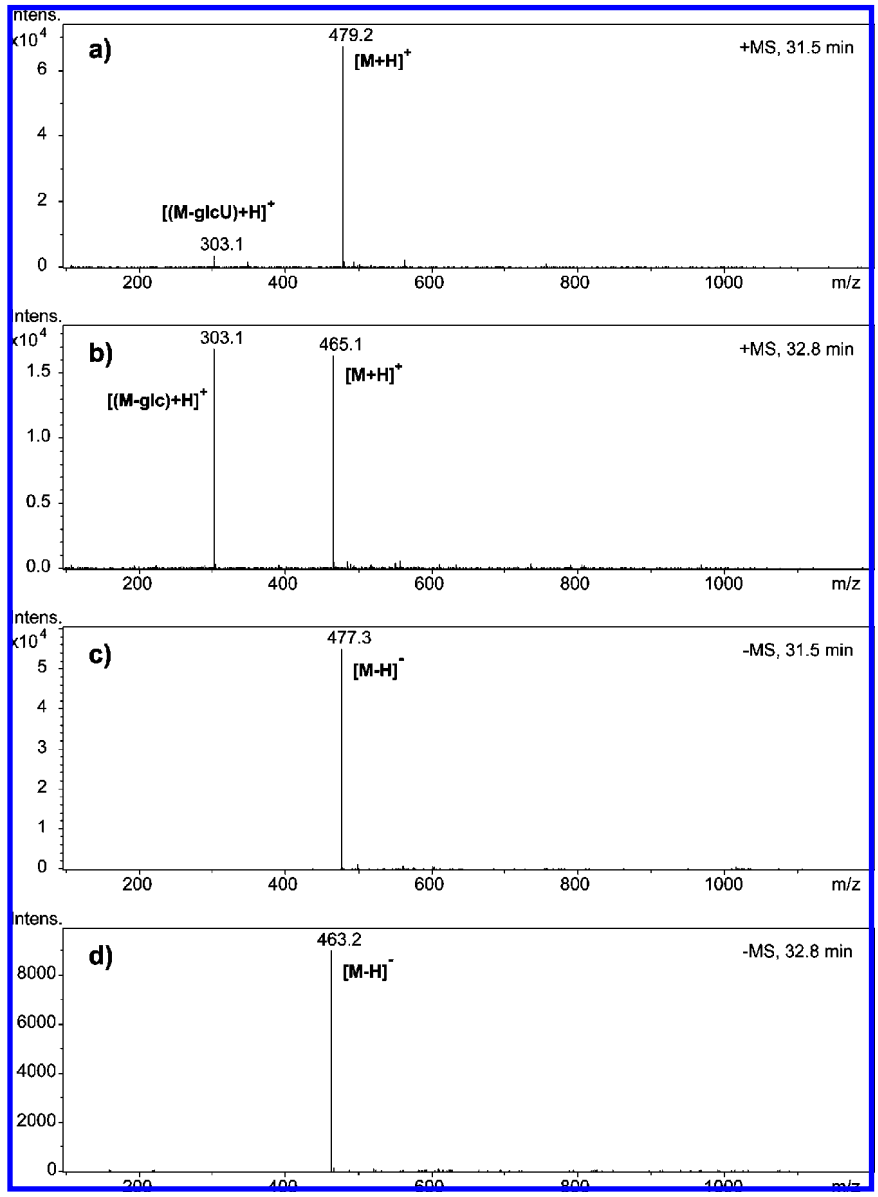

Figure 4. MS and MS^2 spectra of flavonol 3-*O*-glycosides obtained under different ionization conditions: quercetin 3-*O*-glucuronide in positive (**a**) and negative (**c**) ionization modes and quercetin 3-*O*-glucoside in positive (**b**) and negative (**d**) ionization modes.

methoxylated-4′,5′-dihydroxylated, laricitrin; and the 3′,5′-dimethoxylated-4′-hydroxylated, syringetin), the series of 3-*O*-glucoside, 3-*O*-galactoside, and 3-*O*-glucuronide derivatives were found (**Figure 2a**−**c**).

The assignment of chromatographic peaks was initially based on the extracted ion chromatograms (EIC) obtained under positive ionization mode at the characteristic *m*/*z* value corresponding to the pseudomolecular ion for every flavonol aglycone resulting from the loss of the 3-*O*-glycoside moiety ([(M-gly) + H]$^+$, **Figure 3**). The examination of the mass spectra (MS and MS^2) obtained for both positive and negative ionization modes confirmed the suggested assignments (**Table 1**). Under the positive ionization conditions used, the 3-*O*-glucoside and 3-*O*-galactoside derivatives tended to give pseudomolecular ions ([M + H]$^+$) that easily underwent fragmentation by loss of the 3-*O*-glycoside moiety before reaching the mass analyzer. Therefore, the resulting product ions ([(M-gly) + H]$^+$, showing a *m*/*z* value diminished in 162 amu that corresponds to a loss of a hexose unit) were also observable in the MS spectra together with the pseudomolecular ions ([M + H]$^+$) and frequently appeared as the predominant ions (**Figure 4b**). In contrast, the 3-*O*-glucuronide derivatives gave more stable pseudomolecular ions ([M + H]$^+$), and only little fragmentation by loss of the 3-*O*-glucuronide moiety (*m*/*z* value diminished in 176 amu) was appreciated in their MS spectra (**Figure 4a**). For this reason, the peaks assigned as flavonol 3-*O*-glucuronides in the EICs (positive ionization mode) at *m*/*z* values corresponding to the resulting flavonol aglycones by fragmentation of their corresponding flavonol 3-*O*-glycosides were of much lower intensity than their respective peaks in the related DAD spectra recorded at 360 nm, especially in the case of quercetin 3-*O*-glucuronide (**Figure 3b**, EIC at *m*/*z* = 303). This behavior is reminiscent of the higher trend to hydrolysis shown by quercetin 3-*O*-glucoside when compared to quercetin 3-*O*-glucuronide that has been reported for red wine flavonols (*17*). In addition, the 3-*O*-glucuronide derivatives of laricitrin, isorhamnetin, and syringetin showed no appreciable signals in the positive EIC chromatograms because of their low signals at the selected *m*/*z* values and also by their partial coelution with other compounds. Using negative ionization mode, all flavonol 3-*O*-glycosides gave stable pseudomolecular ions ([M − H]$^-$), which were only present in the MS spectra (**Figure 4c**,**d**), and further underwent

