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Identification of the Astringent Taste Compounds in Black Tea Infusions by Combining Instrumental Analysis and Human Bioresponse

SUSANNE SCHARBERT,† NADINE HOLZMANN,† AND THOMAS HOFMANN*,#

Deutsche Forschungsanstalt für Lebensmittelchemie, Lichtenbergstrasse 4, D-85748 Garching, Germany, and Institut für Lebensmittelchemie, Universität Münster, Corrensstrasse 45, D-48149 Münster, Germany

Application of taste dilution analyses on freshly prepared black tea infusions revealed neither the high molecular weight thearubigen-like polyphenols nor the catechins and theaflavins, but a series of 14 flavon-3-ol glycosides as the main contributors to the astringent taste perceived upon black tea consumption. Among these glycosides, the apigenin-8-*C*-[α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside] was identified for the first time in tea infusions. Depending on the structure, the flavon-3-ol glycosides were found to induce a velvety and mouth-coating sensation at very low threshold concentrations, which were far below those of catechins or theaflavins; for example, the threshold of 0.001 μmol/L found for quercetin-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside] is 190,000, or 16,000 times below the threshold determined for epigallocatechin gallate or theaflavin, respectively. Moreover, structure/activity considerations revealed that, besides the type of flavon-3-ol aglycon, the type and the sequence of the individual monosaccharides in the glycosidic chain are key drivers for astringency perception of flavon-3-ol glycosides.

KEYWORDS: Tea; astringency; taste; taste dilution analysis; catechins; theaflavins; flavon-3-ol glycosides; rutin; half-tongue test

INTRODUCTION

The infusion of the dried leaves and buds of the plant *Camellia sinensis* has been consumed by humans for thousands of years as a desirable beverage. Besides green tea and oolong tea, black tea is one of the major tea products, accounting for >75% of the world tea production. One of the key criteria used by tea tasters to describe the quality of tea liquors is their astringent taste, which is perceived as a puckering, shrinking, rough, and drying sensation in the mouth and can enhance the complexity and palate length of the tea. Terms such as "strong", "hard", and "harsh" are used by professional tea tasters to describe the intensity and quality of the astringent sensation perceived. Although multiple attempts have been made to find a correlation between the sensory results of tea tasters and the chemical species imparting the typical astringent sensation in tea infusions, the data reported in the literature on the astringent key components are very contradictory.

More than 40 years ago, the orange low molecular weight theaflavins as well as the red-brown polymeric thearubigins, both generated during tea fermentation upon polyphenol oxidase catalyzed oxidation of flavan-3-ols, were believed to be responsible for the briskness and astringency of black tea infusions (*1*−*4*). Aqueous solutions of theaflavin isolated from black tea were reported to exhibit an astringent taste that was close to the sensation giving the briskness of the tea's liquor character (*5*). Sensory analysis revealed that theaflavins taste bitter and astringent with threshold concentrations of 300−1000 mg/L for bitterness and 125−800 mg/L for astringency (*6*). As these substances are believed to be desirable for tea quality, the analysis of theaflavin was recommended as a measure of tea quality (*7*, *8*).

In contradiction to the reported importance of theaflavins, quantitative and sensory studies could not find any statistical correlation between the overall astringent taste of tea infusions and the theaflavin concentration, but indicated good correlations between astringency and some flavan-3-ols, in particular, epigallocatechin-3-gallate and epicatechin-3-gallate (*9*). Due to the threshold concentrations of 180−200 mg/L determined for these galloylated flavan-3-ols (*10*), these flavan-3-ols were believed to be the key tastants of tea. In line with this hypothesis, analysis of flavan-3-ol content before and after tea fermentation revealed a correlation of the concentrations of epicatechin and catechin gallates in fresh leaves with the quality of its black tea (*11*). Very recent quantitative studies on green and black teas showed that besides caffeine and amino acids, in particular, the flavan-3-ols might be important for the taste, whereas the theaflavins affected not only the tea's taste but also its color.

* Author to whom correspondence should be addressed (telephone +49-251-83-33-391; fax +49-251-83-33-396; e-mail thomas.hofmann@uni-muenster.de).
† Deutsche Forschungsanstalt für Lebensmittelchemie.
# Institut für Lebensmittelchemie.

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In contrast to the flavan-3-ols, flavon-3-ol glycosides are not believed to be important for tea taste (*8*).

Although the knowledge available on the sensory activity of individual astringent phenolics is very fragmentary, molecular weights between 500 and 3000 Da were believed to be essential for the astringent sensation of water-soluble phenols. These polyphenols are thought to reduce the saliva lubrication either upon precipitating proline-rich salivary proteins or upon forming unprecipitated complexes (*12*−*15*). In consequence, the effectiveness of salivary lubrication is believed to be decreased, and astringency is perceived as the friction between two nonlubricated surfaces. The increased friction induces a tactile sensation by activating mechanoreceptors in the mouth, thus leading to the perception of astringency (*16*−*18*). It could be shown that subjects with low saliva flow rate evaluated astringency of black tea as significantly higher than high-flow subjects over eight successive sips (*19*). In conflict with these data, no effect of the salivary flow on the perception of astringency was observed for some astringent mono-, di-, and trimeric flavanols (*20*) and hydroxybenzoic acids (*21*). In summary, these findings support the assumption that the key compounds inducing this sensation in tea infusions are not yet defined on a molecular level (*22*).

To answer the puzzling question as to which nonvolatile, sapid key taste compounds are responsible for the attractive taste generated during food processing, we have recently developed the so-called taste dilution analysis (TDA), which is based on the determination of the taste threshold in serial dilutions of taste-active fractions and which is a powerful screening procedure for the detection of yet unknown taste-active compounds (*23*, *24*). The application of this technique led to the identification of bitter compounds in thermally processed mixtures of sugars and amino acids (*23*), cooling compounds in dark malt (*24*), bitter compounds in carrots and carrot products (*25*), and, very recently, taste enhancers in beef bouillon (*26*).

To bridge the gap between pure structural chemistry and human taste perception, the objectives of the present investigation were therefore to screen a black tea infusion for the key astringent compounds by application of taste dilution techniques, to isolate and identify the compounds inducing the strongest human bioresponse for astringency, and to compare their astringency power on the basis of their human threshold concentrations.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: caffeine (Fluka, Neu-Ulm, Germany); catechin, catechin gallate, epicatechin, epicatechin gallate, epigallocatechin-3-gallate, gallocatechin, gallocatechin-3-gallate, gallustannic acid (Sigma, Steinheim, Germany); kaempferol-3-*O*-*β*-D-glucopyranoside, kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-*β*-D-glucopyranoside], quercetin-3-*O*-*β*-D-galactopyranoside, quercetin-3-*O*-*β*-D-glucopyranoside, and quercetin-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-*β*-D-glucopyranoside] (Roth, Karlsruhe, Germany). Solvents were of HPLC grade (Merck, Darmstadt, Germany). Deuterated solvents were supplied by Euriso-Top (Gif-Sur-Yvette, France). Theaflavin, theaflavic acid, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate were synthesized by closely following the procedures reported recently (*27*).

Sensory Analyses. *Panel Training.* Using triangle tests, 15 assessors were trained to evaluate the taste of solutions (5 mL each) of the following standard compounds in bottled water (Vittel, low mineralization: 405 mg/L) adjusted to pH 6.0 with aqueous hydrochloric acid (0.1 mol/L): sucrose (50 mmol/L) for sweet taste; lactic acid (20 mmol/L) for sour taste; NaCl (12 mmol/L) for salty taste; caffeine (1 mmol/L) for bitter taste; and monosodium glutamate (8 mmol/L, pH 5.7) for umami taste. For the astringent/rough or the velvety, mouth-drying oral sensation, the panel was trained by using tannin (gallustannic acid; 0.001%) or quercetin-3-*O*-*β*-D-galactopyranoside, respectively, in a half-tongue test. Sensory analyses were performed in a sensory panel room at 19−22 °C in three different sessions.

