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Orosensory-Directed Identification of Astringent Mouthfeel and Bitter-Tasting Compounds in Red Wine

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Application of sequential solvent extraction, followed by HPLC combined with the taste dilution analysis, enabled the localization of the most intense velvety astringent, drying, and puckering astringent, as well as bitter-tasting, compounds in red wine, respectively. Isolation of the taste components involving gel adsorption chromatography, ultrafiltration, and synthesis revealed the identification of 26 sensory-active nonvolatiles, among which several 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 astringent components. In contradiction to literature suggestions, flavan-3-ols were found to be not of major importance for astringency and bitter taste, respectively. Surprisingly, a series of hydroxybenzoic acid ethyl esters and hydroxycinnamic acid ethyl esters were identified as bitter compounds in wine. Taste qualities and taste threshold concentrations of the individual wine components were determined by means of a three-alternative forced-choice test and the half-mouth test, respectively.

KEYWORDS: Red wine; wine; astringency; bitterness; taste; taste dilution analysis; polyphenolic acid ethyl esters; half-mouth test

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. Aroma-active volatiles as well as nonvolatile chromophores of red wine were thoroughly investigated in recent decades, but only a small number of studies were targeted toward the nonvolatile taste compounds in wine and, in particular, those eliciting an astringent and/or bitter oral sensation. The typical astringent mouthfeel, which is perceived as a long-lasting trigeminal sensation in the oral cavity, can be classified into several subqualities such as velvety, grainy, drying, or puckering (*1*) and, together with bitterness, is of crucial importance for the palatability of red wines. Whereas velvety astringency is perceived as a silky and finely textured 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 (*1*). Despite being aware of the sensory importance of nonvolatiles in wines, the key inducers of astringency and bitter taste in red wines are still unclear on a molecular level.

Although many attempts have been made to correlate analytical data on distinct wine components with the sensory data obtained from human subjects, the reports on the chemical species imparting the typical bitter and astringent taste of red wines are rather contradictory. It is believed that astringency is due to the polyphenol-induced complexation and/or precipitation of proline-rich salivary proteins in the oral cavity (*2–4*), thus inducing a tactile sensation perceived by touch via mechanoreceptors (*5*). Consistent with this hypothesis, more than 40 years ago water-soluble phenols with molecular masses from 500 to 3000 Da were found to be required for imparting astringency (*6*), and bioassays based on protein complexation have been developed for the measurement of polyphenols such as tannins (*7*). However, first attempts trying to predict astringency by analyzing the turbidity of solutions containing polyphenol−protein complexes gave rather contradictory data (*8*). Furthermore, simple polyphenols containing 1,2-dihydroxybenzene or 1,2,3-trihydoxybenzene groups were reported to cross-link and precipitate proteins (*9*). On the other hand, low molecular weight compounds were found to elicit astringency by complexing salivary proteins without precipitation (*10*).

Flavan-3-ol monomers and higher oligomers, so-called procyanidins, are well accepted to induce an astringent oral sensation as well as bitter taste (*11–13*). Although the threshold concentrations of these compounds were found to be rather high, for example, 46.1 and 17.3 mg/L for (+)-catechin and procyanidin B3 (*14*), respectively, the procyanidins were intensively

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investigated for the time/intensity course of the astringency perceived as well as for interactions in mixtures (*15–17*). More recent studies reported that highly polymeric, galloylated tannins induce a puckering astringent mouthfeel (*18*). In contradiction, studies performed on crude fractions isolated from red wines (*19–21*) revealed that gallic acid and, in particular, flavan-3-ols exhibiting molecular masses below 500 Da elicit astringent and bitter taste qualities.

Besides the polyphenols, organic acids were reported to impart astringency (*22*). On the contrary, other studies found that organic acids are able to influence but, with the exception of malic acid, do not evoke astringent taste sensations (*23, 24*). Although more recent studies showed some correlations between the concentrations of polyphenolic compounds and sensory descriptive analysis, the key inducers of astringency could not be unequivocally identified on a molecular level (*25*).

Similar to astringency, also the data available on bitter taste compounds in wines are rather inconsistent. Multiple investigations suggest procyanidins as bitter stimuli in wines (*11–13, 16, 19, 20*) and, moreover, ethanol was reported to enhance the bitter intensity perceived (*11, 26*). In addition, terpene glycosides were reported to contribute to the bitter taste of Muscat wines (*27*), but studies aimed at correlating chemical and sensory data of red wines were not successful in generating a predictive model for bitterness on wines (*28*).

To molecularize the sensometabolites driving the attractive taste of foods, we have recently developed sensomics tools such as the so-called taste dilution analysis (TDA) to screen for sensory active nonvolatiles (*29*). Application of this approach led to the identification of bitter compounds in thermally processed sugar/amino acid mixtures (*29*), cooling compounds in roasted malt (*30*), bitter off-tastants in carrots (*31*), a taste enhancer in beef bouillon (*32*), the astringent and bitter key compounds in tea infusions (*33*) as well as in roasted cocoa (*34*), and, recently, the astringent ellagitannins migrating into spirits and wines upon oak treatment (*35*).

To bridge the gap between the sensory perception of red wine and the chemical structure of the corresponding taste stimuli, the objectives of the present investigation are to screen for the key astringent and bitter compounds in a red wine by means of the taste dilution analysis, to isolate and identify the chemical structure, and to determine the sensory thresholds of the compounds evaluated with the highest gustatory response. Because Amarone della Valpollicella is considered to be one of the most prestigious wines of excellent taste quality, this wine was selected as the target for analysis.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: (+)-catechin, (−)-epicatechin, gallic acid, caffeic acid, syringic acid, ferulic acid ethyl ester, protocatechuic acid ethyl ester, formic acid, acetic acid, lactic acid, (*E*)-aconitic acid, (*Z*)-aconitic acid, glutaric acid, tartaric acid, succinic acid, malic acid, citric acid, isocitric acid, galacturonic acid, and phosphoric acid (Sigma, Steinheim, Germany); vanillic acid ethyl ester, caftaric acid (Apin Chemicals, Oxon, U.K.); *p*-coumaric acid, vanillic acid, silica gel 60, gallic acid ethyl ester (Fluka Chemika, Taufkirchen, Germany); isorhamnetin-3-*O*-*β*-D-glucopyranoside, quercetin-3-*O*-*β*-D-galactopyranoside, syringetin-3-*O*-*β*-D-glucopyranoside, procyanidin B1, procyanidin B2 (Extrasynthese, Genay Cedex, France); and ethyl acetate (EtOAc), ethanol (EtOH), methanol (MeOH), acetonitrile (CH_3CN), and acetone (Me_2CO) of HPLC grade (Merck, Darmstadt, Germany). Deuterated solvents were from Eurisotop (Saarbruecken, Germany). Deionized water used for chromatography was purified by means of a Milli-Q Gradient A10 system (Millipore, Billerica, MA). For sensory analyses, bottled water (Evian) was adjusted to pH 4.5 with trace amounts of formic acid prior to use. Reference materials of procyanidin B3 and procyanidin C1 were isolated from nonfermented cocoa beans following the procedure reported recently (*34*).

The red wine used for the study was an Amarone della Valpolicella DOC, vintage year 1997, 15% by volume ethanol; the grapes were grown on a clay and limestone soil with a vine density of 2500 vines per hectare; grape varieties used for manufacturing were 30% Corvina, 30% Corvinone, 30% Rondinella, and 10% a mixture of Molinara, Rossignola, Oseleta, Negrara, and Dindarella. For its manufacturing, grapes were harvested by hand at the end of September to the beginning of October, placed in wooden trays, and then dried for about 4 months at controlled temperature and humidity conditions. After the grapes had lost about 40% of their weight, the grapes were pressed and the must obtained was fermented at 15 °C in stainless steel tanks for about 40 days. The young wine was then matured for about 2 years in barriques and another year in the bottle.

Sensory Analyses. *Training of the Sensory Panel.* Ten subjects (five women and five men, ages 22–39 years), who gave informed consent to participate in the sensory tests of the present investigation and had no history of known taste disorders, were trained in sensory experiments with purified reference compounds at regular intervals for at least 2 years as described earlier (*34, 35*) and were, therefore, familiar with the techniques applied. Sensory analyses were performed in a sensory panel room at 22 °C in three different sessions under red light.

Pretreatment of Fractions. Prior to sensory analysis, the fractions or compounds isolated were suspended in water, and, after removal of the volatiles under high vacuum (<5 mPa), were freeze-dried twice. GC-MS and ion chromatographic analysis revealed that food fractions treated by that procedure are essentially free of the solvents and buffer compounds used.

Taste Profile Analysis. A freshly opened bottle of wine was kept at room temperature for at least 2 h prior to sensory analysis. Freeze-dried fractions isolated from red wine were taken up in water in "natural" concentrations prior to analysis. The samples (5 mL) were presented to the sensory panelists, who wore nose clips and who were asked to briefly swirl the sample in the mouth and, then, to expectorate. Using this sip-and-spit method, the panelists were asked to score the taste qualities astringent, bitter, sour, sweet, salty, umami, and mouthfulness/body on a scale from 0 (not detectable) to 5.0 (strong taste impression).