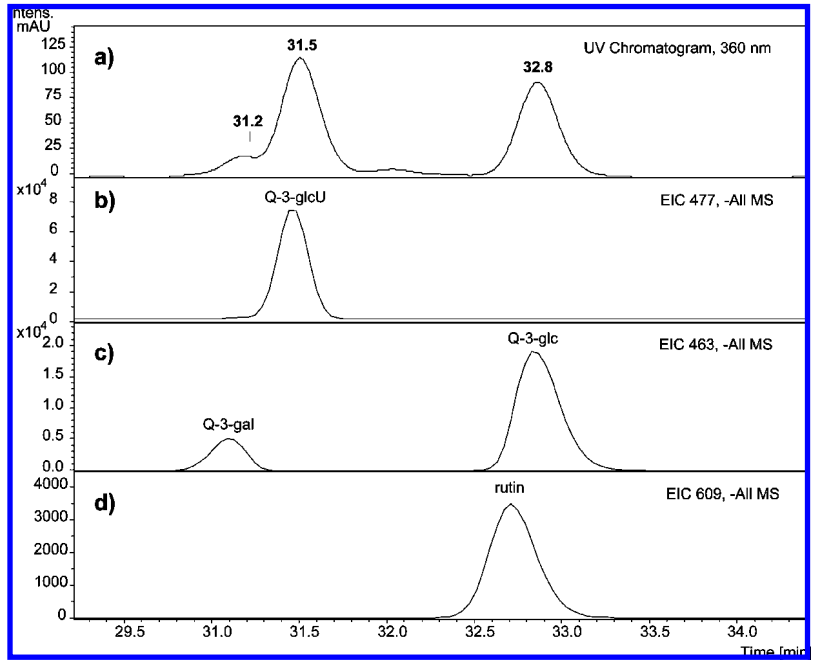

Figure 5. Expanded HPLC chromatogram corresponding to the elution zone of quercetin 3-*O*-glycosides found in Petit Verdot grape skins: (**a**) DAD chromatogram (detection at 360 nm), (**b**) EIC chromatogram at the *m*/*z* value for the pseudomolecular ion (negative ionization mode) corresponding to quercetin 3-*O*-glucuronide (Q-3-glcU); (**c**) EIC chromatogram at the *m*/*z* value for the pseudomolecular ion (negative ionization mode) corresponding to the 3-*O*-galactoside and the 3-*O*-glucoside of quercetin (Q-3-gal and Q-3-glc, respectively), and (**c**) EIC chromatogram at the *m*/*z* value for the pseudomolecular ion (negative ionization mode) corresponding to quercetin 3-*O*-(6″-rhamnosyl)-glucoside (rutin).

the loss of the 3-*O*-glycoside moiety in the ion trap, giving rise to the corresponding flavonol aglycone related ions that were the dominant ones in the MS^2 spectra ([(M-gly) − H]$^-$, **Table 1**). No evidence of 3-*O*-(6″-acetyl)-glucoside derivatives for any of the flavonol structure was found this time in the analyzed Petit Verdot grape skin flavonols, thus indicating that the suggested presence of such a kind of flavonol 3-*O*-glycoside derivative must be carefully revised (*17*).

The presence of quercetin 3-*O*-(6″-rhamnosyl)-glucoside (also known as rutin, **Figure 2d**) in grapes and wines is still a matter of controversy. It has been very common to assign rutin instead of quercetin 3-*O*-glucuronide (*24*), and recent reviews still refer to rutin as one of the major grape and wine flavonol 3-*O*-glycosides (*25*). In a recent paper, it has been demonstrated that rutin is a very unstable 3-*O*-glycoside under the wine acidic conditions and easily and quickly suffers hydrolysis, thus releasing free quercetin (*26*). A standard of rutin eluted only 0.1−0.2 min before quercetin 3-*O*-glucoside under our chromatographic conditions (data not shown). We were only able to detect traces of rutin in our samples of Petit Verdot grape skin flavonols when we selected an EIC in negative ionization mode, corresponding to the pseudomolecular ion of rutin ([M − H]$^-$, *m*/*z* = 609; **Figure 5d**). Rutin ionizes in negative mode conditions, giving rise to only its pseudomolecular ion that further fragments in the ion trap, thus affording the free aglycone related ion (**Figure 6d**,**e**), as similarly happens to quercetin 3-*O*-glucoside (**Figure 6b**,**c**). Therefore, we can suggest that peak 6 eluting at 32.8 min mainly contained quercetin 3-*O*-glucoside (**Figure 6a**, signal at *m*/*z* = 463), together with a little amount of rutin that eluted a little bit faster (**Figure 6a**, signal at *m*/*z* = 609). For quantification of rutin coeluting with quercetin 3-*O*-glucoside, we obtained the ratio of the peak areas shown by standard solutions of both compounds in their corresponding DAD chromatograms at 360 nm as well as in their respective EICs at the two characteristic pseudomolecular *m*/*z* values (609 and 463, respectively). The ratio of the EIC peak areas attributed to rutin and quercetin 3-*O*-glucoside in the sample of grape skin (**Figure 5c**,**d**, respectively) suggested that rutin accounted for no more than 8% of the overall DAD peak that was assigned as quercetin 3-*O*-glucoside (**Figure 5a**, peak at 32.8 min). It is noteworthy that no other 3-*O*-(6″-rhamnosyl)-glucoside derivative analogous to rutin was detected for any of the other flavonol structures.