Half-tongue Test. Taste dilution factors as well as human astringency recognition thresholds, the concentrations at which the typical taste quality of a compound was just detectable, were determined by means of a half-tongue test using bottled water as the solvent. Serial 1:1 dilutions of the samples were presented in order of increasing concentrations to a trained panel of 12 persons in three different sessions, using the sip-and-spit method. At the start of the session and before each trial, the subject rinsed with water and expectorated. Using a half-tongue test, an aliquot (1 mL) of the aqueous solution containing the astringent compound was applied with a pipet on one side of the tongue, whereas pure water was applied on the other side of the tongue for control. The sensory panelists were then asked to move their tongue forward and backward toward the palate for 30 s and to identify the place of astringent sensation by comparison of both sides. After indicating which part of the tongue showed the typical astringent sensation induced by the tastant, the participant rinsed with water and, after 10 min, received another set of one blank and one taste-active sample. To prevent excessive fatigue, tasting began at a concentration level two steps below the threshold concentration that had been determined in a preliminary taste experiment. Whenever the panelist selected incorrectly, the next trial took place at the next higher concentration step. When the panelist selected correctly, the same concentration was presented again besides one blank as a proof for the correctness of the data. The geometric mean of the last and the second last concentration was calculated and taken as the individual recognition threshold. The values between individuals and between five separate sessions differed by not more than plus or minus one dilution step; that is, a threshold value of 0.001 μmol/L for quercetin-3-*O*-rutinoside represents a range from 0.0005 to of 0.002 μmol/L.

Omission Experiments. To evaluate the taste contribution of individual molecular weight fractions isolated from tea, two samples of a recombinate containing all of the fractions in their natural concentrations were compared to a partial recombinate lacking in one fraction by means of a triangle test.

Tea Infusion. The black tea (Darjeeling Gold-Auslese, TGFOP, Summer) (Tee-Handelskontor, Bremen, Germany) was infused with boiling tap water (1 g/100 mL) and maintained for 4 min prior to filtration using a cellulose filter. For sensory experiments the tea infusion was used directly; for identification experiments the tea infusion was stabilized by the addition of ascorbic acid (50 mg/100 mL).

Multiple-Step Ultrafiltration. The freshly prepared tea infusion was cooled in an ice bath and fractionated by means of an ultrafiltration cell (Amicon, Witten, Germany), which was cooled in an ice bath, using sequentially the filters YM 10 and YM 1 (Millipore, Bedford, MA) with cutoffs of 10 and 1 kDa at a nitrogen pressure of 3 bar. The individual filtrates obtained were freeze-dried, and the residues were used for the taste dilution analysis.

HPLC/Taste Dilution Analysis of the Low Molecular Weight Ultrafiltrate of the Tea Infusion. The freeze-dried ultrafiltrate <1 kDa was dissolved in water using a ultrasonic bath and membrane filtered, and aliquots (100 μL) were then separated by a semipreparative 250 × 10 mm i.d. Grom Sil 120 octyl-5-CP HPLC column (Grom, Rottenburg-Hailfingen, Germany). Chromatography was performed with aqueous formic acid (0.1% in water; pH 3.5) and methanol as eluent. Starting with aqueous formic acid, the methanol content was increased to 60% within 65 min and then to 100% within 5 min; thereafter the column was flushed with methanol for 5 min. The effluent was separated into 43 fractions, which were separately collected in ice-cooled glass vials. The corresponding fractions obtained from 10 HPLC runs were collected, combined, and, after freeze-drying, dissolved in exactly 2.0 mL of bottled water (Vittel) and, then, stepwise 1+1-diluted with bottled water. The serial dilutions of each of these fractions were then presented to the sensory panel in the order of increasing concentration, and each dilution was sensorially judged using the comparative duo test described above. The dilution at which a taste difference between the diluted fraction and two blanks (tap water) could just be detected was defined as the taste dilution (TD) factor. The TD

factors evaluated by three different assessors at three different sessions were averaged.

Identification of Catechins. The tea infusion stabilized with ascorbic acid was membrane filtered, and aliquots (50−200 μL) were applied on the semipreparative HPLC column. Starting with a mixture (10:90; v/v) of methanol and aqueous formic acid (0.1% in water, pH 3.5), the methanol content was increased to 20% within 8.5 min, to 60% within 31 min, and, finally, to 100% within 5 min. By monitoring the effluent in the wavelength range from 220 to 500 nm, individual peaks were collected in several runs and the corresponding eluates were combined and freeze-dried. The residues obtained were analyzed by means of UV−vis, LC-MS, and NMR spectroscopy as well as through sensory studies. Finally, the identity of the catechins was confirmed by cochromatography with reference compounds.

Catechin: UV−vis (MeOH) λ_{max} = 278; LC-MS (ESI^+), *m/z* 603 (66; $[M_2 + Na]^+$), 598 (100; $[M_2 + H_2O]^+$), 291 (80; $[M]^+$). Catechin-3-gallate: UV−vis (MeOH) λ_{max} = 277; LC-MS (ESI^+), *m/z* 907 (52; $[M_2 + Na]^+$), 902 (100; $[M_2 + H_2O]^+$), 466 (8; $[M + Na]^+$), 443 (80; $[M]^+$). Epicatechin: UV−vis (MeOH) λ_{max} = 278; LC-MS (ESI^+), *m/z* 603 (60; $[M_2 + Na]^+$), 598 (100; $[M_2 + H_2O]^+$), 291 (83; $[M]^+$). Epicatechin-3-gallate: UV−vis (MeOH) λ_{max} = 277; LC-MS (ESI^+), *m/z* 907 (50; $[M_2 + Na]^+$), 902 (100; $[M_2 + H_2O]^+$), 466 (14; $[M + H_2O]^+$), 443 (85; $[M]^+$). Epigallocatechin: UV−vis (MeOH) λ_{max} = 278; LC-MS (ESI^+), *m/z* 635 (30; $[M_2 + Na]^+$), 630 (100; $[M_2 + H_2O]^+$), 307 (58; $[M]^+$). Epigallocatechin-3-gallate: UV−vis (MeOH) λ_{max} = 278; LC-MS (ESI^+), *m/z* 939 (56; $[M_2 + Na]^+$), 934 (100; $[M_2 + H_2O]^+$), 476 (16; $[M + H_2O]^+$), 459 (80; $[M]^+$). Gallocatechin: UV−vis (MeOH) λ_{max} = 278; LC-MS (ESI^+), *m/z* 635 (32; $[M_2 + Na]^+$), 630 (100; $[M_2 + H_2O]^+$), 307 (61; $[M]^+$); UV−vis (MeOH) λ_{max} = 278; LC-MS (ESI^+), *m/z* 939 (58; $[M_2 + Na]^+$), 934 (100; $[M_2 + H_2O]^+$), 476 (11; $[M + H_2O]^+$), 459 (82; $[M]^+$); ^{1}H NMR data of these catechins were identical with those measured for the reference compounds.

Identification of Theaflavins. The tea infusion stabilized with ascorbic acid was membrane filtered, and aliquots (50−200 μL) were applied on the semipreparative HPLC column. Starting with aqueous formic acid (0.1% in water, pH 3.5), the methanol content was increased to 50% within 30 min, to 80% within 10 min, and, finally, to 100% within 5 min. Monitoring the effluent at 270, 370, and 450 nm, the theaflavins were identified by comparing the spectroscopic (UV−vis, LC-MS) and chromatographic data (retention times on RP-18) with those obtained for the synthetic reference compounds. Finally, the identities of theaflavin, theaflavic acid, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate were confirmed by cochromatography with reference compounds.

