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Compositional and Sensory Characterization of Red Wine Polymers

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Supporting Information

ABSTRACT: After isolation from red wine by means of ultrafiltration and gel adsorption chromatography, the composition of the highly astringent tasting high-molecular weight polymers was analyzed by means of HPLC−MS/MS, HPLC−UV/vis, and ion chromatography after thiolytic, alkaline, and acidic depolymerization and, on the basis of the quantitative data obtained as well as model incubation experiments, key structural features of the red wine polymers were proposed. The structural backbone of the polymers seems to be comprised of a procyanidin chain with (−)-epicatechin, (+)-catechin, (−)-epicatechin-3-*O*-gallate units as extension and terminal units as well as (−)-epigallocatechin as extension units. In addition, acetaldehyde was shown to link different procyanidins at the A-ring via an 1,1-ethylene bridge and anthocyanins and pyranoanthocyanins were found to be linked to the procyanidin backbone via a C−C-linkage at position C(6) or C(8), respectively. Alkaline hydrolysis demonstrated the polymeric procyanidins to be esterified with various organic acids and phenolic acids, respectively. In addition, the major part of the polysaccharides present in the red wine polymeric fraction were found not to be covalently linked to procyanidins. Interestingly, sensory evaluation of individual fractions of the red wine polymers did not show any significant difference in the astringent threshold concentrations, nor in the astringency intensity in supra-threshold concentrations and demonstrated the mean degree of polymerization as well as the galloylation degree not to have an significant influence on the astringency perception.

KEYWORDS: *red wine, polymers, procyanidins, thiolysis, acetaldehyde, astringency*

INTRODUCTION

Besides sourness, sweetness, and bitterness, the astringent mouthfeel is one of the prime oro-sensations perceived during consumption of red wine.[1,2] Molecular knowledge on the chemical structures of the key components imparting astringent sensation is considered a prerequisite for tailoring the taste by means of a knowledge-based optimization of wine manufacturing.[3]

In order to determine the key players driving the attractive taste of a red wine on a molecular level, we recently applied the so-called sensomics approach to red wines.[4,5] Whereas the velvety astringent on-set was imparted by three flavon-3-ol glucosides and dihydroflavon-3-ol rhamnosides, the puckering astringent lingering orosensation was caused by a polymeric fraction exhibiting molecular weights >5 kDa. Very recently, sensomics analysis of the key odor and taste compounds in a Dornfelder red wine, followed by full flavor re-engineering and omission experiments demonstrated that this polymeric fraction was not only impacting the astringent perception but also affecting the perception of volatile aroma compounds.[6]

It is well accepted in literature that the oral astringency is perceived less puckering and that the color changes from pink-red hues to more brick-red hues upon wine storage.[7−9] Various analytical methods have been used to get some semiquantitative data on proanthocyanidins and to separate high-molecular-weight polymers from wines.[10−13] Moreover, targeted proanthocyanidin depolymerization by means of thiolysis or phloroglucinolysis gave some first insights into the composition of red wine polymers.[14,15] This analytical approach was complemented by constructive model experiments targeted toward understanding of the chemical reactions leading to polymer formation under wine-like conditions.[16−19] As acetaldehyde was found to react promptly and to covalently bridge flavanols and anthocyanins, this aldehyde is assumed to be an important polymerization agent in wines.[20,21]

A series of investigations tried to gain an insight into the relationship between proanthocyanidin composition and its bitterness as well as astringency perception, but the data are rather contradictory. For example, the astringency of synthesized procyanidins was reported to increase with chain length whereas the bitterness decreased.[2,22] Sensory evaluation of extracted tannins from grape seeds and skins showed an increase of astringency perception with degree of polymerization.[22] Furthermore, the degree of galloylation was reported to have no influence on the overall astringency but the chalky and coarse grain perception of astringency correlated with galloylation and was significantly higher in tannins with a higher degree of galloylation.[23] In comparison, no significant difference was found in the astringency of compositionally divers grape seed and skin extracts differing in their degree of polymerization and galloylation.[1,22] Moreover, differences in astringency perception of red wine fractions were reported to be mainly due to different overall amounts of tannins.[1,24]

Up to now, comprehensive investigations on the correlation of the composition of red wine polymers with its sensory impact are rather fragmentary. The objectives of the present study were, therefore, (i) to fractionate the polymers isolated

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from red wine, (ii) to perform a compositional analysis on the polymer fractions after hydrolytic depolymerization, (iii) to investigate their recognition thresholds and impact of astringency by means of human sensory analysis, and (iv) to study the role of acetaldehyde-mediated flavan-3-ol oligomerization in wine polymer formation.

MATERIALS AND METHODS

Chemicals. The following reference compounds were obtained commercially: acetaldehyde, arabinose, ascorbic acid, benzyl mercaptan, caffeic acid, (+)-catechin hydrate, cochineal red A, ethylenediaminetetraacetic acid, *p*-coumaric acid, (-)-epicatechin, D-fructose, D-galactose, galacturonic acid monohydrate, gallic acid, gentisic acid, glycerol, D-glucose, *p*-hydroxy benzoic acid, lactic acid, malic acid, D-mannose, succinic acid, syringic acid, and vanillic acid were from Sigma-Aldrich (Taufkirchen, Germany); ethanol abs., hydrochloric acid (32%, 1 N), sulfuric acid (98%), and sodium hydroxide were from Merck (Darmstadt, Germany); (−)-epicatechin-gallate, (−)-epigallocatechin, L(+)-tartaric acid were from Carl Roth (Karlsruhe, Germany); (+)-catechin, cyanidin-3-*O*-glucoside chloride, malvidin-3-*O*-glucoside chloride, peonidin-3-*O*-glucoside chloride were from Extrasynthese (Genay Cedex, France). Solvents were of HPLC- or LC-MS grade (J.T. Baker, Deventer, Netherland), water for chromatographic separations was purified with a Milli-Q Advantage A10 system (Millipore, Molsheim, France). Deuterated NMR solvents were from Euroiso-top (Giv-sur-Yvette, France). The red wine used for the study was a Bordeaux wine (12.5% ethanol by volume, vintage 2003) from Chateau German (Appellation Côtes de Castillon Contrôlée, France). The grapes used for this wine were a mixture of Merlot, Cabernet Sauvignon and Cabernet Franc. The wine was aged in oak barrels for 12 months. Fresh must from red Dornfelder grapes was obtained from a German wine grower in Rheinhessen. Deoiled grape seed powder (oil content 5−10%, approximately 80 μm, topfruits Naturprodukte, Germany) was purchased from a local organic supermarket.

Isolation of High-Molecular Weight Polymers from Red Wine. The polymeric fraction was isolated from red wine by means of ultrafiltration using a VIVACELL 250 static gas pressure filtration system equipped with a 5 kDa molecular weight cutoff VIVACELL 250 5000 MWCO PES membrane (Vivascience, Göttingen, Germany) precisely following the protocol reported recently.[6] The retentate was lyophilized to afford the high-molecular weight polymers (HMW >5 kDa) in a yield of 4.5 g/L. The polymers were kept at −18 °C until used for further experiments.

Gel Adsorption Chromatography (GAC). An aliquot (800 mg) of the HMW polymers (>5 kDa) was dissolved in methanol/water (20/80, v/v; pH 4.5) acidified with traces of formic acid and, then, placed on top of a 100 × 5 cm XK50/100 glass column (GE Healthcare Bio-Science AB, Uppsala, Sweden) filled with a slurry of Sephadex LH 20 (GE Healthcare, Munich, Germany) in methanol/water (20/80, v/v; pH 4.5). Using a peristaltic pump (P-1 type, GE Healthcare Bio-Science AB, Uppsala, Sweden) operating at a flow rate of 1.8 mL/min, chromatography was performed by rinsing the water-cooled column sequentially with the methanol/water mixtures 20/80 (v/v; 7 h), 40/60 (v/v; 16 h), 60/40 (v/v; 7 h), 80/20 (v/v; 16 h), 100/0 (v/v; 16 h), followed by a mixture of acetone/water (70/30, v/v; pH 4.5) for 16 h. Monitoring the effluent at 272 nm by means of an UV/vis detector (UV-2075 plus, Jasco, Großumstadt, Germany), fractions were collected by means of a 7000 Ultrorac fraction collector (LKB, Bromma, Sweden) to give eight GAC fractions, namely I-XII, as displayed in Figure 2. Separation from solvent in vacuum and freeze-drying afforded the GAC fractions I (1.6 g/L), II (0.3 g/L), III (0.4 g/L), IV (0.5 g/L), V (0.7 g/L), VI (0.2 g/L), VII (0.4 g/L), and VIII (0.3 g/L) as amorphous powders in the yields given in parentheses (calc. as conc. in wine). Data processing was done using the LabVIEW Signal Express software (National Instruments, Munich, Germany).

Precipitation of Polysaccharides. Aliquots (50 mg) of polymeric fractions were dissolved in water (10 mL) at 80 °C, ethanol (96%, v/v, 50 mL) was added, and the mixture was kept overnight at room temperature. After separating the precipitate by centrifugation (10 min, 10000 rpm), isolated material was washed with ethanol/water (60/40, v/v; 2 × 20 mL) and, then, freeze-dried. Ethanol precipitation of the HMW (>5 kDa) fraction yielded 22 mg/100 mg of a pale gray polysaccharide fraction.

Thiolytic Depolymerization. *Analytical Scale.* Following a literature protocol,[14,25] an aliquot (0.5 mL) of methanolic hydrochloric acid (3.3% conc. HCl in MeOH) and an aliquot (1 mL) of a methanolic solution of benzyl mercaptan (5% in MeOH) were added to a solution (0.5 mL) of the polymeric fraction in methanol (4 mg/mL) in a brown glass vial. After flushing with nitrogen and sealing, the mixture was kept for 2 h at 40 °C while stirring. The mixture was then cooled to room temperature, solvents were removed with a stream of nitrogen, the residue was taken up in 250 μL (for HPLC-UV analysis) or 2 mL (for LC-MS/MS analysis) of methanol/water (30/70, v/v) and centrifuged prior to analysis. Analytical separation was done by means of HPLC-UV using the following gradient of 1% aqueous formic acid as solvent A and acetonitrile as solvent B: starting at 0% B, the content of B was increased to 10% within 10 min, to 45% within 55 min, to 100% within additional 5 min, then kept at 100% for 3 min, and, finally, decreased again to 0% within 5 min.

Semipreparative Scale. An aliquot (80 mg) of the polymer fraction and grape seed powder, respectively, were dissolved in methanol (2.5 mL), 2.5 mL methanolic hydrochloric acid (3.3% HCl in methanol) and 5 mL benzyl thiol (5% in methanol) were added and, after flushing with nitrogen and sealing, the mixtures was kept for 2 h at 40 °C. After cooling, the solvents were removed with a stream of nitrogen, the residue was taken up in methanol/water (30/70, v/v; 5 mL), centrifuged, and separated by means of semipreparative HPLC using the following gradient of 1% aqueous formic acid (solvent A) and methanol (solvent B): starting at 0% B, solvent B was increased to 20% within 5 min, to 50% within 15 min, to 60% within 20 min, to 100% within additional 5 min, then kept at 100% for 3 min, and, finally, decreased again to 0% within 5 min. Main reaction products detected at 272 nm were collected, separated from solvent in vacuum, freeze-dried, and identified in their chemical structure as epicatechin-4β-benzylthioether (**5**), epigallocatechin-4β-benzylthioether (**6**), catechin-4β-benzylthioether (**7a**), and epicatechin-3-*O*-gallat-4β-benzylthioether (**8**). LC-MS and 1D/2D-NMR data of **5**−**8** are given as Supporting Information.

Analysis of Carbohydrates and Glycerol After Acidic Hydrolysis. Using the Seaman procedure,[26] aliquots (5 mg) of polymeric fractions were mixed with an aliquot (0.82 g) of 72% (w/w) H_2SO_4, kept at room temperature for 2 h, then diluted with distilled water (5.8 g), and incubated at 100 °C for 2 h. After cooling in an ice-bath, the reaction mixture was neutralized with aqueous sodium hydroxide (1 mol/L) and placed on top of a water-conditioned C18 SPE-cartridge (1000 mg, Strata C18-E, 1000 mg, Phenomenex). After rinsing with water (15 mL), the effluent was made up to 20 mL with water and used for carbohydrate and polyol analysis. Aliquots (25 μL) were analyzed by means of HPIC using an already published method with pulsed amperometric detection.[6] By comparison of retention times and cochromatography with reference compounds of fructose, glucose, arabinose, galactose, rhamnose, mannose, xylose, and glycerol, the compounds were quantified using a 6-point external standard calibration. Galacturonic acid was quantified by analyzing aliquots (5 μL) of the obtained aqueous solutions with LC-MS/MS and external calibration according to literature.[6] For analysis of HMW fractions without acidic hydrolysis, aliquots (10 mg) of polymeric fractions were dissolved in 2 mL water, applied on top of a preconditioned C-18 SPE cartridge (1000 mg, Strata C18-E, Phenomenex), eluted with 6 mL water and, finally, the obtained aqueous fractions were filled up to 10 mL prior to HPIC and LC-MS/MS analysis, respectively.