Taste Dilution Analysis (TDA). A taste dilution (TD) factor (*29*) was determined for each individual HPLC fraction isolated from red wine using bottled water (pH 4.5) as the solvent. To achieve this, aliquots of the individual fractions were dissolved in their "natural" concentration ratio in exactly 10 mL of water and were then sequentially 1 + 1 diluted with bottled water. The serial dilutions of each of these fractions were then presented to the sensory panel in order of ascending concentrations, and taste impressions in each dilution was evaluated by means of the recently developed half-tongue test (*33–35*).

Sequential Solvent Extraction of Red Wine. An aliquot (100 mL) of red wine was partially freed from EtOH under vacuum (10 kPa) and then extracted with *n*-pentane (5 × 300 mL) at room temperature. The combined organic layer was freed from solvent under vacuum, thus achieving the pentane-soluble components (fraction A). Thereafter, the aqueous residue was extracted five times with EtOAc (300 mL each) at room temperature, the organic phases were combined, the solvent was removed under vacuum, and after the addition of water (30 mL), the EtOAc extractables (fraction B) were obtained after freeze-drying. The remaining aqueous layer was lyophilized to give the water solubles (fraction C). The individual fractions, stored at – 26 °C until use, were used for sensory and chemical analysis.

HPLC/TDA of Fraction B. A sample (200 mg) of fraction B was dissolved in a mixture (20:80, v/v; 2 mL) of MeOH and aqueous HCOOH (0.1% in water; pH 2.5) using an ultrasonic bath. After membrane filtration (0.45 μm, Satorius Hannover, Germany), portions of 200 μL were analyzed by semipreparative RP-HPLC/UV–vis. Monitoring the effluent at 272 nm, chromatography was performed starting with a mixture (95:5, v/v) of aqueous HCOOH (0.1%) and CH_3CN, then increasing the CH_3CN content to 17% within 35 min, to 20% within 15 min, and then to 50% within an additional 25 min, and finally to 100% within 5 min. The eluent was separated into 30 fractions,

namely, fractions B-1–B-30, which were individually collected into ice-cooled, brown glass vials. The corresponding effluents obtained from 10 HPLC runs were combined and, after the solvent had been removed under vacuum, freeze-dried and were directly used for the TDA.

Gel Adsorption Chromatography (GAC). A sample (600 mg) of fraction B was dissolved in a solution of MeOH/water (20:80, v/v; 10 mL) and placed onto the top of a water-cooled 400 × 50 mm XK 26/70 glass column (Amersham Pharmacia Biotech, Uppsala, Sweden) filled with a slurry of Sephadex LH 20 (GE Healthcare, Munich, Germany), which was conditioned with a solution of MeOH/water (20:80, v/v) and then adjusted to pH 4 with a 1% aqueous solution of HCOOH. For chromatography, the column was eluted sequentially with solutions of MeOH/water containing 20, 40, 60, or 80% MeOH (400 mL each), with MeOH (1200 mL), and finally with an Me_2CO/water (70:30, v/v; pH 4; 800 mL), keeping a flow rate of 1.3 mL/min by means of a P1-type pump (Pharmacia Biotech). With the effluent monitored at 272 nm by means of a UV-2575-type UV−vis detector (Jasco), the GAC fractions were collected every 10 min by means of an LKB Bromma 7000 Ultrorac fraction collector and combined to give 13 GAC fractions (I−XIII). The fractions were then freed from solvent under vacuum, freeze-dried, and used for isolation and identification of the taste-active compounds located by means of HPLC/TDA.

Synthesis of Phenolic Acid Ethyl Esters. A solution of caffeic acid, *p*-coumaric acid, or syringic acid (15 mmol) in EtOH (100 mL) and H_2SO_4 (95%; 0.75 mL) was stirred for 12 h at 60 °C. After cooling, water (300 mL) was added, the mixture was freed of ethanol under vacuum, and the remaining aqueous layer was extracted four times with EtOAc (200 mL each). The combined organic layer was concentrated under vacuum to about 15 mL, and aliquots (5 mL) were added on top of a water-cooled glass column (500 × 30 mm) filled with a slurry of silica gel 60 (6% water) in toluene/EtOAc (60:40, v/v). Chromatography was performed with toluene/EtOAc (60:40, v/v) with stepwise increasing amounts of EtOAc. Fractions were collected every 150 mL, organic solvents were removed under vacuum, and the isolates obtained were analyzed for the target compounds by means of RP-HPLC/DAD, LC-MS, and NMR spectroscopy.

Caffeic acid ethyl ester: UV−vis (CH_3CN/water; pH 2.5), λ_{max} = 241, 297, 323; LC-MS (ESI^+), *m/z* 209 (100; $[M + 1]^+$), 163 (53; $[M - C_2H_5 - H_2O + 1]^+$), 181 (20; $[M - C_2H_5 + 1]^+$); ^1H NMR (400 MHz; CD_3OD), δ 1.36 [t, 3H, H-C(2′)], 4.23 [dd, 2H, H-C(1′)], 6.26 [d, 1H, H-C(2)], 6.79 [d, 1H, H-C(8)], 6.96 [dd, 1H, H-C(9)], 7.05 [d, 1H, H-C(5)], 7.55 [d, 1H, H-C(3)]; ^{13}C NMR (100 MHz; CD_3COD), δ 13.3 [C(2′)], 60.1 [C(1′)], 113.6 [C(5)], 113.9 [C(2)], 115.0 [C(8)], 121.5 [C(9)], 126,4 [C(4)], 145.3 [C(3)], 145.4 [C(7)], 148.1 [C(6)], 176.9 [C(1)].

p-Coumaric acid ethyl ester: UV−vis (CH_3CN/water; pH 2.5), λ_{max} = 234, 323; LC-MS (ESI^+), *m/z* 193 (100; $[M + 1]^+$), 147 (53; $[M - C_2H_5 - H_2) + 1]^+$), 165 (21; $[M - C_2H_5 + 1]^+$); ^1H NMR (400 MHz; CD_3OD), δ 1.35 [t, 3H, H-C(2′)], 4.23 [dd, 2H, H-C(1′)], 6.33 [d, 1H, H-C(2)], 6.82 [d, 2H, H-C(6), H-C(8)], 7.47 [d, 2H, H-C(5), H-C(9)], 7.62 [d, 1H, H-C(3)]; ^{13}C NMR (100 MHz; CD_3COD), δ 13.2 [C(2′)], 60.0 [C(1′)], 113.9 [C(2)], 115.4 [C(6), C(8)], 125.8 [C)4)], 129.7 [C(5), C(9)], 144.9 [C(3)], 159.9 [C(7)], 167.9 [C(1)].

Syringic acid ethyl ester: UV−vis (CH_3CN/water; pH 2.5), λ_{max} = 275; LC-MS (ESI^+), *m/z* 227 (100; $[M + 1]^+$), 199 (28; $[M - C_2H_5 + 1]^+$); ^1H NMR (400 MHz; DMSO-d_6), δ 1.31 [t, 3H, H-C(2′)], 3.81 [s, 6H, H-C(1″), H-C(2″)], 4.28 [dd, 2H, H-C(1′)], 7.21 [s, 2H, H-C(3), H-C(7)]; ^{13}C NMR (100 MHz; DMSO), δ 14.8 [C(2′)], 56.5 [C(1″), C(2″)], 60.9 [C(1′)], 107.1 [C(3), C(7)], 119.9 [C(2)], 141.0 [C(5)], 147.9 [C(4), C(6)], 166.0 [C(1)].

Identification of Flavan-3-ols. Analytical HPLC analysis of the GAC fractions revealed that the taste compounds detected in HPLC fractions B-12 and B-14 by means of HPLC/TDA were present in GAC fractions X and XI. Samples (200 mg) of the individual fractions were dissolved in 1% aqueous HCOOH (20 mL), and after membrane filtration, aliquots (2 mL) of the solution were used for preparative RP-HPLC. The chromatographic column was eluted consecutively with a solution of 1% aqueous HCOOH and CH_3CN (95:5, v/v), then increasing the CH_3CN content to 17% within 35 min, keeping the CH_3CN content constant for 15 min, thereafter increasing the CH_3CN content to 100% within 5 min, monitoring the eluent at 272 nm with a UV–vis detector. After the most active astringent compounds had been located by means of HPLC degustation, individual peaks were collected in several runs and the corresponding eluates were combined and freeze-dried. Spectroscopic data (UV−vis, LC-MS, NMR) of the taste compounds were identical with those measured for the corresponding reference compounds. Finally, the identity of the taste compounds as (+)-catechin (HPLC fraction B-10), (−)-epicatechin (HPLC fraction B-13), procyanidin B1 (HPLC fraction B-10), procyanidin B2 (HPLC fraction B-12), procyanidin B3 (HPLC fraction B-1), and procyanidin C1 (HPLC fraction B-14) was confirmed by cochromatography with the reference compounds.

(+)-Catechin: UV−vis (CH_3CN/water, pH 2.5), λ_{max} = 237, 277; LC-MS (ESI^+), *m/z* 291 (100; $[M + H]^+$), 598 (35; $[M_2 + H + H_2O]^+$); LC-MS (ESI^-), *m/z* 289 (100; $[M]^-$).