Theaflavin: UV−vis (MeOH) λ_{max} = 289, 374, 457; LC-MS (ESI^+), *m/z* 581 (22; $[M + H_2O]^+$, 565 (100; $[M]^+$). Theaflavic acid: UV−vis (MeOH) λ_{max} = 274, 398; LC-MS (ESI^+), *m/z* 429 (100; $[M]^+$). Theaflavin-3-gallate: UV−vis (MeOH) λ_{max} = 271, 373, 450; LC-MS (ESI^+), *m/z* 739 (100; $[M + Na]^+$); LC-MS ($APCI^-$), *m/z* 716 (100; $[M]^-$), 548 (29; [M − gallate]$^-$). Theaflavin-3′-gallate: UV−vis (MeOH) λ_{max} = 271, 373, 450; LC-MS (ESI^+), *m/z* 739 (100; $[M + Na]^+$); LC-MS ($APCI^-$), *m/z* 716 (100; $[M]^-$), 548 (29; [M − gallate]$^-$). Theaflavin-3,3′-digallate: UV−vis (MeOH) λ_{max} = 274, 374, 450; LC-MS (ESI^-), *m/z* 867 (100; $[M]^-$), 697 (17; [M − gallate]$^-$).

Isolation of the Flavon-3-ol Glycoside Fraction from a Tea Drug or Infusion. Either the standard tea infusion (500 mL) was directly freeze-dried or the powdered tea (2.5 g) was extracted at 40 °C twice with a methanol/water mixture (75:25, v/v; 200 mL) for 30 min, and after filtration the solvent was removed in vacuo. The crude tea extracts were taken up in water and then fractionated by chromatography on a 250 × 40 mm polyamide column (SC 6 material 150 mm; 0.05−0.16 mm) (Macherey-Nagel, Düren, Germany) as the stationary phase. After application of the crude tea extract on the top of the column, chromatography was performed with water (300 mL), followed by methanol (3 × 200 mL, PA-I−III) as the effluent. The fraction PA-I was freed from methanol in vacuo, decaffeinated by extraction with dichloromethane (5 × 50 mL), and then freeze-dried to obtain the fraction of flavonol glycosides as a yellow powder. Aliquots of this material were used for the taste dilution analysis as well as for isolation and identification experiments.

HPLC/Taste Dilution Analysis on the Flavon-3-ol Glycoside Fraction. The yellowish glycosidic tea extract isolated from tea infusion was dissolved in methanol (5 mL) and, after membrane filtration, aliquots (100 μL) were further fractionated by HPLC using a 250 × 8 mm i.d. ODS-Hypersil 100-5C18 column (ThermoHypersil, Kleinostheim, Germany). Monitoring the effluent at 345 nm, chromatography was performed starting with a mixture (87:13; v/v) of aqueous formic acid (0.1%, pH 3.5) and acetonitrile, then increasing the acetonitrile content to 17% within 35 min, maintaining the acetonitrile content for an additional 15 min, and, finally, increasing the acetonitrile content to 100% within 5 min. All of the peaks were collected individually in several runs, the eluates of the corresponding fractions were combined and freeze-dried, and the residues obtained were then taken up in exactly 2.0 mL of water and, then, stepwise 1+1-diluted with bottled water. The serial dilutions of each of these fractions were then presented to the sensory panel in the order of increasing concentrations and the TD factor was determined for each fraction by means of the comparative duo test described above. The TD factors evaluated by three different assessors at three different sessions were averaged.

Purification and Structure Elucidation of Flavon-3-ol Glycosides. The glycosidic extract isolated from the tea was further fractionated by chromatography on a 100 × 40 mm Lichroprep 25-40 μm column (Merck, Darmstadt, Germany) conditioned with a mixture (95:5; v/v) of aqueous formic acid (0.1%, pH 3.5) and acetonitrile. After application of the crude material, the chromatography (flow rate = 1.1 mL/min) started with the same solvent mixture (50 mL); the acetonitrile content was then increased to 8%, after 200 mL increased to 10%, after 500 mL was increased by steps of 50 mL to 13, 17, 20, 40, 60, and finally 100%. The fractions containing the compounds detected at 345 nm were collected and freeze-dried, and the target compounds were then isolated by HPLC using the ODS-Hypersil 100-5C18 column and the mobile phase reported above, thus affording 14 flavon-3-ol glycosides, each in purity of >99%.

Kaempferol-3-*O*-β-D-galactopyranoside: UV−vis (acetonitrile) λ_{max} = 255, 339; LC-MS (ESI^+), *m/z* 449 (100; $[M]^+$), 287 (47 [M − gal]$^+$); ^{1}H NMR (600 MHz; CD_3COD) δ 3.43 [ddd, 1H, H−C(5″)], 3.5 [m, 2H, H−C(6b″), H−C(3″)], 3.62 [dd, 1H, H−C(6a″)], 3.78 [dd, 1H, H−C(2″)], 3.81 [dd, 1H, H−C(4″)], 5.2 [d, 1H, *J* = 7.8 Hz, H−C(1″)], 6.21 [d, 1H, H−C(6)], 6.42 [d, 1H, H−C(8)], 6.88 [d, 2H, H−C(3′), H−C(5′)], 8.09 [d, 2H, H−C(2′), H−C(6′)]; ^{1}H NMR data were in line with data reported in the literature (*28*); ^{13}C NMR (150 MHz; CD_3COD) δ 60.6 [C-6″], 68.7 [C-4″], 71.7 [C-2″], 73.7 [C-3″], 75.8 [C-5″], 93.5 [C-8], 98.8 [C-6], 103.7 [C-1″], 104.6 [C-4a], 114.9 [C-2, C-6′], 121.5 [C-1′], 134.6 [C-3], 131.1 [C-3′, C-5′], 157.5 [C-8a], 158.0 [C-2], 160.6 [C-4′], 162.1 [C-5], 165.0 [C-7], 179.8 [C-4].

Kaempferol-3-*O*-β-D-glucopyranoside: UV−vis (acetonitrile) λ_{max} = 255, 339; LC-MS (ESI^+), *m/z* 449 (100; $[M]^+$), 287 (47 [M − glc]$^+$); ^{1}H and ^{13}C NMR data were identical with those measured for the commercially available reference compound.

Kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside]: UV−vis (acetonitrile) λ_{max} = 255, 339; LC-MS (ESI^+), *m/z* 595 (100; $[M]^+$), 449 (13 [M − rha]$^+$), 287 (19 [M − glc − rha]$^+$); ^{1}H and ^{13}C NMR data were identical with those measured for the reference compound.

