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Structure Determination and Sensory Analysis of Bitter-Tasting 4-Vinylcatechol Oligomers and Their Identification in Roasted Coffee by Means of LC-MS/MS

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Aimed at elucidating intense bitter-tasting molecules in coffee, various bean ingredients were thermally treated in model experiments and evaluated for their potential to produce bitter compounds. As caffeic acid was found to generate intense bitterness reminiscent of the bitter taste of a strongly roasted espresso-type coffee, the reaction products formed were screened for bitter compounds by means of taste dilution analysis, and the most bitter tastants were isolated and purified. LC-MS/MS as well as 1-D/2-D NMR experiments enabled the identification of 10 bitter compounds with rather low recognition threshold concentrations ranging between 23 and 178 μmol/L. These bitter compounds are the previously unreported 1,3-bis(3′,4′-dihydroxyphenyl) butane, *trans*-1,3-bis(3′,4′-dihydroxyphenyl)-1-butene, and eight multiply hydroxylated phenylindanes, among which five derivatives are reported for the first time. In addition, the occurrence of each of these bitter compounds in a coffee brew was verified by means of LC-MS/MS (ESI$^-$) operating in the multiple reaction monitoring (MRM) mode. The structures of these bitter compounds show strong evidence that they are generated by oligomerization of 4-vinylcatechol released from caffeic acid moieties upon roasting.

KEYWORDS: Coffee; bitter taste; taste dilution analysis; caffeic acid; chlorogenic acid; 5-*O*-caffeoylquinic acid; phenylindanes; 4-vinylcatechol; polyphenols

INTRODUCTION

In addition to its stimulatory effect, the popularity of a freshly prepared coffee beverage is mainly due to the sheer enjoyment of the alluring aroma as well its attractive and well-balanced taste profile. Within the last 15 years, modern aroma research strategies involving the sensory-based screening of odorants by means of gas chromatography/olfactometry, their accurate quantification by means of stable isotope dilution analysis, followed by aroma reconstitution and omission experiments, have impressively demonstrated that out of several hundreds of coffee volatiles, not more than 30 odorants are essentially required to mimic the typical odor of a freshly brewed cup of coffee (*1*−*3*). As compared to the aroma active volatiles, the knowledge of the molecular basis of the typical coffee bitter taste, developed during appropriate roasting of the beans, is rather fragmentary.

More than 20 years ago, the first systematic analytical and sensory studies revealed that the alkaloids caffeine and trigonelline, already present in raw coffee beans, account for a maximum of 10−30 and 1%, respectively, of the total bitter taste intensity of a coffee beverage (*4*). The author suggested that molecules thermally generated from precursors during bean roasting are the key elicitors of the bitter taste of a coffee brew. In particular, thermally generated compounds such as furfurylalcohol (*5*), 5-hydroxymethyl-2-furanaldehyde (*6*), various pyrazines (*6*), as well as *cis*- and *trans*-configured 2,5-diketopiperazines (*7*) were suggested as potential bitter-tasting agents in roasted coffees but for none of these heterocyclic compounds could a contribution to the coffee bitter taste be confirmed so far.

Besides such heterocyclics, *O*-caffeoylquinic acids (*8*), also known as chlorogenic acids, and their corresponding thermally generated lactones (*9*) were discussed as a cause of the observed bitter taste of coffee, but neither have the purified compounds been sensorially evaluated in these studies nor have any human bitter detection thresholds been determined. In addition, quinic acid liberated from chlorogenic acid upon thermohydrolysis was reported to exhibit an aspirin-like bitter taste at a threshold concentration of 10 ppm. As the concentration of this compound in roasted coffee exceeded its threshold concentration by a factor

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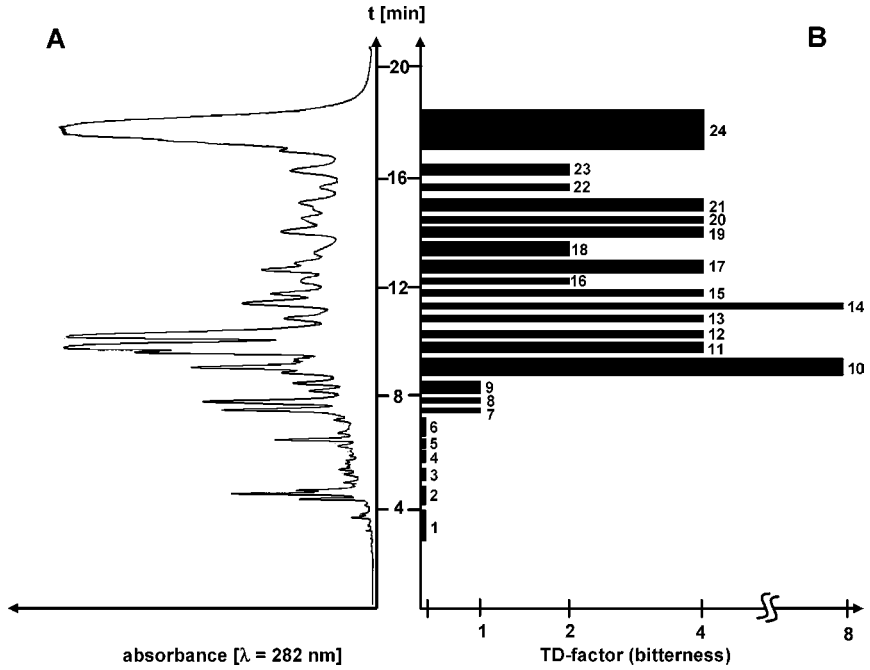

Figure 1. RP-HPLC chromatogram (**A**) and taste dilution (TD) chromatogram (**B**) of the solvent-extractable compounds isolated from thermally treated caffeic acid (15 min, 220 °C).

of 20, quinic acid was suggested as one of the contributors to coffee bitter taste (*10*, *11*).

Very recently, sensory guided analysis of a medium roasted coffee brew, followed by LC-MS/MS and 2-D NMR spectroscopy, as well as synthesis led to the unequivocal identification of 3-*O*-caffeoyl-γ-quinide, 4-*O*-caffeoyl-γ-quinide, 5-*O*-caffeoyl-*epi*-δ-quinide, 4-*O*-caffeoyl-*muco*-γ-quinide, 5-*O*-caffeoyl-*muco*-γ-quinide, 3-*O*-feruloyl-γ-quinide, and 4-*O*-feruloyl-γ-quinide as intensely bitter-tasting compounds in roasted coffee (*12*). Besides these individual bitter compounds, we were able to isolate a highly complex and bitter RP-18 fraction and identified 3,4-*O*-dicaffeoyl-γ-quinide, 3,5-*O*-dicaffeoyl-*epi*-δ-quinide, and 4,5-*O*-dicaffeoyl-*muco*-γ-quinide as strongly bitter-tasting compounds in that fraction (*12*). Sensory experiments revealed that, depending on their chemical structure, the bitter threshold levels of these lactones ranged between 9.8 and 180 μmol/L (water) (*12*). Comparison of the human bitter threshold concentration of the total complex RP-18 fraction with those of the dicaffeoylquinides demonstrated that, besides the compounds already identified, additional substances play an important role in the coffee bitter taste. As the enormous complexity of that RP-18 fraction did not allow the successful isolation of additional bitter compounds, the objectives of the present study were to evaluate the bitter taste precursor potential of major bean ingredients, to screen for the most bitter compounds by applying the recently developed taste dilution analysis (*13*), to identify the compounds inducing the highest human bitter taste response, and, finally, to confirm the occurrence of these bitter compounds in a freshly prepared coffee brew by means of LC-MS/MS experiments.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained commercially: caffeic acid, 5-*O*-caffeoylquinic acid, L-glutamic acid, L-proline, L-alanine, L-asparagine, L-aspartic acid, quinic acid, sucrose, and trigonelline (Aldrich, Steinheim, Germany), and the solvents were of HPLC grade (Merck, Darmstadt, Germany). DMSO-d_6, $CDCl_3$, and methanol-d_4 were obtained from Euriso-top (Saarbrücken, Germany). Roasted Arabica coffee beans (CTN value 68) from Columbia were obtained from the coffee industry.

Sensory Analyses. Twelve assessors (five male and seven female), who gave the informed consent to participate in the sensory tests of the present investigation and who had no history of known taste disorders, were trained in sensory experiments at regular intervals for at least 2 years as described earlier (*13*−*17*) and were, therefore, familiar with the techniques applied. Sensory analyses were performed in a sensory panel room at 22−25 °C in three different sessions.

Prior to sensory analysis, the fractions or compounds isolated were suspended in water, and, after removing the volatiles in 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.

Using a triangle test (*18*), taste recognition threshold concentrations were determined in water (Evian, low mineralization: 405 mg/L; pH 5.2). The values between the 12 individuals and three separate sessions differed by not more than one dilution step (i.e., a threshold value of 0.5 mmol/L for caffeine represents a range from 0.25 to 1.0 mmol/L).

