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Matrix-Calibrated LC-MS/MS Quantitation and Sensory Evaluation of Oak Ellagitannins and Their Transformation Products in Red Wines

Timo Stark,[§] Nadine Wollmann,[§] Kerstin Wenker, Sofie Lösch, Arne Glabasnia,[‡] and Thomas Hofmann*,[†]

[†]Chair of Food Chemistry and Molecular Sensory Science, Technische Universität München, Lise-Meitner Strasse 34, D-85354 Freising, Germany, and [‡]Institut für Lebensmittelchemie, Universität Münster, Corrensstrasse 45, D-48149 Münster, Germany.
[§]These authors contributed equally to this work.

Aimed at investigating the concentrations and taste contribution of the oak-derived ellagitannins castalagin and vescalagin as well as their transformation products acutissimin A/B, epiacutissimin A/B, and β-1-*O*-ethylvescalagin in red wine, a highly sensitive and accurate quantification method was developed on the basis of LC-MS/MS-MRM analysis with matrix calibration. Method validation showed good recovery rates ranging from 102.4 ± 5.9% (vescalagin) to 113.7 ± 15.2% (epiacutissimin A). In oak-matured wines, castalagin was found as the predominant ellagitannin, followed by β-1-*O*-ethylvescalagin, whereas the flavano-*C*-ellagitannins (epi)acutissimin A/B were present in significantly lower amounts. In contrast to the high threshold concentration levels (600−1000 μmol/L) and the puckering astringent orosensation induced by flavan-3-ols, all of the ellagitannin derivatives were found to induce a smooth and velvety astringent oral sensation at rather low threshold concentrations ranging from 0.9 to 2.8 μmol/L. Dose/activity considerations demonstrated that, among all the ellagitannins investigated, castalagin exclusively exceeded its threshold concentration in various oak-matured wine samples.

KEYWORDS: Ellagitannins; red wine; acutissimin; epiacutissimin; castalagin; vescalagin; LC-MS/MS; astringency; barrique

INTRODUCTION

As one of life's finest pleasures, the alluring aroma, the desirable taste, and the typical color of red wines have been attracting consumers for more than 2000 years. The fraction of odor-active volatiles evoking the aroma as well as the various anthocyanins and anthocyanidins contributing to the typical color of red wine was thoroughly investigated in recent decades (*1*), but only a comparatively small number of molecular studies were targeted toward the taste-active nonvolatiles in wines and, in particular, those eliciting oral astringency and/or bitterness. The typical long-lasting astringent mouthfeel, which can be classified into several subqualities such as velvety, grainy, drying, or puckering (*2*), together with bitterness, is of crucial importance for the palatability of red wines. Whereas velvety astringency is perceived as a finely textured and silky kind of astringent sensation, puckering astringency is understood as a reflexive action of cheek surfaces being brought together and released in an attempt to lubricate mouth surfaces (*2*).

Recently, a number of 26 sensory active nonvolatiles comprising hydroxybenzoic acids, hydroxycinnamic acids, flavon-3-ol glycosides, and dihydroflavon-3-ol rhamnosides as well as a structurally undefined polymeric fraction (> 5 kDa) were identified as the key inducers of astringent mouthfeel of red wines by means of a molecular sensory science approach (*3*, *4*). Whereas these molecules are known to originate from grapes and/or to evolve during fermentation and aging of the wine, the knowledge of the orosensory activity of ellagitannin derivatives extracted from the oak barrels and chemically transformed upon barrique maturation is rather fragmentary.

Several ellagitannins such as, e.g., castalagin (**1**, **Figure 1**) and vescalagin (**2**), were identified in hydroalcoholic extracts of oak wood chips (*Quercus alba L.*) (*5*) as well as in bourbon whiskey and oak-matured red wines (*6*). Besides these native ellagitannins, β-1-*O*-ethylvescalagin (**3**, **Figure 1**), a transformation product of vescalagin and castalagin evolving during storage in hydroalcoholic solutions, was identified in red wine (*7*). In addition, the flavano-ellagitannins acutissimin A (**4a**, **Figure 1**) and B (**4b**), first isolated from wood chips of *Quercus acutissima* and *Castanea* species (*8*), were identified in red wine and were found to be generated by a nucleophilic substitution reaction between the ellagitannin vescalagin and the flavan-3-ol (+)-catechin (*7*, *9*). In addition, the reaction between vescalagin and (−)-epicatechin was found to afford epiacutissimin A (**5a**, **Figure 1**) and B (**5b**) (*7*, *9*). Recently, acutissimin A was identified as an active inhibitor of human DNA topoisomerase II and was found to be 250 times

*To whom correspondence should be addressed. Phone: +49-8161/71-2902. Fax: +49-8161/71-2949. E-mail: thomas.hofmann@wzw.tum.de.

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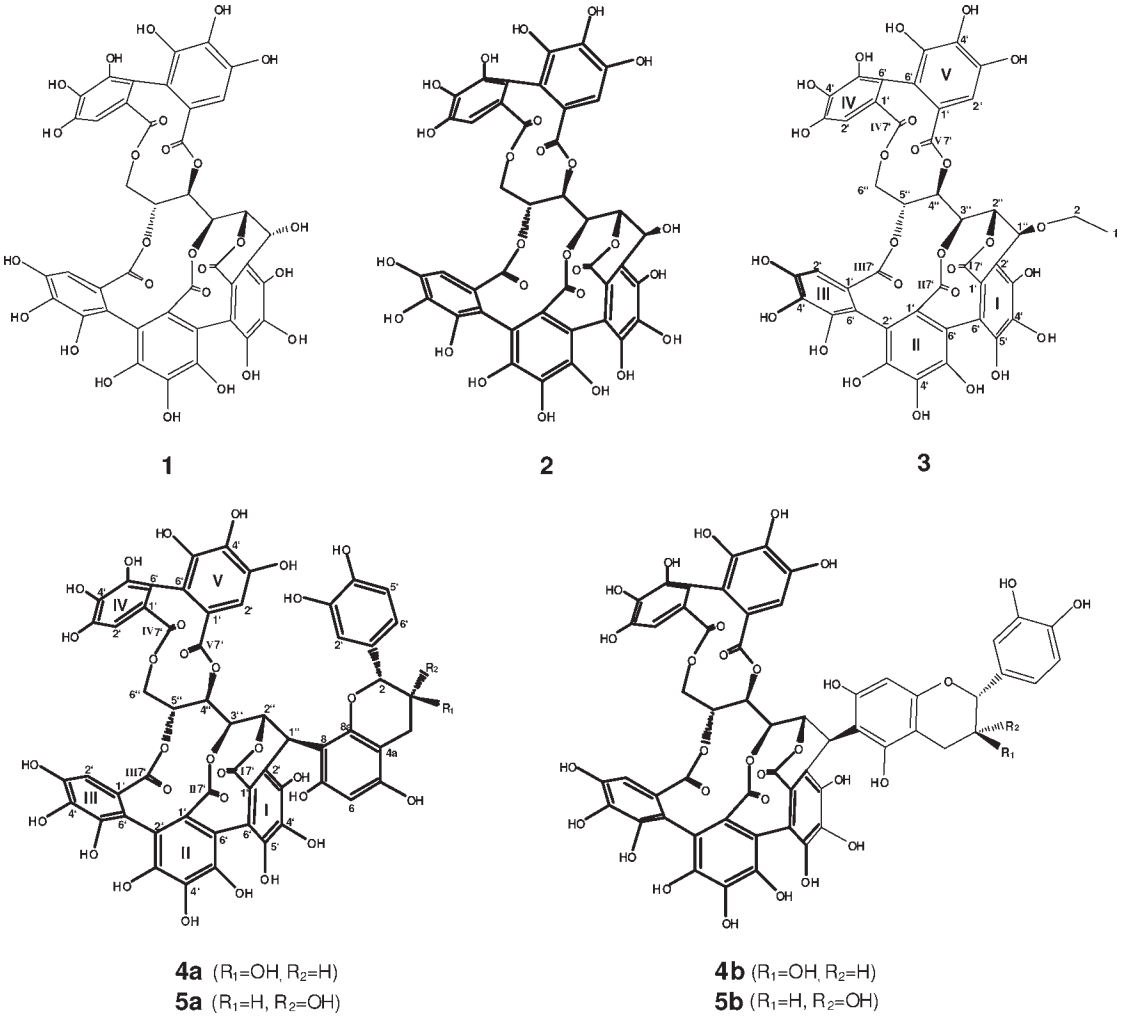

Figure 1. Chemical structures of castalagin (**1**), vescalagin (**2**), β-1-*O*-ethylvescalagin (**3**), acutissimin A (**4a**) and B (**4b**), and epiacutissimin A (**5a**) and B (**5b**).

more potent in vitro than the clinically used anticancer drug etoposide (VP-16) (*10*).

In order to gain some first insight into the content of flavano-ellagitannins in wines, a procedure for their extraction and LC/MS-based detection in red wine was reported using negative electospray ionization in the single ion recording mode and chlorogenic acid as the internal standard for quantitation (*11*). But this procedure is rather laborious and nonspecific and requires that the wine is prefractionated at least two times by column chromatography and that the fractions obtained thereof are evaporated at least three times to dryness prior to LC/MS analysis. Such laborious sample cleanup procedures have been shown to induce artifact formation, e.g., the acutissimins might be further oxidized on the galloyl unit and, then, might undergo nucleophilic attack from the flavanol unit as shown for the generation of mongolicain A (*12*−*14*). Preliminary studies of our group (data not published) revealed that sample workup procedures such as concentration, solid phase extraction, or freeze-drying have a major impact on the amounts of ellagitannins detected in wine. Therefore, a more straightforward quantification procedure avoiding either the degradation or the artificial formation of flavano ellagitannins upon sample workup is needed in order to obtain reliable data on the concentration of acutissimin A and B as well as epiacutissimin A and B in wines. Moreover, no data are yet available on the sensory activity of these ellagitannin transformation products.