Analysis of Amino Acids After Acidic Hydrolysis. Aliquots (5 mg) of polymeric fractions were dissolved in aqueous hydrochloric acid (6 mol/L; 3 mL) and heated for 24 h at 110 °C. After cooling and neutralizing with aqueous NaOH (12 mol/L), the solution was made up to 5 mL with water and used for quantitation using a stable isotope dilution analysis.[6,27] Aliquots (990 μL) of the solutions after hydrolysis

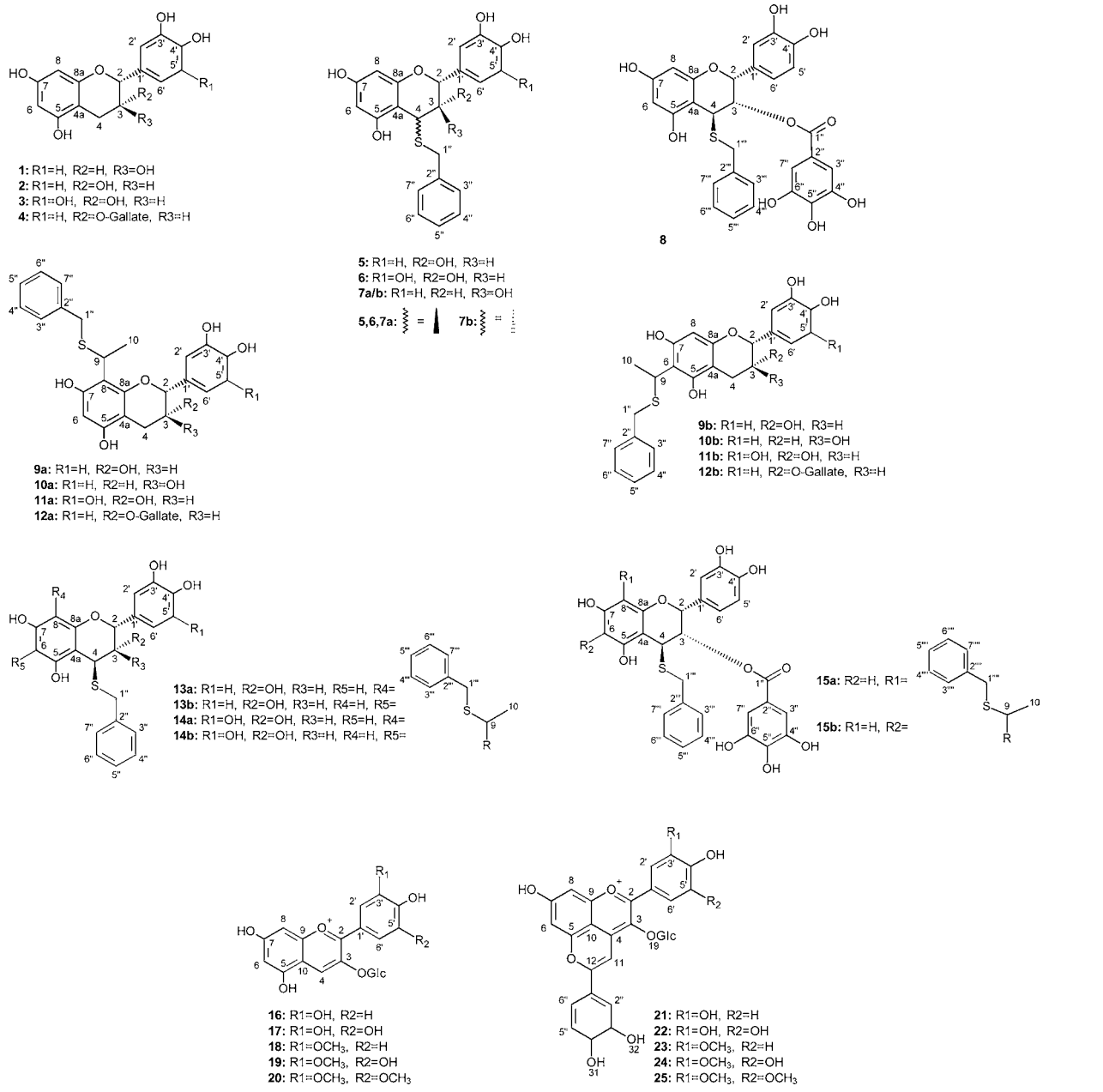

Figure 3. Chemical structures of compounds **1**−**25**.

were mixed with 10 μL of the internal standard and analyzed by LC-MS/MS.

Analysis of Flavan-3-ols, Organic Acids and Phenolic Acids after Alkaline Hydrolysis. Following a literature procedure with some modifications,[28] an aliquot (1 mL) of a solution of sodium hydroxide (8 g), ascorbic acid (1 g), and ethylenediaminetetraacetic acid (1 mg) in water (100 mL) was added to aliquots (10 mg) of the polymeric fractions and heated for 60 min at 40 °C under a nitrogen atmosphere while stirring. After neutralizing with aqueous hydrochloric acid (4 mol/L; 0.5 mL), the solutions were analyzed by means of RP-HPLC using a gradient of 1% aqueous formic acid (solvent A) and acetonitrile (solvent B): starting with 0% B, the content of solvent B was increased to 35% B within 17 min, to 50% within 15 min, to 100% within 5 min, then kept at 100% for additional 5 min, and, finally, decreased again to 0% within 5 min. By comparison of retention times and cochromatography with references, released phenolic acids and flavan-3-ols were identified.

Quantitative analysis of phenolic acids was performed by means of LC-MS/MS and external calibration as reported recently.[6] Flavan-3-ols were quantified by means of HPLC-UV at 272 nm using external calibration.[6] In addition to the solutions obtained after alkaline hydrolysis, blank solutions (2 mg/mL) of polymeric fractions in MeOH/water (20/80, v/v) were also analyzed.

An already published ion chromatography method was used for the identification of organic acids after alkaline hydrolysis.[6] Aliquots (25 μL) of the alkaline hydrolysates were analyzed by means of ion chromatography and concentrations of organic acids calculated using a 6-point external calibration curve of reference compounds. For analysis of HMW fractions without alkaline hydrolysis aliquots (10 mg) of polymeric fractions were dissolved in 2 mL water, applied on top of a preconditioned C-18 SPE cartridge (1000 mg, Strata, Phenomenex), eluted with 6 mL water and the aqueous fraction filled up to 10 mL with water prior to HPIC analysis.

Preparation of Pinotin A (25). According to literature,[29] a solution of malvidin-3-*O*-glucoside (1 mg) and caffeic acid (4 mg) in aqueous ethanol (15%, v/v; 2 mL) was adjusted to pH 3.0 with aqueous hydrochloric acid (1 mol/L), incubated at 40 °C for 4 days and, then, kept at room temperature for 3 months. Pinotin A (**25**) was

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isolated by RP-HPLC, LC-MS and 1D/2D-NMR experiments (in MeOD-d_3/TFA-d_1; 19/1, v/v) were identical to those reported in the literature.[30]

Preparation of Acetaldehyde-Bridged Flavan-3-ols and Identification of Thiolytic Cleavage Products (9a/b, 10a/b). An aliquot (2 mmol each) of (−)-epicatechin (**2**) or (+)-catechin (**1**) was dissolved in ethanol/water (13/87, v/v; 50 mL), the pH value was adjusted to 3.2 with acetic acid and, after the addition of acetaldehyde (20 mmol), the solution was kept under nitrogen at room temperature in the dark. After 7 days, the reaction mixture was separated by means of flash chromatography (column 150 × 40 mm i.d., filled with LiChroprep RP-18 material, 25−40 μm) using the following gradient of 0.1% aqueous formic acid (solvent A) and methanol (solvent B) at a flow rate of 40 mL/min: starting with 1% solvent B, the content of B was increased to 5% within 5 min, to 50% within 20 min, to 80% within additional 5 min, and, then, was kept constant for 5 min. The fractions containing the oligomerized flavan-3-ols were freeze-dried and further analyzed by means of MALDI-TOF-MS (Figure 5). Semipreparative thiolysis of the obtained material, followed by HPLC separation and LC-MS and NMR spectroscopic structure determination as described above revealed 8-*C*-(*R*/*S*)-(1-benzylthioethyl)-(−)-epicatechin (**9a**) and 6-*C*-(*R*/*S*)-(1-benzylthioethyl)-(−)-epicatechin (**9b**) as main cleavage products of the polymer prepared from **2** and 8-*C*-(*R*/*S*)-(1-benzylthioethyl)-(+)-catechin (**10a**) and 8-*C*-(*R*/*S*)-(1-benzylthioethyl)-(+)-catechin (**10b**) as main cleavage products of the polymer prepared from **1**.

*8-C-(R/S)-(1-Benzylthioethyl)-(−)-epicatechin, **9a**, Figure 3.* UV/vis (acetonitrile/1% HCOOH): λ_{max} = 244, 280 nm; LC-TOF-MS (ESI$^-$): found *m*/*z* 411.0906 (411.0902 calc. for $[C_{24}H_{23}O_6S]^-$); LC/MS (ESI$^-$): *m*/*z* (%) 439.0 (100, $[M-H]^-$), MS/MS: *m*/*z* (%) 314.9 (100), 150.7 (20), 122.8 (10), 108.9 (15); ^{1}H NMR (500 MHz, CD_3OD, COSY): δ (ppm) 1.54 [dd, 3H, *J* = 4.1, 7.2 Hz, H−C(10)], 2.77 [td, 1H, *J* = 2.6, 15.2 Hz, H−C(4α)], 2.90 [ddd, 1H, *J* = 4.7, 10.5, 15.3 Hz, H−C(4β)], 3.57 [d, 1H, *J* = 2.0 Hz, H−C(1″α)], 3.66 [dd, 1H, *J* = 13.1, 45.6 Hz, H−C(1″β)], 4.19 [d, 1H, *J* = 12.9 Hz, H−C(3)], 4.69 [dq, 1H, *J* = 1.9, 7.2 Hz, H−C(9)], 4.80 [d, 1H, *J* = 5.0 Hz, H−C(2)], 6.00 [d, 1H, *J* = 6.3 Hz, H−C(6)], 6.78 [m, 2H, H−C(5′), H−C(6′)], 6.97 [dd, 1H, *J* = 1.5, 6.6 Hz, H−C(2′)], 7.05 [m, 1H, H−C(5″)], 7.15−7.22 [m, 4H, H−C(4″), H−C(6″), H−C(3″), H−C(7″)]; ^{13}C NMR (125 MHz, CD_3OD): δ (ppm) 18.95/19.50 [C-10], 28.01/28.26 [C-4], 34.19/35.00 [C-9], 35.66/35.99 [C-1″], 65.64/65.90 [C-3], 78.54/78.58 [C-2], 95.43/95.77 [C-6], 98.90/99.07 [C-4a], 107.38/107.82 [C-8], 113.74/113.94 [C-2′], 114.48/114.53 [C-5′], 117.78/118.14 [C-6′], 126.12/126.14 [C-5″], 127.78/127.88 [C-4″, C-6″]/[C-3″, C-7″], 128.41/128.59 [C-4″, C-6″]/[C-3″, C-7″], 130.84/130.91 [C-1′], 138.92/138.15 [C-2″], 144.26/144.32/144.58 [C-3′, C-4′], 153.29/153.64 [C-8a], 155.07/155.22 [C-5, C-7].

*6-C-(R/S)-(1-Benzylthioethyl)-(−)-epicatechin, **9b**, Figure 3.* UV/vis (Acetonitrile/1% HCOOH): λ_{max} = 244, 280 nm; LC-TOF-MS (ESI$^-$): found *m*/*z* 439.1216 (439.1215 calc. for $[C_{24}H_{23}O_6S]^-$); LC/MS (ESI$^-$): *m*/*z* (%) 439.0 (100, $[M-H]^-$), MS/MS: *m*/*z* (%) 314.9 (100), 150.7 (20), 122.8 (10), 108.9 (15); ^{1}H NMR (500 MHz, CD_3OD, COSY): δ (ppm) 1.44 [dt, 3H, *J* = 6.6, 13.2 Hz, H−C(10)], 2.78 [ddd, 1H, *J* = 2.8, 16.8, 36.1 Hz, H−C(4α)], 2.88 [td, 1H, *J* = 4.5, 16.7 Hz, H−C(4β)], 3.57 [dd, 2H, *J* = 6.0, 11.9 Hz, H−C(1″)], 4.20 [m, 1H, H−C(3)], 4.70 [qd, 1H, *J* = 4.2, 7.2 Hz, H−C(9)], 4.92 [d, 1H, *J* = 6.6 Hz, H−C(2)], 6.03 [d, 1H, *J* = 7.8 Hz, H−C(8)], 6.76 [dd, 1H, *J* = 3.5, 8.1 Hz, H−C(5′)], 6.81 [td, 1H, *J* = 1.9, 8.0 Hz, H−C(6′)], 6.98 [dd, 1H, *J* = 1.9, 10.1 Hz, H−C(2′)], 7.21 [m, 5H, H−C(3″), H−C(4″), H−C(5″), H−C(6″), H−C(7″)]; ^{13}C NMR (125 MHz, CD_3OD): δ (ppm) 19.97/20.04 [C-10], 29.35/29.62 [C-4], 36.14/36.28 [C-9], 36.73/36.94 [C-1″], 67.46/67.49 [C-3], 79.88/79.93 [C-2], 96.49/96.57 [C-8], 101.40 [C-4a], 108.25/108.33 [C-6], 115.39 [C-2′], 115.94/115.95 [C-5′], 119.40/119.46 [C-6′], 127.87/127.93 [C-5″], 129.35/129.42 [C-4″, C-6″]/[C-3″, C-7″], 129.98/130.04 [C-4″, C-6″]/[C-3″, C-7″], 132.23/132.30 [C-1′], 139.33/139.73 [C-2″], 145.89/145.03 [C-3′, C-4′], 155.80 [C-7], 156.07/156.17 [C-8a], 162.08 [C-5].

*8-C-(R/S)-(1-Benzylthioethyl)-(+)-catechin, **10a**, Figure 3.* UV/vis (Acetonitrile/1% HCOOH): λ_{max} = 244, 280 nm; LC-TOF-MS (ESI$^-$): found *m*/*z* 439.1216 (439.1215 calc. for $[C_{24}H_{23}O_6S]^-$); LC/MS (ESI$^-$): *m*/*z* (%) 439.0 (100, $[M-H]^-$), MS/MS: *m*/*z* (%) 314.9 (100), 150.7 (20), 122.8 (10), 108.9 (15); ^{1}H NMR (500 MHz, CD_3OD, COSY): δ (ppm) 1.46 [t, 3H, *J* = 7.1 Hz, H−C(10)], 2.52 [ddd, 1H, *J* = 2.5, 8.6, 16.2 Hz, H−C(4α)], 2.91 [ddd, 1H, *J* = 2.5, 5.7, 16.3 Hz, H−C(4β)], 3.60 [m, 2H, H−C(1″)], 3.90 [dtd, 1H, *J* = 5.6, 8.3, 28.3 Hz, H−C(3)], 4.50 [dd, 1H, *J* = 7.9, 11.7 Hz, H−C(2)], 4.56 [dq, 1H, *J* = 3.7, 6.9 Hz, H−C(9)], 5.98 [d, 1H, *J* = 3.5 Hz, H−C(6)], 6.69 [dd, 1H, *J* = 2.0, 8.1 Hz, H−C(6′)], 6.76 [m, 1H, H−C(5′)], 6.86 [dd, 1H, *J*=1.8, 9.8 Hz, H−C(2′)], 7.15 [m, 5H, H−C(4″), H−C(5″), H−C(6″), H−C(3″), H−C(7″)]; ^{13}C NMR (125 MHz, CD_3OD): δ (ppm) 20.50/20.85 [C-10], 29.17/29.44 [C-4], 35.75/36.28 [C-9], 37.16/37.41 [C-1″], 68.84/69.04 [C-3], 83.14 [C-2], 96.86/97.09 [C-6], 101.32/101.35 [C-4a], 108.77/109.21 [C-8], 115.52/115.71 [C-2′], 116.06/116.12 [C-5′], 120.24/120.28 [C-6′], 127.58/127.61 [C-5″], 129.24/129.29 [C-4″, C-6″]/[C-3″, C-7″], 129.95/129.96 [C-4″, C-6″]/[C-3″, C-7″], 132.26/132.30 [C-1′], 140.52 [C-2″], 146.21/146.22/146.26/146.29 [C-3′, C-4′], 154.56/154.64 [C-8a], 156.03/156.08 [C-5, C-7].