Model Roasting Experiments. 5-*O*-Caffeoylquinic acid, quinic acid, caffeic acid, ferulic acid, trigonelline, and sucrose (2.5 mmol each) and a mixture containing sucrose (2.5 mmol), L-glutamic acid (0.09 mmol), L-proline (0.04 mmol), L-alanine (0.05 mmol), L-asparagine (0.03 mmol), and L-aspartate (0.02 mmol) were separately suspended in water (3 mL), dried at 80 °C for 30 min, and then roasted at 220 °C for 15 min in a laboratory oven. The roasting products were dissolved in hot water (50 mL, 80 °C, pH 5.2), cooled to room temperature, and then split into two aliquots. One aliquot (20 mL) was used for sensory analysis, and the other aliquot (30 mL) was extracted with ethyl acetate (5 × 50 mL). The combined organic layers were freed from solvent in high vacuum (<5 mPa), and the residue was taken up in a mixture of methanol/water (1:2, v/v; 20 mL) and analyzed by means of taste dilution analysis, HPLC-DAD, and HPLC-MS/MS.

Taste Dilution Analysis (TDA). Aliquots (100 μL) of the solvent extract obtained from the thermally treated caffeic acid were separated by a semipreparative RP-18 HPLC using a 250 mm × 10 mm i.d., 5 μm, Microsorb 100-5 C18 column (Varian, Darmstadt, Germany). Monitoring the effluent at 282 nm, chromatography was performed starting with a mixture (75:25, v/v) of aqueous formic acid (0.1% in water) and acetonitrile, thereafter increasing the acetonitrile content to 28% within 5 min, then to 30% within 20 min, then to 100% within 5

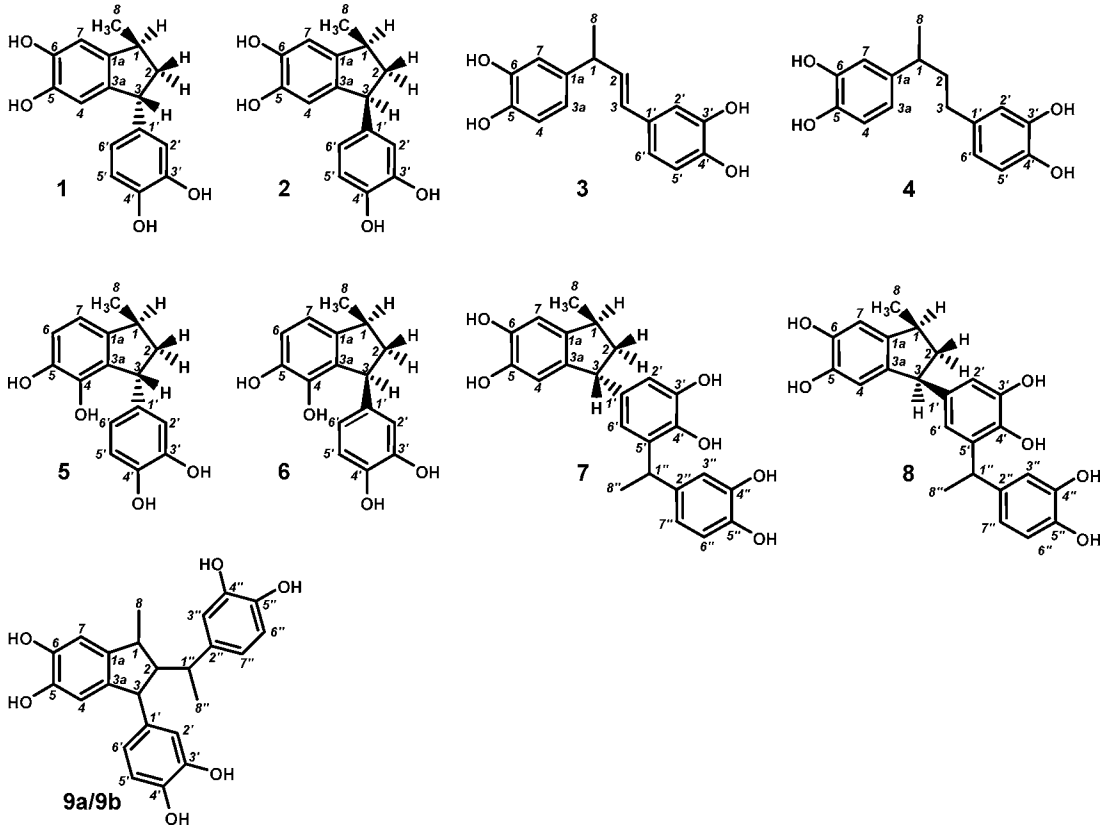

Figure 2. Chemical structures of the bitter-tasting reaction products **1**–**9a**,**b** formed upon thermal treatment of caffeic acid.

min, and, finally, maintaining the acetonitrile content for additional 5 min. As shown in **Figure 1**, the corresponding 24 fractions obtained from 10 runs were combined. After removing the solvent in vacuum, the individual fractions were purified by means of RP-18 solid phase extraction using C-18 E cartridges (1 g) (Phenomenex, Aschaffenburg, Germany), which were conditioned with methanol, followed by water (20 mL each) prior to use. After sample application, the cartridge was rinsed with water (20 mL), nitrogen was sucked through the cartridge for 15 min by means of a vacuum pump, and finally, the target compounds were eluted with methanol (20 mL). After removing the solvent in high vacuum (<5 mPa), the individual fractions were dissolved in natural ratios in exactly 2.0 mL of bottled water adjusted to pH 5.2 by using aqueous hydrochloric acid (0.1 mol/L) and were then used for the taste dilution analysis (*13*−*17*). To achieve this, the individual solutions were sequentially diluted 1:1 with bottled water (pH 5.2), the serial dilutions of each of the fractions were presented to the sensory panel in order of ascending concentrations, and each dilution was evaluated for bitter taste by means of a triangle test. The dilution at which a taste difference between the diluted extract and the blank (control) could just be detected was defined as taste dilution (TD-factor (*13*−*17*). The TD-factors evaluated by four different assessors in three different sessions were averaged. The TD-factors between individuals and separate sessions did not differ by more than one dilution step.

Isolation of Bitter Compounds 1−**9a,b from HPLC Fractions 10**−**15, 17, 20, and 21.** The solvent extractables obtained from the thermally treated caffeic acid were separated by semipreparative RP-18 HPLC using a 250 mm × 10 mm i.d., 5 μm, Microsorb 100-5 C18 column (Varian, Darmstadt, Germany). Monitoring the effluent at 282 nm, chromatography was performed starting with a mixture (75:25, v/v) of aqueous formic acid (0.1% in water) and acetonitrile, thereafter increasing the acetonitrile content to 28% within 5 min, then to 30% within 20 min, thereafter to 100% within 5 min, and, finally, maintaining the acetonitrile content for additional 5 min. The HPLC fractions 10−15, 17, 20, and 21 (**Figure 1**) were collected individually in several runs, and the effluents of the corresponding fractions were combined. To avoid oxidative degradation of phenolic taste compounds, ascorbic acid (0.25 mg/mL) was added to each fraction collected. After removing the solvent in vacuum, each fraction was finally purified by means of solid phase extraction using C-18 E cartridges (1 g) (Phenomenex, Aschaffenburg, Germany) conditioned with methanol, followed by water (20 mL each). After sample application, the cartridge was rinsed with water (20 mL), nitrogen was sucked through the cartridge for 15 min by means of a vacuum pump, and finally, the target compounds were eluted with ethanol (20 mL). The effluent of each individual fraction was split into two aliquots, and after removing the solvent in high vacuum (<5 mPa), one aliquot was used for the determination of the taste threshold concentrations, whereas the second aliquot was used for structure determination. By means of UV/vis, LC-MS/MS, and 1-D/2-D NMR experiments, the following taste compounds (**Figure 2**) were isolated from the HPLC fraction given in brackets in a purity of more the 98%: compound **1** (11), **2** (12), **3** (13), **4** (14), **5** (15), **6** (17), **7** (20), **8** (21), **9a** (10), and **9b** (11).

trans-5,6-Dihydroxy-1-methyl-3-(3′,4′-dihydroxyphenyl) indane, **1** (the arbitrary numbering of the carbon atoms refers to the structure given in **Figure 2**): UV/vis (water/acetonitrile; 65:35, v/v): λ_{max} 286 nm; LC/TOF-MS: $C_{16}H_{16}O_4$; LC/MS (ESI$^-$): *m*/*z* 271.0 (100; [M − H]$^-$); ^{1}H NMR (400 MHz; d_3-MeOD; COSY): δ 1.21 [d, 3H, J = 6.80 Hz, H−C(8)], 2.08 [ddd, 1H, J = 5.2, 8.0, 12.8 Hz, H−C(2_α)], 2.17 [ddd, 1H, J = 6.0, 7.6, 12.8 Hz, H−C(2_β)], 3.14−3.25 [m, 1H, J = 6.7 Hz, H−C(1)], 4.15 [dd, 1H, J = 6.0, 8.0 Hz, H−C(3)], 6.40 [s, 1H, H−C(4)], 6.46 [dd, 1H, J = 2.0, 8.0 Hz, H−C(6′)], 6.50 [d, 1H, J = 2.0 Hz, H−C(2′)], 6.66 [s, 1H, H−C(7)], 6.67 [d, 1H, J = 8.0 Hz, H−C(5′)]; ^{13}C NMR (100 MHz; d_3-MeOD; HMQC, HMBC): δ 19.9 (CH_3, C(8)), 37.4 (CH, C(1)), 45.4 (CH_2, C(2)), 48.6 (CH, C(3)), 109.6 (CH, C(7)), 111.2 (CH, C(4)), 114.2 (CH, C(2′)), 114.6 (CH, C(5′)), 118.7 (CH, C(6′)), 137.3 (C, C(3a)), 138.2 (C, C(1′)), 140.1 (C, C(1a)), 142.9 (C, C(4′)), 143.7 (C, C(5)), 144.0 (C, C(6)), 144.7 (C, C(3′)).