The objective of the present study was, therefore, to prepare pure reference materials for the ellagitannin transformation products acutissimin A/B, epiacutissimin A/B, and β-1-*O*-ethylvescalagin, to validate their structure by means of LC-MS/MS and 1D/2D-NMR spectroscopy, and to develop a straightforward, but highly sensitive and accurate LC-MS/MS method for their robust quantitative analysis in red wines. In addition, the sensory quality and the human taste recognition thresholds of the purified compounds should be determined and compared to their concentrations in red wine samples.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: (−)-epicatechin and (+)-catechin (Sigma, Steinheim, Germany); trifluoroacetic acid (Riedel de Haen, Taufkirchen, Germany); water for chromatographic separations was purified with a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany), and solvents used were of HPLC-grade (Merck, Darmstadt, Germany). Deuterated solvents were obtained from Euriso-Top (Gif-Sur-Yvette, France). For sensory evaluation, bottled water (Evian) was adjusted to pH 4.5 with traces of aqueous formic acid (1% in water). Reference samples of castalagin (**1**) and vescalagin (**2**) were isolated and purified from *Quercus alba* L. following the procedure reported earlier (*6*).

Red wines, namely, Cabernet Sauvignon (2003, Chile), Merlot (2006, Italy), Spaetburgunder (2007, Germany), Bordeaux (2003, France), Barolo (2002, Italy), Shiraz (2005, Australia), and Assemblage (2003, Chile), were obtained from a local wine shop. According to the producer, Assemblage

is a mixture of Syrah which was aged for 12 months in new American oak barrels, Cabernet Sauvignon matured for 12 months in new French oak barrels, and Merlot matured for at least 10 months in second use French oak barrels. The Shiraz was matured in a mixture of oak barrels and stainless steel for 9 months and blended prior to bottling. In addition, four Dornfelder red wines produced differently were obtained from two German vineyards. Two Dornfelder samples (years 2004 and 2005) were aged in barrique barrels for 15 months. These barrels (225 L) were made of French oak wood and were used twice prior to this vintage. Another Dornfelder sample (year 2005) is a blend of the Dornfelder vintage 2005, matured for 15 months in oak-wood barrels, which were already in use for more than 10 years. Finally, a Dornfelder sample from the year 2007 was not matured in wooden barrels.

Sensory Analyses. *Training of the Sensory Panel*. Twelve subjects (seven women and five men, aged 25−38 years), who gave their informed consent to participate in the sensory tests of the present investigation and had no history of known taste disorders, were trained to evaluate the taste of aqueous solutions (3 mL each) of the following standard taste compounds by using a triangle test as described in the literature (*15*): sucrose (12.5 mmol/L) for sweet taste; lactic acid (20 mmol/L) for sour taste; NaCl (12 mmol/L) for salty taste, caffeine (1 mmol/L) for bitter taste; and sodium glutamate (3 mmol/L) for umami taste. For the puckering astringency and the velvety astringent, mouth-drying oral sensation, the panel was trained with aqueous solutions of gallotannic acid (0.05%) and quercetin-3-*O*-*β*-D-glucopyranoside (0.002 mmol/L), respectively, using the half-tongue test (*16*). Sensory analyses were performed in a sensory panel room at 22−25 °C in three different sessions.

Half-Tongue Test. To overcome carry-over effects of astringent compounds, threshold concentrations of astringent compounds were determined in bottled water by means of the recently developed half-tongue test (*16*, *17*). Serial 1:1 dilutions of the samples were presented in order of increasing concentrations to a trained panel of 12 persons in three different sessions, using the sip-and-spit method. At the start of the session and before each trial, the subject rinsed his/her mouth with water and expectorated. An aliquot (1 mL) of the aqueous solution containing the astringent compound was randomly applied with a pipet on one side of the tongue, whereas pure water was applied on the other side of the tongue as the control. The sensory panelists were then asked to move their tongue forward and backward toward the palate for 15 s and to identify the place of astringent sensation by comparison of both sides. After indicating which part of the tongue showed the typical astringent sensation induced by the tastant, the participant rinsed his/her mouth with water, and after 10 min, received another set of one blank and one taste-active sample. To prevent excessive fatigue, tasting began at a concentration level two steps below the threshold concentration that had been determined in a preliminary taste experiment. Whenever the panelist selected incorrectly, the next trial took place at the next higher concentration step. When the panelist selected correctly, the same concentration was presented again besides one blank as a proof for the correctness of the data. The geometric mean of the last and the second to last concentration was calculated and taken as the individual recognition threshold. Values between individuals and three separate sessions differed not more than ±1 dilution step; that is, a threshold value of 0.9 μmol/L for acutissimin A represents a range from 0.45 to 1.8 μmol/L.

Preparation of *β*-1-*O*-Ethylvescalagin (3). Following a procedure reported recently (*7*), a solution of vescalagin (**2**, 0.02 mmol) and ethanol (25 mL) in dry tetrahydrofuran (20 mL) containing 1.5% (v/v) of trifluoroacetic acid was stirred for 5 h at 60 °C. After evaporation of the solvent under reduced pressure, the residue was taken up in water/methanol (10 mL; 95/5, v/v) and, after adjusting the pH to 6.0 by adding aqueous sodium hydroxide (1 mol/L), was chromatographically fractionated on an HPLC system (Jasco, Gross-Umstadt, Germany) consisting of a HPLC-pump system PU 2087, a high-pressure gradient unit, and a PU-2075 UV-detector, equipped with a Luna PhenylHexyl 21.2 × 250 mm, 5 μm column (Phenomenex, Aschaffenburg, Germany) as the stationary phase. Monitoring the effluent (20 mL/min) at 240 nm, we performed chromatography starting with an aqueous solution of formic acid (0.1% in water, pH 2.5) for 3 min, then increasing the methanol content up to 50% within 13 min. Individual fractions were collected, separated from solvent in vacuum, the residue dissolved in water (10 mL), and freeze-dried two times to afford *β*-1-*O*-ethylvescalagin **3** (**Figure 1**) (yield: 82%) at a purity of more than 98%.

β-1-O-Ethylvescalagin (***3***) (***Figure*** **1**). UV−vis (MeOH/0.1% HCOOH, 5/5, v/v): λ_{max} = 233 nm. LC-TOF-MS (ESI$^-$): *m*/*z* 480.0435 (calculated for $(C_{34}H_{30}O_{26}\text{-}2H^-)^2$ *m*/*z* 480.0440). MS (ESI$^-$): *m*/*z* 480.7 (100%, $[M-2H]^{2-}$), 466.8 (53%, $[M-2H\text{-}C_2H_5]^{2-}$), 961.5 (49%, $[M-H]^-$), 932.8 (22%, $[M-H\text{-}C_2H_5]^-$. MS/MS (ESI$^-$, 961) (CE −54 V): *m*/*z* 301 (100), 961 (45), 493 (35), 249 (41), 915 (37), 467 (36) 275 (29). MS/MS (ESI$^-$, 480) (CE −54 V): *m*/*z* 480 (100), 457 (52), 301 (46), 275 (40). ^{1}H NMR (500 MHz, acetone-d_6/D_2O, COSY): δ 1.31 [pt, 3H, $^3J_{H1\text{-}H2\alpha2\beta}$ = 6.97 Hz, H−C(1)], 3.86 [dq, 1H, $^3J_{H2\alpha\text{-}H1}$ = 7.15, $^2J_{H2\alpha\text{-}H2\beta}$ = 14.20 Hz, H−C(2α)], 4.01 [dq, 1H, $^3J_{H2\beta\text{-}H1}$ = 7.15, $^2J_{H2\beta\text{-}H2\alpha}$ = 14.20 Hz, H−C(2*β*)], 4.08 [d, 1H, *J* = 12.91 Hz, H−C(6α″)], 4.54 [d, 1H, *J* = 6.97 Hz, H−C(3″)], 4.77 [s, 1H, H−C(1″)], 5.05 [d, 1H, *J* = 12.80, H−C(6*β*″)], 5.24 [pt, 1H, *J* = 7.19 Hz, H−C(4″)], 5.39 [s, 1H, H−C(2″)], 5.65 [d, 1H, *J* = 7.42 Hz, H−C(5″)], 6.64 [s, 1H, H−C(2′)(IV)], 6.76 [s, 1H, H−C(2′)(V)], 6.80 [s, 1H, H−C(2′)(III)]. ^{13}C NMR (125 MHz, acetone-d_6/D_2O, HMQC, HMBC): 15.8 [C(1)], 64.9 [C(2)], 65.7 [C(6″)], 68.5 [C(3″)], 69.7 [C(4″)], 71.2 [C(5″)], 71.9 [C(1″)], 75.1 [C(2″)], 107.3 [C(2′)(IV)], 108.5 [C(2′)(V)], 108.8 [C(2′)(III)], 113.1, 114.6, 115.8 [C(6′)(I−II), C(2′)(II)], 114.9 [C(6′)(III)], 115.1 [C(6′)(IV), C(2′)(I)], 116.6 [C(6′)(V)], 124.8, 125.2, 126.8, 127.8 [C(1′)(II−V)], 125.3 [C(1′)(I)], 135.2, 137.2 [C(4′)(I−II)], 136.0 [C(4′)(IV)], 136.6 [C(4′)(III)], 137.4 [C(4′)(V)], 144.3, 144.8, 144.9, 145.0, 145.1, 145.3, 145.5, 148.0, [10C, C(3′)(I−V), C(5′)(I−V)], 165.4 [C(7′)(I)], 165.7 [C(7′)(II)], 166.8 [C(7′)(V)], 167.3 [C(7′)(III)], 169.5 [C(7′)(IV)].

Preparation of Flavano-*C*-ellagitannis (4a/b and 5a/b). A binary mixture of (−)-vescalagin (**2**, 0.1 mmol) and (−)-epicatechin or (+)-catechin (0.1 mmol) in dry tetrahydrofuran (50 mL) containing trifluoroacetic acid (1%) was heated at 90 °C for 20 h. After cooling, the solvent was separated under reduced pressure, and the residue was dissolved in acetonitrile/water (10 mL; 10/90, v/v) and, after adjusting the pH to 4.0, was chromatographically fractionated on the HPLC system detailed above using a HyperClone micro ODS (C18), 21.2 × 250 mm, 5 μm RP-18 column (Phenomenex, Aschaffenburg, Germany) as the stationary phase. Monitoring the effluent at 280 nm, we performed chromatography starting with a mixture (10/90, v/v) of acetonitrile and aqueous formic acid (0.1% in water, pH 2.5) for 5 min, then increasing the acetonitrile content to 15% within 10 min at a flow rate of 18.0 mL/min. After separation of the solvents in vacuum, each target compound was taken up in water (10 mL) and freeze-dried two times to afford the flavano-*C*-ellagitannins **4a/b** (yield: 60%/20%) and **5a/b** (yield: 41%/27%) as white, amorphous powders in high purities of more than 98%.