*6-C-(R/S)-(1-Benzylthioethyl)-(+)-catechin, **10b**, Figure 3.* UV/vis (Acetonitrile/1% HCOOH): λ_{max} = 244, 280 nm; LC-TOF-MS (ESI$^-$): found *m*/*z* 439.1216 (439.1215 calc. for $[C_{24}H_{23}O_6S]^-$) ; LC/MS (ESI$^-$): *m*/*z* (%) 439.0 (100, $[M-H]^-$), MS/MS: *m*/*z* (%) 314.9 (100), 150.7 (20), 122.8 (10), 108.9 (15); ^{1}H NMR (500 MHz, CD_3OD, COSY): δ (ppm) 1.45 [d, 3H, *J* = 7.2 Hz, H−C(10)], 2.51 [ddd, 1H, *J* = 8.1, 16.1, 20.0 Hz, H−C(4α)], 2.85 [ddd, 1H, *J* = 5.4, 16.1, 17.6 Hz, H−C(4β)], 3.58 [m, 2H, H−C(1″)], 3.99 [dtd, 1H, *J* = 5.4, 7.8, 20.0 Hz, H−C(3)], 4.58 [dd, 1H, *J* = 7.5, 43.0 Hz, H−C(2)], 4.69 [dq, 1H, *J* = 7.3, 9.8 Hz, H−C(9)], 5.97 [d, 1H, *J* = 5.9 Hz, H−C(8)], 6.73 [ddd, 1H, *J* = 1.9, 8.3, 10.4 Hz, H−C(6′)], 6.77 [dd, 1H, *J* = 5.0, 8.1 Hz, H−C(6′)], 6.84 [dd, 1H, *J* = 1.9, 14.0 Hz, H−C(2′)], 7.19 [m, 5H, H−C(3″), H−C(4″), H−C(5″), H−C(6″), H−C(7″)]; ^{13}C NMR (125 MHz, CD_3OD): δ (ppm) 18.57/18.59 [C-10], 26.89/27.48 [C-4], 34.79/34.91 [C-9], 35.44/35.52 [C-1″], 67.30/67.42 [C-3], 81.31/81.46 [C-2], 94.67/94.68 [C-8], 100.63/100.78 [C-4a], 106.74/106.78 [C-6], 113.73/113.96 [C-2′], 114.68/114.70 [C-5′], 118.49/118.74 [C-6′], 126.37/126.45 [C-5″], 127.88/127.92 [C-4″, C-6″]/[C-3″, C-7″], 128.49/128.54 [C-4″, C-6″]/[C-3″, C-7″], 130.70/130.79 [C-1′], 138.30/138.37 [C-2″], 144.86/144.93 [C-3′, C-4′], 154.12/154.24 [C-8a], 154.44/154.47 [C-5], 154.66/154.70 [C-7].

Preparation of Acetaldehyde-Bridged Grape Seed Procyanidins and Identification of Thiolytic Cleavage Products (13a/b, 15a/b). A mixture of grape seed powder (5 g) and acetaldehyde (1 mL) in ethanol/water (30/70, v/v; 50 mL) was adjusted to pH 3.2 with acetic acid and, then, kept under nitrogen at room temperature in the dark. After 7 days, the mixture was filtered, solvents were separated in vacuum, followed by freeze-drying. An aliquot (1 g) of the residue was dissolved in methanol (10 mL) and, after adding methanolic hydrochloric acid (3.3% HCl in methanol; 10 mL) and a methanolic solution (20 mL) of benzyl mercaptan (5% in methanol), the mixture was kept for 2 h at 40 °C. Thereafter, the solution was concentrated to about 10 mL with a stream of nitrogen, water (20 mL) was added and, after centrifugation, the supernatant was separated by means of flash chromatography (150 × 40 mm i.d., filled with LiChroprep RP-18, 25−40 μm) using the following gradient of aqueous 0.1% formic acid (solvent A) and methanol (solvent B) at a flow rate of 40 mL/min: starting with 20% B for 2 min, the content of solvent B increased to 50% within 18 min, then to 70% within 5 min, to 100% within 5 min, and was, finally, kept at 100% for additional 5 min. The fraction containing **13a/b** and **15a/b** was collected, separated from solvent in vacuum, and purified by means of semipreparative HPLC using the following gradient of aqueous 0.1% formic acid (solvent A) and acetonitrile (solvent B): starting with 45% B for 1 min, the content of solvent B was increased to 58% within 10 min, to 100% with 1 min, and was, then, kept at 100% for additional 2 min. After freeze-drying, the structures of 6/8-*C*-(*R*/*S*)-(1-benzylthioethyl)-(−)-epicatechin 4β-benzylthioether (**13a/b**) and 6/8-*C*-(*R*/*S*)-(1-benzylthioethyl)-

(−)-epicatechin-3-*O*-gallate 4*β*-benzylthioether (**15a/b**) were determined by means of LC-MS and 1D/2D-NMR experiments.

*6/8-C-(R/S)-(1-Benzylthioethyl)-(−)-epicatechin 4β-Benzylthioether, **13a/b**, Figure 3.* UV/vis (acetonitrile/1% HCOOH): λ_{max} = 232, 280 nm; LC-TOF-MS (ESI^-): found *m/z* 561.1415 (561.1406 calc. for $[C_{31}H_{29}O_6S_2]^-$); LC/MS (ESI^-): *m/z* (%) 561.1 (100, $[M - H]^-$), MS/MS: *m/z* (%) 437.0 (65), 313.1 (90), 161.0 (30), 150.8 (100), 107.0 (20); ^{1}H NMR (500 MHz, CD_3OD, COSY): *δ* (ppm) 1.52 [dd, 3H, *J* = 7.2, 14.1 Hz, H−C(10)], 3.62 [m, 2H, H−C(1‴)], 3.85 [ddd, 1H, *J* = 1.1, 2.5, 21.7 Hz, H−C(3)], 3.98 [d, 2H, *J* = 10.4 Hz, H−C(1″)], 4.07 [t, 1H, *J* = 2.2 Hz, H−C(4)], 4.64 [dq, 1H, *J* = 7.2, 29.0 Hz, H−C(9)], 5.21 [d, 1H, *J* = 18.1 Hz, H−C(2)], 6.01 [d, 1H, *J* = 6.3 Hz, H−C(6)], 6.70 [dd, 1H, *J* = 6.1, 1.7 Hz, H−C(6′)], 6.75 [dd, 1H, *J* = 1.4, 8.1 Hz, H−C(5′)], 6.91 [m, 1H, H−C(2′)], 7.16 [m, 5H, H−C(3‴), H−C(4‴), H−C(5‴), H−C(6‴), H−C(7‴), H−C(5″)], 7.31 [m, 2H, H−C(7″), H−C(3″)], 7.43 [m, 2H, H−C(4″), H−C(6″)]; ^{13}C NMR (125 MHz, CD_3OD): *δ* (ppm) 18.78/19.56 [C-10], 33.89/35.01 [C-9], 35.65/36.12 [C-1‴], 36.61/36.68 [C-1″], 42.75/42.88 [C-4], 69.90/69.98 [C-3], 74.24/74.46 [C-2], 95.79/96.05 [C-6], 99.03/99.06 [C-4a], 107.05 [C-8], 113.71/113.97 [C-2′], 114.52 [C-5′], 117.67/118.05 [C-6′], 126.16/126.18 [C-5‴], 126.54/126.55 [C-5″], 127.82/127.85/128.12/128.15/128.45/128.49 [C-4‴, C-6‴]/[C-3″, C-7″]/[C-3‴, C-7‴], 128.59/128.66 [C-4″, C-6″], 130.67/130.71 [C-1′], 139.06/139.10 [C-2″, C-2‴], 144.25/144.36/144.59/144.62 [C-3′, C-4′], 152.89/152.97 [C-8a], 155.99/156.06 [C-5, C-7].

*6/8-C-(R/S)-(1-Benzylthioethyl)-(−)-epicatechin-3-O-gallate 4β-Benzylthioether, **15a/b**, Figure 3.* UV/vis (acetonitrile/1% HCOOH): λ_{max} = 232, 280 nm; LC-TOF-MS (ESI^-): found at *m/z* 713.1514 (713.1515 calc. for $[C_{38}H_{33}O_{10}S_2]^-$); LC/MS ($ESI^-$): *m/z* (%) 713.1 (100, $[M - H]^-$), MS/MS: *m/z* (%) 589.0 (80), 465.1 (30), 437.2 (35), 313.0 (80), 160.9 (30), 150.7 (100), 106.9 (15); ^{1}H NMR (500 MHz, CD_3OD, COSY): *δ* (ppm) 1.58 [dd, 3H, *J* = 7.2, 26.9 Hz, H−C(10)], 3.60 [m, 2H, H−C(1‴′)], 3.84 [dd, 1H, *J* = 2.0, 5.1 Hz, H−C(3)], 4.04 [m, 2H, H−C(1″)], 4.15 [dd, 1H, *J* = 2.2, 7.8 Hz, H−C(4)], 4.65 [dq, 1H, *J* = 7.2, 27.1 Hz, H−C(9)], 5.35 [d, 1H, *J* = 27.7 Hz, H−C(2)], 5.98 [d, 1H, *J* = 3.9 Hz, H−C(6/8)], 6.69 [m, 1H, H−C(6′)], 6.75 [dd, 1H, *J* = 1.0, 8.5 Hz, H−C(5′)], 6.87 [dd, 2H, *J* = 5.1 Hz, H−C(3″), H−C(7″)], 6.93 [dd, 1H, *J* = 1.8, 13.0 Hz, H−C(2′)], 7.23 [m, 6H, H−C(3‴′), H−C(4‴′), H−C(5‴′), H−C(6‴′), H−C(7‴′), H−C(5″)], 7.30 [m, 2H, H−C(3‴), H−C(7‴)], 7.44 [m, 2H, H−C(4‴), H−C(6‴)]; ^{13}C NMR (125 MHz, CD_3OD, HSQC, HMBC): *δ* (ppm) 20.95 [C-10], 35.60 [C-9], 37.45 [C-1‴], 38.09 [C-1″], 41.95 [C-4], 71.74 [C-3], 75.04 [C-2], 97.55 [C-6/8], 98.75 [C-4a], 107.64 [C-6/8], 110.65 [C-3″, C-7″], 115.58 [C-2′], 116.36 [C-5′], 119.56 [C-6′], 127.96 [C-5‴′], 128.34 [C-5‴], 129.95/129.80/130.11 [C-3‴, C-7‴, C-3‴′, C-4‴′, C-6‴′, C-7‴′], 130.43 [C-4‴, C-6‴], 132.28 [C-1′], 139.92 [C-5″], 140.50 [C-2″, C-2‴′], 145.68 [C-3′, C-4′], 146.51 [C-4″, C-6″], 156.81/157.53 [C-8a, C-5, C-7], 167.33 [C-1″].

Quantitative Analysis of Flavan-3-ols after Thiolysis. Quantitative analysis of compounds **1**, **2**, **4**−**6**, and **8** were done by means of HPLC-UV at 272 nm using a 6-point external calibration with the corresponding reference compounds dissolved in methanol/water (50/50, v/v; 0.03−1.0 mg/mL). Purity of isolated reference compounds was determined by means of qNMR prior to quantification.

In addition to HPLC-UV quantification, compounds **5**-**15** were analyzed after thiolysis (as described above) as well as in solutions of polymeric fractions (2 mg/mL) in methanol/water (30/70, v/v) without thiolysis. Prior to LC-MS/MS quantification, concentrations of isolated reference compounds in CD_3OD/D_2O (60/40, v/v) were determined by means of qNMR. These solutions were further diluted with methanol/water (50/50, v/v) and used for external calibration. HPLC-MS/MS was carried out using a 150 × 2.0 mm, 5 *μ*m, Luna C18(2) column (Phenomenex) operated at 40 °C with a flow rate of 0.3 mL/min and coupled to the 4000Qtrap mass spectrometer. Using 0.1% aqueous formic acid as solvent A and 0.1% formic acid in acetonitrile as solvent B, chromatography was performed starting with 10% solvent B for 1 min, then increasing solvent B to 60% within 17 min and, after isocratic elution for 3 min, increasing to 100% within 3 min and, after isocratic elution for 2 min, decreasing again to 10% within 1 min, followed by an equilibration phase at 10% solvent B for 5 min. Using negative electrospray ionization and the multiple reaction monitoring (MRM) mode, the individual target compounds were analyzed for a duration of 30 ms using the mass transitions, declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) as given in parentheses: **6** (*m/z* 427.1 → 124.9; DP, −75 V; EP, −10 V; CE, −26 V; CXP, −1 V), **5**/**7** (*m/z* 411.1 → 287.0; DP, −85 V; EP, −10 V; CE, −12 V; CXP, −9 V), **8** (*m/z* 563.2 → 124.9; DP, −105 V; EP, −10 V; CE, −48 V; CXP, −7 V), **9a**−**10b** (*m/z* 439.0 → 314.9; DP, −75 V; EP, −10 V; CE, −14 V; CXP, −9 V), **11a/b** (*m/z* 455.1 → 331.0; DP, −75 V; EP, −10 V; CE, −12 V; CXP, −19 V), **12a/b** (*m/z* 591.2 → 315.0; DP, −90 V; EP, −10 V; CE, −30 V; CXP, −9 V), **13a/b** (*m/z* 561.2 → 313.1; DP, −85 V; EP, −10 V; CE, −18 V; CXP, −9 V), **14a/b** (*m/z* 577.2 → 453.1; DP, −60 V; EP, −10 V; CE, −14 V; CXP, −9 V), **15a/b** (*m/z* 713.1 → 313.0; DP, −90 V; EP, −10 V; CE, −34 V; CXP, −9 V). Based on the quantitative data of flavan-3-ols (terminal units) and thioethers (extension units), the mean degree of polymerization (mDP) was calculated as the ratio of the sum of terminal units and extension units (in mol/100g) and the terminal units (in mol/100 g).