cis-5,6-Dihydroxy-1-methyl-3-(3′,4′-dihydroxyphenyl) indane, **2** (**Figure 2**): UV/vis (water/acetonitrile; 65:35; v/v): λ_{max} 286 nm; LC/TOF-MS: $C_{16}H_{16}O_4$; LC/MS (ESI$^-$): *m*/*z* 271.0 (100; [M − H]$^-$); ^{1}H NMR (400 MHz; d_3-MeOD; COSY): δ 1.30 [d, 3H, J = 6.8 Hz, H−C(8)], 1.39−1.51 [m, 1H, J = 10.4, 12.0 Hz, H−C(2_α)], 2.60 [ddd, 1H, J = 6.8, 7.2, 12.0 Hz, H−C(2_β)], 2.97−3.08 [m, 1H, J = 6.8, 10.0 Hz, H−C(1)], 3.93 [dd, 1H, J = 7.2, 10.4 Hz, H−C(3)], 6.29 [s, 1H,

H−C(4)], 6.53 [dd, 1H, J = 2.1, 8.1 Hz, H−C(6′)], 6.61 [d, 1H, J = 2.1 Hz, H−C(2′)], 6.65 [s, 1H, H−C(7)], 6.71 [d, 1H, J = 8.1 Hz, H−C(5′)]; ^{13}C NMR (100 MHz; d_3-MeOD; HMQC, HMBC): δ 19.0 (CH_3, C(8)), 37.7 (CH, C(1)), 47.3 (CH_2, C(2)), 49.6 (CH, C(3)), 109.2 (CH, C(7)), 111.1 (CH, C(4)), 114.8 (CH, C(2′)), 114.8 (CH, C(5′)), 119.3 (CH, C(6′)), 138.9 (C, C(3a)), 139.6 (C, C(1′)), 141.2 (C, C(1a)), 143.2 (C, C(5)), 144.5 (C, C(4′)), 145.2 (C, C(6)), 146.2 (C, C(3′)).

trans-1,3-Bis(3′,4′-dihydroxyphenyl)-1-butene, **3** (**Figure 2**): UV/vis (water/acetonitrile; 60:40; v/v): λ_{max} 263, 283 nm; LC/TOF-MS: $C_{16}H_{16}O_4$; LC/MS (ESI$^-$): m/z 271.0 (100; [M − H]$^-$); ^{1}H NMR (400 MHz; d_3-MeOD; COSY): δ 1.37 [d, 3H, J = 6.97 Hz, H−C(8)], 3.40−3.43 [m, 1H, J = 6.72, 6.97 Hz, H−C(1)], 6.12 [dd, 1H, J 6.72, 15.9 Hz, H−C(2)], 6.22 [d, 1H, J = 15.9 Hz, H−C(3)], 6.58 [dd, 1H, J = 2.0, 8.4 Hz, H−C(6′)], 6.29 [s, 1H, H−C(4)], 6.58 [dd, 1H, J = 2.2, 8.2 Hz, H−C(3a)], 6.66−6.73 [m, 4H, J = 2.1 Hz, H−C(4/7/5′/6′)], 6.82 [s, 1H, H−C(2′)]; ^{13}C NMR (100 MHz; d_3-MeOD; HMQC, HMBC): δ 20.8 (CH_3, C(8)), 41.7 (CH, C(1)), 112.1 (CH, C(2′)), 112.3 (CH, C(5′)), 113.8 (CH, C(4)), 114.8 (CH, C(7)), 117.8 (CH, C(6′)), 118.0 (CH, C(3a)), 127.6 (CH, C(3)), 130.1 (C, C(1′)), 132.6 (CH, C(2)), 137.8 (C, C(1a)), 143.0 (C, C(5)), 143.6 (C, C(4′)), 144.0 (C, C(6)), 144.5 (C, C(3′)).

1,3-Bis(3′,4′-dihydroxyphenyl) butane, **4** (**Figure 2**): UV/vis (water/acetonitrile; 65:35; v/v): λ_{max} 286 nm; LC/TOF-MS: $C_{16}H_{18}O_4$; LC/MS (ESI$^-$): m/z 273.0 (100; [M − H]$^-$); ^{1}H NMR (400 MHz; d_3-MeOD; COSY): δ 1.17 [d, 3H, J = 6.97 Hz, H−C(8)], 1.72−1.77 [m, 2H, J = 7.2, 7.4 Hz, H−C(2)], 2.31 [t, 2H, J = 7.2, H−C(3)], 2.48−2.55 [m, 1H, J = 6.97, 7.4, H−C(1)], 6.42 [dd, 1H, J = 2.0, 8.0 Hz, H−C(6′)], 6.50 [dd, 1H, J = 2.0, 8.0 Hz, H−C(3a)], 6.54 [d, 1H, J = 2.0 Hz, H−C(2′)], 6.62 [d, 1H, J = 2.0 Hz, H−C(7)], 6.63 [d, 1H, J = 8.0 Hz, H−C(5′)], 6.69 [d, 1H, J = 8.0 Hz, H−C(4)]; ^{13}C NMR (100 MHz; d_3-MeOD; HMQC, HMBC): δ 21.7 (CH_3, C(8)), 32.9 (CH_2, C(3)), 38.7 (CH, C(1)), 40.4 (CH_2, C(2)), 113.4 (CH, C(7)), 114.8 (CH, C(4)), 114.9 (CH, C(5′)), 115.2 (CH, C(2′)), 118.0 (CH, C(3a)), 119.1 (C, C(6′)), 134.2 (C, C (1′)), 139.2 (C, C(1a)), 142.8 (C, C(3′/4′)), 143.1 (C, C(5/6)), 144.1 (C, C(4′/3′)), 144.9 (C, C(6/5)).

trans-4,5-Dihydroxy-1-methyl-3-(3′,4′-dihydroxyphenyl) indane, **5** (**Figure 2**): UV/vis (water/acetonitrile; 65:35; v/v): λ_{max} = 286 nm; LC/TOF-MS: $C_{16}H_{16}O_4$; LC/MS (ESI$^-$): m/z 271.0 (100; [M − H]$^-$); ^{1}H NMR (400 MHz; d_3-MeOD; COSY): δ 1.20 [d, 3H, J = 6.8 Hz, H−C(8)], 1.99 [ddd, 1H, J = 8.4, 9.3, 12.2 Hz, H−C(2_α)], 2.16 [ddd, 1H, J = 1.96, 6.97, 12.2 Hz, H−C(2_β)], 3.13−3.21 [m, 1H, J = 6.8, 9.3 Hz, H−C(1)], 4.33 [dd, 1H, J = 1.96, 8.4 Hz, H−C(3)], 6.40 [dd, 1H, J = 2.1, 8.1 Hz, H−C(6′)], 6.45 [d, 1H, J = 2.1 Hz, H−C(2′)], 6.55 [d, 1H, J = 7.8, H−C(6)], 6.61 [d, 1H, J = 8.1 Hz, H−C(5′)], 6.69 [d, 1H, J = 7.8, H−C(7)]; ^{13}C NMR (100 MHz; d_3-MeOD; HMQC, HMBC): δ 18.7 (CH_3, C(8)), 36.7 (CH, C(1)), 45.6 (CH, C(3)), 46.0 (CH_2, C(2)), 113.2 (CH, C(6)), 114.2 (CH, C(2′)), 114.7 (CH, C(5′)), 115.0 (CH, C(7)), 118.4 (CH, C(6′)), 132.1 (C, C(3a)), 137.0 (C, C(1′)), 141.2 (C, C(1a)), 143.4 (C, C(4)), 142.8 (C, C(4′)), 143.4 (C, C(5)), 144.5 (C, C(3′)).

cis-4,5-Dihydroxy-1-methyl-3-(3′,4′-dihydroxyphenyl) indane, **6** (**Figure 2**): UV/vis (water/acetonitrile; 65:35; v/v): λ_{max} 286 nm; LC/TOF-MS: $C_{16}H_{16}O_4$; LC/MS (ESI$^-$): m/z 271.0 (100; [M − H]$^-$); ^{1}H NMR (400 MHz; d_3-MeOD; COSY): δ 1.15 [d, 3H, J = 6.91 Hz, H−C(8)], 1.40 [ddd, 1H, J = 7.7, 12.6 Hz, H−C(2_α)], 2.60 [ddd, 1H, J = 8.1, 12.6 Hz, H−C(2_β)], 2.94 [m, 1H, H−C(1)], 4.10 [pt, 1H, J = 7.9 H−C(3)], 6.41 [dd, 1H, J = 2.0, 8.0 Hz, H−C(6′)], 6.46 [d, 1H, J = 2.0 Hz, H−C(2′)], 6.47 [d, 1H, J = 8.0 Hz, H−C(6)], 6.56 [d, 1H, J = 8.0 Hz, H−C(5′)], 6.58 [d, 1H, J = 8.0 Hz, H−C(7)]; ^{13}C NMR (100 MHz; d_3-MeOD; HMQC, HMBC): δ 20.2 (CH_3, C(8)), 38.2 (CH, C(1)), 46.8 (CH_2, C(2)), 47.3 (CH, C(3)), 113.7 (CH, C(6)), 114.2 (CH, C(2′)), 114.4 (CH, C(5′)), 114.7 (CH, C(7)), 118.7 (CH, C(6′)), 131.7 (C, C(3a)), 137.7 (C, C(1′)), 141.4 (C, C(1a)), 143.1 (C, C(4)), 143.3 (C, C(4′)), 144.1 (C, C(5)), 144.5 (C, C(3′)).