Acutissimin A (***4a***) (***Figure*** **1**). UV−vis (ACN/0.1% HCOOH, 5/5, v/v), λ_{max} = 239, 263, 274 nm; exact mass, *m*/*z* 1229.1256 (calculated for $C_{56}H_{38}O_{31}$ + Na^+: 1229.1289). MS (ESI$^-$): *m*/*z* 1205 (100%, $[M-1]^-$), 602 (95%, $[M-2]^{2-}$), 915 (60%, $[M-290]^-$, 457 (45%, $[M-749]^-$, 289 (30%, $[M-916]^-$, 301 (10%). MS/MS (ESI$^-$, 1205) (CE −30 V): *m*/*z* 1205 (100), 915 (87), 301 (35), 467 (18) 493 (17) 249 (15). MS/MS (ESI$^-$, 602) (CE −54 V): *m*/*z* 602 (100), 457 (22), 289 (16), 301 (14). ^{1}H NMR (400 MHz, acetone-d_6/D_2O, COSY): δ 2.36 [d, 1H, *J* = 14.4 Hz, H−C(4α)], 2.84 [d, 1H, *J* = 16.4 Hz, H−C(4*β*)], 4.06 [d, 1H, *J* = 12.0 Hz, H−C(6α″)], 4.54 [s, 1H, H−C(3)], 4.65 [d, 1H, *J* = 12.0 Hz, H−C(6*β*″)], 4.71 [d, 1H, *J* = 6.8 Hz, H−C(3″)], 4.77 [s, 1H, H−C(1″)], 5.15 [s, 1H, H−C(2″)], 5.23 [dd, 1H, *J* = 7.2, 7.6 Hz, H−C(4″)], 5.47 [s, 1H, H−C(2)], 5.60 [d, 1H, *J* = 7.6 Hz, H−C(5″)], 6.00 [s, 1H, H−C(6)], 6.58 [s, 1H, H−C(2′)(V)], 6.73 [s, 1H, H−C(2′)(IV)], 6.76 [d, 1H, *J* = 8.0 Hz, H−C(5′)], 6.85 [s, 1H, H−C(2′)], 6.92 [d, 1H, *J* = 8.0 Hz, H−C(6′)], 7.05 [s, 1H, H−C(2′)(III)]. ^{13}C NMR (125 MHz, acetone-d_6/D_2O, HMQC, HMBC): 24.6 [C(4)], 38.8 [C(1″)], 66.6 [C(6″)], 68.6 [C(3)], 71.9 [C(4″/5″)], 71.9 [C(5″/4″)], 73.1 [C(3″)], 78.3 [C(2″)], 80.9 [C(2)], 97.7 [C(6)], 99.0 [C(4a)], 105.8 [C(8)], 107.8 [C(2′)(V)], 108.1 [C(2′)(III)], 109.7 [C(2′)(IV)], 113.8 [C(2′)], 114.3 [C(6′)(I/II)], 114.4 [C(6′)(II/I)], 115.3 [C(6′)(IV)], 115.9 [C(6′)(V)], 117.1 [C(5′/6′(III))], 117.1 [C(6′)(III)/(5′)], 117.8 [C(2′)(II)], 119.1 [C(6′)], 121.1 [C(2′)(I)], 125.7 [C(1′)(III)], 126.4 [C(1′)(IV)], 127.8 [C(1′)(V)], 128.6 [C-(1′)(I)], 128.9 [C(1′)(II)], 132.4 [C(1′)], 136.2 [C(4′)(II)], 136.5 [C(4′)(V)], 137.2 [C(4′)(IV)], 137.3 [C(4′)(I)], 137.7 [C(4′)(III)], 144.3 [C(3′)(I)], 144.4, 145.2, 145.58, 145.65, 145.8, 146.0, 146.2, 146.3, 146.4 [11C, C(3′)(II−V), C(5′)(I−V), C(3′, 4′)], 153.8 [C(8a)], 156.9 [C(5/7)], 158.1 [C(7/5)], 166.7 [C(7′)(II)], 167.9 [C(7′)(IV)], 168.2 [C(7′)(III)], 168.4 [C(7′)(I)], 170.2 [C(7′)(V)].

Acutissimin B (***4b***) (***Figure*** **1**). UV−vis (ACN/0.1% HCOOH, 5/5, v/v): λ_{max} = 239, 263, 274 nm. LC-TOF-MS (ESI$^+$): *m*/*z* 1229.1256 (calculated for $C_{56}H_{38}O_{31}$ + Na^+: *m*/*z* 1229.1289). MS (ESI$^-$): *m*/*z* 1205

(100%, [M − 1]$^-$), 602 (95%, [M − 2]$^{2-}$), 915 (60%, [M − 290]$^-$, 457 (45%, [M − 749]$^-$, 289 (30%, [M − 916]$^-$, 301 (10%). MS/MS (ESI$^-$, 1205) (CE −30 V): *m/z* 1205 (100), 915 (87), 301 (35), 467 (18) 493 (17) 249 (15); MS/MS (ESI$^-$, 602) (CE −54 V): *m/z* 602 (100), 457 (39), 301 (35), 289 (16). ^{1}H NMR (400 MHz, acetone-d_6/D_2O, COSY): δ 2.57 [dd, 1H, *J* = 9.0, 14.6 Hz, H−C(4)], 4.02 [d, 1H, *J* = 10.8 Hz, H−C(6α″)], 4.03 [s, 1H, H−C(3)], 4.49 [d, 1H, *J* = 8.8, H−C(2)], 4.64 [s, 1H, H−C(1″)], 4.76 [d, 1H, *J* = 6.8 Hz, H−C(3″)], 4.81 [d, 1H, *J* = 10.8, H−C(6β″)], 5.03 [s, 1H, H−C(2″)], 5.19 [dd, 1H, *J* = 7.2, 7.6 Hz, H−C(4″)], 5.57 [d, 1H, *J* = 7.2 Hz, H−C(5″)], 6.06 [br s, 1H, H−C(8)], 6.57 [s, 1H, H−C(2′)(V)], 6.73 [s, 1H, H−C(2′)(III)], 6.74 [dd, 1H, *J* = 1.6, 8.0 Hz, H−C(6′)], 6.79 [d, 1H, *J* = 8.0, H−C(5′)], 6.89 [d, 1H, *J* = 1.6 Hz, H−C(2′)], 7.02 [s, 1H, H−C(2′)(IV)]. ^{13}C NMR (125 MHz, acetone-d_6/D_2O, HMQC, HMBC): 30.3 [C(4)], 38.8 [C(1″)], 66.5 [C(6″)], 69.0 [C(3)], 71.0 [C(4″)], 72.0 [C(5″)], 72.6 [C(3″)], 79.0 [C(2″)], 83.0 [C(2)], 97.0 [C(8)], 102.0 [C(4a)], 108.0 [C(2′)(V)], 108.3 [C(6)], 109.5 [C(2′)(III)], 110.7 [C(2′)(IV)], 114.2 [C(6′)(I/II)], 114.7 [C(6′)(II/I)], 115.3 [C(6′)(V/III)], 115.4 [C(6′)(III/V)], 116.4 [C(2′)], 116.7 [C(5′)], 117.4 [C(6′)(IV)], 117.5 [C(2′)(II)], 121.1 [C(6′)], 121.4 [C(2′)(I)], 125.7 [C(1′)(IV)], 126.0 [C(1′)(III)], 127.3 [C(1′)(I)], 127.7 [C(1′)(V)], 128.6 [C(1′)(II)], 132.2 [C(1′)], 136.1 [C(4′)(II)], 136.5 [C(4′)(V)], 137.3 [C(4′)-(III)], 137.7 [C(4′)(I)], 138.1 [C(4′)(IV)], 144.4 [C(3′)(I)], 144.5, 145.2, 145.3, 145.4, 145.6, 145.9, 146.25, 146.32, 146.5 [11C, C(3′)(II−V), C(5′)-(I−V), C(3′, 4′)], 155.72 [C(8a/5/7)], 155.78 [C(8a/5/7)], 166.7 [C(7′)(II)], 167.9 [C(7′)(IV)], 168.2 [C(7′)(III)], 168.5 [C(7′)(I)], 170.3 [C(7′)(V)].

Epiacutissimin A (***5a***) (***Figure* 1**). UV−vis (ACN/0.1% HCOOH, 5/5, v/v): λ_{max} = 239, 263, 274 nm. LC-TOF-MS (ESI$^+$): *m/z* 1229.1256 (calculated for $C_{56}H_{38}O_{31}$ + Na^+: *m/z* 1229.1289). MS (ESI$^-$): *m/z* 1205 (100%, [M − 1]$^-$), 602 (95%, [M − 2]$^{2-}$), 915 (60%, [M − 290]$^-$, 457 (45%, [M − 749]$^-$, 289 (30%, [M − 916]$^-$, 301 (10%). MS/MS (ESI$^-$, 1205) (CE −30 V): *m/z* 1205 (100), 915 (87), 301 (35), 467 (18) 493 (17) 249 (15). MS/MS (ESI$^-$, 602) (CE −54 V): *m/z* 602 (100), 457 (34), 289 (31), 301 (22). ^{1}H NMR (400 MHz, acetone-d_6/D_2O, COSY): δ 2.61 [dd, 1H, *J* = 5.4, 15.8 Hz, H−C(4α)], 2.96 [d, 1H, *J* = 14.2 Hz, H−C(4β)], 3.98 [d, 1H, *J* = 12.8 Hz, H−C(6α″)], 4.43 [s, 1H, H−C(3)], 4.72−4.75 [m, 3H, *J* = 7.6, 10.8 Hz, H−C(1″, 3″, 6β″)], 5.09 [s, 1H, H−C(2″)], 5.16 [dd, 1H, *J* = 7.2 Hz, H−C(4″)], 5.36 [s, 1H, H−C(2)], 5.58 [d, 1H, *J* = 8.0 Hz, H−C(5″)], 6.01 [s, 1H, H−C(6)], 6.55 [s, 1H, H−C(2′)(V)], 6.61 [d, 1H, *J* = 8.8 Hz, H−C(5′)], 6.70 [s, 1H, H−C(2′)(III)], 6.73 [d, 1H, *J* = 8.8 Hz, H−C(6′)], 6.75 [s, 1H, H−C(IV)], 6.92 [s, 1H, H−C(2′)]. ^{13}C NMR (125 MHz, acetone-d_6/D_2O, HMQC, HMBC): 28.1 [C(4)], 38.4 [C(1″)], 66.8 [C(6″)], 67.4 [C(3)], 70.7 [C(4″)], 72.0 [C(5″)], 73.0 [C(3″)], 78.9 [C(2″)], 80.4 [C(2)], 97.5 [C(6)], 100.5 [C(4a)], 106.3 [C(8)], 108.0 [C(2′)(V)], 109.4 [C(2′)(III)], 110.3 [C(2′)(IV)], 114.3 [C(6′)(I/II)], 115.1 [C(6′)(II/I)], 115.5 [C(6′)(III/V)], 115.5 [C(6′)(V III)], 116.0 [C(2′)], 116.5 [C(5′)], 117.7 [C(6′)(IV)], 117.8 [C(2′)(II)], 120.3 [C(6′)], 122.0 [C(2′)(I)], 125.7 [C(1′)(III)], 126.2 [C-(1′)(IV)], 127.6 [C(1′)(I)], 128.1 [C(1′)(V)], 128.7 [C(1′)(II)], 132.1 [C(1′)], 136.2 [C(4′)(II)], 136.5 [C(4′)(V)], 137.3 [C(4′)(III)], 138.1 [C(4′)(IV)], 138.3 [C(4′)(I)], 143.9 [C(3′)(I)], 144.4, 145.2, 145.3, 145.58, 145.64, 145.68, 146.0, 146.31, 146.37, 146.40 [11C, C(3′)(II−V), C(5′)(I−V), C(3′, 4′)], 154.4 [C(8a)], 156.8 [C(5)], 157.9 [C(7)], 166.7 [C(7′)(II)], 167.2 [C(7′)(IV)], 168.1 [C(7′)(III)], 168.3 [C(7′)(I)], 170.2 [C(7′)(V)].