Kaempferol-3-*O*-[β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside]: UV−vis (acetonitrile) λ_{max} = 255, 339; LC-MS (ESI^+), *m/z* 757 (100; $[M]^+$), 595 (10 [M − glc]$^+$), 449 (4 [M − rha − glc]$^+$), 287 (7 [M − glc − rha − glc]$^+$); ^{1}H NMR (900 MHz; CD_3COD) δ 1.1 [d, 3H, H−C(6‴)], 3.24 [m, 1H, H−C(5‴)], 3.27 [m, 1H, H−C(5″)], 3.28 [m, 1H, H−C(2‴)], 3.39 [m, 1H, H−C(4″)], 3.41 [m, 1H, H−C(4‴)], 3.41 [m, 1H, H−C(3‴)], 3.44 (m, 1H, H−C(3‴)], 3.45 [m, 1H, H−C(6b″)], 3.46 [m, 1H, H−C(4‴)], 3.47 [m, 1H, H−C(2″)], 3.52 [m, 2H, H−C(5‴)], 3.59 [dd, 1H, H−C(3‴)], 3.74 [dd, 1H, H−C(6b‴)], 3.8 [dd, 1H, H−C(6a‴)], 3.83 [dd, 1H, H−C(6a″)], 3.95 [dd 1H, H−C(2‴)], 4.41 [d, 1H, *J* = 7.7 Hz, H−C(1‴)], 4.58 [d, 1H, *J* = 1.3 Hz, H−C(1‴)], 5.15 [d, 1H, *J* = 7.7 Hz, H−C(1″)], 6.25 [d, 1H, H−C(6)], 6.46 [d, 1H, H−C(8)], 6.94 [d, 2H, H−C(3′), H−C(5′)], 8.11 [d, 2H,H−C(2′), H−C(6′)]; ^{1}H NMR data were in line with data reported in the literature (*29*); ^{13}C NMR (225 MHz; CD_3COD) δ 16.8 [C-6‴], 60.4 [C-6‴], 67.4 [C-6″], 67.9 [C-5‴], 69.2 [C-4‴], 69.4 [C-4″], 69.7 [C-2‴], 70.2 [C-5″], 71.0

[C-4‴], 74.3 [C-2″], 75.6 [C-2⁗], 75.8 [C-4⁗], 76.1 [C-3⁗, C-5⁗], 76.9 [C-3″], 81.9 [C-3‴], 93.6 [C-8], 98.5 [C-6], 100.9 [C-1⁗], 103.2 [C-1″], 104.1 [C-1‴], 104.3 [C-4a], 115.1 [C-3′, C-5′], 121.7 [C-1′], 131.4 [C-2′, C-6′], 134.5 [C-3], 157.2 [C-8a], 158.3 [C-4′], 160.5 [C-2], 161.9 [C-5], 165.1 [C-7], 178.5 [C-4].

Kaempferol-3-*O*-[β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-galactopyranoside]: UV−vis (acetonitrile) λ_{max} = 255, 339; LC-MS (ESI$^+$), *m*/*z* 757 (100; [M]$^+$), 595 (10 [M − glc]$^+$), 449 (4 [M − rha − glc]$^+$), 287 (7 [M − gal − rha − glc]$^+$); ^{1}H NMR (900 MHz; CD_3COD) δ 1.2 [d, 3H, H−C(6‴)], 3.21 [m, 1H, H−C(5⁗)], 3.27 [m, 1H, H−C(2⁗)], 3.38 [m, 1H, H−C(3⁗)], 3.39 [m, 1H, H−C(4⁗)], 3.49 [m, 1H, H−C(4‴)], 3.49 [m, 1H, H−C(6b″)], 3.56 [dd, 1H, H−C(3″)], 3.59 [dd, 1H, H−C(3‴)], 3.6 [m, 1H, H−C(5‴)], 3.67 [dd, 1H, H−C(5″)], 3.73 [m, 2H, H−C(6⁗)], 3.74 [m, 1H, H−C(6a″)], 3.79 [dd, 1H, H−C(4″)], 3.82 [dd, 1H, H−C(2″)], 3.92 [dd, 1H, H−C(2‴)], 4.39 [d, 1H, *J* = 7.8 Hz, H−C(1⁗)], 4.58 [d, 1H, *J* = 1.8 Hz, H−C(1‴)], 5.04 [d, 1H, *J* = 7.8 Hz, H−C(1″)], 6.25 [d, 1H, H−C(6)], 6.44 [d, 1H, H−C(8)], 6.92 [d, 2H, H−C(3′), H−C(5′)], 8.15 [d, 2H, H−C(2′), H−C(6′)]; ^{1}H NMR data were in line with data reported in the literature (*30*); ^{13}C NMR (225 MHz; CD_3COD) δ 16.5 [C-6‴], 60.5 [C-6⁗], 66.5 [C-6″], 68.1 [C-5‴], 68.7 [C-4″], 69.4 [C-4⁗], 69.9 [C-2‴], 71.2 [C-4‴], 71.7 [C-2″], 73.5 [C-3″], 73.8 [C-5″], 74.1 [C-2⁗], 76.2 [C-3⁗, C-5⁗], 82.1 [C-3‴], 93.5 [C-8], 98.6 [C-6], 100.9 [C-1‴], 104.3 [C-1″, C-1⁗], 104.5 [C-4a], 114.9 [C-3′, C-5′], 121.4 [C-1′], 131.3 [C-2′, C-6′], 134.4 [C-3], 157.5 [C-8a], 158.3 [C-4′], 160.5 [C-2], 161.7 [C-5], 165.8 [C-7], 178.9 [C-4].

Myricetin-3-*O*-β-D-galactopyranoside: UV−vis (acetonitrile) λ_{max} = 249, 345; LC-MS (ESI$^+$), *m*/*z* 481 (100; [M]$^+$), 319 (10 [M − gal]$^+$); ^{1}H NMR (500 MHz; CD_3COD) δ 3.51 [dd, 1H, H−C(4″)], 3.61 [m, 2H, H−C(6″)], 3.66 [dd, 1H, H−C(5″)], 3.88 [dd, 1H, H−C(2″)], 3.89 [d, 1H, H−C(3″)], 5.23 [d, 1H, *J* = 7.7 Hz, H−C(1″)], 6.23 [d, 1H, H−C(6)], 6.41 [d, 1H, H−C(8)], 7.4 [s, 2H, H−C(2′), H−C(6′)]; ^{1}H NMR data were in line with data reported in the literature (*31*); ^{13}C NMR (125 MHz; CD_3COD) δ 60.9 [C-6″], 66.5 [C-2″], 69.2 [C-3″], 74.3 [C-5″], 76.2 [C-4″], 94.1 [C-8], 99.2 [C-6], 104.5 [C-1″], 104.8 [C-4a], 109.6 [C-6′, C-2′], 120.9 [C-1′], 136.4 [C-3], 137.2 [C-3′, C-5′], 145.4 [C-4′], 157.2 [C-8a], 157.8 [C-2], 162.2 [C-5], 165.4 [C-7], 179.3 [C-4].

Myricetin-3-*O*-β-D-glucopyranoside: UV−vis (acetonitrile) λ_{max} = 249, 345; LC-MS (ESI$^+$), *m*/*z* 481 (100; [M]$^+$), 319 (10 [M − glc]$^+$); ^{1}H NMR (500 MHz; CD_3COD) δ 3.26 [m, 1H, H−C(5″)], 3.41 [dd, 1H, H−C(4″)], 3.46 [dd, 1H, H−C(3″)], 3.54 [dd, 1H, H−C(2″)], 3.64 [dd, 1H, H−C(6b″)], 3.75 [dd, 1H, H−C(6a″)], 5.23 [d, 1H, *J* = 7.7 Hz, H−C(1″)], 6.22 [d, 1H, H−C(6)], 6.41 [d, 1H, H−C(8)], 7.32 [s, 2H, H−C(2′), H−C(6′)]; ^{1}H NMR data were in line with data reported in the literature (*32*); ^{13}C NMR (125 MHz; CD_3COD) δ 61.4 [C-6″], 70.1 [C-4″], 74.8 [C-2″], 77.3 [C-3″], 77.6 [C-5″], 93.9 [C-8], 99.0 [C-6], 103.6 [C-1″], 104.7 [C-4a], 109.1 [C-2′, C-6′], 121.0 [C-1′], 136.1 [C-3], 137.2 [C-3′, C-5′], 145.6 [C-4′], 157.7 [C-8a], 158.1 [C-2], 162.3 [C-5], 165.3 [C-7], 179.3 [C-4].