(−)-Epicatechin: UV−vis (CH_3CN/water, pH 2.5), λ_{max} = 237, 277; LC-MS (ESI^+), *m/z* 291 (100; $[M + H]^+$), 598 (35; $[M_2 + H + H_2O]^+$); LC-MS (ESI^-), *m/z* 289 (100; $[M]^-$).

Procyanidin B1: UV−vis (CH_3CN/water; pH 2.5), λ_{max} = 236, 278; LC-MS (ESI^+), *m/z* 579 (100, $[M + 1]^+$), 601 (39, $[2M + Na]^+$); LC-MS (ESI^-), *m/z* 577 (100; $[M]^-$).

Procyanidin B2: UV−vis (MeOH/water; pH 2.5), λ_{max} = 236, 278; LC-MS (ESI^+), *m/z* 579 (100, $[M + 1]^+$), 601 (39, $[2M + Na]^+$); LC-MS (ESI^-), *m/z* 577 (100; $[M]^-$).

Procyanidin B3. UV−vis (CH_3CN/water; pH 2.5), λ_{max} = 236, 277; LC-MS (ESI^+), *m/z* 579 (100, $[M + 1]^+$), 601 (40, $[2M + Na]^+$); LC-MS (ESI^-), *m/z* 577 (100; $[M]^-$).

Procyanidin C1: UV−vis (CH_3CN/water; pH 2.5), λ_{max} = 236, 278; LC-MS (ESI^+), *m/z* 867 (100, $[M + 1]^+$), 889 (45, $[M + Na]^+$); LC-MS (ESI^-), *m/z* 865 (100; $[M]^-$).

Identification of Flavon-3-ol- and Dihydroflavon-3-ol Glycosides. Analytical HPLC analysis of the GAC fractions revealed that the taste compounds detected in HPLC fractions B-19, B-21, B-23, and B-25 by means of HPLC/TDA were present in GAC fractions VII and VIII. Preparative HPLC, followed by UV−vis, LC-MS/MS, and 1D/2D NMR spectroscopy led to the identification of the key astringent compounds as quercetin-3-*O*-β-D-galactopyranoside (B-19), 2*R*,3*R*-dihydroquercetin-3-*O*-α-L-rhamnoside (B-21), 2*R*,3*R*-dihydrokaempferol-3-*O*-α-L-rhamnoside (B-23), quercetin-3-*O*-β-D-glucuropyranoside (B-21), isorhamnetin-3-*O*-β-D-glucopyranoside (B-23), and syringetin-3-*O*-β-D-glucopyranoside (B-25) in the HPLC fractions given in parentheses. The latter three substances were confirmed by cochromatography with the corresponding reference compound.

Quercetin-3-O-β-D-galactopyranoside: UV−vis (CH_3CN/water; pH 2.5), λ_{max} = 215, 251, 355; LC-MS (ESI^+), *m/z* 465 (100; $[M + 1]^+$), 303 (53; $[M - gal + 1]^+$); ^1H and ^{13}C NMR data were identical with those measured for the reference compound.

Isorhamnetin-3-O-β-D-glucopyranoside: UV−vis (CH_3CN/water; pH 2.5), λ_{max} = 254, 350; LC-MS (ESI^+), *m/z* 479 (100; $[M + 1]^+$), 317 (45; $[M - glc + 1]^+$); ^1H and ^{13}C NMR data were identical with those measured for the reference compound.

Syringetin-3-O-β-D-glucopyranoside: UV−vis (CH_3CN/water; pH 2.5), λ_{max} = 252, 355; LC-MS (ESI^+), *m/z* 509 (100; $[M + 1]^+$), 523 (60; $[M + Na]^+$), 347 (20; $[M - glc + 1]^+$); ^1H and ^{13}C NMR data were identical with those measured for the reference compound.

Quercetin-3-O-β-D-glucuropyranoside: UV−vis (CH_3CN/water; pH 2.5), λ_{max} = 255, 350; LC-MS (ESI^-), *m/z* 477 (100; $[M - 1]^-$), 301 (20; $[M - glu - 1]^-$); ^1H NMR (400 MHz; CD_3COD), δ 3.46 [m, 1H, H-C(3″)], 3.53 [m, 1H, H-C(2″)], 3.58 [dd, 1H, H-C(4″)], 3.74 [d, 1H, H-C(5″)], 5.34 [d, J = 7.6 Hz 1H, H-C(1″)], 6.20 [d, 1H, H-C(6)], 6.39 [d, 1H, H-C(8)], 6.84 [d, 1H, H-C(5′)], 7.62 [dd, 1H, H-C(6′)], 7.64 [d, 1H, H-C(2′)]; ^{13}C NMR (100 MHz; CD_3COD), δ 71.7 [C(4″)], 74.2 [C(2″)], 75.9 [C(5″)], 76.4 [C(3″)], 93.5 [C(8)], 98.7 [C(6)], 103.1 [C(1″)], 104.5 [C(10)], 114.5 [C(5′)], 116.0 [C(2′)], 121.7 [C(1′)], 122.3 [C(6′)], 134.2 [C(3)], 144.8 [C(3′)], 148.7 [C(4′)], 157.2 [C(9)], 157.9 [C(2)], 161.9 [C(5)], 164.8 [C(7)], 171.2 [C(6″)], 178.1 [C(4)].

2R,3R-Dihydrokaempferol-3-O-α-L-rhamnoside, engelitin: UV−vis (CH_3CN), λ_{max} = 290; LC-MS (ESI^+), *m/z* 435 (19; $[M + 1]^+$), 457 (100; $[M + Na]^+$), 311 (10; $[M - rha + Na]^+$), 289 (5; $[M - rha + 1]^+$); ^1H NMR (400 MHz; CD_3COD), δ 1.09 [d, 3H, H-C(6″)], 3.26

[dd, 1H, H-C(4″)], 3.40 [dd, 1H, H-C(2″)], 3.55 [dd, 1H, H-C(3″)], 3.91 [d, J = 1.6 Hz, 1H, H-C(1″)], 4.16 [m, 1H, H-C(5″)], 4.52 [d, 1H, H-C(3)], 5.04 [d, 1H, H-C(2)], 5.79 [d, 1H, H-C(6)], 5.82 [d, 1H, H-C(8)], 6.75 [d, 2H, H-C(3′), H-C(5′)], 7.25 [d, 2H, H-C(2′), H-C(6′)]; ^{13}C NMR (100 MHz; CD_3COD), δ 16.2 [C(6″)], 68.9 [C(5″)], 69.1 [C(2″)], 70.8 [C(3″)], 72.1 [C(4″)], 76.8 [C(3)], 82.7 [C(2)], 94.4 [C(6)], 95.9 [C(8)], 100.4 [C(10)], 100.9 [C(1″)], 127.1 [C(2′), C(6′)], 128.4 [C(3′), C(5′)], 127.7 [C(1′)], 157.8 [C(4′)], 162.9 [C(9)], 165.9 [C(7)], 167.4 [C(5)], 194.2 [C(4)].

2R,3R-Dihydroquercitin-3-O-α-L-rhamnoside, astilbin: UV−vis (CH_3CN/water; pH 2.5), λ_{max} = 290; LC-MS (ESI^+), m/z 473 (100; $[M + Na]^+$), 923 (49; $[2M + Na]^+$), 451 (34; $[M + 1]^+$), 305 (19; $[M - rha + 1]^+$); ^{1}H NMR (400 MHz; CD_3COD), δ 1.19 [d, 3H, H-C(6″)], 3.32 [dd, 1H, H-C(4″)], 3.56 [m, 1H, H-C(2″)], 3.67 [dd, 1H, H-C(3″)], 4.08 [d, J = 1.1 Hz 1H, H-C(1″)], 4.27 [m, 1H, H-C(5″)], 4.61 [d, 1H, H-C(3)], 5.10 [d, 1H, H-C(2)], 5.91 [d, 1H, H-C(6)], 5.94 [d, 1H, H-C(8)], 6.82 [d, 1H, H-C(5′)], 6.86 [dd, 1H, H-C(6′)], 6.97 [d, 1H, H-C(2′)]; ^{13}C NMR (100 MHz; CD_3COD), δ 16.3 [C(6″)], 69.0 [C(5″)], 70.2 [C(2″)], 70.6 [C(3″)], 72.3 [C(4″)], 77.1 [C(3)], 82.4 [C(2)], 94.6 [C(6)], 96.3 [C(8)], 100.9 [C(10)], 105.4 [C(1″)], 113.9 [C(2′)], 114.4 [C(5′)], 118.9 [C(6′)], 127.8 [C(1′)], 145.1 [C(3′)], 145.9 [C(4′)], 162.9 [C(9)],166.5 [C(7)], 167.2 [C(5)], 194.5 [C(4)].