To support the assignations made to the aforementioned flavonol 3-*O*-glycosides, we separated them into two groups according to the acidic character of the 3-*O*-glycoside moiety. By means of solid-phase extraction using a cartridge filled with a material that combines phase-reverse and anionic exchange phenomena, we obtained the neutral (3-*O*-glucoside and 3-*O*-galactoside derivatives) and the acidic (3-*O*-glucuronide derivatives) flavonol fractions. The neutral flavonol 3-*O*-glycosides really have a weak acidic character, due to the phenol groups present in the flavonol structures. For this reason, they were also weakly retained by the cationic sites of the exchanger resin, and they were easily eluted (3-*O*-glucoside and 3-*O*-galactoside derivatives, **Figure 1b**) using methanol weakly acidified with 2% of formic acid instead of only methanol. The acidic flavonol 3-*O*-glycosides were further eluted using strong acidic methanol having 2% HCl (3-*O*-glucuronide derivatives, **Figures 1c** and **7a**). All of the 3-*O*-glycoside derivatives were found for every one of the six possible flavonol aglycones. In the case of neutral flavonol 3-*O*-glycosides (3-*O*-glucoside and 3-*O*-galactoside derivatives), the positive EIC chromatograms at the characteristic *m*/*z* values corresponding to their flavonol aglycones (see additional information) confirmed the assignment made when the total flavonol fraction was analyzed (**Figure 3**). In contrast, the acidic 3-*O*-glucuronide derivatives were better detected using the positive EIC chromatograms obtained at the *m*/*z* values of their corresponding pseudomolecular ions (**Figure 7b**), being now possible the detection of the complete series of the expected flavonol 3-*O*-glucuronides. The assignment of the flavonol 3-*O*-glycosides peaks was based on the characteristic sugar loss of

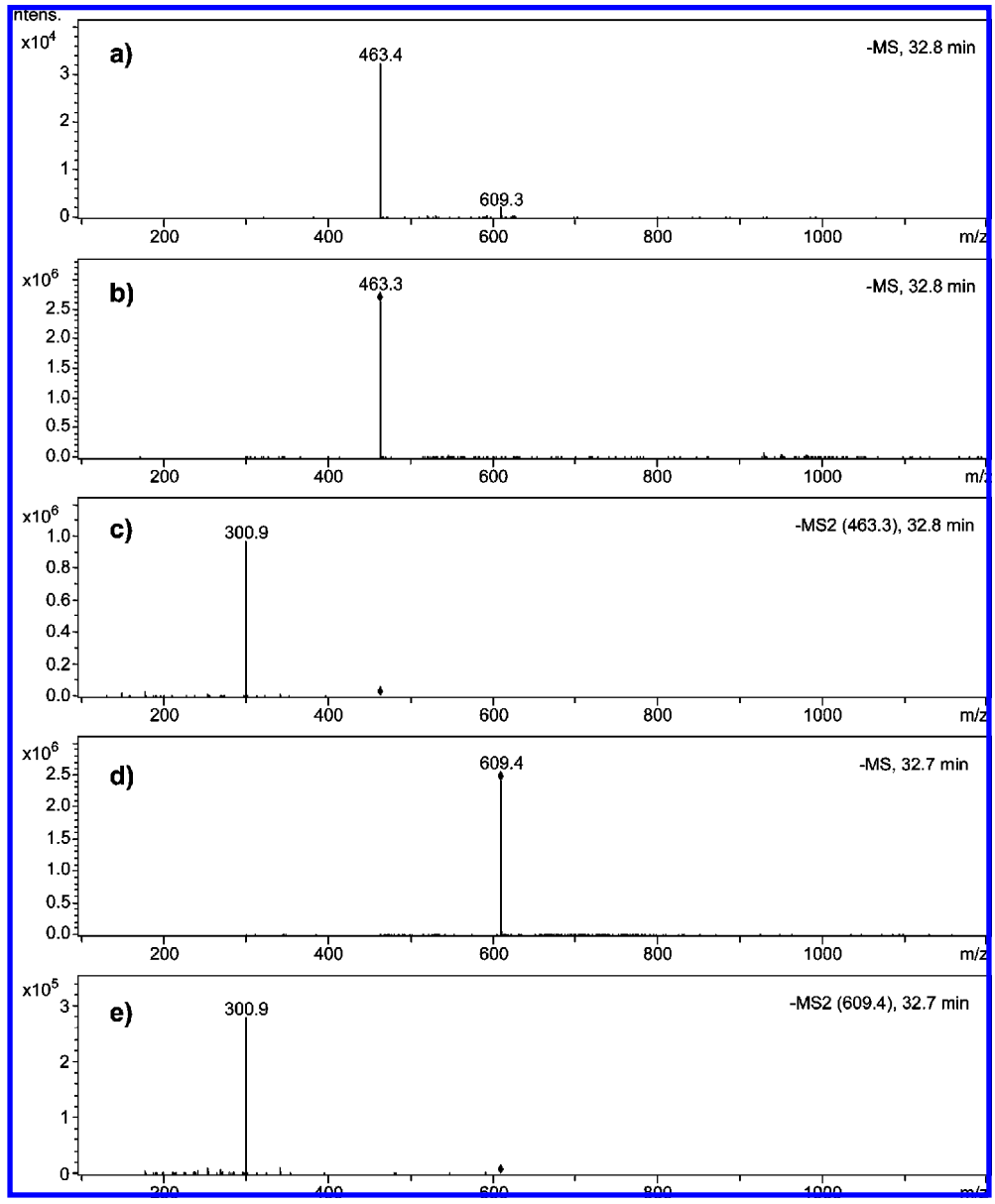

Figure 6. Characteristic MS and MS^2 shown by quercetin 3-*O*-glucoside and rutin under negative ionization mode mass spectrometry: (**a**) mass spectrum of the peak assigned to quercetin 3-*O*-glucoside (m/z = 463 uma) in the HPLC chromatogram of the total flavonol 3-*O*-glycosides from Petit Verdot grape skins, showing the presence of traces of rutin (m/z = 609 uma); (**b** and **c**) MS and MS^2 spectra, respectively, for a standard of quercetin 3-*O*-glucoside; and (**d** and **e**) MS and MS^2 spectra, respectively, for a standard of rutin.