Epiacutissimin B (***5b***) (***Figure* 1**). UV−vis (ACN/0.1% HCOOH, 5/5, v/v): λ_{max} = 239, 263, 274 nm. LC-TOF-MS (ESI$^+$): *m/z* 1229.1256 (calculated for $C_{56}H_{38}O_{31}$ + Na^+: *m/z* 1229.1289). MS (ESI$^-$): *m/z* 1205 (100%, [M − 1]$^-$), 602 (95%, [M − 2]$^{2-}$), 915 (60%, [M − 290]$^-$, 457 (45%, [M − 749]$^-$, 289 (30%, [M − 916]$^-$, 301 (10%). MS/MS (ESI$^-$, 1205) (CE −30 V): *m/z* 1205 (100), 915 (87), 301 (35), 467 (18) 493 (17) 249 (15). MS/MS (ESI$^-$, 602) (CE −54 V): *m/z* 602 (100), 457 (20), 289 (11), 301 (8). ^{1}H NMR (400 MHz, acetone-d_6/D_2O, COSY): δ 2.95 [br s, 2H, H−C(4α, β)], 3.99 [d, 1H, *J* = 12.8 Hz, H−C(6α″)], 4.25 [br s, 1H, H−C(3)], 4.66 [s, 1H, H−C(1″)], 4.80 [d, 1H, *J* = 7.2 Hz, H−C(3″)], 4.82 [d, 1H, *J* = 12.8, H−C(6β″)], 4.85 [s, 1H, H−C(2)], 5.08 [s, 1H, H−C(2″)], 5.22 [dd, 1H, *J* = 7.2, 7.6 Hz, H−C(4″)], 5.60 [d, 1H, *J* = 7.6 Hz, H−C(5″)], 6.15 [br s, 1H, H−C(8)], 6.59 [s, 1H, H−C(2′)(V)], 6.75 [s, 1H, H−C(2′)-(III)], 6.79 [d, 1H, *J* = 8.4, H−C(5′)], 6.84 [d, 1H, *J* = 8.4 Hz, H−C(6′)], 7.04 [s, 1H, H−C(2′)(IV)], 7.06 [s, 1H, H−C(2′)]. ^{13}C NMR (125 MHz, acetone-d_6/D_2O, HMQC, HMBC): 30.6 [C(4)], 39.0 [C(1″)], 66.7 [C(6″)], 67.6 [C(3)], 71.2 [C(4″)], 72.1 [C(5″)], 72.8 [C(3″)], 79.0 [C(2″)], 79.9 [C(2)], 97.4 [C(8)], 100.9 [C(4a)], 108.1 [C(2′)(V)], 108.4 [C(6)], 109.6 [C(2′)(III)], 110.6 [C(2′)(IV)], 114.3 [C(6′)(I/II)], 114.9 [C(6′)(II/I)], 115.5 [C(6′)(III)], 115.7 [C(6′)(V)], 116.1 [C(2′)], 116.4 [C(5′)], 117.6 [C(6′)(IV)], 117.7 [C(2′)(II)], 120.1 [C(6′)], 121.5 [C(2′)(I)], 125.9 [C(1′)(IV)], 126.3 [C(1′)(III)], 127.5 [C(1′)(I)], 127.9 [C(1′)(V)], 128.9 [C(1′)(II)], 132.8 [C(1′)], 136.2 [C(4′)(II)], 136.6 [C(4′)(V)], 137.3 [C(4′)(III)], 137.7 [C(4′)-(I)], 138.1 [C(4′)(IV)], 144.4 [C(3′)(I)], 144.5, 145.3, 145.4, 145.5, 145.7, 145.8, 146.0, 146.18, 146.27, 146.37, 146.44 [11C, C(3′)(II−V), C(5′)(I−V), C(3′, 4′)], 156.2 [C(8a)], 156.4 [C(5/7)], 157.0 [C(7/5)], 166.8 [C(7′)(II)], 168.0 [C(7′)(IV/I/III)], 168.09 [C(7′)(I/III/IV)], 168.13 [C(7′)(III/I/IV)], 170.2 [C(7′)(V)].

High-Performance Liquid Chromatography−Mass Spectrometry (HPLC-MS/MS). LC-MS/MS analysis was performed using an Agilent 1200 HPLC system connected to the API 4000QTrap LC-MS/MS (Applied Biosystems, Darmstadt, Germany) running in the negative electrospray ionization (ESI$^-$) mode. Zero grade air served as the nebulizer gas (45 psi) and as turbo gas (400 °C) for solvent drying (55 psi). Nitrogen served as the curtain (20 psi) and collision gas (4.5×10^{-5} Torr). Both quadrupols were set at unit resolution. ESI$^-$ mass and product ion spectra were acquired with direct flow infusion. For ESI$^-$, the ion spray voltage was set at −4500 V in the negative mode. The MS/MS parameters were tuned for each individual compound, detecting the fragmentation of the [M − H]$^-$ and [M − 2H]$^{2-}$ molecular ions into specific product ions after collision with nitrogen (4.0×10^{-5} Torr). By means of the multiple reaction monitoring (MRM) mode, the individual ellagitannins **1** and **2** (*m/z* 466.0 → 300.9; DP, −65 V; EP, −10 V; CE −36 V; EXP, −11 V), **3** (*m/z* 480.1 → 457.2; DP, −75 V; EP, −10 V; CE, −22 V; EXP, −5 V), and **4a/b** and **5a/b** (*m/z* 602.3 → 457.0; DP, −55 V; EP, −10 V; CE, −26 V; EXP, −29 V) were analyzed for a duration of 50 ms using the mass transitions, declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP), each given in parentheses.

Quantitative Analysis of Ellagitannins 1−5a/b by Means of HPLC-MS/MS. Aliquots (20 μL) of each wine were injected into the HPLC-MS/MS system equipped with a Luna Phenyl-hexyl, 2 × 150 mm, 5 μm, column (Phenomenex). Operated with a flow rate of 0.25 mL/min, we used the following gradients for chromatography: gradient A, starting with a mixture (5/95, v/v) of methanol (1% formic acid) and aqueous formic acid (1%, pH 2.5) for 3 min, the methanol content was increased to 50% within 17 min, then increased to 100% within 8 min, and, finally, held at 100% for 5 min. Gradient B: starting with a mixture (5/95, v/v) of methanol (1% formic acid) and aqueous formic acid (1%, pH 2.5) for 3 min, the methanol content was increased to 60% within 22 min, then increased to 100% within 5 min, and, finally, held at 100% for 10 min. Quantitation was performed by comparing the peak area obtained for the corresponding mass transition of the 2-fold charged pseudomolecular ion with those of defined standard solutions (0.005−5.0 mg/L) of each reference compound dissolved in methanol/water (20/80, v/v) and the zero-analyte wine sample Dornfelder (2007), which was demonstrated in preliminary experiments to be free of the target analytes. Calibration curves, obtained by linear regression analysis of the peak area versus concentration, showed a linear response with correlation coefficients of > 0.99.

Besides external calibration, standard addition was carried out by spiking aliquots of Shiraz wine (950 μL) with increasing amounts of the purified ellagitannins **1−5a/b** dissolved in methanol/water (20/80, v/v; 50 μL), to give wine solutions (1 mL) lacking any ellagitannin (blank sample) or containing 0.001, 0.05, and 0.5 μg/mL of analytes **1** and **2**, 0.005, 0.025, and 0.25 μg/mL of analyte **3**, and 0.15, 0.4, and 0.65 μg/mL of the analytes **4a/b** and **5a/b**. Each experiment was repeated three times.

Recovery experiments were performed using the wine sample Shiraz (2005). An aliquot of red wine (950 μL) was spiked with a solution containing defined amounts of analytes **1−5a/b** dissolved in methanol/water (20/80, v/v; 50 μL) to obtain wine sample containing 0.2, 1.0, and 10.0 mg/L of **1** and **2**, 0.1, 0.5, and 5.0 mg/L of **3**, and 0.2, 0.6, and 0.8 mg/L of **4a/b** and **5a/b**. As the basis for the calculation of the recovery rate, we used the initial concentration of analytes in unspiked wine.