Quantitative Analysis of Native and Modified Anthocyanins (16−25) after Thiolysis. Anthocyanins were analyzed in solutions of polymeric fractions obtained after thiolysis (as described above) and polymeric fractions (2 mg/mL) in methanol/1% formic acid (30/70, v/v) by means of HPLC-MS/MS using a 10 × 2.0 mm, 5 *μ*m, Gemini RP 18 column (Phenomenex) coupled to a API 3200 mass spectrometer operating in the positive electrospray ionization and the MRM mode. Chromatography was performed at a flow rate of 0.25 mL/min using the following gradient of 5% formic acid in water (solvent A) and 5% formic acid in acetonitrile (solvent B): starting at 15% solvent B for 3 min, the content of solvent B increased to 35% within 7 min, then to 100% within 10 min and, after isocratic elution for additional 5 min, solvent B was decreased again to 15% within 5 min, followed by isocratic column equilibrium for 5 min. MS parameters were tuned using reference compounds of the anthocyanins **16**-**20** and pinotin A (**25**), the MS parameters for the pyranoanthocyanins **21**-**24** were retrieved from pinotin A in accordance to literature.[9] External calibration was carried out using **20** and **25** as reference compounds in acetonitrile/1% aqueous formic acid (20/80, v/v, 0.3 to 150 *μ*g/L). The individual mass transitions were analyzed for a duration of 40 ms using the declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP), each given in parentheses: **16** (*m/z* 449.1 → 287.1; DP, 111 V; EP, 6.5 V; CE, 27 V; CXP, 6 V), **17** (*m/z* 465.1 → 303.1; DP, 146 V; EP, 5 V; CE, 29 V; CXP, 6 V), **18** (*m/z* 463.1 → 301.1; DP, 116 V; EP, 5.5 V; CE, 31 V; CXP, 6 V), **19** (*m/z* 479.1 → 317.0; DP, 81 V; EP, 6.5 V; CE, 29 V; CXP, 24 V), **20** (*m/z* 493.1 → 331.0; DP, 141 V; EP, 4 V; CE, 31 V; CXP, 26 V), **21** (*m/z* 581.1 → 419.1; DP, 61 V; EP, 7.5 V; CE, 35 V; CXP, 36 V), **22** (*m/z* 597.1 → 435.1; DP, 61 V; EP, 7.5 V; CE, 35 V; CXP, 36 V), **23** (*m/z* 595.1 → 433.1; DP, 61 V; EP, 7.5 V; CE, 35 V; CXP, 36 V), **24** (*m/z* 611.1 → 449.1; DP, 61 V; EP, 7.5 V; CE, 35 V; CXP, 36 V), and **25** (*m/z* 625.1 → 463.2; DP, 61 V; EP, 7.5 V; CE, 35 V; CXP, 36 V).

Spiking Experiments with Acetaldehyde. Aliquots (20 mL) of must and red wine, respectively, were spiked with 0, 2, 20, or 100 *μ*L acetaldehyde, sealed under nitrogen atmosphere, and stored in brown glass vials in the dark for 7 days at room temperature. The high-molecular weight polymers (>5 kDa) were separated by means of ultrafiltration as described above and were then subjected to analytical thiolysis, followed by LC-MS/MS analysis.

High Performance Liquid Chromatography (HPLC). The HPLC-UV system (Jasco, Groß-Umstadt, Germany) consisted of a HPLC-gradient pump system PU 2087, a degasser DG-2080−53 and a MD 2010 Plus DAD-detector. After sample injection by means of an AS-2055 Plus autosampler (20 *μ*L), analytical HPLC was performed on a 250 × 4.6 mm, 5 *μ*m, ODS C18 HyperClone column (Phenomenex, Aschaffenburg, Germany) operated at a flow rate of 1 mL/min. Semipreparative chromatography was performed after

injection by means of a sample loop (0.4 mL) on a 250 × 10 mm i.d. Microsorb 100−5 C18 column (Varian, Darmstadt, Germany) operated at a flow rate of 4 mL/min.

Gradient Flash Chromatography. The gradient flash chromatography apparatus (Büchi, Flawil, Switzerland) consisted of C-605 type pumps with a C-615 pump manager, a C-635 type photometer, and a C-660 type fraction collector. Chromatography was performed on a self-packed 150 × 40 mm i.d. polypropylene cartridge filled with LiChroprep RP-18 material (25−40, Merck, Darmstadt, Germany) operated with a flow rate of 40 mL/min. Eluent was monitored at 280 nm.

High-Performance Liquid Chromatography−Mass Spectrometry (HPLC-MS/MS). LC-MS/MS analyses were either performed using an Dionex UltiMate 3000 HPLC system (Dionex, Idstein, Germany) connected to a API 4000 QTrap triple quadrupole LC-MS/MS system (ABSciex Instruments, Darmstadt, Germany) or an Agilent 1100 series HPLC system (Agilent Technologies, Frankfurt, Germany) connected to a API 3200 triple quadrupole system (ABSciex Instruments, Darmstadt, Germany). Data acquisition and instrumental control was performed with Analyst 1.5 software (ABSciex Instruments, Darmstadt, Germany). Nitrogen served as the nebulizer gas (45 psi), turbo gas (400 or 425 °C) for solvent drying (55 psi), curtain gas (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. The ion spray voltage was set at −4500 V (ESI-) and 5500 V (ESI+), respectively. The MS/MS parameters were tuned for each individual compound in methanol/water (50/50, v/v) using direct flow injection with a syringe pump (20 μL/min) detecting the fragmentation of the molecular ions into specific product ions after collision with nitrogen using the software tool "compound optimization".

UPLC/Time-of-Flight Mass Spectrometry (UPLC/TOF-MS). High-resolution mass spectra were recorded on a SYNAPT G2 HDMS mass spectrometer (Waters UK Ltd., Manchester, UK) operated in the negative electrospray ionization and resolution modus. Sample aliquots (1−5 μL) in methanol/water (50/50; v/v) were introduced into the instrument via an Acquity UPLC core system (Waters). The UPLC-TOF-MS system was equipped with a BEH C18, 2 × 150 mm, 1.7 μm, column (Waters, Manchester, UK) operating at a flow rate of 0.3 mL/min and a temperature of 40 °C. Following gradient was used for chromatography: starting with a mixture (5/95, v/v) of acetonitrile and aqueous formic acid (0.1% HCOOH), the acetonitrile content was increased to 95% within 3 min and, then, kept constant for 1 min. All data were lock mass corrected on the pentapeptide leucine enkephaline (Tyr-Gly-Gly-Phe-Leu, m/z 554.2615, $[M - H]^-$) in a solution (2 ng/μL) of acetonitrile/0.1% formic acid (1/1, v/v). Data acquisition and interpretation were performed by using MassLynx software version 4.1 (Waters) and the tool "elemental composition".

Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). Aliquots of the solution (0.5 μL) of polymeric red wine fractions (0.5 mg/mL) in methanol/water (50/50, v/v) were mixed with an aliquot (2 μL) of a matrix solution consisting of 2,5-dihydroxybenzoic acid (20 mg/mL) in 30% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid on a stainless steel target (Bruker Daltonik, Bremen, Germany). Mass spectra were acquired in positive reflectron mode on an UltraFlex eXtreme MALDI-TOF/TOF mass spectrometer equipped with a 1 kHz Smartbeam-II laser (Bruker Daltonik, Bremen, Germany). Laser intensity was adjusted to optimize signal-to-noise ratio and resolution. A total of 3000 shots were acquired per MALDI spectrum with random walk mode enabled (50 shots per position) from m/z 900 to 3500. Each spectrum was externally calibrated using the peptide calibration standard II (Bruker Daltonik, Bremen, Germany) and the "cubic enhanced" calibration function. Mass spectra were analyzed using the flexAnalysis software version 3.3 (Bruker Daltonik, Bremen, Germany).

Nuclear Magnetic Resonance Spectroscopy (NMR). 1D- (^{1}H, ^{13}C) and 2D-NMR experiments (COSY, HMQC, HMBC) were performed on a 500 MHz Advance III spectrometer (Bruker, Rheinstetten, Germany). Experiments were carried out using the pulse sequences taken from the Bruker software library. Chemical shifts were referenced to TMS. Data processing and interpretation was performed using Topspin Version 2.1 (Bruker, Rheinstetten, Germany) and MestRe-Nova version 5.0 software (Mestrelab Research, La Coruna, Spain). To determine the concentration of isolated reference compounds used as external standards for quantification, quantitative NMR (qNMR) experiments with these compounds dissolved in methanol-d_4/D_2O (40/60, v/v) were carried out on a DRX 400 spectrometer (Bruker, Rheinstetten, Germany) using a relaxation time of 40 s. Reference solution for quantitation was benzoic acid, control solution was caffeine in methanol-d_4/D_2O (40/60, v/v; 3 and 5 mmol/L) as reported recently.[31]

Sensory Analyses. Ten persons (six women and four men, 20−30 years of age), who gave informed consent to participate in the sensory tests of the present investigation and have no history of known taste disorders, were trained in weekly training sessions (see Supporting Information).

Taste Profile Analysis. For taste profile analysis the trained panelists were asked to judge the taste intensities of the samples using the taste descriptors astringent, bitter, sour, sweet, salty and mouthfullness on a scale from 0 (not perceivable) to 5 (strong taste impression). Fractions of the ultrafiltration were dissolved in their natural concentrations (LMW: 21.4 g/L, HMW: 4.5 g/L) using 12.5% aqueous ethanol and presented to the panelists.

Astringency Threshold Concentrations. Human astringency recognition thresholds of polymeric fractions were determined in 1% aqueous ethanol solutions (1.0 mL; pH 4.5) by means of the half-tongue test following the protocol reported earlier.[32−34] The threshold values between individuals and between two separate sessions differed by not more than plus or minus one dilution step; that is, a threshold value of 3.3 mg/L represents a range from 1.7 to 6.6 mg/L.

Astringency Intensity at Iso-Concentrations. To evaluate the astringency intensity of the GPC fractions at the same concentration level, aliquots (5 mg) were dissolved in 10 mL 1% aqueous ethanol (pH 4.5) and presented to the panel in direct comparison to GPC fraction 5, judged with a score of 2.5 (reference) in preliminary consensus experiments. Using the half-tongue procedure,[32] panelists were asked to evaluate the astringency intensity of the test sample on a scale from 0 (not perceivable) to 5 (strong impression) in comparison to the reference (fraction 5). To adjust the color of each sample pair, traces of caramel color and cochineal red A were added prior to the experiment.

RESULTS AND DISCUSSION

As the high molecular weight polymers (HMW fraction, >5 kDa) were recently demonstrated to exhibit a considerable impact on the puckering astringency of red wine,[4,6] the HMW fraction was isolated from red wine by means of ultrafiltration (5 kDa cutoff), thus affording a slightly rose-colored, low molecular weight (LMW) fraction and a dark-red colored HMW fraction in yields of 21.4 and 4.5 g/L, respectively. Taste profile analysis of both fractions, each in its natural concentration, revealed the most pronounced astringency for the HMW fraction described as a puckering type of astringent orosensation and judged with a score of 3.4 on a 5-point scale (Table 1). In comparison, the panelists described the LMW fraction to impart a more velvety astringent mouthcoating and was evaluated with a lower score of 2.2 only, but showed higher scores for sourness (2.8) and mouthfullness (1.0).

Composition Analysis of the HMW Fraction. In order to analytically characterize the red wine polymers, the total HMW fraction was decomposed by (i) acidic hydrolysis to release monosaccharides, polyols, and amino acids, (ii) alkaline hydrolysis to release ester bound molecules, and (iii) thiolysis, that means by acidic hydrolysis in the presence of benzyl thiol, to analyze procyanidins. In order to ensure the quantification of

Table 1. Taste Profile of the High-Molecular Weight (HMW, >5 kDa) and Low-Molecular Weight Fraction (LMW, <5 kDa) isolated from Red Wine by Means of Ultrafiltration

	intensity of					
fraction[a]	astringency	bitter	sour	sweet	salty	mouthfullness
HMW	3.4	1.1	0.4	0.2	0	0.3
LMW	2.2	0.8	2.8	0.8	0.3	1.0

[a]Fractions were dissolved in 12.5% aqueous ethanol in their natural concentrations (HMW: 4.5 g/L; LMW: 21.4 g/L) and then the individual descriptors were evaluated on a scale from 0 (not perceivable) to 5 (strong taste impression).

released and not already present compounds in the fractions, also unhydrolyzed solutions of the corresponding polymeric fractions were analyzed. All concentrations given in Table 2 were calculated as the difference between the concentration of the compound in the fraction before and after hydrolysis.

The monosaccharides glucose, arabinose, mannose, galactose and xylose, and the polyol glycerol were identified in the acidic hydrolysate of the HMW fraction and the organic acids malic acid, lactic acid, quinic acid, citric acid, tartaric acid, and succinic acid were identified in the alkaline hydrolysate by means of high-performance ion chromatography. Moreover, galacturonic acid as well as the amino acids L-glutamic acid, L-aspartic acid, L-serine, glycine, L-lysine, L-arginin, L-threonine, L-histidine, L-leucine, and L-alanin were identified in the HMW fraction after acidic hydrolysis by means of HPLC-MS/MS.