Identification of Phenolic Acids and Their Esters. Analytical HPLC analysis of the individual GAC fractions, followed by UV−vis, LC-MS/MS, and 1D/2D NMR spectroscopy, led to the identification of a series of astringent phenolic acids as well as their esters. Spectroscopic data of the taste compounds caftaric acid, caffeic acid, *p*-coumaric acid, syringic acid, protocatechuic acid, gallic acid, vanillic acid, gallic acid ethyl ester, vanillic acid ethyl ester, protocatechuic acid ethyl ester, ferulic acid ethyl ester, caffeic acid ethyl ester, *p*-coumaric acid ethyl ester, and syringic acid ethyl ester were identical with those measured for the corresponding reference compounds. The structures of these compounds were confirmed by cochromatography with commercially available reference compounds or with synthetic compounds.

HPLC/TDA of Fraction C. An aliquot (500 mg) of fraction C was dissolved in 0.1% aqueous HCOOH (10 mL; pH 2.5) by means of an ultrasonic bath. After membrane filtration, aliquots (250 μL) were analyzed by semipreparative RP-HPLC/DAD. With the effluent monitored at 272 nm, chromatography was performed starting with 0.1% aqueous HCOOH, then increasing the CH_3CN content to 17% within 35 min, keeping the CH_3CN content constant for additional 15 min, then raising the CH_3CN content to 100% within 15 min and maintaining this solvent for 5 min. The effluent was separated into 17 fractions, namely, C-1–C-17, which were individually collected into ice-cooled brown glass vials. The corresponding fractions obtained from 25 HPLC runs were combined and solvents evaporated under vacuum. After lyophilization, fractions were used for TDA and qualitative studies.

Identification of Organic and Inorganic Acids. Formic acid, acetic acid, lactic acid, (*E*)-aconitic acid, (*Z*)-aconitic acid, glutaric acid, tartaric acid, succinic acid, malic acid, citric acid, isocitric acid, galacturonic acid, hydrochloric acid, and phosphoric acid were identified in HPLC fraction C-1 by means of ion exchange chromatography using a Bio-LC system (Dionex) following the standard protocol (*36*). The chromatographic system consisted of a GS 50 gradient pump with an AS50 autosampler, an AS50 thermal compartment, and an ED50 detector and was operated with a Dionex IonPac AS11-HC column (250 × 2 mm i.d.) and a Dionex IonPac AG11-HC (50 × 2 mm i.d.) guard column. Analysis of data was performed with Chromeleon software v 6.60 SP4.

Isolation of Polymeric Fraction by Means of Ultrafiltration. An aliquot (250 mL) of the red wine was placed into a VIVACELL 250 static gas pressure filtration system (Vivascience) equipped with a 5 kDa molecular weight cutoff VIVACELL 250 5000 MWCO PES membrane (Vivascience). After sealing, a nitrogen pressure of 4 bar was applied using an air pressure controller. During filtration VIVACELL 250 was moved on a type 3005 GFL laboratory shaker (GFL) with 200 rpm at room temperature. After filtration, the retentate was taken up with a 15% aqueous EtOH solution (3 × 100 mL). The solubilized retentates as well as the filtrate were separately freed of ethanol under vacuum and freeze-dried to afford the low molecular mass fraction UF5 (<5 kDa) and the high molecular mass fraction UR5 (>5 kDa), respectively. For sensory analysis, these materials were solubilized in "natural" concentrations in a 15% aqueous EtOH solution.

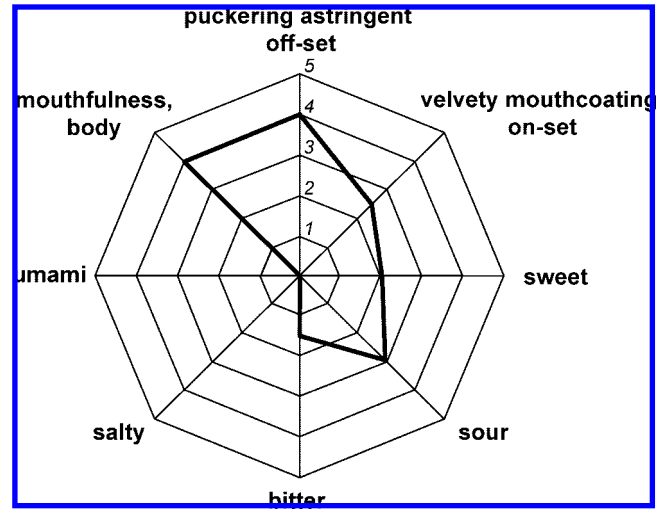


Figure 1. Taste profile of Amarone red wine.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a PU 1580 type pump with a DG-1580-53 degasser, an LG-1580-02 low-pressure gradient unit, and an MD1515 diode array detector (DAD). Chromatographic separations were performed on stainless steel columns packed with ODS-Hypersil, 5 μm, RP-18 material (ThermoHypersil, Kleinostheim, Germany) either in an analytical (250 × 4.6 mm i.d., flow rate = 1.0 mL/min), a semipreparative (250 × 10 mm i.d., flow rate = 3.5 mL/min), or a preparative scale (250 × 21.2 mm i.d., flow rate = 20 mL/min).

Liquid Chromatography−Mass Spectrometry (LC-MS/MS). Spectra were acquired in turbo spray electrospray ionization (ESI) mode on an API 4000 Q-Trap LC-MS/MS system (AB Sciex Instruments, Darmstadt, Germany) connected to an HPLC system (Agilent 1100 series, Karlsruhe, Germany). Samples were dissolved in a mixture (50:50, v/v) of MeOH and 0.1% aqueous HCOOH and were analyzed by direct loop injection (2–20 μL) at a flow rate of 200 μL/min. The ion-spray voltage was set at −4500 V in the ESI^- mode and at +5500 V in the ESI^+ mode, and the temperature was set at 300 °C. Nitrogen served as the curtain gas (20 psi), gas 1 (35 psi), and gas 2 (40 psi). The declustering potential was set at −10 to −30 V in the ESI^- mode and at +60 V in the ESI^+ mode. The mass spectrometer was operated in the full-scan mode monitoring positive or negative ions. Fragmentation of $[M - H]^-$ and $[M + H]^+$ molecular ions into specific product ions was performed in enhanced product ion (EPI) mode induced by collision with nitrogen (4×10^{-5} Torr) with a collision energy of +30 V in the positive and −30 V in the negative mode. Analysis of mass spectroscopic data was performed by means of Analyst software v 1.4.1.

NMR Spectroscopy. The 1D/2D NMR experiments were performed on a Bruker DPX 400 spectrometer (Rheinstetten, Germany) using MeOH-d_4 or DMSO-d_6 as the solvent. Data processing was performed by using the NMR software Mestre-C v 1.4.

RESULTS AND DISCUSSION

Preliminary wine tasting involving wine taste experts revealed the Amarone della Valpolicella as an excellent red wine with a velvety mouthcoating onset and a balanced astringent offset accompanied by a mild bitter taste and pronounced mouthfulness and body. To evaluate the taste profile of that red wine on a scientific basis, a taste profile analysis using a five-point scale was performed (**Figure 1**). A high score of 4.0 was given for body/mouthfulness as well as for the puckering astringent offset, followed by sourness (3.0). The velvety mouthcoating onset,

Table 1. Yields and Sensorial Evaluation of Fractions A−C Isolated from Red Wine

	intensity[a] perceived for				
fraction[b]	astringency	bitterness	sourness	sweetness	mouthfulness/ body
A	0.3	0.2	0.0	0.0	0.0
B	1.8	1.3	2.2	0.2	0.0
C	3.4	0.4	3.3	1.0	4.0

[a] The taste intensity of aqueous solutions of the individual fractions isolated from 100 mL of Amarone red wine in bottled water is rated on a scale from 0 (not detectable) to 5.0 (strongly detectable). [b] Individual fractions contain the *n*-pentane solubles (A), ethyl acetate extractables (B), and water solubles (C) isolated from Amarone red wine.

perceived by the sensory panel in the first part of the sensation, was judged with an intensity of 2.5, closely followed by sweetness (2.0). In comparison, bitterness was perceived with a somewhat lower intensity of 1.5, whereas umami and salty tastes were not detected at all.

To gain first insight into the hydrophobicity of the compounds imparting the typical astringent as well as bitter taste sensation, the red wine was extracted sequentially with solvents of increasing polarity.

Solvent Fractionation of Red Wine. A red wine sample was extracted sequentially with *n*-pentane, followed by EtOAc, and the extracts obtained were freed from solvent under vacuum to give fractions A and B in yields of 0.1 and 4.6%, respectively. The remaining aqueous layer was freeze-dried to give the water-solubles (fraction C) accounting for >95% of the dry mass of the red wine (**Table 1**). Fractions A−C were individually dissolved in water (pH 4.5), each in natural concentration, and the solutions obtained were then sensorially evaluated. Whereas fraction A was found to be nearly tasteless, fractions B and C reflected taste impressions already found for the red wine, respectively (**Table 1**). Fraction C induced an puckering oral astringent sensation as well as sweetness in high intensities of 3.4 and 1.0, respectively. Although fraction B was judged with a somewhat lower score for total astringency (1.8), bitterness was perceived with high intensity (1.3). In addition, sourness was detected predominantly in fractions B and C, whereas body/ mouthfulness was exclusively perceived in fraction C. On the basis of the results of these sensory data, the following identification of astringent and bitter compounds was focused on fractions B and C.