trans-5,6-Dihydroxy-1-methyl-3-[3′,4′-dihydroxy-5′-(1-(3″,4″-dihydroxyphenyl)-1-ethyl)phenyl] indane, **7** (**Figure 2**): UV/vis (water/acetonitrile; 65:35; v/v): λ_{max} 286 nm; LC/TOF-MS: $C_{24}H_{24}O_6$; LC/MS (ESI$^-$): m/z 407.2 (100; [M − H]$^-$); ^{1}H NMR (400 MHz; d_3-MeOD; COSY): δ 1.17 [d, 3H, J = 6.8 Hz, H−C(8)], 1.47 [d, 3H, J = 7.2 Hz, H−C(8″)], 2.02 [ddd, 1H, J = 5.2, 8.0, 12.5 Hz, H−C(2_α)], 2.10 [ddd, 1H, J = 6.0, 7.6, 12.5 Hz, H−C(2_β)], 3.10−3.20 [m, H−C(1)], 4.07 [dd, 1H, J = 6.0, 8.0 Hz, H−C(3)], 4.36 [q, 1H, J = 7.2, H−C(1″)], 6.29 [d, 1H, J = 2.0, H−C(2′/6′)], 6.37 [s, H−C(4)], 6.43 [d, 1H, J = 2.0 Hz, H−C(6′/2′)], 6.57 [dd, 1H, J = 8.2, 2.0 Hz, H−C(7″)], 6.62 [s, H−C(7)], 6.65 [d, 1H, J = 8.2 Hz, H−C(6″)], 6.67 [d, 1H, J = 2.0 Hz, H−C(3″)]; ^{13}C NMR (100 MHz; d_3-MeOD; HMQC, HMBC): δ 19.7 (CH_3, C(8)), 20.11 (CH_3, C(8″)), 36.7 (CH, C(1″)), 37.4 (CH, C(1)), 45.3 (CH, C(2)), 48.8 (CH, C(3)), 109.4 (CH, C(7)), 111.1 (CH, C(2′/6′)), 111.3 (CH, C(4)), 114.5 (CH, C(6″)), 114.6 (CH, C(3″)), 118.0 (CH, C(6′/2′)), 118.6 (CH, C(7″)), 133.3 (C, C(5′)), 137.2 (C, C(1′)), 138.1 (C, C(2″)), 139.6 (C, C(3a)), 140.3 (C, C(1a)), 143.5 (C, C(5/6)), 143.6 (C, C(3′/4′)),143.7 (C, C(6/5)),143.8 (C, C(4″/5″)),144.0 (C, C(4′/3′)),144.4 (C, C(5″/4″))

cis-5,6-Dihydroxy-1-methyl-3-[3′,4′-dihydroxy-5′-(1-(3″,4″-dihydroxyphenyl)-1-ethyl)phenyl] indane, **8** (**Figure 2**): UV/vis (water/acetonitrile; 65:35; v/v): λ_{max} 286 nm; LC/TOF-MS: $C_{24}H_{24}O_6$; LC/MS (ESI$^-$): m/z 407.2 (100; [M − H]$^-$); ^{1}H NMR (400 MHz; d_3-MeOD; COSY): δ 1.26 [d, 3H, J = 6.8 Hz, H−C(8)], 1.38−1.47 [m, 1H, J = 10.4, 12.4 Hz, H−C(2_α)], 1.49 [d, 3H, J = 7.2 Hz, H−C(8″)], 2.54 [ddd, 1H, J = 6.8, 7.2, 12.5 Hz, H−C(2_β)], 2.93−3.03 [m, 1H, J = 6.8, 10.2, H−C(1)], 3.87 [dd, 1H, J = 7.2, 10.4 Hz, H−C(3)], 4.39 [q, 1H, J = 7.2, H−C(1″)], 6.26 [s, H−C(4)], 6.42 [d, 1H, J = 2.0, H−C(2′/6′)], 6.50 [d, 1H, J = 2.0 Hz, H−C(6′/2′)], 6.59 [dd, 1H, J = 8.2, 2.0 Hz, H−C(7″)], 6.62 [s, H−C(7)], 6.65 [d, 1H, J = 8.2 Hz, H−C(6″)], 6.69 [d, 1H, J = 2.0 Hz, H−C(3″)]; ^{13}C NMR (100 MHz; d_3-MeOD; HMQC, HMBC): δ 18.6 (CH_3, C(8)), 20.14 (CH_3, C(8″)), 36.6 (CH, C(1″)), 37.6 (CH, C(1)), 46.6 (CH, C(2)), 49.9 (CH, C(3)), 109.0 (CH, C(7)), 111.0 (CH, C(4)), 111.5 (CH, C(2′/6′)), 114.5 (CH, C(3″)), 114.6 (CH, C(6″)), 118.4 (CH, C(6′/2′)), 118.6 (CH, C(7″)), 133.2 (C, C(5′)), 136.7 (C, C(1′)), 138.3 (C, C(2″)), 139.2 (C, C(3a)), 139.7 (C, C(1a)), 143.2 (C, C(3′/4′)), 143.4 (C, C(4″/5″)), 143.6 (C, C(5/6)), 143.7 (C, C(6/5)), 144.2 (C, C(5″/4″)), 144.4 (C, C(4′/3′)).

5,6-Dihydroxy-1-methyl-2-[1-(3′,4′-dihydroxyphenyl)-1-ethyl]-3-(3″,4″-dihydroxyphenyl) indane, diastereomer **9a** (**Figure 2**): UV/vis (water/acetonitrile; 65:35; v/v): λ_{max} 286 nm; LC/TOF-MS: $C_{24}H_{24}O_6$; LC/MS (ESI$^-$): m/z 407.2 (100; [M − H]$^-$); ^{1}H NMR (400 MHz; d_3-MeOD; COSY): δ 0.94 [d, 3H, J = 6.8 Hz, H−C(8)], 1.22 [d, 3H, J = 7.2 Hz, H−C(8″)], 2.14 [ddd, 1H, J = 6.8, 7.2 Hz, H−C(2)], 2.69 [dq, 1H, J = 7.2 Hz, H−C(1″)], 2.80 [dq, 1H, J = 6.8 Hz, H−C(1)], 3.85 [d, 1H, J = 7.2 Hz, H−C(3)], 6.15 [s, 1H, H−C(4)], 6.50 [s, H−C(7)], 6.52−6.56 [m, 2H, J = 2.0, 8.0 Hz, H−C(6′/7″)], 6.60 [d, 1H, J = 2.0 Hz, H−C(2′)], 6.68 [d, 1H, J = 2.0 Hz, H−C(3″)], 6.70 [d, 1H, J = 8.0 Hz, H−C(5′)], 6.71 [d, 1H, J = 8.0 Hz, H−C(6″)]; ^{13}C NMR (100 MHz; d_3-MeOD; HMQC, HMBC): δ 21.9 (CH_3, C(8″)), 22.4 (CH_3, C(8)), 44.3 (CH, C(1)), 46.4 (CH, C(1″)), 56.6 (CH, C(3)), 66.2 (CH, C(2)), 110.6 (CH, C(7)), 112.4 (CH, C(4)), 116.09 (CH, C(3″/5′/6″)), 116.14 (CH, C(3″/5′/6″)), 116.15 (CH, C(3″/5′/6″)), 116.7 (CH, C(2′)), 120.5 (CH, C(7″)), 121.2 (CH, C(6′)), 138.9 (C, C(3a)), 139.6 (C, C(2″)), 140.2 (C, C(1a)), 140.6 (C, C(1′)), 143.33 (C, C(5/4″)), 143.36 (C, C(5/4″)), 145.2 (C, C(3′)), 145.4 (C, C(6)), 146.0 (C, C(4′/5″)), 146.2 (C, C(5″/4′)); diastereomer **9b** (**Figure 2**): UV/vis (water/acetonitrile; 65:35; v/v): λ_{max} 286 nm; LC/TOF-MS: $C_{24}H_{24}O_6$; LC/MS (ESI$^-$): m/z 407.2 (100; [M − H]$^-$); ^{1}H NMR (400 MHz; d_3-MeOD; COSY): δ 1.32−1.36 [m, 6H, J = 6.8, 7.2 Hz, H−C(8), H−C(8″))], 2.50 [ddd, 1H, J = 6.4, 8.0 Hz, H−C(2)], 2.73 [dq, 1H, J = 6.8 Hz, H−C(1)], 3.11−3.22 [m, 2H, J = 7.2 Hz, H−C(1″), H−C(3))], 6.24 [dd, 1H, J = 2.2, 8.0 Hz H−C(6′/7″)], 6.41 [d, 1H, J = 2.2 Hz, H−C(2′/3″)], 6.49 [d, 1H, J = 2.2 Hz, H−C(3″/2′)], 6.54−6.60 [m, 3H, J = 2.2, 8.0 Hz, H−C(4/7), H−C(5′/6″), H−C(7″/6′)], 6.62 [s, 1H, H−C(7/4)], 6.70 [d, 1H, J = 8.0 Hz (H−C(6″/5′))].