Monitoring the effluent at 272 and 530 nm, chromatographic analysis of the HMW fraction was performed before hydrolysis (Figure 1A), after alkaline hydrolysis (Figure 1B), and after thiolysis (Figure 1C,D). Comparison of retention times and spectroscopic data (UV/vis, MS) with those of reference compounds, followed by cochromatography, enabled the identification of the phenolic acids gallic acid (**26**), syringic acid (**30**), and *p*-coumaric acid (**31**) as well as the flavan-3-ols (+)-catechin (**1**) and (−)-epicatechin (**2**) in the alkaline hydrolysate of the HMW fraction (Figure 1B). Besides (+)-catechin (**1**) and (−)-epicatechin (**2**), thiolytic decomposition of the polymeric fraction revealed (−)-epicatechin-3-*O*-gallate (**4**) and the flavan-3-ol-thioethers **5**−**8** by means of HPLC with UV-detection at 272 nm (Figure 1C). Monitoring the HPLC effluent at 530 nm led to the identification of the anthocyanins cyanidin-3-*O*-glucoside (**16**), delphinidin-3-*O*-glucoside (**17**), peonidin-3-*O*-glucoside (**18**), petunidin-3-*O*-glucoside (**19**), and malvidin-3-*O*-glucoside (**20**) (Figure 1D).

To further separate the puckering astringent HMW fraction, a gel absorption chromatography (GAC) was performed using LH-20 material as the stationary phase. A total of eight fractions, namely GAC I−VIII, were collected with the highest yield of 1.6 g/L found for fraction I and lowest yields of 0.3 and 0.2 g/L for fractions II, VIII, and VI, respectively (Figure 2). The HMW fraction and the individual GAC fractions were decomposed by means of acidic hydrolysis, alkaline hydrolysis as well as thiolysis. Carbohydrates, polyols, and organic acids were then quantified by means of high-performance ion chromatography, phenolic acids, galacturonic acid, amino acids and anthocyanins by means of LC-MS/MS, and flavan-3-ols by means of HPLC-UV/vis (Table 2). Among the carbohydrates, arabinose, galactose, and glucose were found as the dominating monosaccharides in the acidic hydrolysates of the GAC fractions with by far the highest concentration of carbohydrates of 48.5 mg/100 mg in GAC fraction I. In comparison, only minor contents of amino acids were found with a maximum content of 2.0 mg/100 mg in GAC fraction II.

After alkaline hydrolysis, quantitative analysis revealed gallic acid as the predominant phenolic acid with the highest content of 3.0 mg/100 mg found in the late eluting GAC fraction VIII. Other phenolic acids were present at comparatively low levels reaching up to a maximum content of 0.8 mg/100 mg for *p*-coumaric acid found in GAC fraction V (Table 2). Moreover, organic acids were found to be released upon alkaline hydrolysis with galacturonic acid as the most abundant one with levels ranging from 2.7 (GAC fraction I and IV) to 4.7 mg/100 mg (GAC fraction VI) and lactic acid in GAC fraction I (8.3 mg/100 mg) and GAC fraction II (3.1 mg/100 mg). Interestingly, also some amounts of (+)-catechin (**1**) and (−)-epicatechin (**2**) were released from the HMW polymers upon alkaline hydrolysis, e.g. up to 3.4 and 1.5 mg/100 mg of **1** and **2** were found in GAC fraction VI (Table 2). These data suggest an ester-type covalent linkage of these flavan-3-ols in the polymer.

Thiolytic depolymerization revealed the highest flavan-3-ol concentration in GAC fraction VII with 33.2 mg/100 mg followed by GAC fraction VIII with 30 mg/100 mg. In contrast, no flavan-3-ols were found in GAC fraction I. Terminal units, released as flavan-3-ols, were predominantly represented by catechin (**1**), followed by epicatechin (**2**) and epicatechin-3-O-gallate (**4**). There were no epigallocatechin units detected as terminal units within the polymeric fractions. On the contrary, epigallocatechin was detected as extension unit (released as the thioether), but the main extension units were epicatechin and epicatechin-3-O-gallate in all fractions.

In summary, about 50% of the HMW fraction >5 kDa isolated from red wine could be characterized by targeted release of low-molecular weight constituents, followed by quantitative analysis. Carbohydrates and flavan-3-ols were found as the quantitatively predominating constituents in the >5 kDa fraction with yields of 20.2 and 17.7 mg/100 mg. In comparison, amino acids, organic acids, phenolic acids, polyols, and anthocyanins were present in levels below 3.6 mg/100 mg.

Comparison of the quantitative data found for the total HMW fraction (>5 kDa) with those calculated for the sum of the constituents found in the individual GAC fractions and taking into account the natural concentration ratio of the GAC fractions, showed a total of 20.2% carbohydrates, 17.7% flavan-3-ols, 3.6% organic acids, 1.8% phenolic acids, 1.6% anthocyanins, 1.2% polyols and 0.8% amino acids in the HMW fraction (>5 kDa) and 23.9% carbohydrates, 15.3% flavan-3-ols, 4.9% organic acids, 1.8% phenolic acids, 1.1% anthocyanins, 0.8% polyols and 0.8% amino acids as the sum of the individual fractions, thus demonstrating that the GAC fractionation did not lead to an irrevocable loss of material. Taking all the quantitative data into consideration, it was obvious that the carbohydrates were present mainly in GAC fraction I (48.5 mg/100 mg) and then decreased in content with increasing fraction number, whereas the flavan-3-ols increased and reached their highest concentration of 37.0 mg/100 mg in GAC fraction VII.

In order to separate polysaccharides, ethanol was added to an aqueous solution of the HMW fraction (>5 kDa) to afford a pale gray precipitate with a yield of 22%, thus reflecting the amount of carbohydrates determined in >5 kDa fraction (20.2 mg/100 mg). HPIC analysis of the monosaccharide composition of the precipitated polysaccharide after acidic hydrolysis revealed mannose (22%) as the predominant carbohydrate,

Table 2. Composition Analysis of Hydrolysates of HMW (>5 kDa) and GAC Fractions

compound[a]	% (mg/100 mg) in fraction								
	I	II	III	VI	V	VI	VII	VIII	>5 kDa
carbohydrates and polyols after acidic hydrolysis									
arabinose	15.2	3.6	0.1	0.1	0.1	<0.1	<0.1	<0.1	4.1
galactose	14.0	3.7	0.2	0.1	<0.1	<0.1	<0.1	<0.1	4.1
glucose	3.7	7.1	8.0	6.9	5.5	3.4	2.6	2.1	4.3
xylose	1.1	0.8	<0.1	n.d.	n.d.	n.d.	n.d.	n.d.	0.2
mannose	10.9	2.7	0.1	n.d.	n.d.	n.d.	n.d.	n.d.	4.0
galacturonic acid	0.9	0.5	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.4
glycerol	1.4	1.2	0.2	0.2	0.1	0.1	0.2	0.3	0.8
amino acids after acidic hydrolysis									
L-glutamic acid	0.3	0.6	0.3	0.2	0.1	0.1	0.1	0.1	0.2
L-aspartic acid	0.2	0.4	0.1	0.1	<0.1	<0.1	<0.1	<0.1	0.1
L-serine	0.4	0.3	0.1	0.1	<0.1	<0.1	<0.1	<0.1	0.2
glycine	0.1	0.2	0.1	0.1	<0.1	<0.1	<0.1	<0.1	0.1
L-lysine	0.2	0.2	0.1	0.1	<0.1	<0.1	<0.1	<0.1	0.1
L-arginine	<0.1	0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
L-threonine	0.1	0.1	<0.1	<0.1	<0.1	n.d.	n.d.	n.d.	<0.1
L-histidine	<0.1	<0.1	<0.1	<0.1	n.d.	<0.1	n.d.	n.d.	<0.1
L-leucine	<0.1	<0.1	<0.1	<0.1	<0.1	n.d.	n.d.	n.d.	<0.1
L-isoleucine	<0.1	<0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
L-alanine	<0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<0.1
phenolic acids after alkaline hydrolysis									
gallic acid (**26**)	<0.1	0.2	0.7	1.2	1.7	1.3	1.9	3.0	0.7
p-hydroxy benzoic acid (**27**)	n.d.	n.d.	n.d.	n.d.	n.d.	<0.1	<0.1	<0.1	<0.1
gentisic acid (**28**)	n.d.	0.4	0.3	0.6	0.4	0.3	0.3	0.2	0.3
caffeic acid (**29**)	n.d.	0.5	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
syringinc acid (**30**)	n.d.	0.4	0.3	0.6	0.4	0.3	0.3	0.2	0.3
p-coumaric acid (**31**)	n.d.	0.2	0.5	0.6	0.8	0.6	0.4	0.3	0.5
ferulic acid	n.d.	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
vanillic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
protocatechuic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
flavan-3-ols after alkaline hydrolysis									
catechin (**1**)	n.d.	1.2	2.9	1.6	2.2	3.4	3.4	1.1	1.9
epicatechin (**2**)	n.d.	0.8	1.1	0.9	1.2	1.6	1.5	0.8	1.0
organic acids after alkaline hydrolysis									
galacturonic acid	2.7	2.9	2.9	2.7	3.4	4.7	3.7	4.6	3.1
malic acid	0.2	0.1	0.1	0.1	n.d.	n.d.	n.d.	0.1	0.3
lactic acid	8.3	3.1	1.1	1.0	0.8	1.0	1.3	1.5	2.0
quinic acid	0.3	0.6	0.5	0.6	0.7	0.8	0.6	0.9	0.7
citric acid	0.1	0.1	0.1	<0.1	0.1	0.1	0.1	<0.1	0.1
tartaric acid	0.3	0.5	0.1	0.1	0.2	0.1	0.1	0.1	0.4
succinic acid	0.1	0.1	1.4	0.1	0.1	n.d.	n.d.	n.d.	0.1
flavan-3-ols after thiolysis									
catechin (**1**)	n.d.	0.2	0.8	0.8	1.2	1.9	1.3	1.2	0.8
epicatechin (**2**)	n.d.	0.2	0.7	0.7	1.0	1.4	1.0	0.7	0.5
epicatechin-3-*O*-gallate (**4**)	n.d.	<0.1	<0.1	<0.1	0.1	0.1	0.1	0.1	<0.1
EC-4β-thioether (**5**)	n.d.	3.2	7.8	12.1	15.5	19.0	22.3	19.4	10.1
ECG-4β-thioether (**8**)	n.d.	0.6	1.2	2.8	4.2	3.4	5.7	6.7	2.2
EGC-4β-thioether (**6**)	n.d.	0.7	1.6	0.8	0.6	0.7	1.4	0.7	1.2
C-4β-thioether (**7a**)	n.d.	0.4	0.5	0.5	0.8	0.9	1.2	1.3	0.9
anthocyanins after thiolysis									
malvidin-3-*O*-glc (**20**)	n.d.	3.33	0.88	0.60	0.33	0.19	0.10	0.06	0.79
petunidin-3-*O*-glc (**19**)	n.d.	2.14	0.44	0.22	0.11	0.07	0.04	0.02	0.41
peonidin-3-*O*-glc (**18**)	n.d.	3.01	0.42	<0.01	0.02	<0.01	<0.01	<0.01	0.32
cyanidin-3-*O*-glc (**16**)	n.d.	0.39	0.08	0.05	0.05	0.02	<0.01	<0.01	0.09
delphinidin-3-*O*-glc (**17**)	n.d.	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
pyranoanthocyanins after thiolysis									
pinotin A (**25**)	n.d.	<0.01	0.01	0.01	0.01	0.02	0.28	0.21	0.01
peonidin-3-*O*-glc 4-VC adduct (**23**)	n.d.	<0.01	<0.01	<0.01	<0.01	<0.01	0.05	0.11	<0.01
petunidin-3-*O*-glc 4-VC adduct (**24**)	n.d.	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.07	<0.01

Table 2. continued

compound[a]	% (mg/100 mg) in fraction								
	I	II	III	VI	V	VI	VII	VIII	>5 kDa
pyranoanthocyanins after thiolysis									
cyanidin-3-*O*-glc 4-VC adduct (**21**)	n.d.	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.07	<0.01
delphinidin-3-*O*-glc 4-VC adduct (**22**)	n.d.	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.04	<0.01

[a]Numbers given in brackets refer to peaks in Figure 1. EGC, epigallocatechin; C, catechin; EC, epicatechin; ECG, epicatechin-3-*O*-gallate; 4-VC, 4-Vinylcatechol; n.d., not detected.

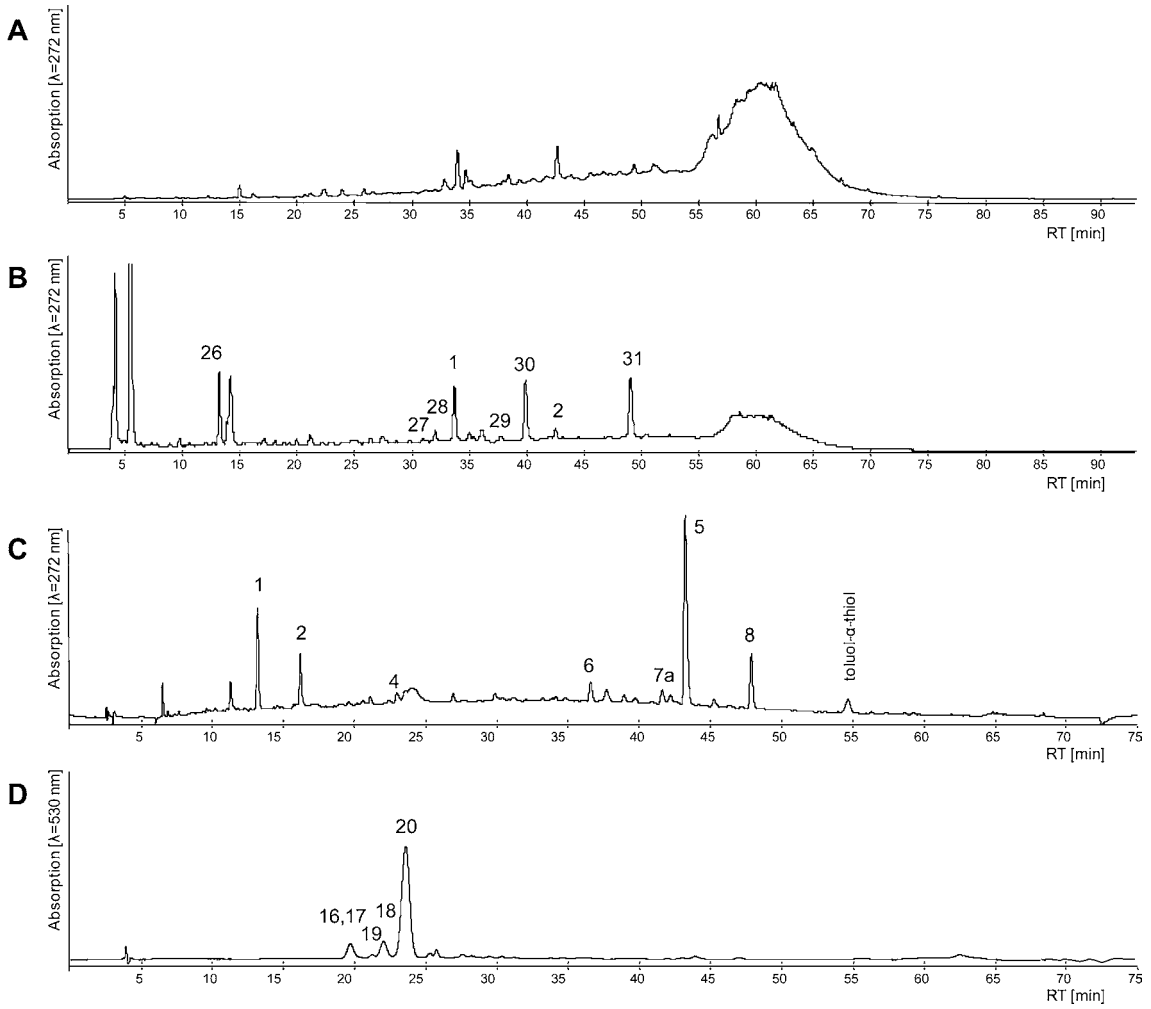

Figure 1. HPLC analysis of HMW fraction (A) prior to hydrolysis, (B) after alkaline hydrolysis, and (C, D) after thiolysis. Numbers according to compounds in Figure 3 and Table 3.