Taste-Active Compounds in Fraction B. To separate the bulk of tasteless or less taste-active components from the intensely tasting compounds, fraction B was fractionated by means of RP-HPLC/DAD to give 30 fractions, namely, fractions B-1−B-30 (**Figure 2**). To evaluate their taste impact, these HPLC fractions were freeze-dried, taken up in water, and then analyzed by means of the TDA using the half-tongue test (*33*–*35*). The highest taste impact was located in fraction B-2 judged with a TD factor of 256 for astringency, followed by fractions B-6, B-12, and B-14 evaluated with a TD factor of 128 (**Table 2**). Fractions B-16 and B-29 still showed an astringent oral sensation after a dilution of 1:32, and fractions B-1, B-11, B-21, B-25, and B-27 were taste-active up to a dilution of 1:16. Besides astringency, sourness was detected in fractions B-1–B-8, and a bitter taste quality was found for fractions B-14, B-27, and B-29 evaluated with a TD factor of 4 (**Table 2**).

To gain a first insight into the compounds inducing the taste impression in the most active HPLC fractions, the fractions evaluated with the highest TD factors for astringency and bitterness were investigated by means of HPLC-MS operating in the ESI^+ mode. Fractions B-2 and B-6, both judged with high TD factors for astringency, each contained only a single component showing the pseudomolecular ions *m*/*z* 171 and 155, respectively. Upon comparison of the UV−vis, LC-MS, and NMR spectroscopic data with those found for the reference compound, the key tastants in fractions B-2 and B-6 were

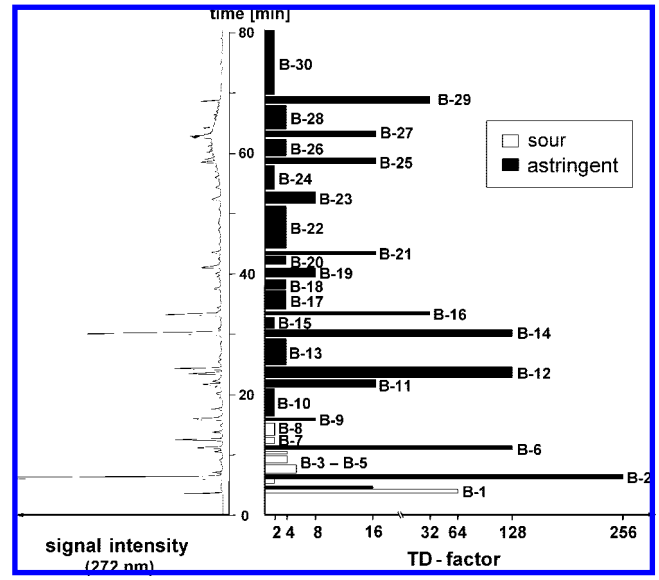


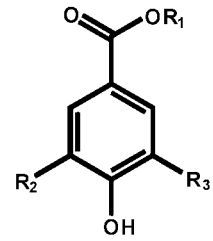
Figure 2. RP-HPLC chromatogram (left side) and taste dilution analysis (right side) of fraction B isolated from red wine.

Table 2. Yields, Taste Qualities and Taste Dilution (TD) Factors of HPLC Fractions Isolated from Amarone Red Wine Fraction B

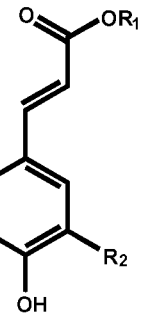
fraction[a]	taste quality[b]	TD factor[b]	compounds identified[c]
B-1	sour	64	
	puckering astringent	16	
B-2	puckering astringent	256	**1**
	sour	2	
B-3	sour	4	
B-4	sour	4	
B-5	sour	4	
B-6	puckering astringent	128	**6**
B-7	sour	2	
B-8	sour	2	
B-9	puckering astringent	8	**16**
B-10	puckering astringent	2	**23**, **24**, **25**
B-11	puckering astringent	16	**11**
B-12	puckering astringent	128	**3**, **4**, **5**
B-13	puckering astringent	4	**26**
B-14	puckering astringent	128	**6**, **7**
	bitter	4	**6**, **7**
B-15	velvety astringent	2	
B-16	puckering astringent	32	**8**
B-17	puckering astringent	4	
B-18	puckering astringent	4	
B-19	velvety astringent	8	**22**
B-20	velvety astringent	4	
B-21	puckering astringent	16	**12**, **13**, **14**
B-22	puckering astringent	4	
B-23	velvety astringent	8	**17**, **18**
B-24	puckering astringent	2	
B-25	velvety astringent	16	**15**
B-26	puckering astringent	4	
B-27	puckering astringent	16	**19**, **20**, **21**
	bitter	4	**19**, **20**, **21**
B-28	velvety astringent	4	
B-29	puckering astringent	32	**9**, **10**
	bitter	4	**9**, **10**
B-30	puckering astringent	2	

[a] Number of HPLC fraction referring to **Figure 2**. [b] The taste quality and TD factor were determined by using a half-tongue duo test. [c] The structures of the compounds given as numbers are displayed in **Figure 3**.

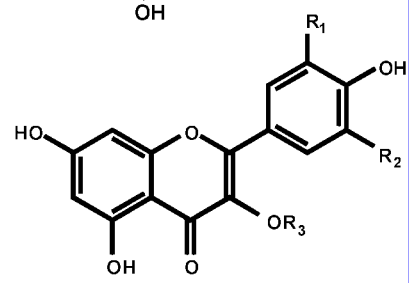
hydroxybenzoic acids	R_1	R_2	R_3
gallic acid (**1**)	H	OH	OH
gallic acid ethyl ester (**6**)	C_2H_5	OH	OH
protocatechuic acid (**2**)	H	OH	H
protocatechuic acid ethyl ester (**14**)	C_2H_5	OH	H
vanillic acid (**11**)	H	OCH_3	H
vanillic acid ethyl ester (**20**)	C_2H_5	OCH_3	H
syringic acid (**4**)	H	OCH_3	OCH_3
syringic acid ethyl ester (**21**)	C_2H_5	OCH_3	OCH_3



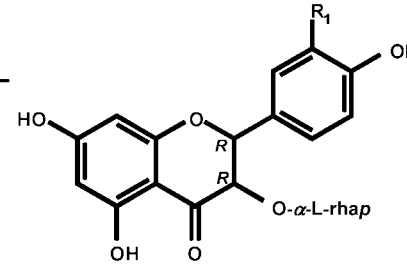
hydroxycinnamic acids	R_1	R_2
caffeic acid (**3**)	H	OH
caffeic acid ethyl ester (**19**)	C_2H_5	OH
p-coumaric acid (**8**)	H	H
p-coumaric acid ethyl ester (**10**)	C_2H_5	H
ferulic acid ethyl ester (**9**)	C_2H_5	OCH_3
(*E*)-caftaric acid (**16**)	tartaric acid	OH



flavonol glycosides	R_1	R_2	R_3
quercitin-3-*O*-ß-D-glu*p* (**12**)	OH	H	glucuronide
isorhamnetin-3-*O*-ß-D-glc*p* (**17**)	OCH_3	H	glucoside
syringetin-3-*O*-ß-D-glc*p* (**15**)	OCH_3	OCH_3	glucoside
quercitin-3-*O*-ß-D-gal*p* (**22**)	OH	H	galactoside



dihydroflavonol glycosides	R1
dihydroquercetin-3-*O*-*a*-L-rha*p* (**13**)	OH
dihydrokaempferol-3-*O*-*a*-L-rha*p* (**18**)	H



flavan-3-ols/procyanidins	R_1	R_2	R_3
(+)-catechin (**23**)	H	OH	H
(-)-epicatechin (**26**)	OH	H	H
procyanidin B1 (**24**)	OH	H	(4ß→8)-(+)-catechin
procyanidin B2 (**5**)	OH	H	(4ß→8)-(-)-epicatechin
procyanidin B3 (**25**)	H	OH	(4ß→8)-(+)-catechin
procyanidin C1 (**7**)	OH	H	[(4ß→8)-(-)-epicatechin]$_2$

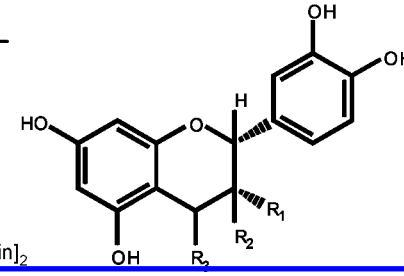


Figure 3. Chemical structures of phenolic taste compounds identified in Amarone red wine.

identified as gallic acid (*1*) and protocatechuic acid (*2*), respectively (**Figure 3**).