Preparation of the Coffee Beverage. After grinding the coffee beans by means of an ultra centrifuge mill (Retsch, Haan, Germany) equipped with a sieve (2 mm pore diameter), aliquots (54 g) of the coffee powder were placed in a coffee filter (No. 4, Melitta, Minden, Germany) and percolated with hot water by using a Melitta Look coffee maker (Melitta, Minden, Germany) until the filtrate reached a volume of 1 L. The resulting beverage (54 g/L) had a temperature of 75 °C and was rapidly cooled in an ice-bath prior to HPLC-MS/MS analysis.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Kontron, Eching, Germany) consisted of two 422-type pumps, an M800-type gradient mixer, a 100 μL Rheodyne injector, and a 520-type diode array detector, monitoring the effluent in a range

between 220 and 500 nm. Separations were performed on a semi-preparative 250 mm × 10 mm i.d., 5 μm, Microsorb 100-5 C18 column (Varian, Darmstadt, Germany) operating with a flow rate of 5.2 mL/min or an analytical 250 mm × 4.6 mm i.d., 5 μm, Microsorb 100-5 C18 column operating with a flow rate of 1.0 mL/min.

LC/Time-of-Flight Mass Spectrometry (LC/TOF-MS). High-resolution mass spectra of the isolated compounds **1**−**9** were measured on a Bruker Micro-TOF mass spectrometer (Bruker Daltronics, Bremen, Germany) and referenced on sodium formate.

High-Performance Liquid Chromatography/Tandem Mass Spectrometry (HPLC-MS/MS). The Agilent 1100 Series HPLC system consisted of a pump, a degasser, and an autosampler (Agilent, Waldbronn, Germany) and was connected to a 4000 Q Trap triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex, Darmstadt, Germany) with an electrospray ionization (ESI) device running in negative ionization mode with a spray voltage of −4500 V. The quadrupoles operated at unit mass resolution. Nitrogen served as a curtain gas (20 psi), and the declustering potential was set at −10 to −85 V in the ESI^- mode. The mass spectrometer was operated in the full scan mode monitoring negative ions. Fragmentation of $[M - H]^-$ molecular ions into specific product ions was induced by collision with nitrogen (5×10^{-5} Torr) and a collision energy of −15 to −70 V. For instrumentation control and data aquisition, the Sciex Analyst software (v1.4) was used.

For HPLC-MS/MS analysis of the bitter compounds **1**−**9a**,**b** in coffee, an analytical 150 mm × 2.1 mm i.d., 5 μm, Zorbax Eclipse XDB C18 column (Agilent, Waldbronn, Germany) was connected to the mass spectrometer operating in the multiple reaction monitoring mode (MRM) detecting negative ions. For a duration of 150 ms, the mass transitions *m*/*z* 271 → 109 and 271 → 146 were used for the analysis of **1** and **2**, *m*/*z* 271 → 109 and 271 → 135 for compound **3**, *m*/*z* 273 → 109 and 273 → 135 for the analysis of **4**, *m*/*z* 271 → 109 and 271 → 161 for analysis of **5** and **6**, *m*/*z* 407 → 109 and 407 → 297 for analysis of **7** and **8**, and *m*/*z* 407 → 109 and 407 → 161 for analysis of compounds **9a** and **9b**. Nitrogen served as nebulizer gas (35 psi) and as turbo gas (350 °C) for solvent drying (45 psi). After injection of the sample (20 μL), chromatography was performed with a flow rate of 250 μL/min using a gradient starting with a mixture (75:25, v/v) of aqueous formic acid (0.1% in water) and acetonitrile and increasing the acetonitrile content to 28% within 5 min, then to 30% within 20 min, and, finally, to 100% within 5 min, thereafter maintaining the acetonitrile content for an additional 5 min.

Nuclear Magnetic Resonance Spectroscopy (NMR). 1-D and 2-D NMR experiments were performed on a Bruker DMX-400 (Bruker, Rheinstetten, Germany). Chemical shifts were determined using tetramethylsilane (TMS) as the internal standard in the proton dimension and from the carbon signal of d_3-MeOD (49.3 ppm) in the carbon dimension.

RESULTS AND DISCUSSION

Aimed at evaluating the potential of various raw coffee ingredients to generate bitter-tasting compounds upon bean roasting, 5-*O*-caffeoylquinic acid, quinic acid, caffeic acid, ferulic acid, sucrose, and trigonelline, respectively, were separately roasted for 15 min at 220 °C in a laboratory oven. To study the influence of amino acids on the generation of Maillard-type bitter compounds from sucrose, in addition, sucrose was heated in the presence of amino acids that are quantitatively predominant in raw coffee beans, namely, L-glutamic acid, L-proline, L-alanine, L-asparagine, and L-aspartate used in their natural concentration ratios (*19*). After cooling, the roasted materials were taken up in hot water, and after cooling to room temperature, the aqueous extractables of the individual roasted precursor mixtures were sensorially evaluated in aqueous solution (pH 5.2), and their bitter taste intensities were rated on a 5-point scale from 0 (not bitter) to 5 (extremely bitter). The results, given in **Table 1**, show that roasting of caffeic acid produced the most intense bitter-tasting compounds, exhibiting a very harsh type of bitterness reminiscent of the bitter quality of a strongly roasted, espresso-type coffee. Also, the reaction products formed from 5-*O*-caffeoylquinic acid showed a rather intense bitter taste rated with an intensity of 2.5. The coffee-like bitter taste profile of the roasting products of 5-*O*-caffeoylquinic acid was very close to the bitter quality of the caffeoylquinic acid lactones reported recently (*12*). In comparison, thermal treatment of trigonelline, sucrose, and the sucrose/amino acid mixture, respectively, led to the development of a non-coffee-like bitter taste rated with significantly lower intensity scores between 0.5 and 1.5 (**Table 1**). Interestingly, quinic acid as well as ferulic acid, respectively, did not generate any bitter compounds under the roasting conditions applied (**Table 1**).

Table 1. Bitter Taste of Aqueous Solutions of Thermally Treated Precursor Components

precursor	bitter taste intensity[a]	bitter taste quality
5-*O*-caffeoyl quinic acid	2.5	coffee-like bitter taste
quinic acid	0	nb[b]
caffeic acid	5	harsh bitter taste, like strongly roasted coffee
ferulic acid	0	nb
trigonelline	1.5	bitter taste, but not coffee-like
sucrose	1.0	bitter taste, but not coffee-like
sucrose/amino acids	0.5	bitter taste, but not coffee-like

[a] Taste intensity of aqueous mixtures of the individual model mixtures in bottled water (50 mL; pH 5.2) was rated on a scale from 0 (not bitter) to 5.0 (extremely bitter). [b] No bitterness perceived.

Taste Dilution Analysis of Thermal Degradation Products of Caffeic Acid. Aimed at characterizing the intensely harsh bitter-tasting compounds formed from caffeic acid, the hydrophobic reaction products were separated by means of RP-HPLC connected to a diode array detector operating in a wavelength range from 220 to 500 nm. As shown in **Figure 1**, a huge multiplicity of reaction products was detectable. To sort out the strongly bitter compounds from the bulk of less taste active or tasteless substances, we first screened these reaction products by application of the recently developed taste dilution analysis (*12*−*17*). To achieve this, the effluent of the HPLC separation was collected in 24 fractions (**Figure 1**), and the taste compounds were purified by means of solid phase extraction using RP-18 cartridges. The isolated compounds were taken up with the same amount of bottled water (pH 5.2), and each fraction was then stepwise diluted 1:1 with water (pH 5.2) and presented in order of ascending concentrations to the trained sensory panel. The panelists were asked to evaluate the taste quality and to determine the detection threshold by means of a triangle test. As reflected by their high TD-factors, the HPLC fractions 10 and 14 were evaluated with the highest bitter impacts, closely followed by the fractions 11−13, 15, 17, 19−21, and 24, respectively (**Figure 1**). All the other fractions were evaluated with somewhat lower taste impacts or were entirely tasteless. The following identification experiments were focused on the bitter compounds in HPLC fractions: 10−15, 17, 20, and 21.

Structure Determination of Bitter Compounds. To gain first insight into the molecules imparting the intense bitter taste sensation in the fractions 10−15, 17, 20, and 21, the individual taste compounds were isolated by means of semipreparative RP-HPLC, and their chemical structures were elucicated by means of UV/vis, LC-MS/MS, and 1-D/2-D NMR experiments.

The UV/vis spectra of both **1** and **2** isolated from HPLC fractions 11 and 12, respectively, showed the same absorption

maximum at 286 nm, whereas the taste compound **3** obtained from HPLC fraction 13 revealed a spectrum with two absorption maxima at 263 and 283 nm. In addition, LC-MS analysis revealed an intense pseudo-molecular ion $[M - H]^-$ with *m*/*z* 271 for **1**−**3**. Analysis of the spectroscopic data obtained by ^{1}H NMR spectroscopy as well as homonuclear (*gs*-COSY) and heteronuclear 2-D NMR experiments (HMQC and HMBC) and comparison of the data obtained with those reported earlier for caffeic acid degradation products in the literature (*20*, *21*) led to the unequivocal identification of the bitter compounds in HPLC fractions 11−13 as *trans*-5,6-dihydroxy-1-methyl-3-(3′,4′-dihydroxyphenyl) indane (**1**), *cis*-5,6-dihydroxy-1-methyl-3-(3′,4′-dihydroxyphenyl) indane (**2**), and *trans*-1,3-bis(3′,4′-dihydroxyphenyl)-1-butene (**3**) (**Figure 2**). Although these compounds have been reported earlier as condensation products of 4-vinylcatechol liberated from caffeic acid upon thermal treatment (*20*, *21*), the bitter taste activity of these molecules has not been reported so far.