followed by galactose (17.8%), arabinose (10.2%), rhamnose (1.9%), and glucose (1.3%), respectively. This finding is well in line with literature data on red wine polysaccharides.[36] Mannoproteins, released by yeast during fermentation, and grape-derived arabinogalactan-proteins as well as rhamnogalacturonans I and II account for 35, 42, 4, and 19%, respectively, of the total red wine polysaccharides,[36] thus matching the monosaccharide distribution found in the ethanol precipitate .

Thiolysis. In order to gain a more clear picture on flavan-3-ol units, the HMW fraction was decomposed by means of thiolysis using benzylthiol in MeOH/HCl as reported earlier.[25,37,38] HPLC-UV/vis analysis revealed a series of cleavage products (**1**, **2**, **4**, **5**−**8**) detected at 272 nm (Figure 1C), besides compounds **16**−**20** detected at 530 nm (Figure 1D). The flavan-3-ols **1**, **2**, and **4** were identified by comparison of spectroscopic (MS, UV/vis) data and retention times with those found for the reference compounds (Figure 1). As thiolysis products **5**−**8** could not be identified by means of reference compounds, these compounds were isolated and purified by means of semipreparative HPLC and, then, their chemical structure determined by means of LC-MS/MS and NMR experiments.

MS analysis in the ESI negative mode revealed pseudo molecular ions ($[M\text{-}H]^-$) with *m*/*z* 411.1 (**5**, **7a**), 427.1 (**6**) and 563.2 (**8**), respectively, and a characteristic loss of 124 amu, corresponding to the loss of a benzylthiol moiety. 1D/2D-NMR spectroscopic analysis led to the identification of epicatechin-4β-benzylthioether (**5**), epigallocatechin-4β-benzylthioether (**6**), catechin-4β-benzylthioether (**7a**), and epi-

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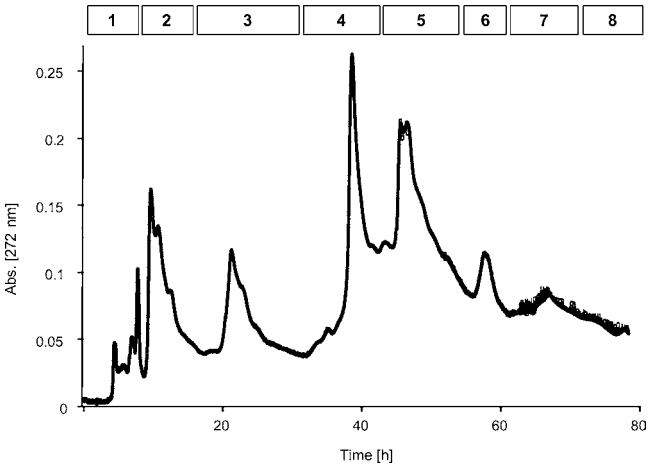

Figure 2. Chromatogram of the GPC fractionation (λ = 272 nm) of the HMW fraction (>5 kDa).

catechin-3-*O*-gallate-4β-benzylthioether (**8**) by comparison of spectroscopic data with those reported in literature.[25,38−41] The 2(*R*),3(*S*)-configuration of compounds **5**, **6** and **8** as well as the 2(*R*),3(*R*)-configuration of compound **7a** was determined from the chemical shifts and coupling constants of H−C(2) and H−C(3), respectively.[41,42] HMBC experiments between the methylene protons H−C(1″) of the benzylthiol moiety and carbon atom C(4) of compounds **5**−**8**, demonstrated the linkage of the benzylthiol to the flavanol aglycon and analysis of the coupling constants of H−C(3) and H−C(4) revealed the β-position of the benzylthioether moiety at C(4), thus confirming literature data.[37,41,43,46]

As the overall astringency of procyanidins is discussed to increase with the polymerization degree and a more rough and puckering astringency impression was reported to correlate with the degree of galloylation,[2,22] compounds **5**−**8** were quantitatively determined by HPLC/UV in the GAC fractions after thiolytic degradation. On the basis of the content of terminal units (released as flavan-3-ols) and extension units (released as thioethers), the mean degree of polymerization (mDP) was calculated. The percentage of galloylation (%G) was determined as the amount of epicatechin-3-*O*-gallate units present in the polymers (Table 3). With the exception of GAC fraction I, the mDP calculated for fractions II−VIII ranged from 5.8 to 10.1 units with the highest values found for the later eluting fractions VII and VIII. These mDP values were in good accordance with published data, e.g. mDPs of 6 to 18 units were reported for proanthocyanidin fractions isolated from Shiraz wine.[47] The degree of galloylation was widely distributed over fractions II − VIII, but again GPC VIII showed the highest content of 17.3% epicatechin gallate units.

HPLC-MS/MS analysis of the compounds **16**−**20** detected at 530 nm (Figure 1D) revealed *m*/*z* 449.1, 465.1, 463.1, 479.1, and 493.1 as molecular ions ($[M]^+$) and a characteristic loss of 162 amu corresponding to the loss of a hexosyl moiety. By means of cochromatography with reference compounds, these compounds were unequivocally identified as the anthocyanins cyanidin-3-*O*-glucoside (**16**), delphinidin-3-*O*-glucoside (**17**), peonidin-3-*O*-glucoside (**18**), petunidin-3-*O*-glucoside (**19**), and malvidin-3-*O*-glucoside (**20**). As only trace amounts of these anthocyanins were detectable in the polymeric fractions prior thiolysis (data not shown), their release upon thiolytic degradation suggests the covalent attachment of these polyphenols in the polymer structures. This finding confirms earlier reports on the release of malvidin-3-*O*-glucoside from red wine fractions upon thiolysis.[48]

Besides the anthocyanins, so-called pyranoanthocyanins generated by the reaction of 4-vinylcatechol with cyanidin- (**21**), delphinidin- (**22**), peonidin- (**23**), petunidin- (**24**) and malvidin-3-*O*-glucoside (**25**) and exhibiting an absorption maximum at about 515 nm, have been reported in matured red wines.[9] For the analysis of pyranoanthocyanins, pinotin A (**25**) was synthesized as a reference compound for calibration through reaction of malvidin-3-*O*-glucoside (**20**) with caffeic acid at pH 3.0 in 15% aqueous ethanol.[29] In order to gain insights into the amounts and distribution of anthocyanins (**16**−**20**) and pyranoanthocyanins (**21**−**25**) in each GAC fraction, compounds **16**−**25** were quantitatively determined by means of HPLC-MS/MS (Table 2). In all fractions, malvidin-3-*O*-glucoside (**25**) was the predominant anthocyanin, followed by petunidin- (**19**) and peonidin-3-*O*-glucoside (**18**). Accordingly to the concentration of anthocyanins, pinotin A (**25**), the vinylcatechol adduct of malvidin-3-*O*-glucoside, was found in highest concentrations among the pyranoanthocyanins. In contrast to the anthocyanins showing their maximum content in GAC fraction II with a total amount of 8.9 mg/100 mg, the highest amount of pyranoanthocyanins (0.5 mg/100 mg) was found in the late eluting GAC fraction VIII.

Acetaldehyde-Mediated Polymerization of Flavan-3-ols. Formed as a metabolic byproduct of fermentation [50] or an autoxidation product of ethanol involving *o*-quinones as the

Table 3. Composition Analysis of Polymeric Flavan-3-ols in GAC Fractions and >5 kDa Fraction after Thiolysis

				molar percentage												
				% of extension subunits[a]				% of terminal subunits[a]			% of ethyl-bridged subunits[a]					
GAC fraction	conc. [g/100 g]	mDP	%G	**5**	**6**	**7**	**8**	**1**	**2**	**4**	**9a/b**	**11a/b**	**12a/b**	**13a/b**	**14a/b**	**15a/b**
I	<0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
II	5.8	8.5	11.6	57.0	11.6	6.3	8.1	6.2	4.9	0.0	0.4	0.1	3.5	1.7	0.1	<0.1
III	13.2	6.0	8.4	58.6	11.9	3.6	6.5	8.7	7.5	0.1	0.3	<0.1	1.8	0.9	0.1	<0.1
IV	17.9	8.2	12.0	68.2	4.4	2.9	11.4	6.6	5.3	0.2	0.2	<0.1	0.4	0.4	<0.1	<0.1
V	23.6	7.3	13.7	66.5	2.5	3.5	13.3	7.5	5.8	0.3	0.2	<0.1	0.1	0.2	<0.1	<0.1
VI	27.5	5.8	9.1	67.9	2.5	3.1	8.9	9.7	7.3	0.2	0.1	<0.1	<0.1	0.2	<0.1	<0.1
VII	33.3	9.3	13.2	68.4	4.3	3.6	12.8	5.8	4.5	0.4	0.1	<0.1	<0.1	0.1	<0.1	<0.1
VIII	30.1	10.1	17.3	66.6	2.2	4.3	16.8	6.1	3.3	0.5	0.1	<0.1	<0.1	0.1	<0.1	<0.1
>5 kDa	16.0	8.1	10.8	63.6	7.2	5.5	10.2	7.0	4.9	0.2	0.4	<0.1	0.3	0.6	<0.1	<0.1

[a]Numbers of compounds refer to structures in Figure 3. mDP, mean degree of polymerization; %G, percentage of galloylation; n.d., not determined.

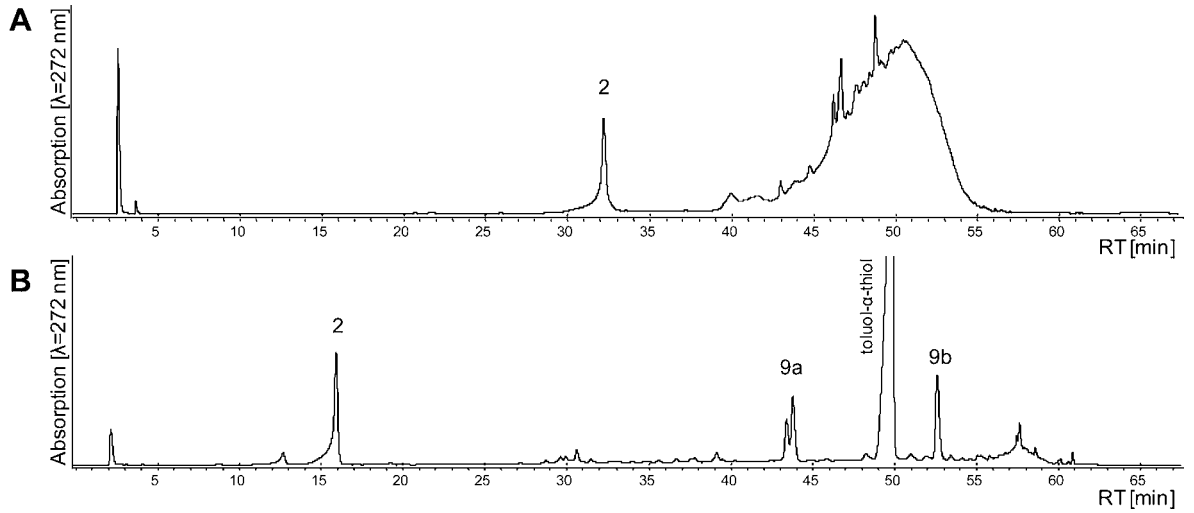

Figure 4. HPLC chromatogram of the model solution containing (−)-epicatechin (**2**) and acetaldehyde (A) after one week of storage and (B) after thiolysis. Numbers according to structures in Figure 3.

oxidation agent,[7,51] acetaldehyde is suggested as a putative polymerization agent via its cross-linking reaction with flavan-3-ols and anthocyanins, respectively.[18] By reducing the concentration of free flavan-3-ols and anthocyanins, this reaction might contribute to changes in astringency perception and color of red wine during aging. In addition, acetaldehyde-mediated polymerization of flavan-3-ols is not only discussed in red wine but was also highlighted to play a key role for the decrease in astringency during ripening of persimmon fruits.[52] Therefore, the following experiments were done to gain insight into the role of acetaldehyde in red wine polymer formation.

Aimed at generating reference compounds for LC-MS/MS analysis of the GAC fractions, binary solutions of (−)-epicatechin or (+)-catechin, respectively, and acetaldehyde in 13% aqueous ethanol (pH 3.2) were incubated in the dark at room temperature. The reaction was monitored by means of HPLC-UV until a characteristic broad peak of oligomeric procyanidins was detected after one week of incubation (Figure 4A). To remove the nonreacted flavan-3-ol, the oligomeric fraction was isolated by flash chromatography on RP18 material and freeze-dried to afford the oligomers of **1** or **2**, respectively, each as light yellow powder. Exemplified for the oligomer produced from (−)-epicatechin and acetaldehyde, MALDI-TOF-MS analysis revealed the characteristic latter of ions differing by the incorporation of m/z 288 corresponding to one epicatechin unit (Figure 5). In total, up to 10 flavan-3-ol units (**1**, **2**) could be detected with MALDI-TOF-MS in the oligomers isolated from the reactions of epicatechin or catechin with acetaldehyde.