a fragment of m/z = 162 for both 3-*O*-galactoside and 3-*O*-glucoside derivatives, as well as the characteristic sugar loss of a fragment of m/z = 176 attributed to the 3-*O*-glucuronide derivatives. In addition, we were able to compare standards of the 3-*O*-glucoside derivatives of all of the flavonol structures with the exception of laricitrin and also standards of 3-*O*-galactoside derivatives of quercetin and syringetin and of 3-*O*-glucuronides of quercetin and kaempferol. Further isolation and NMR structure elucidation allowed us to assign without doubt the peaks corresponding to myricetin 3-*O*-galactoside, quercetin 3-*O*-glucuronide, laricitrin 3-*O*-glucoside, and syringetin 3-*O*-glucoside. The results showed that 3-*O*-galactoside derivatives of flavonols always elute before their corresponding 3-*O*-glucoside derivatives under the reversed-phase HPLC conditions used. In contrast, the 3-*O*-glucuronide derivatives of flavonls sometimes eluted before their corresponding 3-*O*-glucoside derivatives (this is the case of myricetin, quercetin, laricitrin, and kaempferol 3-*O*-glucuronides), but others eluted after (case of isorhamnetin and syringetin 3-*O*-glucuronides), and only myricetin 3-*O*-glucuronide also eluted before its corresponding 3-*O*-galactoside derivative. This behavior can be explained by the interaction between the acidic character of the 3-*O*-glucuronide derivatives and the pH of the solvent used for elution. The relative elution position of 3-*O*-glucuronide flavonols can be strongly affected by the solvent pH and very likely is one of the reasons of some of the mistakes in flavonol assignation that can be found in the literature data, especially when peak assignment is only based in UV−vis spectra and the elution order given by different HPLC methods. For instance, in a previous work using another chromatographic system, we observed that quercetin 3-*O*-glucuronide eluted after, and not before, quercetin 3-*O*-glucoside (*27*). Quercetin 3-*O*-glucuronide was the main acidic flavonol 3-*O*-glycoside found in Petit Verdot grape skin, its assignment supported by comparison to a standard and also by NMR structure elucidation after its isolation.

We were also able to isolate some of the newly described and most interesting flavonol 3-*O*-glycosides to approach their structural elucidation, thus confirming their assignation. Because