The astringent fraction B-12, judged with a TD factor of 128, was more complex and contained three individual compounds. Rechromatography by means of RP-HPLC revealed two components showing the mass transitions *m/z* 181 → 163 and *m/z* 199 → 155, respectively, and were identified as caffeic acid (*3*) and syringic acid (*4*) by comparison of the chromatographic, spectroscopic, and sensory data with those obtained for the reference compounds (**Figure 3**), thus confirming earlier literature reports (*37*). The third component of fraction B-12 showed a pseudomolecular ion of *m/z* 579 in the ESI^+ mode, thus suggesting the presence of a procyanidin. By comparison of spectroscopic (LC-MS/MS, UV−vis), chromatographic, and sensory data with those obtained for the reference compound, this compound was unequivocally identified as procyanidin B2 (*5*) (**Figure 3**), fitting well with data reported in the literature (*38*).

Due to its high TD factor (**Table 2**), HPLC fraction B-14 was investigated next. LC-MS analysis of this fraction revealed the presence of two compounds, one showed a pseudomolecular ion of *m/z* 199 in the ESI^+ mode, whereas the second compound exhibited *m/z* 865 as the $[M - H]^-$ ion in the ESI^- mode. Fragmentation of the ion *m/z* 199 resulted in a daughter ion with *m/z* 171 corresponding to the $[M + 1]^+$ ion expected for gallic acid. The loss of 28 amu as well as the galloyl-type

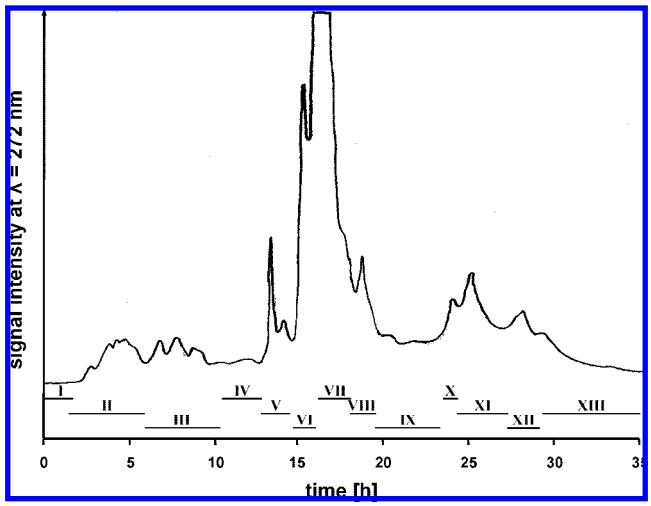


Figure 4. Gel adsorption chromatography of fraction B isolated from red wine.

UV−vis spectrum of this compound suggested this astringent and bitter compound to be gallic acid ethyl ester (*6*) (**Figure 3**). This was confirmed by comparison of spectroscopic and chromatographic data, followed by cochromatography with the reference compound. Although this compound was recently identified in red wine (*39*), its bitter taste quality has not been reported so far. In addition, the second taste compound eluting in fraction B-14 was identified as the trimeric procyanidin C1 (*7*) by comparison with the reference compound (**Figure 3**), thus confirming earlier reports in the literature (*40*).

Judged with a TD factor of 32, the astringent compound in fraction B-16 was analyzed by means of LC-MS/MS, NMR, and UV−vis spectroscopy, as well as by sensory analysis. On the basis of comparison of the data obtained with those found for the reference compounds, this astringent compound was identified as *p*-coumaric acid (*8*) (**Figure 3**), which was identified in red wine already 50 years ago (*41*).

Furthermore, fraction B-29, evaluated as astringent and bitter, was analyzed by means of LC-MS. By using the ESI^+ mode, two compounds were detected showing pseudomolecular ions of *m*/*z* 193 and 223, respectively. Fragmentation of both of these pseudomolecular ions revealed a neutral loss of 28 amu, thus implying the presence of one ethyl ester moiety. After isolation and purification, the taste compound LC-MS/MS and NMR data revealed the structure of the two taste compounds as ferulic acid ethyl ester (*9*) and *p*-coumaric acid ethyl ester (*10*) (**Figure 3**). The identity of both compounds was confirmed by comparison of spectroscopic and chromatographic data with those obtained for the corresponding reference compound. Although the *p*-coumaric acid ethyl ester has already been isolated from red and white wines (*42, 43*) and ferulic acid ethyl ester has been identified in white wines (*44*), the bitter taste quality of both esters has not been reported so far.

Evaluated with a TD factor of 16, fraction B-11 was investigated next. LC-MS experiments revealed a pseudomolecular ion with *m*/*z* 169 $[M + 1]^+$ and a daughter ion with *m*/*z* 151, most likely due to the cleavage of one molecule of water. To isolate suitable amounts for NMR spectroscopic structure elucidation, fraction B was fractionated in a preparative scale by means of GAC on Sephadex LH 20 to give the fractions I–VIII (**Figure 4**). By comparison of spectroscopic data and chromatographic data, this taste compound was identified to be present in high amounts in GAC fraction V (**Figure 5**). After isolation, NMR as well as LC-MS analysis identified the

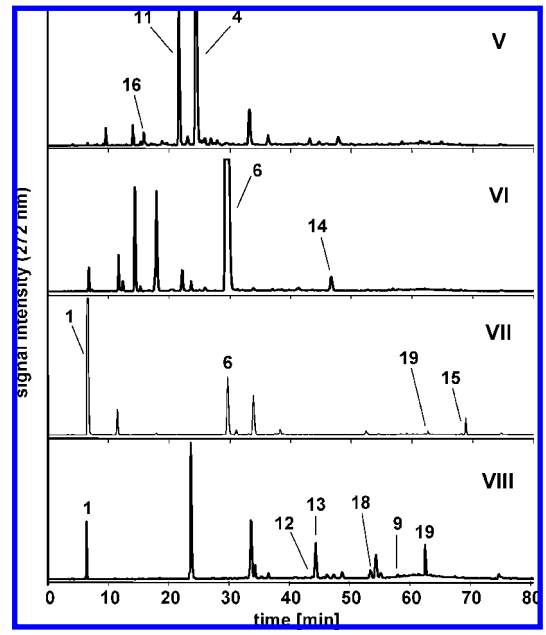


Figure 5. RP-HPLC chromatograms of GAC fractions V−VIII isolated from red wine fraction B.

astringent compound as vanillic acid (*11*) (**Figure 3**), well-known as a red wine constituent (*39*).

The identification of the astringent compounds in fraction B-21 seemed to be more challenging. LC-MS analysis of the key astringent compound revealed several ions. In the ESI^+ mode, the predominant ion detected showed m/z_1 451, followed by m/z_2 183. In addition, the ion m/z_3 477 was detected in the ESI^- mode. By HPLC degustation as well as comparison of the LC-MS data obtained, two compounds, exhibiting pseudomolecular ions with *m*/*z* 451 $[M + 1]^+$ and *m*/*z* 477 $[M - 1]^-$, respectively, were found to be enriched in GAC fraction VIII (**Figure 5**). After purification by means of analytical RP-HPLC, the structure of both compounds was elucidated by means of NMR spectroscopy. Fragmentation of the minor compound, showing *m*/*z* 477 $[M - 1]^-$ in the ESI^- mode, revealed a daughter ion of *m*/*z* 301 as expected for a quercetin aglycone. This was supported by the adsorption maxima observed at 255 and 350 nm in the UV−vis spectrum. Also, NMR data confirmed the presence of a quercetin aglycon as well as a glycoside moiety, but the methylene proton signals expected for H-C(6″) of the hexose moiety were lacking. Due to the unusually high ^{13}C chemical shift of 171.2 ppm observed for carbon C(6″) as well as the neutral loss of 176 amu upon MS/MS analysis, the taste compound was identified as quercetin-3-*O*-*β*-D-glucuropyranoside (**12**) (**Figure 3**). Although this glucuronic acid derivative was identified earlier (*45*), its astringent activity was not reported so far. The major compound, exhibiting the pseudomolecular ions *m*/*z* 451 $[M + 1]^+$ and *m*/*z* 473 $[M + Na^+]^+$ in the LC-MS spectrum, showed a rather different UV−vis spectrum with only one absorption maximum centering around 290 nm. Fragmentation of the pseudomolecular ion $[M + 1]^+$ resulted in a daughter ion with *m*/*z* 305, indicating the cleavage of one molecule of rhamnose. The difference of 2 amu between the aglycone of this taste compound and quercetin suggested a dihydroquercetin as the aglycon. 1D/2D NMR experiments and comparison of the spectroscopic data with those reported earlier in the literature (*46*) enabled the determination of the structure of that astringent compound as 2*R*,3*R*-dihydroquercitin-3-*O*-α-L-rhamnoside (*13*), known as astilbin (**Figure 3**) (*46*). The third compound present in fraction B-21 was

detected in GAC fraction VI (**Figure 5**). After isolation, NMR, LC-MS/MS, and UV−vis analyses resulted in the identification of that taste compound as protocatechuic acid ethyl ester (*14*) (**Figure 3**). Although this ester has already been identified in wines (*47*), its bitter taste quality has not been reported so far.