In order to gain a first insight into the nature of the acetaldehyde-induced cross-linking, the oligomers generated from **1** and **2** were degraded by thiolysis and the compounds released were analyzed by means of HPLC-UV/vis and HPLC-MS/MS. Besides the flavan-3-ol (terminal unit), compounds **9a**/**9b** and **10a**/**10b** were found as thiolysis products (extension units) of the oligomer produced from **2** and **1**, respectively (Figure 4B). These compounds were isolated and purified by semipreparative HPLC and their chemical structure determined by means of LC-MS/MS, LC-TOF-MS, and NMR experiments. LC-MS/MS analysis of compounds **9a**/**b** and **10a**/**b** revealed m/z 439.0 as the pseudo molecular ion ($[M - H]^-$) and showed a characteristic loss of 124 amu corresponding to the benzylthiol moiety. Furthermore, 1D/2D NMR analysis revealed a double signal set of **9a**, **9b**, **10a**, and **10b**, thus indicating the existence of diastereomers as outlined for compound **9a** in Figure 6. Compared to (−)-epicatechin, the resonance signal of the aromatic proton H−C(8) was lacking in the spectrum of compound **9a**, thus suggesting a linkage of benzylthiol at position C(8). Moreover, the appearance of a doublet methyl (1.5 ppm) and a quartet methine signal (4.7 ppm) indicated the presence of a 1-substituted ethyl group within the molecule. Both signals showed a heteronuclear correlation (HMBC) to the A-ring of the flavan-3-ol (C-8, C-7, C-8a) as well as the carbon atom C-1″ of the benzylthiol moiety. Therefore, the thioether was linked via an ethyl-bridge to position C-8 of the flavanol aglycon. The double signal set in the NMR spectra suggested a 50:50 mixture of the 9(*R*)- and 9(*S*)-configuration of the ethylbridge, hence confirming the previously observed *R*/*S*-isomerization of 8-*C*-(*R*/*S*)-(1-benzylthioethyl)-(−)-epigallocatechin 4*β*-benzylthioether.[52] Based on the careful assignment of all 1D/2D-NMR data, the thiolysis products were identified as 8-*C*-(*R*/*S*)-(1-benzylthioethyl)-(−)-epicatechin (**9a**), 6-*C*-(*R*/*S*)-(1-benzylthioethyl)-(−)-epicatechin (**9b**), 8-*C*-(*R*/*S*)-(1-benzylthioethyl)-(+)-catechin (**10a**), 6-*C*-(*R*/*S*)-(1-benzylthioethyl)-(+)-catechin (**10b**). To the best of our knowledge,

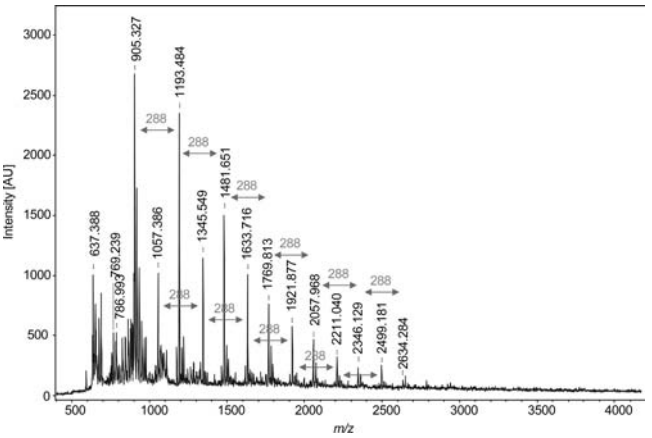

Figure 5. MALDI-TOF-MS spectrum of the oligomeric fraction of the model solution of (−)-epicatechin (**2**) and acetaldehyde.

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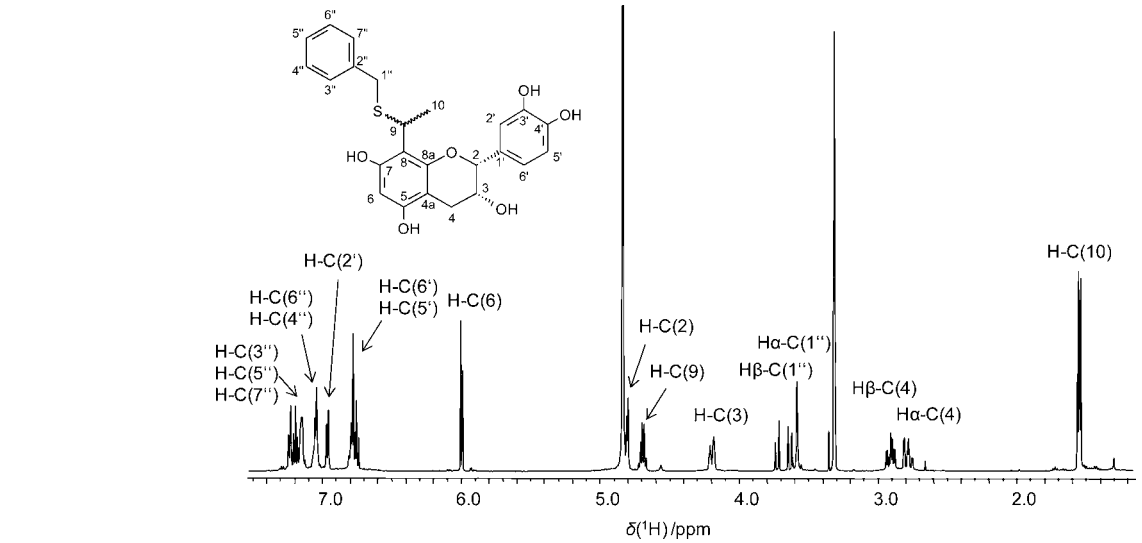

Figure 6. ^{1}H NMR spectrum of compound **9a**.

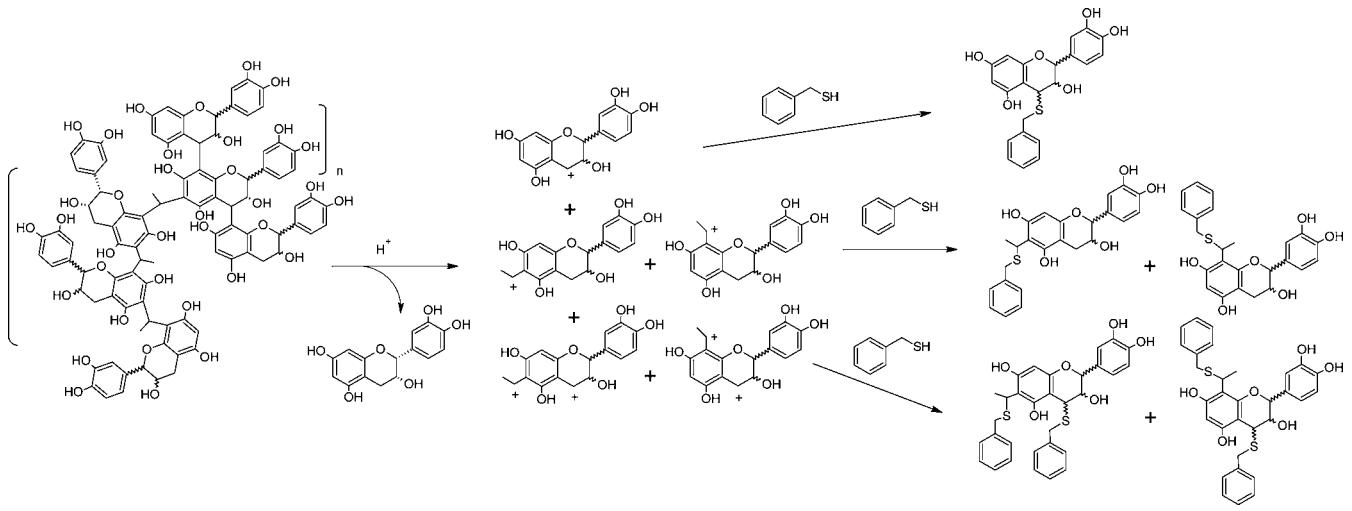

Figure 7. Reaction pathway of thiolytoc depolymerization of proanthocyanidins with 1,1-ethylene-bridged units.

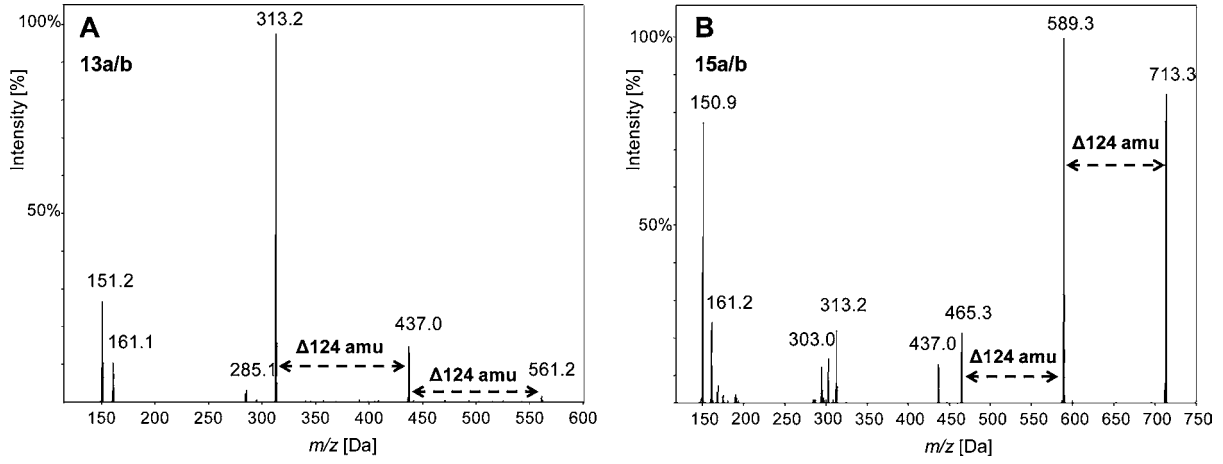

Figure 8. LC-MS/MS chromatograms of compounds (A) **13a/b** and (B) **15a/b** showing the characteristic loss of *m*/*z* 124.

these benzylthioethyl derivatives of catechin and epicatechin have not been previously reported in literature.

This model reaction demonstrated that acetaldehyde-induced ethyl-bridging of flavan-3-ols can be monitored by HPLC analysis of **9a**/**9b** and **10a**/**10b** after thiolysis. In theory, thiolysis of a more complex flavan-3-ol polymer exhibiting ethyl as well as procyanidin linkages, should release up to three different types of thioethers (Figure 7). These are the

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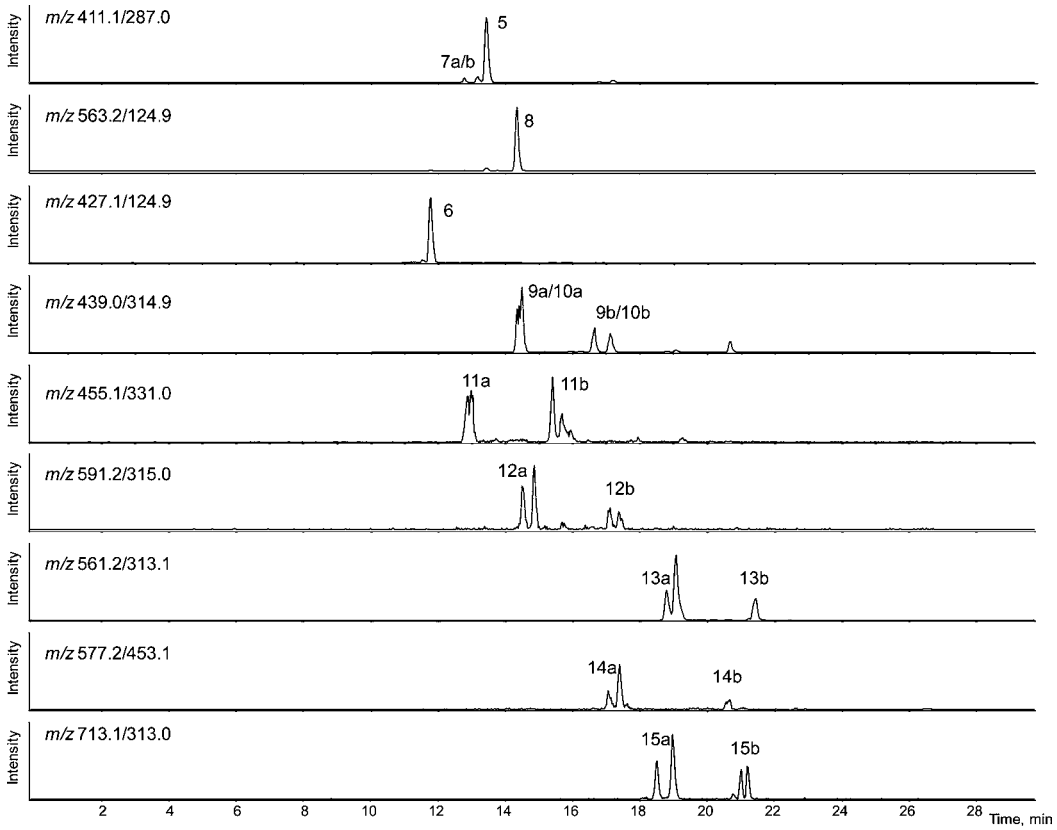

Figure 9. HPLC-MS/MS chromatogram of the red wine polymer (>5 kDa), spiked with 1% acetaldehyde, after thiolysis. Selected mass transitions for compounds **5**-**15** monitored in the MRM-mode.

thioethers representing the linkage at C(4) of the C-ring (as in **5** and **7**), the thioethers **9a/b** and **10a/b** indicating the ethyl-bridging of flavan-3-ols at C(6) or C(8) of the A-ring, and compounds **13a/b** bearing a benzylthio moiety at C(4) of the C-ring plus a 1-benzylthioethyl group at C(6) or C(8) at the A-ring.