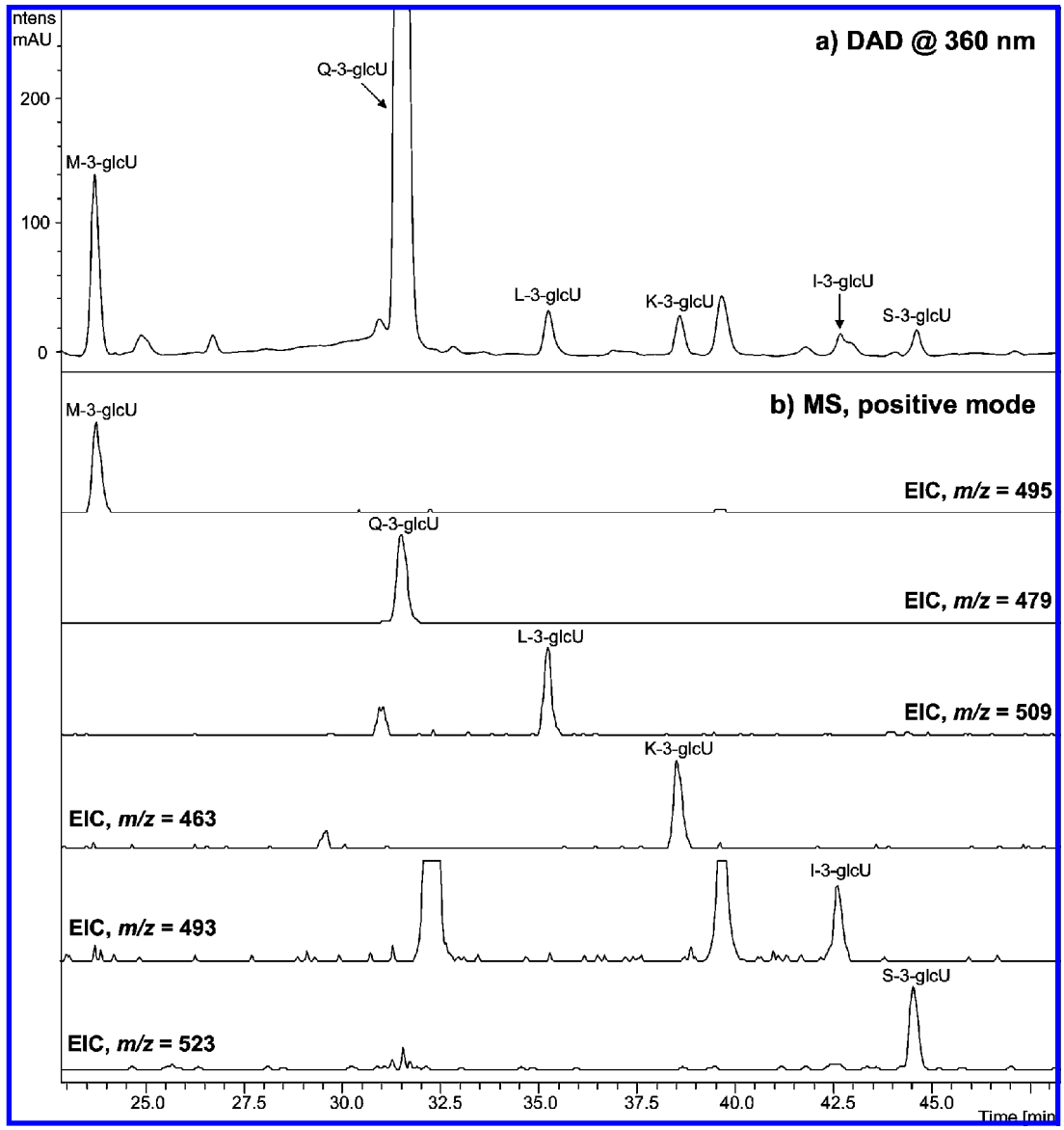

Figure 7. HPLC chromatograms corresponding to the fraction of acidic flavonol 3-*O*-glycosides isolated from Petit Verdot grape skins: (**a**) DAD chromatogram (360 nm) and (**b**) EIC chromatograms in positive ionization mode at the *m*/*z* values of the flavonol 3-*O*-glucuronide pseudomolecular ions, $[M - H]^-$. Peak assignation: M, myricetin; Q, quercetin; L, laricitrin; K, kaempferol; I, isorhamnetin; S, syringetin; and glcU, glucuronide.

of the high number of compounds to be separated, many of them occurring at very low concentrations, we opted to perform a repeated analytical HPLC separation with the help of a fraction collector. We only isolated a few micrograms of some of the less-studied flavonol 3-*O*-glycosides, paying special attention to the recently reported laricitrin and syringetin derivatives. Using cryogenic NMR probe technology, it was possible to obtain enough ^{1}H and ^{13}C NMR data for a complete structural elucidation in the case of myricetin 3-*O*-galactoside, quercetin 3-*O*-glucuronide, laricitrin 3-*O*-glucoside, and syringetin 3-*O*-glucoside (**Table 2**). The NMR data were in agreement with those available in the literature (*28*−*33*), and some of them have been reported for the first time in this work (^{1}H and ^{13}C NMR data for myricetin 3-*O*-galactoside; ^{13}C NMR data for laricitrin 3-*O*-glucoside). The shielding of the ^{13}C signal for C-3 (133.7−134.5 ppm) when compared to the corresponding signal for C-5 (161.7−162.0 ppm) and C-7 (164.5−165.0 ppm) supported the fact that the glycosidic linkage is by the C-3 position of the flavonoid structure in all of the studied flavonol glycosides. The measured value for the coupling constant (J_{HH}) between the sugar protons H-1″ and H-2″, ranging within 7−8 Hz for all of the analyzed compounds, indicated a configuration β for the 3-*O*-glycosidic linkage. The assignment of the sugar moiety as glucose (laricitrin and syringetin 3-*O*-glucosides) and glucuronic acid (quercetin 3-*O*-glucuronide) was based on the high values found for the coupling constants (J_{HH}) involving every consecutive couple of the sugar protons H-1″, H-2″, H-3″, H-4″, and H-5″, ranging within 7−9 Hz. In contrast, the lower J_{HH} values found for the couples of sugar protons H-3″ and H-4″ (4 Hz), the rest of the J_{HH} values being similar to that found for 3-*O*-glucoside derivatives, suggested that compound **2** was a 3-*O*-galactoside. The ^{1}H and ^{13}C signal assignments were based on one-dimensional ^{1}H NMR and two-dimensional NMR experiments (COSY, HMQC, and HMBC).

To the best of our knowledge, this is the first time that the complete series of the 3-*O*-galactosides, 3-*O*-glucosides, and 3-*O*-glucuronides derivatives for all six possible flavonol aglycones have been reported for *V. vinifera* red grapes. In addition, the presence of some of the aforementioned flavonol 3-*O*-glycosides in *V. vinifera* grape skins has been demonstrated for the first time in this work: the 3-*O*-galactosides of myricetin, quercetin, and isorhamnetin and the 3-*O*-glucuronides of the

Table 1. HPLC Chromatographic and UV−Vis and Mass Spectral Data of Flavonol 3-*O*-Glycosides Identified in *V. vinifera* Red Grape Skins

					molecular and product ions (*m*/*z*)	
peak	flavonol assignation	HPLC t_R (min)	UV−vis maxima (nm)	molecular weight	positive ionization	negative ionization
1	myricetin 3-*O*-glucuronide	23.8	257 (sh), 260, 305 (sh), 354	494	495, 319	493, 317
2	myricetin 3-*O*-galactoside	24.2	254 (sh), 261, 305 (sh), 356	480	481, 319	479, 317
3	myricetin 3-*O*-glucoside	25.1	255 (sh), 261, 305 (sh), 355	480	481, 319	479, 317
4	quercetin 3-*O*-galactoside	31.2	256, 264 (sh), 302 (sh), 354	464	465, 303	463, 301
5	quercetin 3-*O*-glucuronide	31.5	254, 264 (sh), 300 (sh), 353	478	479, 303	477, 301
	rutin	32.7	256, 264 (sh), 300 (sh), 354	610	611, 303	609, 301
6	quercetin 3-*O*-glucoside	32.8	256, 264 (sh), 300 (sh), 354	464	465, 303	463, 301
7	laricitrin 3-*O*-galactoside	34.5	256, 264 (sh), 305 (sh), 358	494	495, 333	493, 331
8	laricitrin 3-*O*-glucuronide	35.3	251, 261 (sh), 305 (sh), 355	508	509, 333	507, 331
9	laricitrin 3-*O*-glucoside	35.4	255, 262 (sh), 305 (sh), 357	494	495, 333	493, 331
10	kaempferol 3-*O*-galactoside	36.7	265, 298 (sh), 325 (sh), 349	448	449, 287	447, 285
11	kaempferol 3-*O*-glucuronide	38.6	265, 300 (sh), 325 (sh), 348	462	463, 287	461, 285
12	kaempferol 3-*O*-glucoside	39.6	264, 300 (sh), 325 (sh), 349	448	449, 287	447, 285
13	isorhamnetin 3-*O*-galactoside	41.0	355[a]	478	479, 317	477, 315
14	isorhamnetin 3-*O*-glucoside	42.5	254, 264 (sh), 300 (sh), 354	478	479, 317	477, 315
15	isorhamnetin 3-*O*-glucuronide	42.6	254, 264 (sh), 300 (sh), 355	492	493, 317	491, 315
16	syringetin 3-*O*-galactoside	43.2	254, 262 (sh), 305 (sh), 359	508	509, 347	507, 345
17	syringetin 3-*O*-glucoside	44.0	253, 264 (sh), 305 (sh), 357	508	509, 347	507, 345
18	syringetin 3-*O*-glucuronide	44.6	253, 264 (sh), 305 (sh), 357	522	523, 347	521, 345