LC-MS analysis of the bitter compound **4** isolated from HPLC fraction 14 showed a pseudo-molecular ion with *m*/*z* 273 in the ESI^- mode, thus indicating two additional protons in the molecule when compared to **1**−**3**. The ^{1}H NMR spectrum showed 10 resonance signals, among which one signal integrated for three protons, two signals for two protons, and seven signals for one proton only. In the chemical shift range between 6.4 and 6.7 ppm, the typical coupling pattern of a 4-substituted 1,2-dihydroxybenzene moiety was observed twice. In addition, the *gs*-COSY spectrum showed strong couplings between the methylene protons H−C(2) resonating at 1.75 ppm and the protons H−C(1) and H−C(3). Moreover, the proton H−C(1) showed homonuclear connectivity with the methyl protons H−C(8), and due to its downfield shift, C(1) should be linked in the 4-position to one of the 1,2-dihydroxybenzene moieties. As H−C(3) showed only coupling with H−C(2) and a similar downfield shift was found as observed for H−C(1), the methylene group C(3) has to be linked to the second 1,2-dihydroxybenzene system. These findings were further strengthened by heteronuclear multiple bond correlation spectroscopy (HMBC) optimized for $^2J_{C,H}$ and $^3J_{C,H}$ coupling constants and heteronuclear multiple quantum correlation (HMQC) optimized for $^1J_{C,H}$ coupling constants, respectively. The HMBC experiment revealed correlations between the methylene group H−C(3) and the adjacent carbon atoms C(2) and C(1′) as well as the aromatic carbons C(2′) and C(6′), thus corroborating the structure proposed in **Figure 2**. In addition, the long-range correlations between H−C(1) and the carbon atoms C(8), C(2), and C(3), as well as the coupling to the aromatic carbons C(1a), C(3a), and C(7), visualized the connection between C(1) and the second 1,2-dihydroxybenzene moiety. Taking all the spectroscopic data into consideration, the bitter compound isolated from HPLC fraction 14 could be identified as the previously unreported 1,3-bis(3′,4′-dihydroxyphenyl) butane (**4**) (**Figure 2**).

LC-MS analysis of bitter compound **5** isolated from HPLC fraction 15 revealed the same $[M - H]^-$ ion *m*/*z* 271 as found for **1**−**3**. In addition, the UV/vis spectrum of **5** was identical to the spectrum obtained for **1** and **2**. Comparing the 1-D/2-D NMR data of compound **5** with those obtained for tastant **1** revealed some major similarities. The *gs*-COSY experiment showed the same coupling pattern for the protons of the carbon skeleton C(8)/C(1)/C(2)/C(3) as observed for compound **1**. But, the coupling pattern of one of the 1,2-dihydroxybenzene substituents showed major differences in the NMR data of **1** and **5**. Whereas two singlets could be detected for the two aromatic protons H−C(4) and H−C(7) of the phenylindane system in **1**, the protons H−C(6) and H−C(7) of the phenylindane structure in **2** were observed as two doublets with a coupling constant of 7.8 Hz as expected for vicinal protons. This coupling pattern indicates that the five-membered carbocycle of the phenylindane is attached in the α- and *β*-positions to C(4). On the basis of careful consideration of all the NMR data, the bitter compound **5** isolated from HPLC fraction 15 could be identified as the previously unreported *trans*-4,5-dihydroxy-1-methyl-3-(3′,4′-dihydroxyphenyl) indane (**5**) (**Figure 2**).

Table 2. Human Bitter Recognition Thresholds of Caffeic Acid Derived Bitter Taste Compounds **1**–**9a,b**

compound[a]	bitter recognition threshold concentration (μmol/L)[b]
1	178
2	148
3	145
4	23
5	45
6	32
7	67
8	60
9a,b	37

[a] Compound numbering refers to the chemical structures given in **Figure 2**. [b] Taste threshold concentrations were determined by means of a triangle test in bottled water (pH 5.2).

The taste compound **6** isolated from HPLC fraction 17 exhibited identical UV/vis and LC-MS data as found for **1**, **2**, and **5**, respectively. The analysis of the ^{1}H NMR spectrum revealed a total of 10 resonance signals, among which one integrated for three protons and nine integrated for one proton only. The chemical shifts and the coupling constants of the aromatic protons were similar to those obtained for **5**, indicating the same substitution pattern in the dihydroxybenzene moieties, whereas the chemical shifts in the five-membered carbocycle were similar to those found for **2**. This could be further strengthened by the *gs*-COSY with the correlation signals of the geminal protons H−C(2_α) and H−C(2_β), which showed nearly the same shifts and similar coupling constants as the corresponding protons in **2**, thus indicating a *cis*-configuration in molecule **6**. The homonuclear correlations of H−C(2_α) and H−C(2_β) with H−C(1) and H−C(3) as well as the couplings of H−C(1) with the methyl group H−C(8) led to the identification of the taste compound isolated from HPLC fraction 17 as the *cis*-4,5-dihydroxy-1-methyl-3-(3′,4′-dihydroxyphenyl) indane (**6**) (**Figure 2**). To the best of our knowledge, compound **6** has not been previously reported in the literature.

LC-MS (ESI^-) analysis of **7** isolated from HPLC fraction 20 revealed the pseudo-molecular ion $[M - H]^-$ with *m*/*z* 407. LC-MS/MS experiments revealed a daughter ion with *m*/*z* 297, most likely corresponding to the loss of a pyrocatechol moiety, and a daughter ion with *m*/*z* 109, representing a pyrocatechol ion. In addition, **7** showed an almost identical UV/vis spectrum as **1**, **2**, **5**, and **6**, respectively, thus giving strong evidence that **7** also exhibits a phenylindane structure. The ^{1}H NMR spectrum showed a total of 14 resonance signals with one additional methyl and methine group and two additional aromatic protons as compared to the phenylindanes **1**, **2**, **5**, and **6**. The coupling pattern of the protons H−C(8), H−C(1), H−C(2_α), H−C(2_β), and H−C(3) as well as their chemical shifts were nearly identical to those observed for **1**. In addition, the para-configured aromatic protons H−C(4) and H−C(7) in **1** were detectable at 6.37 and 6.62 ppm in the NMR spectrum of **7**, thus confirming the phenylindane structure of **1** in the molecule. In contrast to **1**, the dihydroxybenzene residue attached to C(3) of the phenylin-