As compounds **13a/b** are expected to be formed upon thiolysis of acetaldehyde-polymerized proanthocyanidins, grape seed procyanidins were ethyl-bridged by the reaction with acetaldehyde, followed by thiolysis and semipreparative purification of the cleavage products released. A total of four compounds (**13a/b**, **15a/b**) could be isolated and their structures determined by means of LC-MS and NMR experiments. LC-MS analysis revealed *m/z* 561.1 and 731.1 as the pseudo molecular ions ($[M - H]^-$) of the regio-isomers **13a/b** and the regio-isomers **15a/b**, respectively, and showed twice a characteristic cleavage of 124 amu as expected for the loss of two benzylthiol moieties (Figure 8). Structure determination was achieved by means of 1D/2D-NMR. Comparison of chemical shifts and coupling constants to those reported in literature[41,42] led to the assignment of the 2(*R*),3(*S*)-configuration of the epicatechin-type compounds **13a/b** and the 2(*R*),3(*S*)-configuration of **15a/b** exhibiting a epicatechin-3-*O*-gallate backbone, both with a *β*-benzylthioether moiety at C(4). As found for **9a/b** and **10a/b**, a diastereomeric signal splitting was observed due to (*R*/*S*)-isomerization of the 1-benzylthioethyl group at C(6) or C(8) of the target compounds. Taking all spectroscopic data into account, the target compounds were identified as 6-*C*- and 8-*C*-(*R*/*S*)-(1-benzylthioethyl)-(−)-epicatechin 4*β*-benzylthioether (**13a** and **13b**) and 6-*C*- and 8-*C*-(*R*/*S*)-(1-benzylthioethyl)-(−)-epicatechin-3-*O*-gallate 4*β*-benzylthioether (**15a** and **15b**).

To gain a first insight into the involvement of acetaldehyde-mediated ethyl-bridging of procyanidins in the HMW fraction of red wine, the mass spectrometer was tuned for the mass transitions of the target thioethers **9a/b**, **10a/b**, **13a/b**, and **15a/b**, respectively, and, after thiolytic degradation, the HMW fraction was analyzed by means of HPLC-MS/MS operating in the multiple reaction monitoring mode (Figure 9). Whereas only the 4*β*-isomer was detected for the epicatechin-type benzylthioethers **5**, **6**, and **8**, the catechin-4-benzylthioether was present in the 4-*α*- (**7b**) as well as the 4-*β*-configuration (**7a**), thus confirming literature data.[39] The traces of the mass transitions recorded for 1,1-ethylene-bridged thioethers showed always two separated peak clusters, the earlier peaks corresponding to the 6-*C*-(*R*)- and the 6-*C*-(*S*)-(1-benzylthioethyl) isomers and the later eluting ones corresponding to the 8-*C*-(*R*)- and the 8-*C*-(*S*)-(1-benzylthioethyl) isomers. As the C(8) regio-isomer **9a** of compound **9** was found to elute earlier than the corresponding C(6)-isomer **9b**, the same elution order was assumed for the regio-isomers of the structurally related compounds **11**-**15**. Although several analytical columns were tested, no further separation of the R/S-isomers of **9a** and **11a** could be obtained.

Quantitation of **9**−**15** by means of external calibration using the reference compounds revealed low concentrations of these acetaldehyde bridged flavanols in the GAC fractions (Table 3). The highest absolute concentration of 0.6 g/100 g ethyl-bridged thioethers was found in GAC fraction III, whereas the maximum molar percentage of 5.9% was calculated for GAC fraction II. In the total HMW fraction (>5 kDa), ethyl-bridged

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flavan-3-ols accounted for only 1.4% of the total interflavonoid linkages on a molar ratio, thus strengthening literature data obtained by phloroglucinolysis of red wine polymers.[20] Compared to phloroglucinolysis, thiolysis showed the advantage to distinguish between flavanol units polymerized solely with acetaldehyde at the A-ring and units linked with acetaldehyde at the A-ring as well as via a procyanidin-linkage between the A and the C-ring. Thus, it was possible to unambiguously proof the polymerization, not only of monomeric flavanol units, but of intact proanthocyanidin chains. Bearing in mind, that acetaldehyde can link complete procyanidin chains, the real mDP of the fractions could be higher than the mDP based on the calculation of extension and terminal units only.

In order to verify the active incorporation of acetaldehyde into the HMW fraction of wine, spiking experiments with red wine and grape must were carried out. Samples of must and red wine were incubated in the absence or the presence of acetaldehyde at levels of 0.01, 0.1, and 0.5%, respectively, under nitrogen in the dark for 7 days at room temperature. The HMW fraction (>5 kDa) of each model solution was isolated by means of ultrafiltration and was used for thiolysis, followed by LC-MS/MS analysis of the released ethyl bridged thioethers. Quantitation by means of external calibration using reference compounds revealed the highest amounts and the biggest increase after spiking with acetaldehyde for ethyl bridged thioethers (**9a/b−15a/b**); that is, an increase from 0.25% **13a/b** in the HMW fraction of Bordeaux spiked with no acetaldehyde up to 0.51% in the HMW fraction after spiking with 0.1% acetaldehyde (Table 4). In general, the total amount of acetaldehyde linked flavan-3-ols (**9a/b−15a/b**) increased with the amount of acetaldehyde in the must and red wine. Whereas in must the highest concentration was found at a spiking level of 0.5% acetaldehyde, there was no increase of ethyl bridged flavanols in red wine after adding 0.5% acetaldehyde in comparison to 0.1%, indicating that a further increase of acetaldehyde levels had no influence on the formation of the target compounds.

Table 4. Concentration of 1,1-Ethylene Bridged Thioethers after Thiolysis of the HMW Fractions of Must and Bordeaux, Spiked with Different Amounts of Acetaldehyde

	% (mg/100 mg)						
HMW fraction of	**9a/b/ 10a/b**	**11a/b**	**12a/b**	**13a/b**	**14a/b**	**15a/b**	*sum*[b]
Must 0%[a]	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Must 0.01%	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1
Must 0.1%	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	0.1
Must 0.5%	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	0.2
Bordeaux 0%	0.1	<0.1	<0.1	0.3	<0.2	<0.1	0.5
Bordeaux 0.01%	0.3	<0.1	<0.1	0.4	<0.1	<0.1	0.8
Bordeaux 0.1%	0.4	<0.1	<0.1	0.5	<0.1	<0.1	1.0
Boreaux 0.5%	0.4	0.1	<0.1	0.5	<0.1	<0.1	1.0

[a]Amount of spiked acetaldehyde. [b]Sum of compounds **9a/b − 15a/b**.

Sensory Analysis. In order to answer the question as to whether the variation in the chemical composition of GAC fractions I−VIII is reflected by differences in their astringency, their threshold concentration was determined in 1% aqueous ethanol (Table 5). By far the highest threshold concentration of 42.1 mg/L was found for GAC fraction I, whereas the threshold concentrations of all the other fractions ranged between 1.7 and 4.2 mg/L and were not significantly different.

Table 5. Results of the Sensory Evaluation of GPC Fractions and Threshold Concentrations for Astringency and Astringency Intensity at Iso-Concentration

	threshold [mg/L][a]	intensity at iso-concentration[b]
GPC 1	42.1 (±29.0)	n.d.
GPC 2	2.9 (±2.4)	2.1 (±0.7)
GPC 3	3.1 (±5.6)	2.4 (±0.6)
GPC 4	3.3 (±2.6)	3.0 (±1.2)
GPC 5	4.3 (±2.6)	2.5
GPC 6	3.3 (±5.5)	2.8 (±0.6)
GPC 7	1.7 (±1.1)	3.4 (±0.9)
GPC 8	4.2 (±6.9)	3.6 (±0.7)

[a]Threshold concentration for astringency determined in 1% ethanol in bottled water (pH 4.5) using the half-tongue-test. Numbers in parentheses are 95% confidence levels. [b]Intensities of solutions (0.5 mg/L) were rated on a scale from 0 (not perceivable) to 5 (strong impression) using GPC 5 as reference with a given intensity of 2.5. Numbers in parentheses are 95% confidence levels. n.d., not determined.

In order to compare the sensory impact of the GAC fractions at supra-threshold concentrations, fractions II−VIII were evaluated at iso-concentration levels (500 mg/L) by means of the half-tongue test. To achieve this, the trained panelists were asked to evaluate the perceived astringency intensity of each GAC fraction in comparison to fraction V defined with an astringency intensity of 2.5 (Table 5). Although the sensory data might indicate a slight trend of increasing astringency from GAC fraction II (2.1) to VIII (3.4), ANOVA analysis did not show any significant differences between the fractions. Comparing these sensory data with the composition of the individual GAC fractions revealed by far the lowest astringency for GAC fraction I, consisting of about 50% polysaccharides. These data confirm previous literature reports showing that wine polysaccharides do not evoke any astringent orosensation and can even decrease the overall astringency of wines.[49] Interestingly, GAC fractions II−VIII did not differ significantly in threshold concentrations as well as in the astringency intensity when evaluated at iso-concentrations, despite varying mDP and %G values.

In summary, hydrolytic experiments, followed by quantitative analysis revealed that flavan-3-ols, polysaccharides, phenolic acids, amino acids, organic acids, and anthocyanins account for about 50% of the puckering astringent HMW fraction and the GAC subfractions isolated from red wine. The remaining 50% might be explained by the fact that some linkages are not sensitive to hydrolysis, for example, biphenyl linkages derived from nucleophilic attack of polyphenols to the B-ring quinines or A-type linkages of flavan-3-ols.[53] With the exception of a polysaccharide rich GAC fraction I, sensory analysis did not demonstrate any significant difference in the astringency threshold and impact of the various GAC fractions collected. It might therefore be concluded that the degree of polymerization as well as galloylation does not significantly influence the astringent orosensation of red wine fractions.

On the basis of the data obtained, a hypothetical structure of a red wine polymer was proposed in Figure 10. As the main proportion of the HMW fraction was found to be represented by flavan-3-ols upon thiolytic depolymerization, the structural

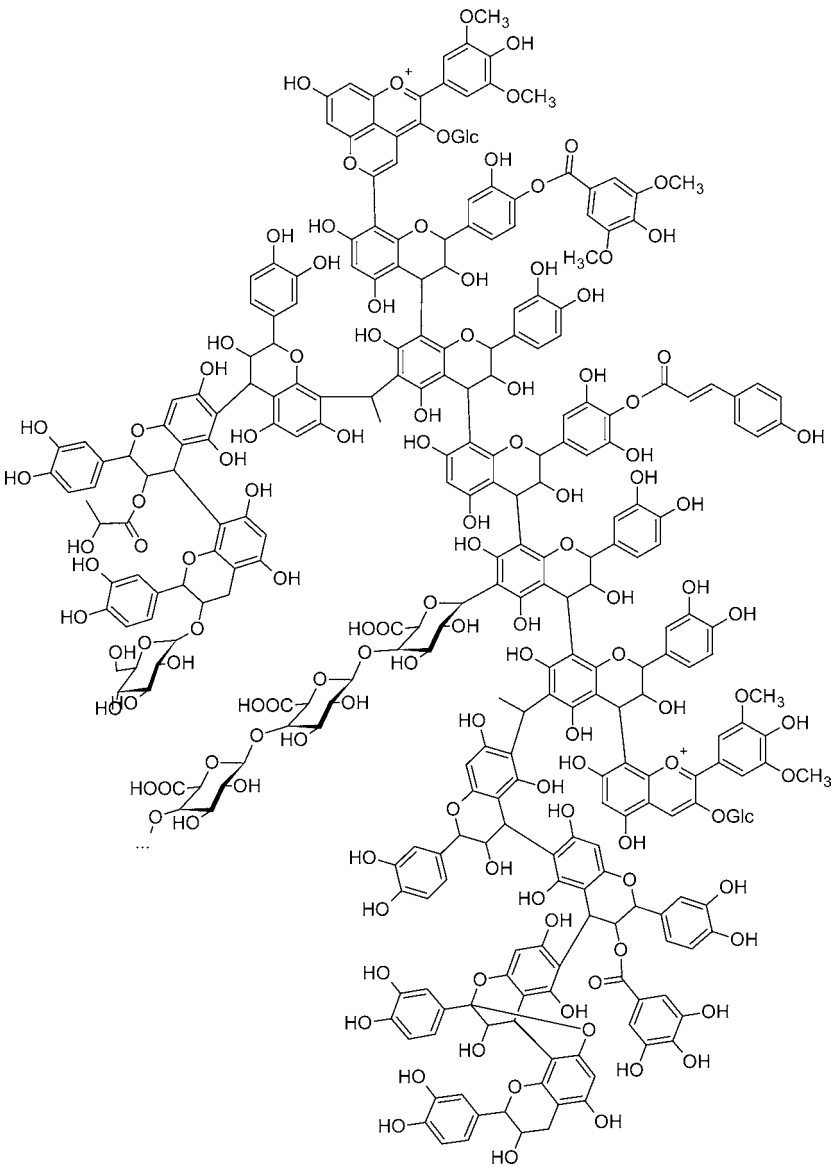

Figure 10. Hypothetical chemical structure of a red wine high molecular weight polymer.

backbone of the polymers seems to be comprised of a procyanidin chain with (−)-epicatechin, (+)-catechin, (−)-epicatechin-3-*O*-gallate units as extension and terminal units as well as (−)-epigallocatechin as extension units. In addition, acetaldehyde was shown to link different procyanidins at the A-ring via an 1,1-ethylene bridge and anthocyanins and pyranoanthocyanins were found to be linked to the procyanidin backbone via a C−C-linkage at position C(6) or C(8), respectively. Moreover, alkaline hydrolysis demonstrated the polymeric procyanidins to be esterified with various organic acids and phenolic acids, respectively. Although the major part of the polysaccharides present in the HMW fraction were found to be not covalently linked to procyanidins, some carbohydrates are expected to be linked *O*-glycosidically or *C*-glycosidically at the A-ring of procyanidins. As amino acids were only present in trace amounts, they were not considered as a fundamental part of the proposed polymer structure. The complexity of the proposed structure might even be increased by $\pi-\pi$ stacking complexes between anthocyanins and procyanidins. The unequivocal verification of structural domains of the polymer structure requires model wine incubations with stable-isotope labeled precursors, followed by LC-MS and NMR diagnostics to monitor the flux of these candidate precursors into the target polymer.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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