Investigation of Matrix Effects during HPLC-MS/MS Analysis. In order to investigate the effect of matrix components in LC-MS/MS analysis, the same LC-MS/MS parameter settings were used as detailed above. Aliquots (20 μL) of red wine samples (Cabernet Sauvignon, Dornfelder) were injected, but, in addition, a constant flow of 10 μL/min of solutions of either vescalagin (**2**; 100 μmol/L), β-1-*O*-ethylvescalagin (**3**; 100 μmol/L), or acutissimin A (**4a**; 10 μmol/L) were introduced by means of a PHD 4400 Hpsi type syringe pump (Harvard Apparatus, Massachusetts, United States of America) connected to the solvent flow via a three-way valve.

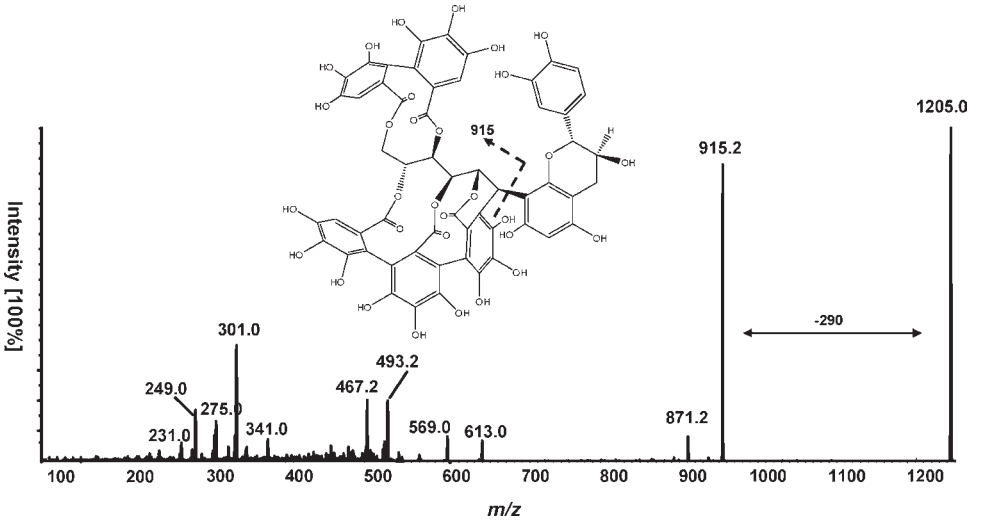

Figure 2. MS/MS spectrum (ESI$^-$, collision energy −30 V) of acutissimin A (**4a**).

LC-Time-of-Flight Mass Spectrometry (LC/TOF-MS). Mass spectra of the compounds were measured on a Bruker Micro-TOF mass spectrometer (Bruker Daltronics, Bremen, Germany) with flow injection and on a Quattro LCZ mass spectrometer (Waters-Micromass, Manchester, UK) with NanoSpray interface and referenced on sodium formate and polyethylene glycol (PEG) 600, respectively. The compounds were dissolved in 1 mL of methanol, and 10 μL of a saturated solution of sodium formate in methanol was added to measure the exact mass of the sodium adducts.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, *gs*COSY, *gs*HMQC, and *gs*HMBC NMR measurements were performed on a DMX 400 spectrometer (Bruker, Rheinstetten, Germany) and ^{13}C NMR measurements on a 500 MHz INOVA spectrometer (Varian, Darmstadt, Germany). β-1-*O*-Ethylvescalagin (**3**) was measured on an Avance 3 DRX 500 MHz spectrometer (Bruker, Rheinstetten, Germany). Chemical shifts were referenced to the solvent signal. Data processing was performed by using Topspin version 1.3 (Bruker, Rheinstetten) and MestReNova version 5.2.3 software (Mestrelab Research, Santiago de Compostela, Spain).

RESULTS AND DISCUSSION

To accurately determine the amounts of the ellagitannins **1** and **2** as well as their transformation products **3**−**5a/b** in red wine, a sensitive and robust HPLC-MS/MS analysis should be developed. Since no pure standard substances were commercially available, first the identity of these ellagitannins reported in the literature needed to be verified, and reference materials needed to be isolated and purified for their use as reliable standards.

Preparation of Reference Compounds 1−**5a/b.** Castalagin (**1**) and vescalagin (**2**) were isolated from an ethanol/water extract prepared from oak wood chips by means of adsorption chromatography using LH-20 material, followed by preparative RP-HPLC as described previously (*6*). β-1-*O*-Ethylvescalagin (**3**) was prepared by heating vescalagin (**2**) and ethanol in dry tetrahydrofuran containing 1% trifluoroacetic acid at 60 °C. LC-MS analysis with electrospray ionization (ESI$^-$) revealed *m*/*z* 480 ([M − 2H]$^{2-}$) and *m*/*z* 961 ([M − H]$^-$) as the pseudomolecular ions. Besides the protons of vescalagin, five additional protons, representing the ethyl group, could be observed in the ^{1}H NMR spectrum of **3**. The HMBC experiment revealed a heteronuclear correlation between the singlet proton H−C(1″) of the carbohydrate skeleton resonating at 4.77 ppm and the neighboring methylene carbon atom C(2) of the ethyl residue and vice versa, a correlation between the methylene protons H−C(2$\alpha\beta$) and the carbon atom C(1″). Comparison of the ^{13}C NMR spectrum of the educt vescalagin and the target compound **5** revealed a downfield shift of carbon C(1″) from about 63 to 72 ppm thus confirmed the *O*-linkage of the ethyl residue and demonstrated the position of the ethoxy group at C(1″) of the vescalagin moiety.

In order to prepare the acutssimins **4a/4b**, a mixture of (−)-vescalagin and (+)-catechin in tetrahydrofuran containing 1% trifluoroacetic acid was heated at 90 °C for 24 h (*7*, *9*). After cooling, acutissimin A (**4a**) and B (**4b**) were isolated by preparative RP-HPLC and, then, analyzed by means of UV−vis, LC-MS, and NMR spectroscopy. LC-MS/MS analysis by electrospray ionization (ESI$^-$) revealed an [M − H]$^-$ ion with *m*/*z* 1205 as well as a main fragment ion with *m*/*z* 915 (**Figure 2**). LC-TOF-MS analysis confirmed the target compound to have the molecular formula $C_{56}H_{39}O_{31}$.

The ^{1}H NMR spectrum of compound **4a** showed an aromatic singlet for H−C(6) at 6.00 ppm and three aromatic protons resonating at 6.76, 6.85, and 6.92 ppm showing an ABX coupling system. In addition, the four aliphatic protons H−C(4α), H−C(4β), H−C(3), and H−C(2) were observable at 2.36, 2.84, 4.54, and 5.47 ppm, respectively, coupling with each other and confirming a flavan-3-ol moiety. Moreover, the ^{1}H NMR spectrum exhibited seven aliphatic protons resonating at 4.06 [H−C(6α'')], 4.65 [H−C(6β'')], 4.71 [H−C(3″)], 4.77 [H−C(1″)], 5.15 [H−C(2″)], 5.23 [H−C(4″)], and 5.60 ppm [H−C(5″)] as expected for a carbohydrate skeleton. Additionally, three one-proton aromatic singlets resonating at 6.58, 6.73, and 7.05 ppm could be observed indicating the aromatic part of (−)-vescalagin. Considering all of the coupling constants of the carbohydrate moiety in the molecule and, in particular, the COSY-coupling between H−C(1″) and H−C(2″), and comparing these data with those reported for *C*-glycosidic hydrolyzable tannins (*8*, *9*, *18*), the β-linkage of the catechin residue could be deduced, thus demonstrating the α-orientation of H−C(1″) in the molecules.

A comparison of the ^{13}C NMR spectrum with the results of the HMQC experiment showing 16 signals revealed 40 signals corresponding to quaternary carbon atoms. Unequivocal assignment of these quaternary carbon atoms and the hydrogen-substituted carbon atoms, respectively, could be successfully achieved by means of heteronuclear multiple bond correlation spectroscopy (HMBC) optimized for $^2J_{C,H}$ and $^3J_{C,H}$ coupling constants and heteronuclear multiple-quantum correlation spectroscopy (HMQC) optimized for $^1J_{C,H}$ coupling constants, respectively. With the exception of the marker carbon atoms C(1″) and C(8), the ^{13}C NMR data were in good agreement with those of the corresponding educts vescalagin (*6*) and (+)-catechin (*19*). Of capital importance, the HMBC experiment revealed a correlation between the