Fraction B-25, evaluated with a TD factor of 16 for astringency, showed UV−vis absorption maxima at 252 and 355 nm and two pseudomolecular ions with *m/z* 509 $[M + 1]^+$ and 523 $[M + Na^+]^+$, respectively, indicating a molecular mass of 508 Da. LC-MS/MS (ESI^+) experiments revealed a neutral loss of 162 amu between the parent ion *m/z* 509 and the daughter ion *m/z* 347 as expected for the cleavage of one molecule of hexose. HPLC-DAD and HPLC degustation identified the same tastant to be present in GAC fraction VII (**Figure 5**), from which the target compound could be isolated in amounts suitable for NMR experiments. The ^{1}H NMR data revealed two methoxy groups located at a flavonol-like aglycone. By means of homo- (gCOSY) and heteronuclear (HMBC) chemical shift correlation experiments, the taste compound was identified as syringetin-3-*O*-β-D-glucopyranoside (**15**; **Figure 3**). Although this compound has already been detected in red wine (*48*), the astringent activity of this glycoside has not been reported so far. Furthermore, quercetin-3-*O*-β-D-galactopyranoside (**22**; **Figure 3**) was identified as the velvety astringent compound detected in fraction B-19, thus confirming data published earlier for Spanish wines (*49*).

The astringent fraction B-9, evaluated with a TD factor of 8, showed a pseudomolecular ion of *m/z* 311 in the LC-MS (ESI^-) spectrum. Detection of the same compound in GAC fraction V (**Figure 5**), followed by isolation, NMR analysis, and comparison of the spectroscopic and chromatographic data with those observed for the reference compound, led to the identification of the taste-active substance in fraction B-9 as caftaric acid (**16**; **Figure 3**), the taste contribution of which was declined to be of interest in former studies (*50*).

HPLC-MS analysis of the astringent fraction B-23 gave two compounds, **17** and **18**, showing the pseudomolecular ions *m/z* 479 and *m/z* 435, respectively. MS/MS analysis of compound **17** indicated the cleavage of one molecule of hexose (162 amu) from *m/z* 479 to give the daughter ion *m/z* 317, thus indicating the presence of isorhamnetin as aglycone. Finally, cochromatography and comparison of UV−vis spectra as well as MS data identified this substance as isorhamnetin-3-*O*-β-D-glucopyranoside (**17**; **Figure 3**), thus confirming earlier literature reports (*51*). The astringent compound detected with the pseudomolecular ion *m/z* 435 showed a loss of 146 amu to give the daughter ion *m/z* 289, thus demonstrating the presence of a molecule of rhamnose. In addition, this compound exhibited a UV−vis spectrum which was rather similar to that observed for astilbin. Assuming this compound to be a dihydroflavonol rhamnoside, ^{1}H NMR analysis of the compound, isolated from GAC fraction VIII (**Figure 5**), showed two strongly coupling protons resonating at 5.04 and 4.52 ppm, respectively, similar to those found in the NMR spectrum of astilbin (**Figure 6**). Careful interpretation of all NMR data obtained led to the identification of this taste compound as 2*R*,3*R*-dihydrokaempferol-3-*O*-α-L-rhamnoside (**18**; **Figure 3**), known as engelitin. Although engelitin has been identified earlier in grapes as well as white wines (*46, 52*), this compound has not been previously reported as a constituent of red wine, nor has its taste activity been reported before.

Fraction B-27 was evaluated as one of three bitter-tasting HPLC fractions. LC-MS/MS analysis revealed three compounds exhibiting the pseudomolecular ions *m/z* 227, *m/z* 197, and *m/z* 209, respectively, and upon a neutral loss of 28 amu the daughter ions *m/z* 199, 179, and 181, thus suggesting the presence of ethyl esters. ^{1}H NMR analysis of the main compound **19**, isolated from GAC fractions VII and VIII (**Figure 5**), showed a triplet at 1.36 ppm integrating for three protons and coupling with a double duplet resonating at 4.23 ppm, thus confirming the ethyl ester function in the molecule. In addition, NMR spectroscopy showed the typical signal pattern expected for caffeic acid and allowed the identification of that taste compound as caffeic acid ethyl ester. Furthermore, spectroscopic analysis of the other two compounds eluting in fraction B-27 led to syringic acid ethyl ester and vanillic acid ethyl ester as the proposed structures. To verify the identity of these ethyl esters, vanillic acid ethyl ester (**20**; **Figure 3**) and syringic acid ethyl ester (**21**; **Figure 3**) were synthesized, and the spectroscopic, chromatographic, and sensory data were compared to those obtained from the isolates. On the basis of the identity of the data obtained, the three bitter compounds detected in fraction B-27 were unequivocally identified as the ethyl esters of caffeic acid (**19**), vanillic acid (**20**), and syringic acid (**21**). Although compounds **19** (*53–55*), **20** (*56*), and **21** (*57*) have already been identified in wine as well as wine vinegar, the bitter taste activity of these esters was not known.

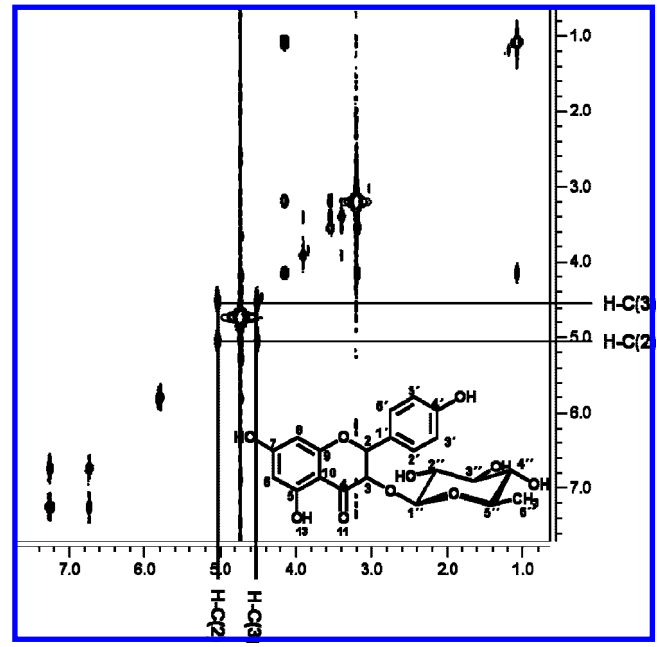


Figure 6. COSY NMR spectrum (400 MHz, MeOD) of 2*R*,3*R*-dihydrokaempferol-3-*O*-α-L-rhamnoside (**18**).

As literature studies (*19*) reported on the flavan-3-ol monomers (+)-catechin and (−)-epicatechin as well as the dimeric procyanidins B1 and B3, respectively, as important taste compounds in red wine, additionally the HPLC fractions B-1−B-30 were screened for these compounds by means of HPLC-MS/MS using the corresponding reference compounds for MS tuning experiments. This MS-based screening led to the identification of (+)-catechin (**23**), procyanidin B1 (**24**), and procyanidin B3 (**25**) in fraction B-10 and (−)-epicatechin (**26**; **Figure 3**) in fraction B-13. However, it is interesting to note that the flavan-3-ol-containing fractions B-10 and B-13 were not evaluated as tasting bitter by means of the TDA. In contrast, the bitter-tasting fractions B-14, B-27, and B-29 were found to contain the bitter-tasting ethyl esters of hydroxybenzoic and hydroxycinnamic acids.

Taste-Active Compounds in Fraction C. To locate the most taste-active compounds in the water-soluble fraction isolated

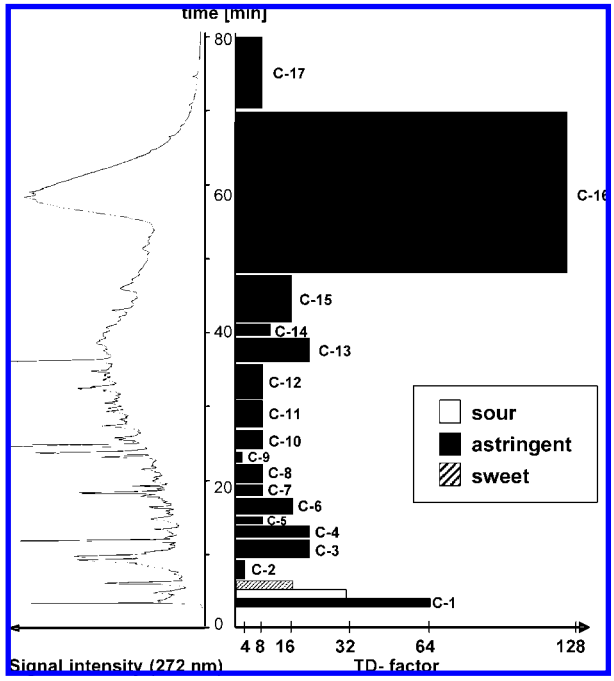


Figure 7. RP-HPLC chromatogram (left side) and taste dilution analysis (right side) of red wine fraction C.

from red wine, fraction C was separated by means of RP-HPLC and the TDA was applied onto the collected fractions C-1−C-17 (**Figure 7**). Interestingly, only two fractions, namely, fractions C-1 and C-16, were evaluated with TD factors above 16. Fraction C-16 was judged with a TD factor of 128 for astringency, and fraction C-1 was evaluated with TD factors of 64, 32, and 16 for astringency, sourness, and sweetness, respectively.