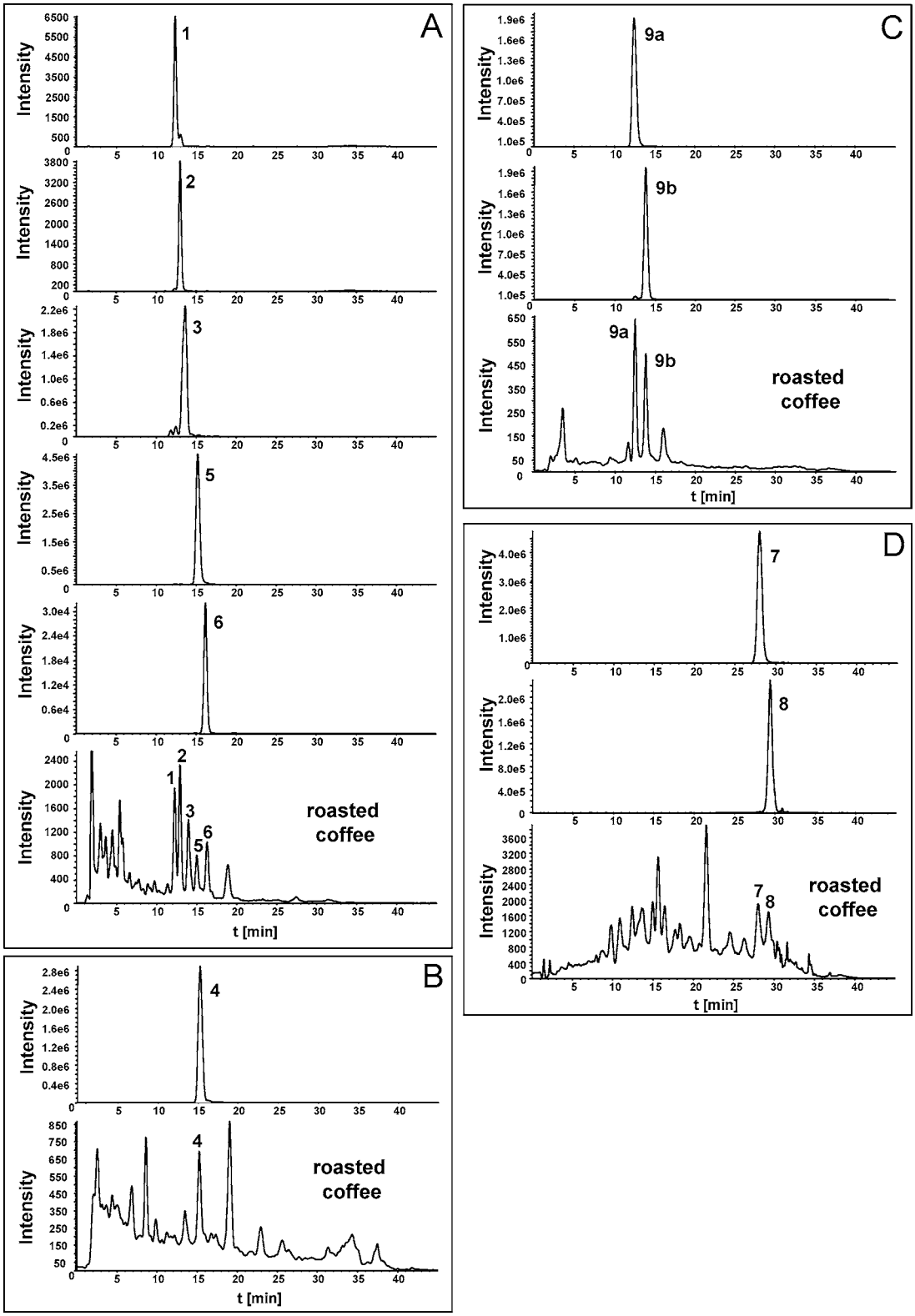

Figure 3. LC-MS/MS (ESI$^-$) analysis of the reference compounds **1**–**9a**,**b** and of the same bitter compounds in a freshly prepared coffee brew: (**A**) mass transition *m*/*z* 271 → 109 for the analysis of **1**–**3**, **5**, and **6**; (**B**) mass transition *m*/*z* 273 → 109 for the analysis of **4**; (**C**) mass transition *m*/*z* 407 → 161 for the analysis of **9a** and **9b**; (**D**) mass transition *m*/*z* 407 → 297 for the analysis of **7** and **8**.

dane structure showed only the two aromatic protons H−C(2′) and H−C(6′) with long-range coupling (2 Hz). The observed long-range coupling constant is just visible, when another substituent is connected at position C(5′). This assumption could be further strengthened by HMBC experiments demonstrating heteronuclear coupling between C(5′) and additional methyl and methine protons resonating at 1.47 and 4.36 ppm, respectively. The latter signals appeared as a duplet and a quartet with a coupling constant of 7.2 Hz and showed couplings to the quaternary carbon atoms C(5′) and C(2″) resonating at 133.3 and 138.1 ppm. Additional connectivity between H−C(1″) and the aromatic protons H−C(6′) and H−C(7″) confirmed the structure of the compound isolated from HPLC fraction 20 as the previously unreported *trans*-5,6-dihydroxy-1-methyl-3-[3′,4′-

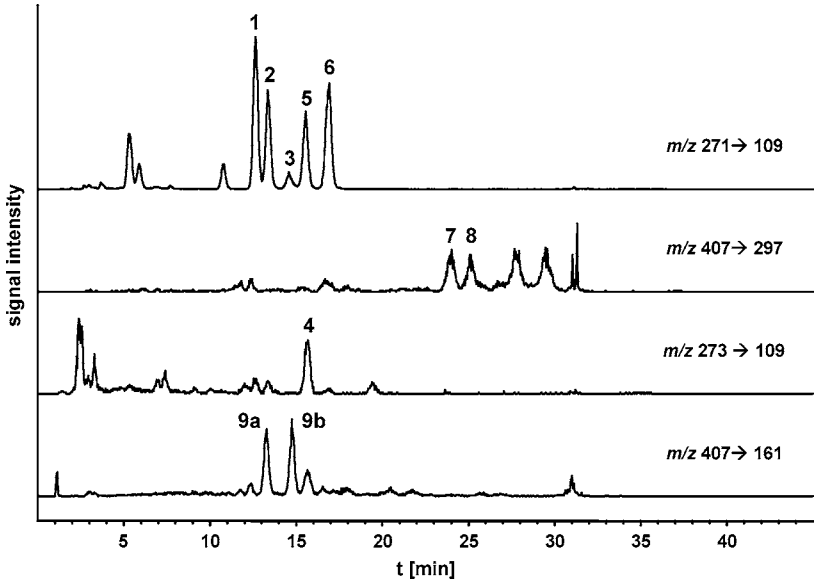

Figure 4. LC-MS/MS (MRM) analysis of bitter compounds **1**–**9a**,**b** in a solvent extract obtained from 5-*O*-caffeoylquinic acid heated for 15 min at 220 °C.

dihydroxy-5′-(1-(3″,4″-dihydroxyphenyl)-1-ethyl)phenyl] indane (**7**) (**Figure 2**).

Also, the taste compound **8** obtained from HPLC fraction 21 revealed a $[M - H]^-$ ion with *m*/*z* 407, the same MS/MS fragmentation pattern as well as a proton NMR spectrum with 14 resonance signals as found for compound **7**. The chemical shifts and the coupling pattern of the phenylindane moiety were comparable to those found for **2**, whereas the additional signals of the aromatic protons, the methyl protons, as well as the methine proton were comparable to those of **7**. Careful consideration of all the NMR data led to the unequivocal assignment of the taste compound isolated from HPLC fraction 21 as the previously unreported *cis*-5,6-dihydroxy-1-methyl-3-[3′,4′-dihydroxy-5′-(1-(3″,4″-dihydroxyphenyl)-1-ethyl)phenyl] indane (**8**) (**Figure 2**).

Bitter compound **9a** isolated from HPLC fraction 10 showed a quasi-molecular ion with *m*/*z* 407 in the LC-MS spectrum and a total of 14 resonance signals in the ^{1}H NMR spectrum as already found for **7** and **8**. Also, chemical shifts and coupling topology of the aliphatic protons were consistent with the phenylindane structure of **7** and **8**. In contrast to these taste compounds, tastant **9a** showed a 1-(3,4-dihydroxyphenyl)-1-ethyl residue linked to carbon C(2) of the phenylindane system. This was strengthened by the data obtained from the *gs*-COSY spectrum demonstrating homonuclear couplings between H−C(2) and H−C(1) as well as H−C(1″) and correlations between these methine protons and the adjacent methyl groups C(8) and C(8″). In addition, heteronuclear correlations optimized for $^{2,3}J$ coupling constants revealed coupling of H−C(1″) and H−C(8″) with the quaternary carbon atom C(2″) and correlations between H−C(2) with C(1″), thus confirming the attachment of the 1-(3,4-dihydroxyphenyl)-1-ethyl residue to position 2 of the phenylindane system. Taking all spectroscopic data into consideration, the bitter compound **9a** isolated from HPLC fraction 10 could be identified as a 5,6-dihydroxy-1-methyl-2-[1-(3′,4′-dihydroxyphenyl)-1-ethyl]-3-(3″,4″-dihydroxyphenyl) indane (**Figure 2**), which has previously not been reported in the literature. In addition, **9b** could be isolated from HPLC fraction 11, showing an identical LC-MS and UV/vis spectrum and rather similar NMR data, thus indicating the presence of a diastereomer of taste compound **9a**. Unfortunately, the spectroscopic data obtained did not allow a more precise assignment of the exact stereochemistry of the bitter compounds **9a** and **9b**.

Bitter Recognition Threshold Concentrations. Prior to sensory analysis, the purity of the taste compounds **1**−**9a**,**b** was checked by ^{1}H NMR spectroscopy as well as HPLC-MS. To determine the human threshold concentrations for bitter taste, aqueous solutions (pH 5.2) of **1**−**9a**,**b** were evaluated by means of a triangle test (**Table 2**). Independent from their structure, all the compounds imparted a bitter taste with threshold concentrations ranging from 23 to 178 μmol/L. The lowest thresholds with values below 45 μmol/L were found for **4**−**6** and **9a**,**b**, whereas the phenylindane **1** showed the highest threshold concentration of 178 μmol/L (**Table 2**). In addition, the sensory panel evaluated the taste profile of these phenolic compounds as being significantly different from the bitter quality of the recently identified caffeoyl quinides (*12*). Whereas the caffeoyl quinides were evaluated with a pleasant coffee-like bitter taste (*12*), **1**−**9a**,**b** exhibited a more harsh bitter taste reminiscent of the bitter quality of a strongly roasted, espresso-type coffee.

Identification of Bitter Compounds 1−**9a,b in Coffee Brew.** To verify the presence of the caffeic acid derived bitter compounds in coffee beverages, a freshly prepared coffee brew was screened for these compounds by means of LC-MS/MS (ESI^-) operating in the multiple reaction monitoring (MRM) mode. Prior to analysis, characteristic mass transitions were selected for each taste compound in tuning runs. Thereafter, the retention time as well as the characteristic mass transitions of the taste compounds in coffee was compared to those of the reference compounds. As shown in **Figure 3**, for one mass transition for each of the target compounds **1**−**9a**,**b**, each of these caffeic acid derived bitter compounds could be detected in coffee brew by means of HPLC-MS/MS (MRM). In addition, the identity of the bitter tastants **1**−**9a**,**b** in coffee was confirmed by means of co-chromatography with the corresponding reference compounds. To the best of our knowledge, these bitter compounds have not been previously reported in the literature as bitter tastants in coffee beverages.