[a] Overlapped with another nonflavonol compound (λ_{max} around 275 nm and shoulder over 290 nm), it is the only flavonol type maximum that could be measured for this flavonol 3-*O*-glycoside.

Table 2. ^{1}H and ^{13}C Chemical Shifts (ppm) and Proton−Proton Coupling Constants (J_{HH}, Hz) for Some Flavonol 3-*O*-Glycosides Isolated from the Skins of *V. vinifera* cv. Petit Verdot Grapes

	myricetin 3-β-*O*-galactoside		quercetin 3-β-*O*-glucuronide		laricitrin 3-β-*O*-glucoside		syringetin 3-β-*O*-glucoside	
position	^{1}H (J_{HH})	^{13}C	^{1}H (J_{HH})	^{13}C	^{1}H (J_{HH})	^{13}C	^{1}H (J_{HH})	^{13}C
				benzopyrano ring				
2		ND		157.6		157.1		157.1
3		134.5		134.2		134.1		133.7
4		ND		ND		ND		ND
5		161.7		161.7		162.0		162.0
6	6.23	98.4	6.23 (2)	98.5	6.20 (2)	98.1	6.23 (2)	98.4
7		165.0		164.6		164.5		164.7
8	6.42	93.2	6.42 (2)	93.3	6.42 (2)	93.2	6.44 (2)	93.4
9		157.1		157.3		157.4		157.1
10		104.1		ND		104.2		104.4
				flavonoid B ring				
1′		120.1		121.3		ND		120.4
2′	7.40	108.4	7.70 (2)	115.9	7.55 (2)	105.2	7.55	106.9
3′		145.0		148.6		147.8		147.4
4′		136.9		144.3		137.5		138.6
5′		145.0	6.87 (2, 8)	114.5		144.7		147.4
6′	7.40	108.4	7.65 (8)	121.9	7.30 (2)	109.7	7.55	106.9
OCH_3					3.90	56.0	3.95	55.8
				sugar moiety				
1″	5.23 (8)	102.4	5.37 (8)	102.8	5.40 (8)	102.4	5.50 (7)	101.8
2″	3.87 (8, 10)	71.7	3.54 (8, 9)	74.0	3.51 (8, 9)	74.4	3.49−3.48	74.5
3″	3.59 (10, 4)	73.6	3.51−3.47	76.3	3.47 (9, 9)	76.7	3.48−3.47	76.6
4″	3.89 (4)	68.7	3.60 (9, 9)	71.4	3.33[c]	70.0	3.32[c]	70.1
5″	3.51 (6, 6)	75.8	3.75 (9)	75.7	3.27 (9, 6, 2)	77.2	3.27 (9, 6, 2)	77.1
6″	3.68[a] (6, 12)	60.6		171.2[b]	3.77[a] (6, 12)	61.0	3.77[a] (2, 12)	61.1
	3.60[a] (6, 12)				3.61[a] (2, 12)		3.58[a] (6, 12)	

[a] H-6″a and H-6″b signals of the CH_2OH group. [b] Signal of the COOH group. [c] H-4″ signal overlaps with CD_3OD signal. ND, not detected.

methoxylated flavonol structures, namely, isorhamnetin, laricitrin, and syringetin. The presence of quercetin 3-*O*-galactoside has been reported for grapes of the *Vitis labrusca* cultivar Concord (*34*), and no references have been found reporting its presence in *V. vinifera* grape skins (*25*). Myricetin and isorhamnetin 3-*O*-galactosides are known flavonols found in different plant materials (*35*), but they have been never reported for *V. vinifera* grape skins (*25*). In a recent work, isorhamnetin and laricitrin 3-*O*-glucuronides have been detected in bilberries (*36*), and previously, only isorhamnetin 3-*O*-glucuronide has also been found in other plant materials different to grapes (*35*). Finally, to the best of our knowledge, syringetin 3-*O*-glucuronide has never been reported, not only for the case of grapes but also for any plant material (*35*).