Myricetin-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside]: UV−vis (acetonitrile) λ_{max} = 249, 345; LC-MS (ESI$^+$), *m*/*z* 627 (100; [M]$^+$), 481 (9 [M − rha]$^+$), 319 (13 [M − glc − rha]$^+$); ^{1}H NMR (500 MHz; CD_3COD) δ 1.11 [d, 3H, H−C(6‴)], 3.26 [dd, 1H, H−C(4″)], 3.31 [dd, 1H, H−C(4‴)], 3.32 [ddd, 1H, H−C(5″)], 3.42 [dd, 1H, H−C(6b″)], 3.43 [m, 1H, H−C(3″)], 3.46 [m, 1H, H−C(5‴)], 3.50 [dd, 1H, H−C(2″)], 3.56 [dd, 1H, H−C(3‴)], 3.65 [dd, 1H, H−C(2‴)], 3.83 [dd, 1H, H−C(6a″)], 4.55 [d, 1H, *J* = 1.1 Hz, H−C(1‴)], 5.11 [d, 1H, *J* = 7.9 Hz, H−C(1″)], 6.24 [d, 1H, H−C(6)], 6.43 [d, 1H, H−C(8)], 7.32 [s, 2H, H−C(2′), H−C(6′)]; ^{1}H NMR data were in line with data reported in the literature (*32*); ^{13}C NMR (125 MHz; CD_3COD) δ 16.9 [C-6‴], 67.5 [C-6″], 68.7 [C-5‴], 70.2 [C-4″], 71.03 [C-2‴], 71.11 [C-3‴], 72.93 [C-4‴], 74.6 [C-2″], 76.2 [C-5″], 77.2 [C-3″], 93.9 [C-8], 99.1 [C-6], 101.4 [C-1⁗], 103.7 [C-1″], 104.6 [C-4a], 109.3 [C-2′, C-6′], 121.3 [C-1′], 135.6 [C-3], 137.2 [C-3′, C-5′], 145.4 [C-4′], 157.4 [C-8a], 162.1 [C-5], 165.0 [C-7], 165.4 [C-2], 178.4 [C-4].

Quercetin-3-*O*-β-D-galactopyranoside: UV−vis (acetonitrile) λ_{max} = 243, 345; LC-MS (ESI$^+$), *m*/*z* 465 (100; [M]$^+$), 303 (53 [M − gal]$^+$); ^{1}H and ^{13}C NMR data were identical with those measured for the reference compound.

Quercetin-3-*O*-β-D-glucoside: UV−vis (acetonitrile) λ_{max} = 243, 345; LC-MS (ESI$^+$), *m*/*z* 465 (100; [M]$^+$), 303 (53 [M − glc]$^+$); ^{1}H and ^{13}C NMR data were identical with those measured for the reference compound.

Quercetin-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside]: UV−vis (acetonitrile) λ_{max} = 243, 345; LC-MS (ESI$^+$), *m*/*z* 611 (100 [M]$^+$), 465 (10 [M − rha]$^+$) 303 (14 [M − glc − rha]$^+$); ^{1}H and ^{13}C NMR data were identical with those measured for the reference compound.

Quercetin-3-*O*-[β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-galactopyranoside]: UV−vis (acetonitrile) λ_{max} = 243, 345; LC-MS (ESI$^+$), *m*/*z* 773 (100 [M]$^+$), 611 (3 [M − glc]$^+$), 465 (4 [M − glc − rha]$^+$), 303 (6 [M − glc − rha − glc]$^+$); ^{1}H NMR (900 MHz; CD_3COD) δ 1.2 [d, 3H, H−C(6‴)], 3.22 [m, 1H, H−C(5⁗)], 3.27 [d, 1H, H−C(2⁗)], 3.38 [ddd, 1H, H−C(3⁗)], 3.39 [ddd, 1H, H−C(4⁗)], 3.49 [dd, 1H, H−C(4‴)], 3.52 [dd, 1H, H−C(6a″)], 3.59 [m, 1H, H−C(3‴)], 3.59 [m, 1H, H−C(3″)], 3.61 [m, 1H, H−C(5‴)], 3.70 [dd, 1H, H−C(5″)], 3.72 [dd, 1H, H−C(6⁗)], 3.75 [dd, 1H, H−C(6⁗)], 3.76 [dd, 1H, H−C(6b″)], 3.84 [dd, 1H, H−C(4″)], 3.86 [dd, 1H, H−C(2″)], 3.91 [dd, 1H, H−C(2‴)], 4.4 [d, 1H, *J* = 7.7 Hz, H−C(1⁗)], 4.56 [d, 1H, *J* = 1.8 Hz, H−C(1‴)], 5.04 [d, 1H, *J* = 7.7 Hz, H−C(1″)], 6.22 [d, 1H, H−C(6)], 6.42 [d, 1H, H−C(8)], 6.87 [d, 1H, H−C(5′)], 7.62 [dd, 1H, H−C(6′)], 7.87 [d, 1H, H−C(2′)]; ^{1}H NMR data were in line with data reported in the literature (*30*); ^{13}C NMR (225 MHz; CD_3COD) δ 16.8 [C-6‴], 60.6 [C-6⁗], 66.4 [C-6″], 68.1 [C-5‴], 68.8 [C-4‴], 69.5 [C-4⁗], 69.8 [C-2‴], 71.2 [C-4‴], 71.8 [C-2″], 73.6 [C-3″], 73.8 [C-5″], 74.0 [C-2⁗], 76.1 [C-3⁗], 76.3 [C-5⁗], 81.9 [C-3‴], 93.5 [C-8], 98.9 [C-6], 100.5 [C-1‴], 104.3 [C-4a], 104.4 [C-1⁗], 104.7 [C-1″], 116.6 [C-2′], 121.3 [C-1′], 121.6 [C-6′], 134.5 [C-3], 144.6 [C-3′], 148.9 [C-4′], 157.3 [C-8a], 157.8 [C-2], 161.9 [C-5], 164.9 [C-7], 179.9 [C-4].

Quercetin-3-*O*-[β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside]: UV−vis (acetonitrile) λ_{max} = 243, 345; LC-MS (ESI$^+$), *m*/*z* 773 (100 [M]$^+$), 611 (5 [M − glc]$^+$), 465 (6 [M − glc − rha]$^+$), 303 (7 [M − glc − rha − glc]$^+$); ^{1}H NMR (900 MHz; CD_3COD) δ 1.13 [d, 3H, H−C(6‴)], 3.27 [m, 1H, H−C(5⁗)], 3.29 [m, 1H, H−C(2⁗)], 3.30 [m, 1H, H−C(5″)], 3.38 [m, 1H, H−C(4″)], 3.41 [m, 1H, H−C(4⁗)], 3.43 [m, 1H, H−C(3⁗)], 3.44 [m, 1H, H−C(3″)], 3.47 [m, 1H, H−C(4‴)], 3.48 [m, 1H, H−C(6b″)], 3.51 [m, 1H, H−C(2″)], 3.52 [m, 1H, H−C(5‴)], 3.64 [dd, 1H, H−C(3‴)], 3.74 [dd, 1H, H−C(6b⁗)], 3.77 [dd, 1H, H−C(6a⁗)], 3.81 [dd, 1H, H−C(6a″)], 3.97 [dd, 1H, H−C(2⁗)], 4.45 [d, 1H, *J* = 7.8 Hz, H−C(1⁗)], 4.59 [d, 1H, *J* = 1.4 Hz, H−C(1‴)], 5.12 [d, 1H, *J* = 7.9 Hz, H−C(1″)], 6.24 [d, 1H, H−C(6)], 6.45 [d, 1H, H−C(8)], 6.91 [d, 1H, H−C(5′)], 7.65 [dd, 1H, H−C(6′)], 7.72 [d, 1H, H−C(2′)]; ^{1}H NMR data were in line with data reported in the literature (*29*); ^{13}C NMR (225 MHz; CD_3COD) δ 16.6 [C-6‴], 60.8 [C-6⁗], 67.5 [C-6″], 68.1 [C-5‴], 69.6 [C-4″], 69.9 [C-2‴], 70.1 [C-5″], 71.3 [C-4‴], 74.2 [C-2⁗], 74.5 [C-2″], 75.65 [C-4⁗], 76.1 [C-5⁗], 76.2 [C-3⁗], 76.8 [C-3⁗], 81.7 [C-3‴], 93.5 [C-8], 98.6 [C-6], 100.9 [C-1‴], 103.7 [C-1″], 104.2 [C-1⁗], 104.3 [C-4a], 114.9 [C-5′], 116.4 [C-2′], 121.7 [C-1′], 122.1 [C-6′], 134.5 [C-3], 144.5 [C-3′], 148.6 [C-4′], 157.3 [C-8a], 158.2 [C-2], 161.8 [C-5], 164.8 [C-7], 178.2 [C-4].