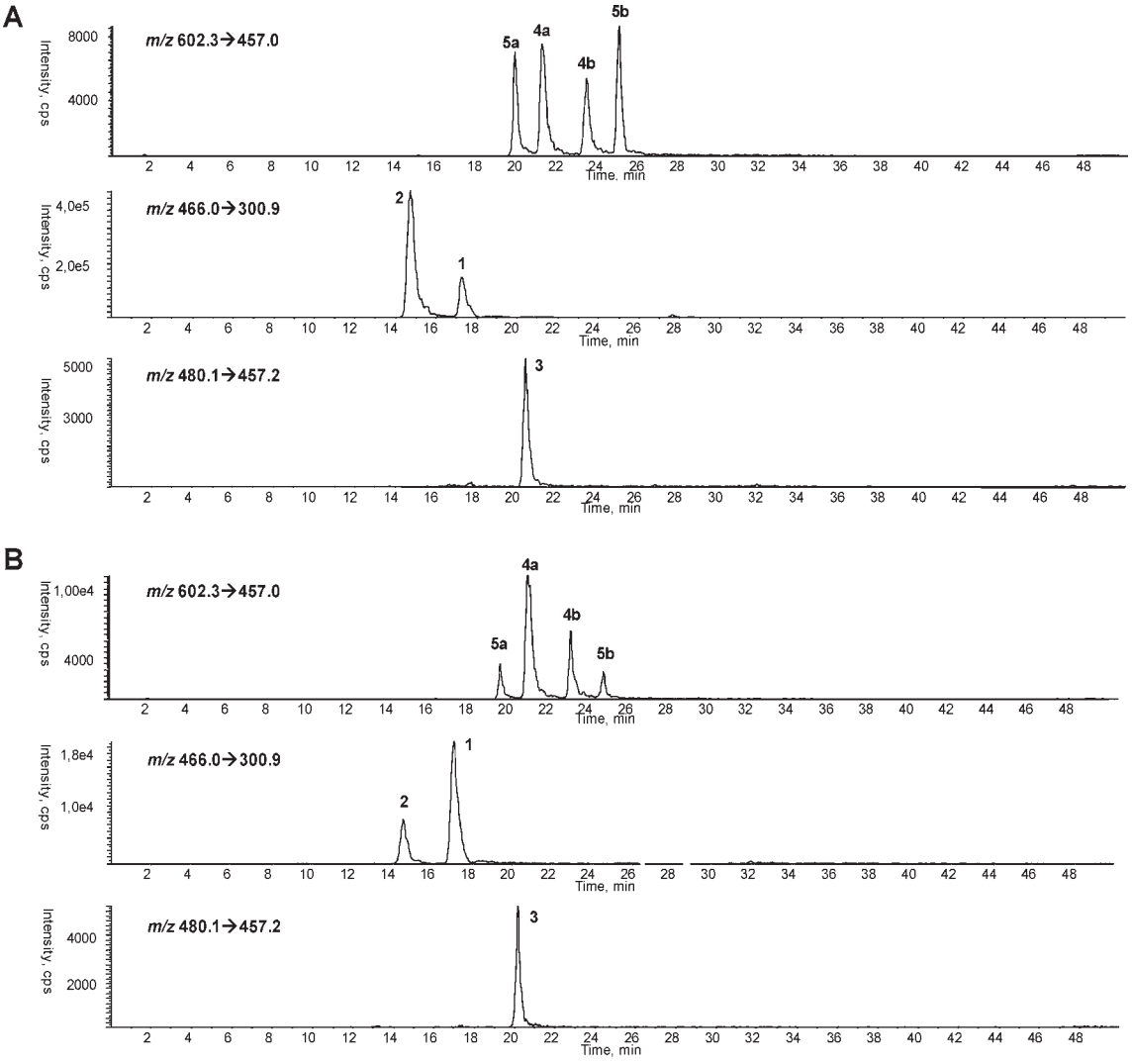

Figure 3. HPLC-MS/MS (ESI$^-$) chromatogram of (**A**) a reference mixture containing compounds **1**−**5a/5b** and (**B**) a red wine sample (Cabernet Sauvignon) recorded by using the multiple reaction monitoring (MRM) mode.

proton H−C(1″) of the carbohydrate skeleton resonating at 4.77 ppm and neighboring carbon atoms C(7), C(8), and, in particular, C(8a), thus demonstrating clearly the intramolecular 8-*C*-linkage of the (+)-catechin unit. The chemical upfield shift of the carbon atom C(1″) resonating at 38.8 ppm in comparison to its chemical shift in vescalagin or castalagin (63−65 ppm) (*6*) confirmed the *C*-linkage of the polyalcohol residue (*8*). In addition, the significant downfield shift of about 8 ppm of the carbon atom C(8) of the A-ring of the flavan-3-ol indicated a substituent at position 8. Taking all of this spectroscopic data into consideration, the identity of compound **1** was confirmed by comparison of exact mass measurement, 1D- and 2D NMR, and LC-MS data with those published in the literature (*7*−*9*, *11*).

Using a similar strategy for signal assignment, we confirmed the carbohydrate skeleton to be linked via 6-*C*-linkage to the (+)-catechin unit in acutissimin B as indicated by heteronuclear multiple bond correlations of the proton H−C(1″) to the carbon atoms C(7), C(6), and C(5).

Using the same hemisynthetic approach, we prepared epiacutissimin A (**5a**) and B (**5b**) from (−)-vescalagin and the flavan-3-ol (−)-epicatechin and confirmed the structure by means of LC-TOF-MS, LC-MS/MS, and 1D/2D-NMR experiments.

Development of an HPLC-MS/MS Procedure for the Quantitation of Compounds 1−5a/b. In order to develop a highly sensitive and direct LC-MS/MS based detection as well as an accurate quantitative analysis of the ellagitannins **1**−**5a**/**5b** in wine samples without the need of prior sample workup, compounds **1**−**5a**/**5b** were analyzed by LC-MS/MS for specific mass transitions. As an example, the MS/MS spectrum of acutissimin A (**4a**) is presented in **Figure 2**, thus demonstrating the cleavage of the flavan-3-ol unit at C-1 of the vescalagin core to give the characteristic fragment ion *m*/*z* 915. To achieve the best selectivity and sensitivity for their detection, the mass spectrometer was tuned on the 1-fold negatively charged pseudomolecular ion of the ellagitannins as well as on the 2-fold charged ion, which revealed a significant increase of intensity. Therefore, the mass transitions of the 2-fold charged pseudomolecular ion were used for ellagitannin quantification.

After optimization, the instrument settings and the mass transition *m*/*z* 602.3 → 457.0 were recorded for the acutissimins and allowed the detection of four peaks in the injected wine sample (**Figure 3**). Comparison of the retention times (RP-HPLC) and mass spectrometric fragmentation data with those obtained for the corresponding reference compounds, followed by cochromatography with the synthetic standards demonstrated that the flavano-8-*C*-ellagitannins acutissimin A (**4a**) and epiacutissimin A (**5a**) elute prior to the flavano-6-*C*-ellagitannins acutissimin B (**4b**) and epiacutissimin B (**5b**). The assignment of the elution

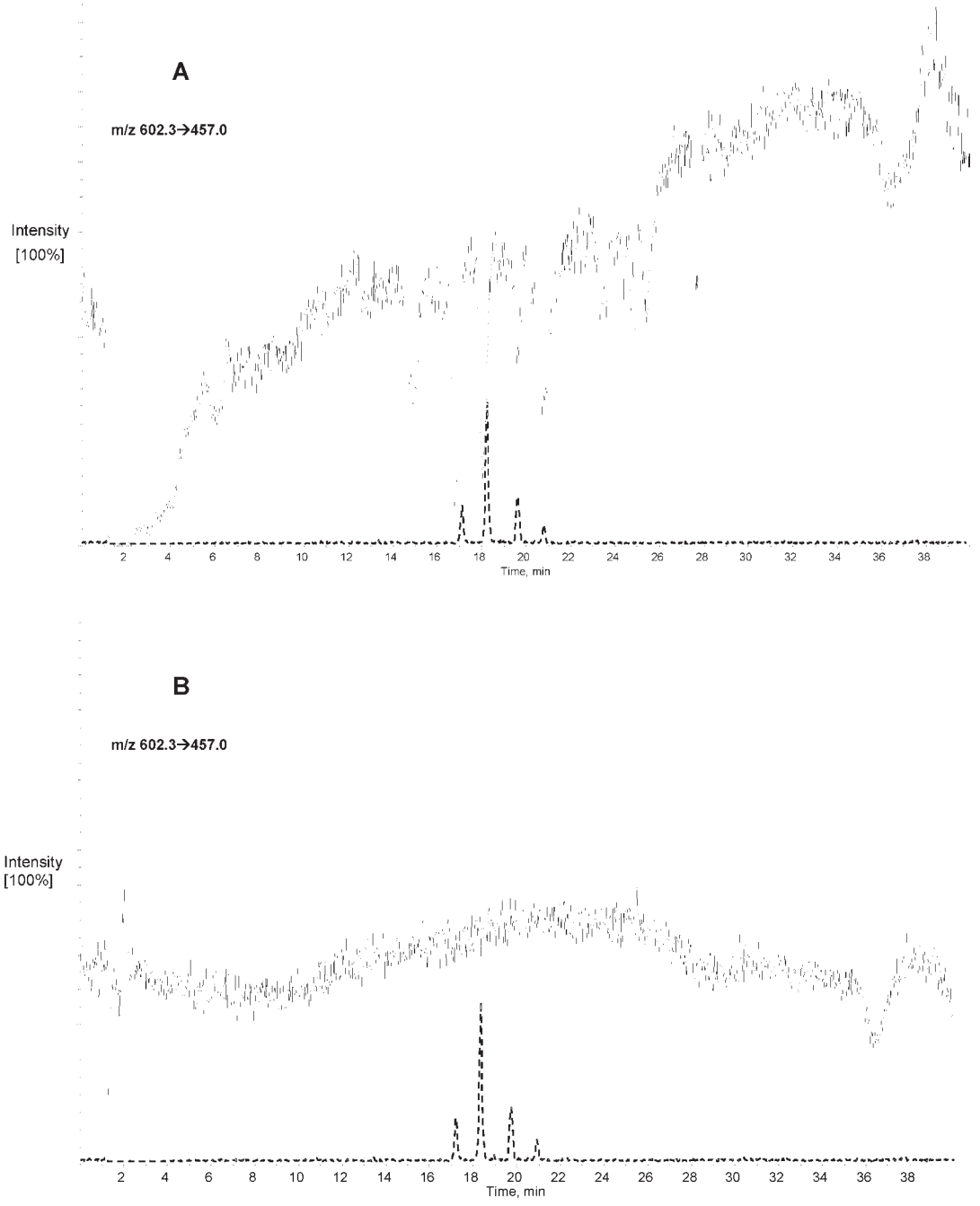

Figure 4. Influence of the red wine matrix components on the ionization of acutissimin A (**4a**). LC-MS/MS (MRM) chromatograms were recorded for (**A**) Dornfelder (2007) and (**B**) water/methanol (80/20, v/v), while a constant flow of acutissimin A was introduced into the LC-MS/MS system by a syringe pump. The dotted line shows the elution pattern of the ellagitannins **4a/b** and **5a/b**.

order of **4a/b** and **5a/b** is contradictory to previous reports (*7*, *9*, *11*) but is well in line with the elution order of flavan-3-ol-*C*-mono-, -di-, and -oligoglycosides, among which the 8-*C*-glycoside always elutes earlier than the corresponding 6-*C*-glycoside (*19*, *20*).

Monitoring the mass transition *m*/*z* 480.1 → 457.2 revealed one compound in red wine (**Figure 3**), which was identified by retention time and cochromatography as β-1-*O*-ethylvescalagin (**3**). In addition, vescalagin (**2**), followed by castalagin (**1**), was detected in the red wine sample by recording the mass transition *m*/*z* 466.0 → 300.9 (**Figure 3**).