Analysis of fraction C-1 by means of HPLC-DAD as well as ion chromatography revealed a broad spectrum of 14 organic acids and inorganic salts, namely, formic acid, acetic acid, lactic acid, (*E*)-aconitic acid, (*Z*)-aconitic acid, glutaric acid, tartaric acid, succinic acid, malic acid, citric acid, isocitric acid, galacturonic acid, chloride, and phosphate. Sensory analysis revealed that, among these organic acids, exclusively (*E*)/(*Z*)-aconitic acid (**27**) induced a strongly puckering astringent sensation.

Fraction C-16, exhibiting a rather shrinking and puckering type of astringency, exhibited a UV−vis spectrum which was similar to that of procyanidins. As LC-MS/MS experiments did not reveal any reliable results, this fraction was suggested to be composed of polymeric structures. To gain further insight into the molecular weight of the components inducing the puckering taste sensation, an aliquot of fraction C was separated by means of ultrafiltration using a molecular weight cutoff of 5 kDa. The low molecular weight fraction (LMW) and the fraction containing high molecular weight components (HMW, >5 kDa) were freeze-dried and, after the residues had been dissolved in 15% aqueous EtOH in their "natural" concentration, were evaluated by means of a taste profile analysis (**Table 3**). The panelists judged the LMW fraction to exhibit the entire sweetness of the wine judged with a score of 2.0. Furthermore, sourness, bitterness, and mouthfulness/body induced by the LMW fraction were evaluated with only slightly lower intensities when compared to the red wine. The astringency imparted by the LMW fraction was judged with a rather high intensity of 3.1, but the quality was described to be more velvety and silky when compared to the HMW fraction, which was evaluated as more puckering astringent with an intensity of 3.7. The

Table 3. Sensorial Evaluation of Fractions Isolated by Means of Ultrafiltration from Amarone Red Wine

	intensity[b] perceived for				
sample[a]	astringency	bitterness	sourness	sweetness	mouthfulness/body
red wine	4.0	1.5	3.0	2.0	4.0
LMW fraction[a]	3.1	1.5	2.7	2.0	3.7
HMW fraction[c]	3.7	0.2	0.3	0.0	0.3

[a] Low molecular weight fraction (<5 kDa) isolated from red wine by means of ultrafiltration. [b] High molecular weight fraction (≥5 kDa) isolated from red wine by means of by ultrafiltration. [c] The taste intensity of solutions of the individual fractions in 15% aqueous EtOH (50 mL, pH 4.5) was rated on a scale from 0 (not detectable) to 5.0 (strongly detectable).

mouthfulness/body as well as the other taste qualities bitterness, sourness, and sweetness either were evaluated with rather low scores or were not detectable at all. These data clearly demonstrate that besides the LMW compounds identified by means of HPLC-TDA, also the HMW components contribute to the astringent taste of red wine, thus fitting well with literature data (*11–13, 18*).

Sensory Activity of Taste-Active Compounds. Prior to sensory analysis, the purity of all compounds was checked by HPLC-MS as well as ^{1}H NMR spectroscopy. To determine the human threshold concentrations for bitter taste and the astringent oral sensation, aqueous solutions of the target compound were evaluated by means of the three-alternative forced-choice test (*35*) and half-tongue test (*33–35*), respectively. As given in **Table 4**, the individual taste compounds have been grouped into three classes: a group of velvety astringent compounds, a group of puckering astringent compounds, and a group of bitter and astringent compounds, respectively.

Among the velvety astringent compounds, syringetin-3-*O*-*β*-D-glucopyranoside (**15**) and quercitin-3-*O*-*β*-D-galactopyranoside (**22**) were evaluated with the lowest recognition threshold concentrations of 0.2 and 0.4 μmol/L, followed by the other flavon-3-ol and dihydroflavon-3-ol glycosides **12**, **17**, **13**, and **18** with about 10-fold higher taste thresholds. The low threshold concentrations of these velvety astringent compounds are well in line with those reported for other flavon-3-ol glycosides (*33, 34*).

The group of puckering astringent compounds contained various hydroxybenzoic acids, cinnamic acid derivatives, and the polymeric fraction (UR5) isolated from red wine, as well as (*E*)/(*Z*)-aconitic acid (**Table 4**). The lowest taste thresholds were found for the aconitic acid inducing an astringent sensation already at the low concentration of 0.5 μmol/L, whereas its typical sour taste was perceived not below a concentration of 500 μmol/L. All of the other compounds in that group were significantly less taste active, and vanillic acid was judged with the highest taste threshold of 315 μmol/L. The threshold found for the polymeric fraction was 22.0 mg/L, which is in the range of the low molecular weight phenols **1**−**4** (**Table 4**).

The group of compounds inducing a bitter and astringent oral sensation consisted of monomeric and dimeric flavan-3-ols and various hydroxybenzoic acid and cinnamic acid ethyl esters (**Table 4**). The human threshold concentrations for the astringency of these flavan-3-ols ranged from 200 to 930 μmol/L and, with the exception of (−)-epicatechin, were always below the recognition thresholds determined for their bitter taste. The astringent taste threshold decreased from the flavan-3-ol monomers (+)-catechin and (−)-epicatechin over the dimeric procyanidins B1, B2, and B3, to the trimeric procyanidin C1, thus being well in line with data published on procyanidins from cocoa (*Theobroma cocoa* L.) (*34*). The bitter taste thresholds

Table 4. Human Taste Recognition Thresholds of Compounds Isolated from Red Wine

	taste threshold[b] for			
	astringency		bitterness	
compound[a] (no.)	μmol/L	mg/L	μmol/L	mg/L
Velvety Astringent Compounds				
dihydrokaempferol-3-*O*-α-L-rha*p* (**18**)	4.8	2.1	nd	nd
dihydroquercitin-3-*O*-α-L-rha*p* (**13**)	3.7	1.7	nd	nd
isorhamnetin-3-*O*-β-D-glc*p* (**17**)	2.4	1.1	nd	nd
quercetin-3-*O*-β-D-glcA*p* (**12**)	2.0	1.0	nd	nd
quercetin-3-*O*-β-D-gal*p* (**22**)	0.4	0.2	nd	nd
syringetin-3-*O*-β-D-glc*p* (**15**)	0.2	0.1	nd	nd
Puckering Astringent Compounds				
vanillic acid (**11**)	315	53	nd	nd
gallic acid (**1**)	292	50	nd	nd
syringic acid (**4**)	263	52	nd	nd
protocatechuic acid (**2**)	206	32	nd	nd
p-coumaric acid (**8**)	139	23	nd	nd
ferulic acid	67	13	nd	nd
caffeic acid (**3**)	72	13	nd	nd
(*E*)-caftaric acid (**16**)	16	5	nd	nd
(*Z*)/(*E*)-aconitic acid (**27**)[c]	0.5	0.1	nd	nd
polymeric fraction (>5 kDa)		22	nd	nd
Bitter and Astringent Compounds				
(−)-epicatechin (**26**)	930	270	930	270
(+)-catechin (**23**)	410	119	1000	290
procyanidin C1 (**7**)	300	260	400	347
caffeic acid ethyl ester (**19**)	277	58	1100	229
procyanidin B1 (**24**)	240	139	400	231
procyanidin B3 (**25**)	200	116	500	289
procyanidin B2 (**5**)	190	110	485	280
gallic acid ethyl ester (**6**)	185	37	2200	438
p-coumaric acid ethyl ester (**10**)	143	27	715	137
vanillic acid ethyl ester (**20**)	125	25	1500	294
ferulic acid ethyl ester (**9**)	67	15	710	158
protocatechuic acid ethyl ester (**14**)	49	9	1000	182
syringic acid ethyl ester (**21**)	18	4	576	130

[a] The structures of the compounds are displayed in **Figure 3**. [b] Taste threshold concentrations were determined in bottled water by means of a triangle test for bitterness and by means of the half-tongue test for astringency. [c] Taste threshold for sour is 500 μmol/L.

of these procyanidins were about 2-fold below the values found for the flavan-3-ol monomers.

The sensory properties of phenolic acid ethyl esters were described as primarily puckering astringent and were evaluated with taste thresholds ranging from 18 to 185 μmol/L; for example, syringic acid ethyl ester was found with the lowest astringency threshold of 18 μmol/L in that group. It is interesting to note that in contrast to the procyanidins the bitter taste thresholds of all the phenolic acid ethyl esters were significantly higher than their astringency threshold concentrations; for example, the bitter threshold for protocatechuic acid ethyl ester was about 20 times above the astringency threshold of that compound (**Table 4**).

In conclusion, the data obtained by application of a sensomics approach give strong evidence that velvety astringent and puckering astringent as well as bitter and astringent compounds contribute to the typical astringent and bitter taste of red wine. In contrast to previous studies; most surprisingly, the procyanidin monomers, dimers, and trimers seem not to be the key elicitors of the astringent taste of the red wine, but a variety of different low molecular weight polyphenols as well as a polymeric fraction were found with highest taste impacts. Quantitative studies as well as taste reconstruction and omission experiments in a wine-like matrix are currently ongoing to get a lead on the importance of the individual substance classes for wine taste and, in particular, the different kinds of astringency perceived.

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