Apigenin-8-*C*-[α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside] (vitexin-2″-*O*-[α-L-rhamnopyranoside]): UV−vis (acetonitrile) λ_{max} = 219, 255, 327; LC-MS (ESI$^+$), *m*/*z* 579 (100 [M]$^+$), 433 (5 [M − rha]$^+$); ^{1}H NMR (900 MHz; DMSO-d_6) δ 0.43 [d, 3H, *J* = 6.2 Hz, H−C(6‴)], 2.15 [ddd, 1H, *J* = 6.2, 9.4 Hz, H−C(5‴)], 2.92 [dd, 1H, *J* = 9.4 Hz, H−C(4‴)], 3.11 [dd, 1H, *J* = 3.0, 9.4 Hz, H−C(3‴)], 3.25 [m, 1H, *J* = 1.0, 6.2; 8.8 Hz, H−C(5″)], 3.41 [d, 1H, *J* = 8.8 Hz, H−C(4″)], 3.45 [dd, 1H, *J* = 8.8 Hz, H−C(3″)], 3.53 [dd, 1H, *J* = 6.2, 12 Hz, H−C(6b″)], 3.58 [d, 1H, *J* = 1.1, 3.0 Hz, H−C(2‴)], 3.78 [d, 1H, *J* = 1.0, 12 Hz, H−C(6a″)], 4.07 [dd, 1H, *J* = 8.8, 10 Hz, H−C(2″)], 4.76 [d, 1H, *J* = 10 Hz, H−C(1″)], 4.99 [d, 1H, *J* = 1.1 Hz, H−C(1‴)], 6.27 [s, 1H, H−C(6)], 6.79 [s, 1H, H−C(3)], 6.93 [d, 2H, *J* = 8.8 Hz, H−C(3′) and H−C(5′)], 8.05 [d, 2H, *J* = 8.8 Hz; H−C(2′) and H−C(6′)]; ^{13}C NMR (225 MHz; DMSO-d_6) δ 17.1 [C-6‴], 61.6 [C-6″], 68.7 [C-5‴], 70.6 [C-3⁗], 70.7 [C-2‴], 71.1 [C-4″], 71.8 [C-4⁗], 72.1 [C-1″], 75.5 [C-2″], 80.3 [C-3″], 82.4 [C-5″], 98.9 [C-6], 100.8 [C-1⁗], 102.7 [C-3], 104.5 [C-4a], 104.8 [C-8], 116.3

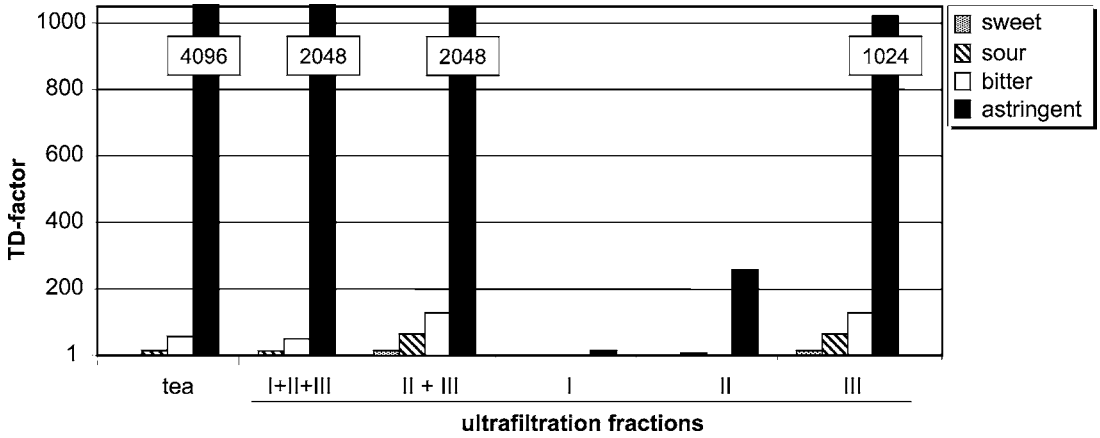

Figure 1. Influence of the molecular weight of tea fractions on the intensity of individual taste qualities.

[C-3′, C-5′], 122.0 [C-1′], 129.6 [C-2′, C-6′], 156.5 [C-8a], 161.2 [C-5], 162.1 [C-4′], 163.4 [C-7], 164.5 [C-2], 182.3 [C-4].

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Bio-Tek Kontron Instruments, Eching, Germany) consisted of two pumps (type 522), a Rheodyne injector (100 μL loop), and a diode array detector (DAD type 540), monitoring the effluent in a wavelength range between 220 and 500 nm.

Liquid Chromatography−Mass Spectrometry (LC-MS). A Grom Sil 120 octyl-5-CP analytical HPLC column (Grom, Rottenburg-Hailfingen, Germany) was coupled to an LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) using electrospray and atmospheric pressure chemical ionization, respectively. After injection of the sample (2−100 μL), the analysis was performed using a gradient starting with a solution of aqueous formic acid (0.1%; pH 3.5) and increasing the methanol content. For chromatography of catechins and theaflavins, the methanol content was increased from 0 to 60% within 65 min and then to 100% within 5 min. For separation of flavonol glycosides, chromatography started with a solution of 14% acetonitrile in aqueous formic acid (0.1%; pH 3.5), increasing the acetonitrile content to 21% within 55 min and, finally, to 100% within 5 min.

Nuclear Magnetic Resonance Spectroscopy (NMR). The ^{1}H, ^{13}C, DEPT-135, COSY, HMQC, eHSQC, HMBC, and NOESY spectroscopic experiments were performed on Bruker Advance-500, Bruker Advance-600, and Bruker-Advance-900 spectrometers (Bruker, Rheinstetten, Germany). Acetone-d_6, DMSO-d_6, and MeOH-d_4 were used as solvents, and tetramethylsilane was used as internal standard.