Formation Pathways of Bitter Compounds 1−**9a**,**b.** As free caffeic acid is known to be present in coffee in rather small quantities, the question arose as to whether also bound caffeic

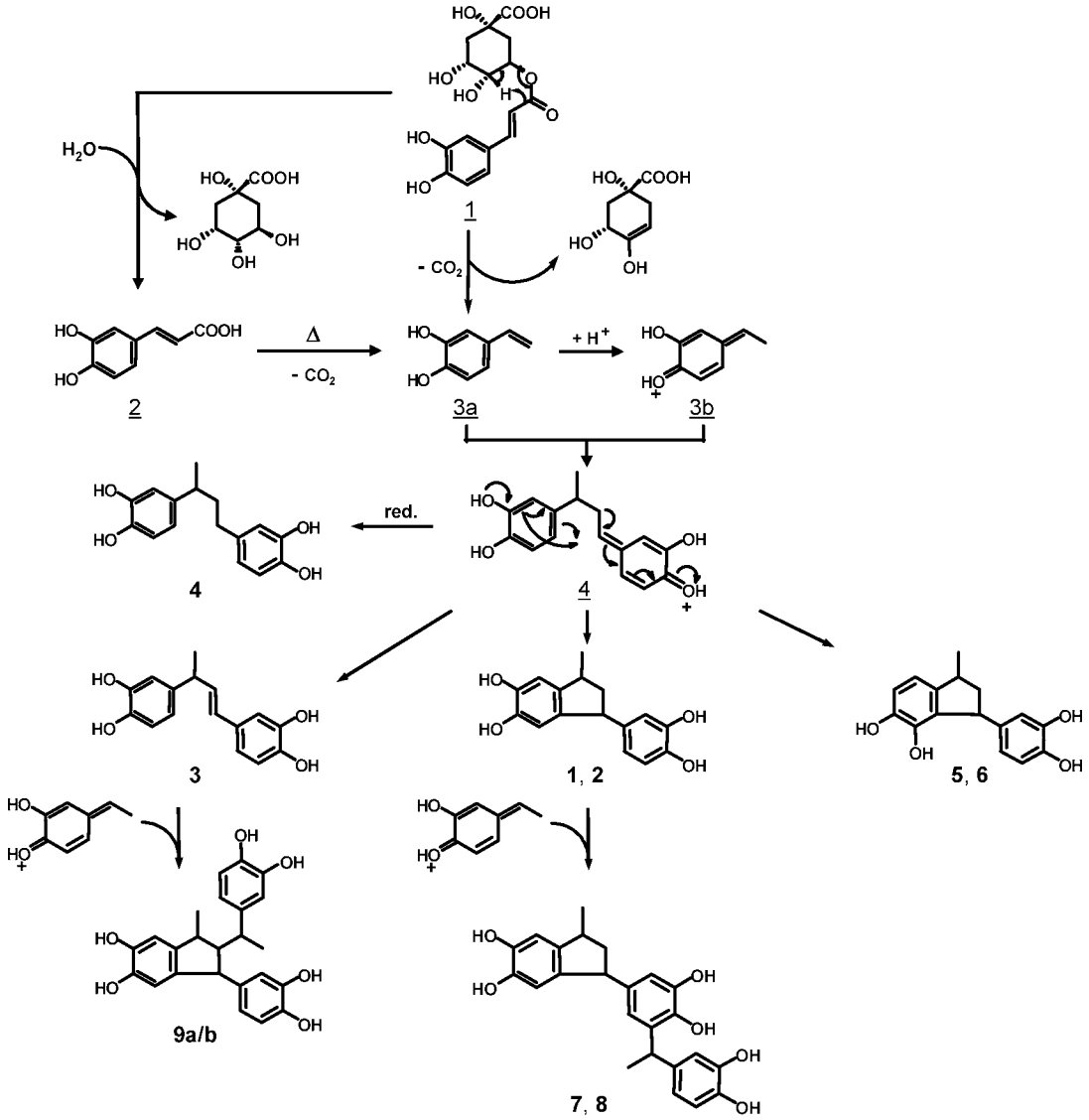

Figure 5. Proposed route map of reactions leading to the formation of the bitter compounds **1**–**9a**,**b** from 5-*O*-caffeoylquinic acid (**1**) and caffeic acid (**2**) via 4-vinylcatechol (**3a**) as the key intermediate.

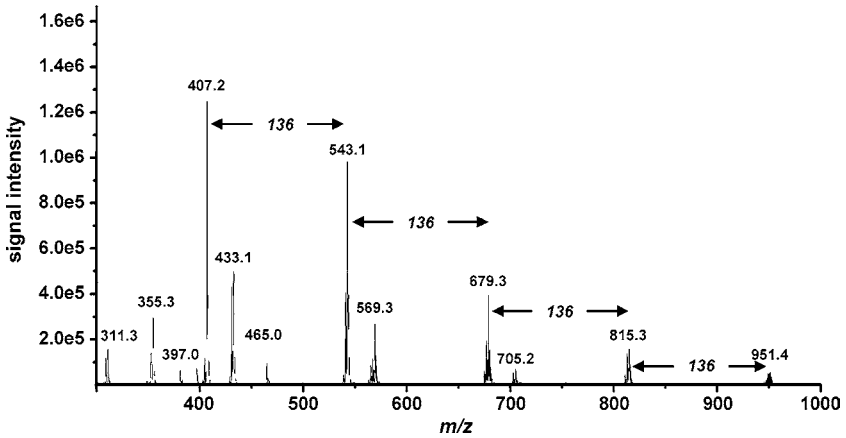

Figure 6. LC-MS (ESI$^-$) spectrum of the bitter-tasting HPLC fraction 24 isolated from thermally generated caffeic acid.

acid, for example, esterified as found for 5-*O*-caffeoylquinic acid, would be able to generate the bitter compounds **1**−**9a**,**b** upon roasting. To check that assumption, 5-*O*-caffeoylquinic acid was thermally treated in a model experiment, and the roasting products formed were analyzed for the bitter tastants **1**−**9a**,**b** by means of LC-MS/MS operated in the MRM mode. As shown in **Figure 4**, all bitter compounds were identified based on their retention times, mass transitions, as well as co-chromatography with the corresponding reference compounds. These results clearly also demonstrate 5-*O*-caffeoylquinic acid, the major phenol in coffee, as a precursor for the formation of the bitter compounds **1**−**9a**,**b**.

On the basis of these findings, a route map of reactions leading to the bitter taste compounds **1**−**9** from thermal degradation of 5-*O*-caffeoylquinic acid is outlined in **Figure 5**. 5-*O*-Caffeoylquinic acid (**1**) can hydrolytically liberate caffeic acid (**2**) that, upon decarboxylation, was reported to generate the highly reactive 4-vinylcatechol, **3a** (*20*−*22*). Alternatively, thermal syn-elimination of 5-*O*-caffeoylquinic acid (**1**) might directly give rise to 4-vinylcatechol (**3a**). Protonation of **3a** at the vinyl function results in the electrophile **3b**, which can dimerize with the nucleophile **3a**, thus giving rise to the key intermediate **4**. The oxonium ion **4** can then either be reduced to the bitter compound **4**, for example, by a redox reaction with the 1,2-dihydroxybenzene moiety of one molecule of **1** and **2**, respectively, or can generate bitter compound **3** upon re-aromatization. Alternatively, an intramolecular ring-closure reaction between the unsaturated Michael system of the oxonium ion and one of the ortho-positions of the other 1,2-dihydroxybenzene moiety gives rise to the phenylindanes **1**, **2**, **5**, and **6**, respectively. Starting from these bitter-tasting 4-vinylcatechol dimers, the formation of the trimers **7**, **8**, and **9a**,**b** is easily explainable. Condensation of compound **3** with another molecule of the vinylcatechol oxonium ion (**3b**) upon ring-closure leads to the phenylindane **9a**,**b**, whereas reaction of phenylindanes **1** and **2** with **3b** gives rise to the 4-vinylcatechol trimers **7** and **8**.

LC-MS Studies on the Bitter-Tasting HPLC Fraction 24. Besides the bitter taste compounds identified previously, HPLC fraction 24 also was evaluated with a high TD-factor for bitterness (**Figure 1**). However, any attempt to isolate and further purify individual bitter taste compounds in that complex fraction failed so far. To gain a more general insight into the chemical nature of the bitter compounds in that fraction, the total HPLC fraction 24 was used for LC-MS (ESI^-) studies. As given in **Figure 6**, the MS spectrum showed a predominant ion with *m*/*z* 407 fitting well with the pseudo-molecular ion found for the bitter-tasting trimers **7**−**9a**,**b**, thus indicating the existence of additional 4-vinylcatechol trimers. In addition, pseudo-molecular ions with *m*/*z* 543, 679, 815, and 951 were observed in the mass spectrum. These ions indicate that, besides dimers and trimers, also higher 4-vinylcatechol oligomers at least up to the heptamer are formed upon roasting by proton-catalyzed oligomerization of 4-vinylcatechol liberated from caffeic acid and caffeoylquinic acids, respectively.

Studies on the determination of the exact molecular structures of the higher 4-vinylcatechol oligomers, on their sensory activity, as well as on the contribution of these 4-vinylcatechol derived reaction products to coffee bitterness are currently in progress.

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