From a biosynthetic point of view, the results suggest differences in substrate selectivity of the glycosyl-transferase enzymes that might be involved in the biosynthesis of *V. vinifera* grape flavonol 3-*O*-glycosides. First, the 3-*O*-glucosides are the main flavonol derivatives synthesized in Petit Verdot grape skins, accounting for a percentage of 77% of the overall flavonol 3-*O*-glycosides, whereas the 3-*O*-galactoside and 3-*O*-glucuronide derivatives account for similar lower percentages (around 12% each). In addition, the results suggest that the selectivity of every glycosyl-transferase toward the different flavonols also

differs depending upon the flavonol structure. First, the proportion of every kind of flavonol structure (kaempferol, quercetin, isorhamnetin, myricetin, laricitrin, and syringetin) differs within a particular class of flavonol 3-*O*-glycoside: Quercetin and, in a lesser but closer extent, myricetin structures predominate within the 3-*O*-galactoside and 3-*O*-glucoside derivatives, but their importance decreases for the 3-*O*-glucosides, especially in the case of quercetin (from 45 to 28% and from 34 to 28% in the case of myricetin); in contrast, quercetin 3-*O*-glucuronide accounts for 89% of the overall flavonol 3-*O*-glucuronides, and the importance of the myricetin derivative (6%) falls to similar levels than for the other flavonol structures (1−2%). Second, regarding the number of substituting groups in the B ring of the flavonol structure, monosubstituted flavonols have always been minor compounds (they account for 1.4−8.0% depending upon the kind of 3-*O*-glycoside), whereas disubstituted and trisubstituted flavonols predominate, although some differences are remarkable: Disubstituted flavonols slightly predominate for 3-*O*-galactoside derivatives (48 vs 44% for trisubstituted flavonols) and are the main kind of flavonols for 3-*O*-glucuronide derivatives (90%); in contrast, trisubstituted flavonols predominate over the disubstituted ones for 3-*O*-glucoside derivatives (57 vs 35%). Finally, the enzymes that might be involved in the biosynthesis of rutin in *V. vinifera* cv. Petit Verdot grapes seem to act selectively only on quercetin, since no similar 3-*O*-glycoside derivatives have been detected for any other flavonol structure.

Supporting Information Available: HPLC chromatograms corresponding to the fraction of neutral flavonol 3-*O*-glycosides isolated from Petit Verdot grape skins. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Price, S. F.; Breen, P. J.; Valladao, M.; Watson, B. T. Cluster sun exposure and quercetin in Pinot Noir grapes and wine. *Am. J. Enol. Vitic*. **1995**, *46*, 187–194.

(2) Haselgrove, L.; Botting, D.; van Heeswijck, R.; Høj, P. B.; Dry, P. R.; Ford, C.; Illand, P. Canopy microclimate and berry composition: The effect of bunch exposure on the phenolic composition of *Vitis vinifera* L. cv. Shiraz grape berries. *Aust. J. Grape Wine Res*. **2000**, *6*, 141–149.

(3) Downey, M. A.; Harvey, J. S.; Robinson, S. P. The effect of bunch shading on berry development and flavonoid accumulation in Shiraz grapes. *Aust. J. Grape Wine Res*. **2004**, *10*, 55–73.

(4) Boulton, R. The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *Am. J. Enol. Vitic*. **2001**, *52*, 67–87.

(5) Schwarz, M.; Picazo-Bacete, J. J.; Winterhalter, P.; Hermosín-Gutiérrez, I. Effect of copigments and grape cultivar on the color of red wines fermented after the addition of copigments. *J. Agric. Food Chem*. **2005**, *53*, 8372–8381.

(6) Burda, S.; Olezsek, W. Antioxidant and antiradical activities of flavonols. *J. Agric. Food Chem*. **2001**, *49*, 2774–2779.

(7) De Beer, D.; Joubert, E.; Gelderblom, W. C. A.; Manley, M. Antioxidant activity of South African red and white cultivar wines and selected phenolic compounds: In vitro inhibition of microsomal lipid peroxidation. *Food Chem*. **2005**, *90*, 569–577.

(8) Montoro, P.; Braca, A.; Pizza, C.; de Tommasi, N. Structure-antioxidant activity relationships of flavonoids isolated from different plant species. *Food Chem*. **2005**, *92*, 349–355.

(9) Gardner, P. T.; McPhail, D. B.; Crozier, A.; Duthie, G. G. Electron spin resonance (ESR) spectroscopic assessment of the contribution of quercetin and other flavonols to the antioxidant capacity of red wines. *J. Sci. Food Agric*. **1999**, *79*, 1011–1014.

(10) Fernández-Pachón, M. S.; Villaño, D.; García-Parrilla, M. C.; Troncoso, A. M. Antioxidant activity of wines and relation with their polyphenolic composition. *Anal. Chim. Acta* **2004**, *513*, 113–118.

(11) De Beer, D.; Joubert, E.; Marais, J.; Manley, M. Unravelling the total antioxidant capacity of Pinotage wines: Contribution of phenolic compounds. *J. Agric. Food Chem*. **2006**, *54*, 2897–2905.

(12) Rodríguez Montealegre, R.; Romero Peces, R.; Chacón Vozmediano, J. L.; Martínez Gascueña, J.; García Romero, E. Phenolic compounds in skins and seeds of ten grape *Vitis vinifera* varieties grown in a warm climate. *J. Food Compos. Anal*. **2006**, *19*, 687–693.

(13) Mattivi, F.; Guzzon, R.; Vrhovsek, U.; Stefanini, M.; Velasco, R. Metabolite profiling of grape: Flavonols and anthocyanins. *J. Agric. Food Chem*. **2006**, *54*, 7692–7702.

(14) Hermosín-Gutiérrez, I.; García-Romero, E.; Gómez-Alonso, S.; Quintanar-Lara, A. Flavonols from La Mancha varietal red wines: Content, evolution during ageing, and usefulness as varietal differentiation tool. Presented at Euro Food Chem XIII, 2005; pp 21−23.

(15) Hermosín Gutiérrez, I.; Sánchez-Palomo Lorenzo, E.; Vicario Espinosa, A. Phenolic composition and magnitude of copigmentation in young and shortly aged red wines made from the cultivars Cabernet Sauvignon, Cencibel, and Syrah. *Food Chem*. **2005**, *92*, 269–283.

(16) Von Baer, D.; Mardones, C.; Gutiérrez, L.; Hofmann, G.; Becerra, J.; Hitschfeld, A.; Vergara, C. Varietal authenticity verification of Cabernet Sauvignon, Merlot and Carmenere wines produced in Chile by their anthocyanin, flavonol and shikimic acid profiles. *Bull. OIV* **2005**, *78*, 45–57.