RESULTS AND DISCUSSION

Preliminary taste profile analysis of a freshly prepared infusion of Darjeeling tea revealed the highest intensity for an astringent and mouth-coating sensation, followed by bitterness and sourness with significantly lower intensities. In comparison, sweetness was judged with a very low intensity, and saltiness and umami taste were not detectable at all (data not shown). To gain first insight into the chemical compounds imparting the intense astringent sensation perceived in the oral cavity, the influence of the molecular weight of the tea constituents on their contribution to the overall astringency of the tea infusion was elucidated. Therefore, the freshly prepared tea infusion was rapidly cooled in an ice bath and then separated by means of multiple-step ultrafiltration using filters with cutoffs of 10 and 1 kDa in sequence. To inhibit oxidative degradation of phenolic substances, the ultrafiltration cell was cooled with an ice bath and the separation was performed under an atmosphere of nitrogen. Three fractions were obtained, strongly differing in color. The deep brown fraction I contained the thearubigen-type polymers with molecular weights >10 kDa; fraction II, containing the compounds with molecular weights between 1 and 10 kDa, showed a red-brown coloration; and the nearly colorless fraction III contained the tea components with molecular weights <1 kDa. After freeze-drying, each individual fraction, or a recombinate prepared from fractions I−III in their natural concentrations, was dissolved in the same amount of water, and the sensory impressions astringency, bitterness, sourness, and sweetness were then rated by their intensities through application of the taste dilution analysis. To achieve this, the individual solutions were diluted stepwise 1+1 with water, and the dilutions of each fraction were presented to a sensory panel in order of increasing concentrations until the detection threshold was reached. The dilution at which a certain taste modality could just be detected is defined as the TD factor. Although being intensely brown in color, the polymeric fraction I was nearly tasteless and showed only a very faint astringent sensation judged with a TD factor of only 16 (**Figure 1**). In comparison, the low molecular weight fraction III was described by the sensory panel to impart the typical taste profile of the black tea and was evaluated with a high TD factor of 1024 for astringency besides lower TD factors for bitter and sour notes. Also, fraction II induced an astringent sensation in the oral cavity, but the TD factor was 4-fold lower than the one determined for fraction III. Interestingly, the astringency of the recombinate prepared from fractions I−III was judged with the high TD factor of 2048, thus being close to the taste impact evaluated for the authentic tea infusion (**Figure 1**). After omitting fraction I from this recombinate, the sensory panel was not able to differentiate the sample from the total recombinate containing fractions I−III, thus demonstrating that the high molecular weight polyphenols do not contribute to the typical taste of the black tea infusion (**Figure 1**). As these data showed that not the polymeric thearubigens but the low molecular weight compounds were the main contributor to tea astringency, the following tastant mapping was focused on fraction III.

Mapping of Key Compounds Contributing to Tea Astringency. To get a first insight into the chemical composition of the low molecular weight fraction III, this fraction was further analyzed by HPLC using cyanopropyl-modified RP-8 material as the stationary phase. As outlined in **Figure 2A**, fraction III consisted of a tremendous multiplicity of different substances of which only a limited number of compounds were expected to contribute significantly to the overall astringent and mouth-coating sensation imparted by the tea infusion. To focus the challenging identification experiments on these key tastants, it was therefore necessary to sort out the strongly astringent compounds from the less active or tasteless substances. Aimed at rating the tea compounds in their relative taste contribution, fraction III was analyzed by HPLC (**Figure 2A**). The effluent

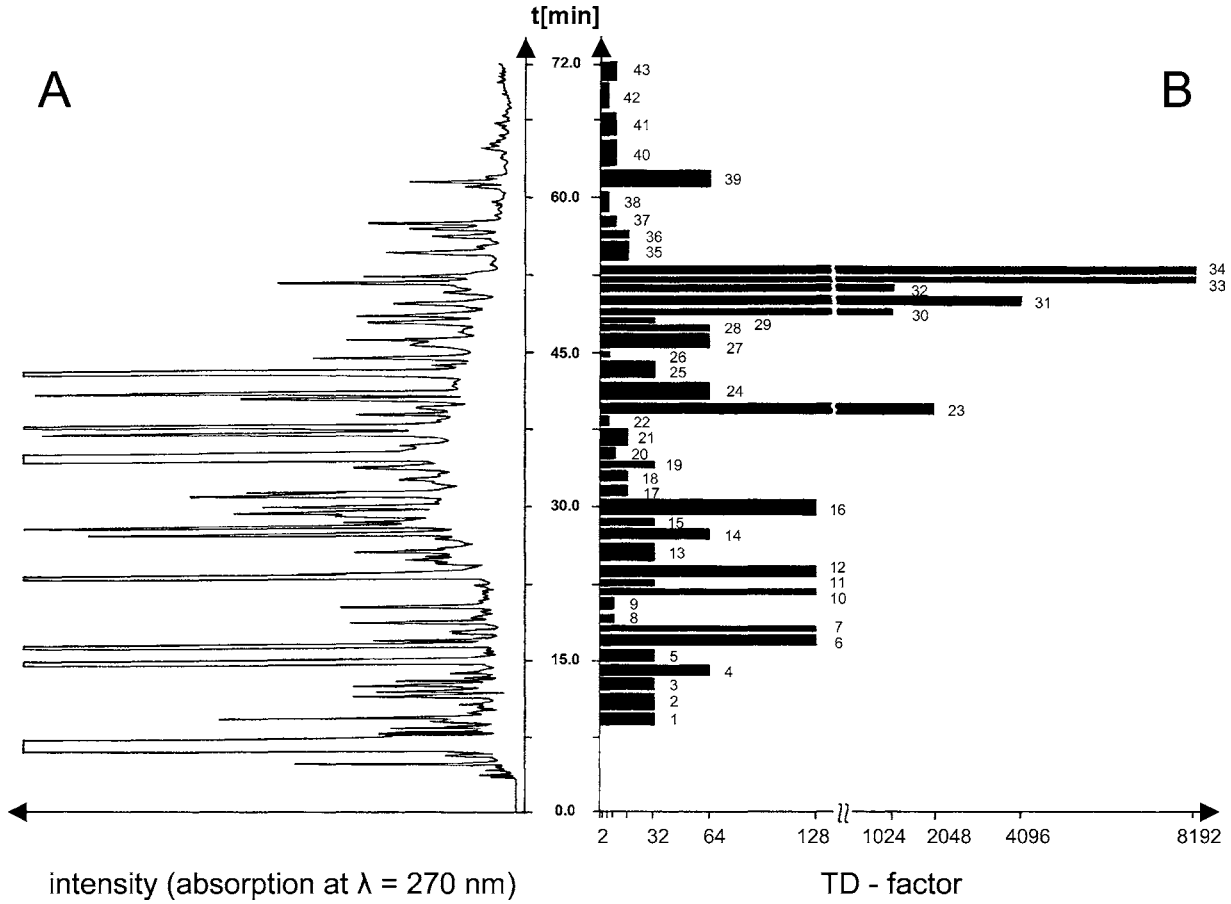

Figure 2. (**A**) RP-HPLC chromatogram and (**B**) taste dilution (TD) chromatogram of the low molecular weight fraction III (MW < 1 kDa) isolated from black tea infusion by means of ultrafiltration.

was separated into 43 fractions, which were freed from solvent and then used for the taste dilution analysis. To achieve this and to overcome the well-known memory effects reported for perception of astringency (*19*), a half-tongue test was developed. The aqueous solution containing the individual HPLC fraction was applied in random order on one side of the tongue, whereas pure water (control) was applied on the other side of the tongue of the subject. After 30 s, the sensory panelists were then asked to identify the side of the tongue where the typical chemesthetic sensation could be perceived. Thereafter, the participants received another set of one blank and one tastant sample. Whenever the panelist selected incorrectly, the next trial took place at the next higher concentration step until the sensory threshold was reached. The TD factor determined rated the 43 HPLC fractions in their relative astringency as shown in **Figure 2B**. Due to their high TD factor of 8192, fractions 33 and 34 were evaluated with by far the highest taste impacts for astringency, closely followed by fractions 30−32 and 23 judged with TD factors between 1024 and 4096 (**Figure 2B**). In comparison, fractions 6, 7, 10−12, and 16 were evaluated with somewhat lower taste impacts, whereas the low TD factors determined for all other fractions excluded major contributions to the perception of tea astringency. To gain first insight into the astringent key compounds within these HPLC fractions, we first investigated the phenolic substances, which were already suggested in the literature as the astringent compounds in black tea, namely, the catechins and the theaflavins.