Before performing quantitative analysis, it was necessary to confirm that coeluting wine matrix components do not affect the ionization of the analytes. In order to visualize such matrix effects, a constant flow of a solution of either vescalagin (**2**), β-1-*O*-ethylvescalagin (**3**), or acutissimin A (**4a**) was introduced into the LC-MS/MS system via a syringe pump during the analysis of red wine samples Dornfelder (2007) (**Figure 4A**) and Cabernet Sauvignon. As given in **Figure 4A**, a strong matrix dependent suppression of almost 100% of the ionization of acutissimin A (**4a**) was observed between the retention times of 1 and 3 min. Such effects are known to be due to the high amount of strongly polar compounds eluting within the dead volume of the column and can be observed in many RP applications (*21*, *22*). After 3 min, the signal intensity regained slowly within 3 min and, then, revealed another signal suppression of about 70% after about 6 min. After 12 min, the original signal intensity was recovered, whereas in the region from 14 to 25 min, strong signal suppression was again observed (**Figure 4A**). In this elution

window, the four flavano-ellagitannins could be detected. After about 26 min, the ionization yield of acutissimin A was observed to rise with increasing retention times, thus demonstrating that red wine matrix components have a strong influence on the ionization of flavano-ellagitannins.

By infusing acutissimin A at a constant flow rate into the LC-MS/MS system by means of a syringe pump with a one-time injection of 20% methanol during analysis, only the influence of the solvent gradient on the ionization could be shown (**Figure 4B**). In contrast to the red wine matrix (**Figure 4A**), only minor influences on the ionization rate could be observed. By syringe infusion of 20% methanol at the same flow rate into the LC-MS/MS system and injection of red wine, the exact elution times of the four flavano-ellagitannins during the infusion experiments described above could be determined (**Figure 4A**,**B**). This allows the exact assignment of the influence of the matrix on the ionization of the analytes to their corresponding elution time. Considering this observation, quantitation by external calibration with standard solutions in 20% methanol ought to result in an overestimation of the flavano-ellagitannin concentration in red wines. Changing the solvent gradient in order to compensate the influence of the matrix by separating the target analytes from coeluting matrix molecules was not successful, and at least, the signal of one of the flavano-ellagitannins **4a/b** and **5a/b** was strongly influenced by the matrix. Similar observations were made for the analysis of vescalagin (**2**) and β-1-*O*-ethylvescalagin (**3**) (data not shown). Taking all of these data into account, external quantitation of ellagitannins in red wine without any matrix compensation is very likely to deliver nonreliable results.

In order to compare different possibilities for ellagitannin quantitation, experiment A was performed using external calibration with standards dissolved in 20% aqueous methanol, experiment B (matrix calibration) was done using external calibration with standards dissolved in the analyte-free red wine sample Dornfelder (2007), and, finally, experiment C was performed using a standard addition procedure. The results obtained by standard addition were rather similar to the data obtained by the matrix calibration using Dornfelder. Regarding the (epi)acutissimins **4a/b** and **5a/b**, the results obtained by external calibration using standard solutions in 20% methanol were found to be considerably higher (**Figure 5A**), thus further substantiating the influence of red wine matrix components on the ionization of the ellagitannins shown above. In contrast to the acutissimins, the quantitative data obtained for castalagin (**1**), vescalagin (**2**), and β-1-*O*-ethylvescalagin (**3**) by using the external matrix calibration matched rather well those found by standard addition, whereas external quantitation without matrix calibration showed somewhat lower amounts in particular of castalagin and vescalagin (**Figure 5B**). In particular, low-abundant analytes such as the (epi)acutissimins seem to be stronger influenced by matrix components when compared to analytes present in higher concentrations such as, e.g., castalagin showing 10 times higher amounts in wine when compared to **4a/b** and **5a/b**. Taking all of these data into consideration, the external matrix-calibration with reference compounds dissolved in an analyte-free matrix such as Dornfelder (2007) was shown to be a straightforward and accurate method for the quantitation of ellagitannins.

To check the accuracy of the HPLC-MS/MS method with matrix calibration, recovery experiments were performed in the following. To achieve this, the samples of the purified ellagitannins were added to a wine sample (Shiraz) at three different concentration levels prior to HPLC-MS/MS analysis, and the amounts determined were compared with those found in the blank wine sample. The recovery rates were found to be 106.1 ± 8.7% for castalagin (**1**), 102.4 ± 5.9% for vescalagin (**2**), 104.6 ± 7.1% for β-1-*O*-ethylvescalagin (**3**), 105.1 ± 4.3% for acutissimin A (**4a**), 111.4 ± 5.6% for acutissimin B (**4b**), 109.7 ± 7.9% for epiacutissimin A

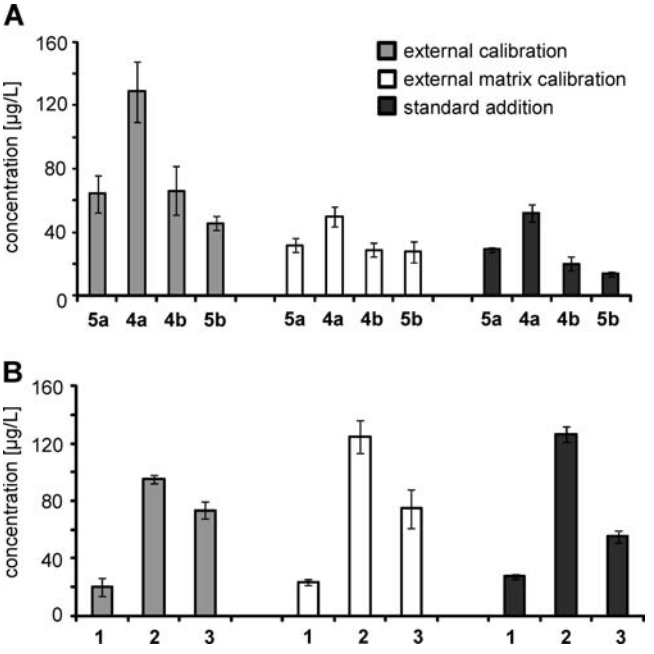

Figure 5. Concentrations of flavano-ellagitannins (**A**) and castalagin, vescalagin, and β-1-*O*-ethylvescalagin (**B**) in red wine (Shiraz) obtained by external calibration (in methanol), external red wine matrix-calibration, and standard addition.

Table 1. Concentrations and Dose-over-Threshold (DoT) Factors of Ellagitannins in Red Wine Samples Obtained by Matrix Calibration with the Analyte-Free Red Wine Sample Dornfelder (2007)

	concentration in mg/L (DoT factor[a])													
wine sample	**5a**		**4a**		**4b**		**5b**		**3**		**1**		**2**	
Shiraz	0.03	(<0.1)	0.05	(0.1)	0.03	(<0.1)	0.02	(<0.1)	1.08	(0.4)	1.58	(1.5)	0.27	(0.3)
Barolo	0.05	(<0.1)	0.16	(0.1)	0.08	(<0.1)	0.04	(<0.1)	1.00	(0.4)	3.19	(3.1)	0.38	(0.4)
Spaetburgunder	<0.01	(<0.1)	0.03	(<0.1)	0.01	(<0.1)	<0.01	(<0.1)	0.10	(0.1)	0.35	(0.34)	0.05	(0.1)
Bordeaux	<0.01	(<0.1)	0.01	(<0.1)	0.01	(<0.1)	0.03	(<0.1)	0.04	(<0.1)	0.20	(0.2)	0.01	(<0.1)
Merlot	0.01	(<0.1)	0.03	(<0.1)	0.02	(<0.1)	0.01	(<0.1)	0.36	(0.1)	0.94	(0.9)	0.13	(0.1)
Cabernet Sauvignon	0.05	(<0.1)	0.17	(0.2)	0.10	(0.1)	0.04	(<0.1)	1.31	(0.5)	3.78	(3.7)	0.43	(0.4)
Assemblage	0.01	(<0.1)	0.02	(<0.1)	0.01	(<0.1)	0.02	(<0.1)	0.03	(<0.1)	0.40	(0.4)	0.01	(<0.1)
Dornfelder 2004 barrique	0.01	(<0.1)	0.03	(<0.1)	0.02	(<0.1)	0.01	(<0.1)	0.26	(0.1)	1.12	(1.1)	0.07	(0.1)
Dornfelder 2005 barrique	0.01	(<0.1)	0.03	(<0.1)	0.01	(<0.1)	<0.01	(<0.1)	0.72	(0.3)	1.35	(1.3)	0.28	(0.3)
Dornfelder 2005	<0.01	(<0.1)	0.01	(<0.1)	0.01	(<0.1)	<0.01	(<0.1)	0.08	(<0.1)	0.33	(0.3)	0.03	(<0.1)
Dornfelder (2007)	0.00	(0)	0.00	(0)	0.000	(0)	0.00	(0)	0.00	(0)	0.00	(0)	0.00	(0)

[a] The DoT factor is calculated as the ratio of the concentration and taste threshold.

(**5a**), and 113.7 ± 15.2% for epiacutissimin A (**5a**), showing a suitable trueness of the developed analytical method.

Using the matrix-calibrated HPLC-MS/MS method, the ellagitannins **1**−**5a/5b** were quantitatively determined in a series of 11 red wine samples (**Table 1**). With the exception of Dornfelder (2007), which has not been in contact with oak, all of the other red wine samples contained the ellagitannins **1**−**5a/b** in varying amounts. By far, the highest amounts of the acutissimins (**4a/b**) and epiacutissimins (**5a/b**) were detected in Cabernet Sauvignon, which was aged in new oak barrels. In contrast, the lowest amounts were found in samples of Bordeaux, Spaetburgunder, and in Dornfelder (2005), which was not aged in barrique barrels. Independent of the wine sample analyzed, castalagin (**1**) was found as the predominant ellagitannin, followed by β-1-*O*-ethylvescalagin (**3**), e.g., 3.78 (**1**) and 1.31 mg/L (**3**), respectively, present in Cabernet Sauvignon (**Table 1**). In comparison, significantly lower amounts were found for the flavano-*C*-ellagitannins **4a/b** and **5a/b** ranging between 0.002 and 0.17 mg/L in the wine samples under investigation. Among the flavano-*C*-ellagitannins, acutissimin A (**4a**) was the predominant isomer, followed by its constitution isomer acutissimin B (**4b**).

Comparison of the two Dornfelder wines 2005, produced by the same wine grower but differing in maturation, revealed that the barrique wine contained double the amounts of acutissimins (**4a/b**) and epiacutissimins (**5a/b**), 4-fold increased levels of castalagin (**1**), and about 10 times higher concentrations of β-1-*O*-ethylvescalagin (**3**) and vescalagin (**2**) (**Table 1**). These differences are due to the different maturation of the two Dornfelder 2005 samples. Both wines were made of the same must but were aged in different barrels. For the production of the barrique-matured Dornfelder 2005, the wine was aged in French oak barrels for 15 months, which had been used twice for maturation before. In contrast, the nonbarrique Dornfelder 2005 was matured in oak barrels repeatedly used for more than 10 years.