(17) Castillo-Muñoz, N.; Gómez-Alonso, S.; García-Romero, E.; Hermosín-Gutiérrez, I. Flavonol profiles of *Vitis vinifera* red grapes and their single-cultivar wines. *J. Agric. Food Chem*. **2007**, *55*, 992–1002.

(18) Cheynier, V.; Moutounet, M.; Sarni-Manchado, P. Los compuestos fenólicos. In *Enología. Fundamentos Científicos y Tecnologicos*, 2nd ed.; Flanzy, C., Ed.; AMV Ediciones and Ediciones Mundi-Prensa: Madrid, Spain, 2003; pp 120−121.

(19) Davies, K. M.; Schwinn, K. E. Molecular biology and biotechnology of flavonoid biosynthesis. In *Flavonoids. Chemistry, Biochemistry and Applications*; Andersen, Ø. M., Markham, K. R., Eds.; CRC Press, Taylor & Francis Group: Boca Raton, FL, 2006; pp 168−169.

(20) Ford, C. M.; Boss, P. K.; Høj, P. B. Cloning and characterization of *Vitis vinifera* UDP-glucose:flavonoid 3-*O*-glucosyltransferase, a homologue of the enzyme encoded by the maize bronze-1 locus that may primarily serve to glucosylate anthocyanidins in vivo. *J. Biol. Chem*. **1998**, *273*, 9224–9233.

(21) Cheynier, V.; Rigaud, J. HPLC separation and characterization of flavonols in the skins of *Vitis vinifera* var. Cinsault. *Am. J. Enol. Vitic*. **1986**, *37*, 248–252.

(22) Styles, P.; Soffe, N. F.; Scott, C. A.; Cragg, D. A.; Row, F.; White, D. J.; White, P. C. J. A high-resolution NMR probe in which the coil and the preamplifier are cooled with liquid nitrogen. *J. Magn. Reson*. **1984**, *60*, 397–404.

(23) Logan, T. M.; Murali, N.; Wang, G.; Jolivet, C. Application of high-resolution superconducting NMR probe in natural product structure determination. *Magn. Reson. Chem*. **1999**, *37*, 512–515.

(24) Betés-Saura, C.; Andrés-Lacueva, C.; Lamuela-Raventós, R. M. Phenolics in white free run juices and wines from Penedès by high-performance liquid chromatography: Changes during vinification. *J. Agric. Food Chem*. **1996**, *44*, 3040–3046.

(25) Makris, D. P.; Kallithraka, S.; Kefalas, P. Flavonols in grapes, grape products and wines: Burden, profile and influential parameters. *J. Food Compos. Anal*. **2006**, *19*, 396–404.

(26) Jeffery, D. W.; Parker, M.; Smith, P. A. Flavonol composition of Australian red and white wines determined by high-performance liquid chromatography. *Aust. J. Grape Wine Res* **2008**, *14*, 153–161.

(27) Gómez-Alonso, S.; García-Romero, E.; Hermosín-Gutiérrez, I. HPLC analysis of diverse grape and wine phenolics using direct injection and multidetection by DAD and fluorescence. *J. Food Compos. Anal*. **2007**, *20*, 618–626.

(28) Slimestad, R.; Andersen, Ø. M.; Francis, G. W.; Marston, A.; Hostettmann, K. Syringetin 3-*O*-(6″-acetyl)-*β*-glucopyranoside and other flavonols from needles of Norway spruce *Picea abies*. *Phytochemistry*. **1995**, *40*, 1537–1542.

(29) Guo, J.; Yu, D. L.; Xu, L.; Zhu, M.; Yang, S. L. Flavonol glycosides from *Lysimachia congestiflora*. *Phytochemistry* **1998**, *48*, 1445–1447.

(30) Vvedneskaya, I. O.; Rosen, R. T.; Guido, J. E.; Rusell, D. J.; Mills, K. A.; Vorsa, N. Characterization of flavonols in cranberry (*Vaccinium macrocarpon*) powder. *J. Agric. Food Chem*. **2004**, *52*, 188–195.

(31) Parejo, I.; Viladomat, F.; Bastida, J.; Schmeda-Hirschmann, G.; Burillo, J.; Codina, C. Bioguided isolation and identification of the non-volatile antioxidant compounds from fennel (*Foeniculum vulgare* Mill.) waste. *J. Agric. Food Chem*. **2004**, *52*, 1890–1897.

(32) Ek, S.; Kartimo, H.; Mattila, S.; Tolonen, A. Characterization of phenolic compounds from lingonberry (*Vaccinium vitis-idaea*). *J. Agric. Food Chem*. **2006**, *54*, 9834–9842.

(33) Gutzeit, D.; Wray, V.; Winterhalter, P.; Jerz, G. Preparative isolation and purification of flavonoids and protocatechuic acid from sea buckthorn juice concentrate (*Hippophaë rhamnoides* L. ssp. *rhamnoides*) by high-speed counter-current chromatography. *Chromatographia* **2007**, *65*, 1–7.

(34) Oszmianski, J.; Lee, C. Y. Isolation and HPLC determination of phenolic compounds in red grapes. *Am. J. Enol. Vitic*. **1990**, *41*, 204–206.

(35) Williams, C. A. Flavone and flavonol *O*-glycosides. In *Flavonoids. Chemistry, Biochemistry and Applications*; Andersen, Ø. M., Markham, K. R., Eds.; CRC Press, Taylor & Francis Group: Boca Raton, FL, 2006; pp 824−856.

(36) Koponen, J. M.; Happonen, A. M.; Auriola, S.; Kontkanen, H.; Buchert, J.; Poutanen, K. S.; Törrönen, A. R. Characterization and fate of black currant and bilberry flavonols in enzyme-aided processing. *J. Agric. Food Chem*. **2008**, *56*, 3136–3144.

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