In order to gain a first insight into the effect of different vintages on the amount of ellagitannins, barrique mature Dornfelder samples (2004 and 2005), produced in the same way but in different years, were analyzed by means of the matrix-calibrated HPLC-MS/MS method. The Dornfelder barrique of the vintage 2005 showed somewhat higher amounts of castalagin (**1**), vescalagin (**2**), and β-1-*O*-ethylvescalagin but exhibited slightly lower amounts of the (epi)acutissmins **4a/b** and **5a/b**, thus indicating that the amount of vescalagin is not the only limiting factor for (epi)acutissimin formation (**Table 1**).

Sensory Activity of Ellagitannins 1−**5a/b.** Prior to sensory analysis, the purity of all compounds was checked by HPLC-MS as well as ^{1}H NMR spectroscopy. To study the sensory activity of the ellagitannins, the human sensory recognition thresholds were determined in bottled water (pH 4.5) using the half-mouth test (*15*−*17*).

Compared to the flavan-3-ols (−)-epicatechin and (+)-catechin exhibiting a puckering astringent as well as bitter taste at threshold concentration levels between 600 and 1000 μmol/L, all of the ellagitannins **1**−**5a/b** were found to induce a smooth astringent and velvety mouth-coating sensation at very low threshold concentrations ranging from 0.9 to 2.8 μmol/L (**Table 2**). In contrast to β-1-*O*-ethylvescalagin (**3**), sensory analysis of the (epi)acutissmins **4a/b** and **5a/b** revealed that a puckering sensation was overlapping the smooth astringency when tested at levels of more than 10-fold above their threshold concentration. In summary, a modification of the vescalagin structure by substitution with flavan-3-ols or ethanol does not significantly influence its taste threshold for astringency, although the (epi)acutissmins **4a/b** and **5a/b** exhibited a puckering quality of astringency when tasted at higher levels.

Table 2. Human Recognition Taste Thresholds for the Oral Sensation Induced by Flavan-3-ols and Ellagitannins

	threshold conc [μmol/L]	
compound	astringency[a]	bitterness[b]
(−)-epicatechin	800.0[c]	800.0
(+)-catechin	600.0[c]	1000.0
castalagin (**1**)	1.1[d]	1690.0
vescalagin (**2**)	1.1[d]	1690.0
β-1-*O*-ethylvescalagin (**3**)	2.8[d]	>1500.0
acutissimin A (**4a**)	0.9[e]	n.d.
acutissimin B (**4b**)	1.6[e]	n.d.
epiacutissimin A (**5a**)	1.5[e]	n.d.
epiacutissimin B (**5b**)	2.4[e]	n.d.

[a] Taste threshold concentrations were determined by means of the half-tongue test in bottled water (pH 4.5). [b] Bitter taste threshold concentrations were determined by means of a three-alternative forced-choice test in bottled water (pH 4.5). [c] The astringent sensation was described as rough and extremely puckering. [d] The astringent sensation was described as very smooth and mouth-drying. [e] The astringent sensation was described as smooth and in higher concentrations as puckering. n.d.: not detectable below 100 μmol/L.

To gain a first insight into the question as to whether these ellagitannins do reach or exceed their taste threshold concentration in red wines, a dose-overthreshold (DoT) factor (*23*) was calculated for each compound as the ratio of its concentration in wine to its threshold concentration (**Table 2**). The data revealed that, among all of the ellagitannins investigated, exclusively, castalagin (**1**) exceeded its threshold concentration in some wine samples, e.g., this compound reached DoT values of 3.8, 3.2, 1.3, and 1.2 in Cabernet Sauvignon, Barolo, Shiraz, and Dornfelder barrique, respectively. Comparing Dornfelder samples 2005, we could demonstrate that aging in barrique leads to higher DoT factors, even exceeding 1.0. Whether the ellagitannins **2**−**5a/b**, although present in subthreshold concentrations, do contribute to wine taste due to additive behavior or synergistic effects between the acutissimins and other classes of wine polyphenols such as procyanidins remains open and needs to be investigated in future human psychophysical studies.

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LITERATURE CITED

(1) Manach, C.; Scalbert, A.; Morand, C.; Rémésy, Ch.; Jiménez, L. Polyphenols: food source and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747.

(2) Gawel, R.; Iland, G. G.; Francis, I. L. Characterizing the astringency of red wine: a case study. *Food Qual. Pref.* **2001**, *12*, 83–94.

(3) Hufnagel, J. C.; Hofmann, T. Orosensory-directed identification of astringent mouthfeel and bitter-tasting compounds in red wine. *J. Agric. Food Chem.* **2008**, *56*, 1376–1386.

(4) Hufnagel, J. C.; Hofmann, T. Quantitative reconstruction of the nonvolatile sensometabolome of a red wine. *J. Agric. Food Chem.* **2008**, *56*, 9190–9199.

(5) Glabasnia, A.; Hofmann, T. Identification and sensory evaluation of dehydro- and deoxy-ellagitannins formed upon toasting of oak wood (*Quercus alba* L.). *J. Agric. Food Chem.* **2007**, *55*, 4109–4118.

(6) Glabasnia, A.; Hofmann, T. Sensory-directed Identification of taste-active ellagitannins (*Quercus alba* L.) and european oak wood (*Quercus robur* L.) and quantitative analysis in bourbon whiskey and oak-matured red wines. *J. Agric. Food Chem.* **2006**, *54*, 3380–3390.

(7) Quideau, S.; Jourdes, M.; Lefeuvre, D.; Montaudon, D.; Saucier, C.; Glories, Y.; Pardon, P.; Pourquier, P. The chemistry of wine polyphenolic *C*-glycosidic ellagitannins targeting human topoisomerase II. *Chem.—Eur. J.* **2005**, *11*, 6503–6513.

(8) Ishimaru, K.; Nonaka, G.-I.; Nishioka, I. Tannins and related compounds. LV. [1] Isolation and characterization of acutissimins A and B, novel tannins from *Quercus* and *Castanea* species. *Chem. Pharm. Bull.* **1987**, *35*, 602–610.

(9) Quideau, S.; Jourdes, M.; Saucier, C.; Glories, Y.; Pardon, P.; Baudry, C. DNA topoisomerase inhibitor acutissimin A and other flavano-ellagitannins in red wine. *Angew. Chem., Int. Ed.* **2003**, *42*, 6012–6014.

(10) Kashiwada, Y.; Nonaka, G.-I.; Nishioka, I.; Lee, K. J.-H.; Bori, I.; Fukushima, Y.; Bastow, K. F.; Lee, K.-H. Tannins as potent inhibitors of DNA topoisomerase II in vitro. *J. Pharm. Sci.* **1993**, *82*, 487–492.

(11) Saucier, C.; Jourdes, M.; Glories, Y.; Quideau, S. Extraction, detection, and quantification of flavano-ellagitannins and ethylvescalagin in a Bordeaux red wine aged in oak barrels. *J. Agric. Food. Chem.* **2006**, *54*, 7349–7354.

(12) Vivas, N.; Laguerre, M.; Glories, Y.; Bourgeois, G.; Vitry, C. Structure simulation of two ellagitannins from *Quercus robur L. Phytochemistry* **1995**, *39*, 1193–1199.

(13) Lefeuve, D.; Saucier, C.; Quideau, S. Remarkable Selective Oxidation of Flavano-Ellagitannins in Wine: First Hemisynthesis of Monogolicain A from Acutissimin A. 232nd ACS National Meeting, San Francisco, CA, 2006.

(14) Nonaka, G.-I.; Ishimaru, K.; Mihashi, K.; Iwase, Y.; Ageta, M.; Nishioka, I. Tannins and related compounds. LXIII. [1] Isolation and characterization of monogolicain a and b, novel tannins from *Quercus* and *Castanopsis* species. *Chem. Pharm. Bull.* **1988**, *36*, 857–869.

(15) Wieser, H.; Belitz, H.-D. Studies on the structure/activity relationship of bitter tasting amino acids and peptides (in German). *Z. Lebensm. Unters. Forsch.* **1975**, *159*, 65–72.

(16) Scharbert, S.; Holzmann, N.; Hofmann, T. Identification of the astringent taste compounds in black tea infusions by combining instrumental analysis and human bioresponse. *J. Agric. Food Chem.* **2004**, *52*, 3498–3508.

(17) Brock, A.; Hofmann, T. Identification of the key astringent compounds in spinach (*Spinacia oleracea*) by means of the taste dilution analysis. *Chem. Percept* **2008**, *1*, 268–281.

(18) Nonaka, G.-I.; Sakai, T.; Tanaka, T.; Mihashi, K.; Nishioka, I. Tannins and related compounds. XCVII. [1] Structure revision of C-glycosidic ellagitannins, castalagin, vescalagin, casuarinin and stachyurin, and related hydrolyzable tannins. *Chem. Pharm. Bull.* **1990**, *38*, 2151–2156.

(19) Stark, T.; Hofmann, T. Application of a molecular sensory science approach to alkalized cocoa (*Theobroma cacao*): structure determination and sensory activity of nonenzymatically C-glycosylated flavan-3-ols. *J. Agric. Food Chem.* **2006**, *54*, 9510–9521.

(20) Stark, T.; Keller, D.; Wenker, K.; Hillmann, H.; Hofmann, T. Nonenzymatic *C*-glycosylation of flavan-3-ols by oligo- and polysaccharides. *J. Agric. Food Chem.* **2007**, *55*, 9685–9697.

(21) Bonfiglio, R.; King, R. C.; Olah, T. V.; Merkle, K. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. *Rapid Commun. Mass Spectrom.* **1999**, *13* (12), 1175–1185.

(22) Hsieh, Y.; Chintala, M.; Mei, H.; Agans, J.; Brisson, J. M.; Ng, K.; Korfmacher, W. A. Quantitative screening and matrix effect studies of drug discovery compounds in monkey plasma using fast-gradient liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2001**, *15* (24), 2481–7.

(23) Scharbert, S.; Hofmann, T. Molecular definition of black tea taste by means of quantitative studies, taste reconstitution and omission experiments. *J. Agric. Food Chem.* **2005**, *53*, 5377–5384.

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