Identification of Catechins and Theaflavins. Analysis of the Darjeeling tea infusion by means of HPLC coupled to either a diode array detector or a mass spectrometer led to the identification of eight catechins and five theaflavins by comparison of chromatographic (RP-HPLC) and spectroscopic data (UV−vis, LC-MS) as well as human taste thresholds with those obtained for reference compounds. Finally, cochromatography confirmed the following compounds to be the key taste compounds in the individual HPLC fractions (**Figure 2**): gallocatechin (fraction 11), epigallocatechin (fraction 15), catechin (fraction 15), epigallocatechin-3-gallate (fraction 20), epicatechin (fraction 22), gallocatechin-3-gallate (fraction 24), epicatechin-3-gallate (fraction 25), catechin-3-gallate (fraction 26), theaflavin (fraction 37), theaflavic acid (fraction 37), theaflavin-3-gallate (fraction 38), theaflavin-3′-gallate (fraction 38), and theaflavin-3,3′-digallate (fraction 39). On comparison of these data with the results of the taste dilution analysis (**Figure 2**) it is obvious that those fractions containing the catechin and theaflavin-type compounds were evaluated with TD factors below 128, whereas the unknown compounds eluting in HPLC fractions 30−34 were judged to have TD factors of up to 8192. On the basis of these findings it might be concluded that neither the catechins nor the theaflavins are the key compounds imparting the astringent taste sensation during tea consumption but that additional compounds eluting in fractions 30−34 seem to be of major importance.

Identification of Key Tastants in Fractions 30−34. Because the intensely tasting fractions 30−34 still consisted of a multiplicity of substances each in low concentrations, the tea infusion was preseparated by column chromatography on polyamide material. After the column had been flushed with water, three methanol fractions (PA-I−III) were collected, decaffeinated by repeated dichloromethane extraction, and then concentrated in vacuo. HPLC analysis revealed that fraction PA-I contained the compounds which have already been

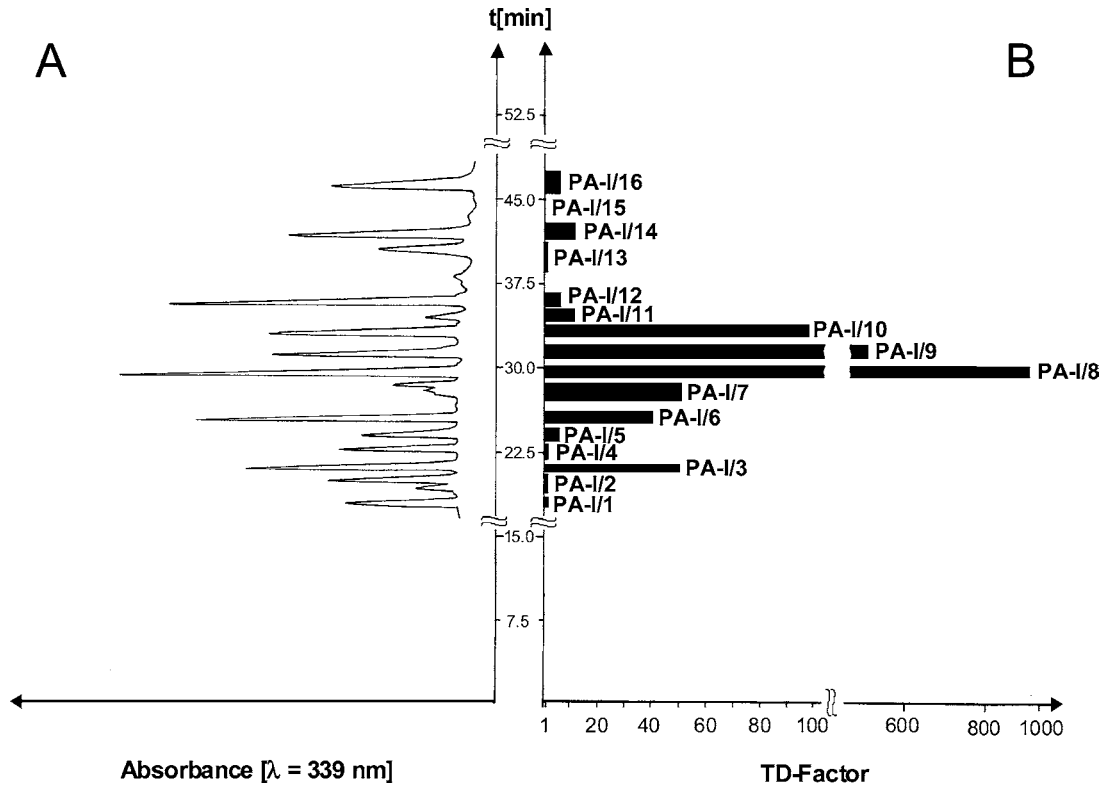

Figure 3. (**A**) RP-HPLC chromatogram and (**B**) taste dilution (TD) chromatogram of the fraction of flavon-3-ol glycosides isolated from black tea infusion by means of polyamide chromatography.

detected in HPLC fractions 30−34 (**Figure 2**). Using a modified solvent gradient, this fraction could be resolved by semipreparative HPLC into 16 individual subfractions, PA-I/1−16 in **Figure 3A**. To rate these 16 compounds by their taste impact, their effluents were collected, and, after removal of the solvent, these fractions were analyzed by taste dilution analysis using the half-tongue test described above (**Figure 3B**). The highest taste impact was found for fraction PA-I/8 with a TD factor of 1024, closely followed by fractions PA-I/9 and PA-I/10 with somewhat lower TD factors.

To enable the structure determination of the astringent compounds by means of LC-MS and NMR studies, these compounds needed to be isolated in higher amounts. To achieve this, the powdered tea was extracted with aqueous methanol, and, after removal of the solvent in vacuo, the aqueous extract was fractionated by polyamide chromatography. To isolate and purify the compounds imparting the astringent and mouth-coating sensation to these HPLC fractions, the polyamide fraction PA-I was fractionated by column chromatography on an RP-18 material on a preparative scale. By monitoring the effluent at 345 nm, the effluents of the individual peaks were collected and freed from solvent, and 14 individual compounds were purified by RP-HPLC and analyzed by UV−vis, LC-MS, and NMR spectroscopy.

Compounds isolated from fractions PA-I/1−3 showed the typical absorption maxima at 249 and 345 nm as reported for myricetin glycosides. LC-MS analysis showed $[M + H]^+$ ions at *m*/*z* 627 for the compound eluting in fraction PA-I/1 and at *m*/*z* 481 for the glycosides detected in fractions PA-I/2 and PA-I/3. MS/MS experiments with the ion at *m*/*z* 481 or 627 further revealed the loss of 162 or 306 Da, corresponding to a hexose or a hexose-methylpentose moiety, respectively, to generate the ion *m*/*z* 319 of the myricetin aglycon. To further confirm the structure of the aglycon and to identify the sugar moiety, 1D- and 2D-NMR experiments were performed. The ^{1}H NMR spectrum showed a characteristic A_2X_2 system for the protons H−C(6) and H−C(8) resonating at 6.41 and 6.22 ppm with a coupling constant of 2 Hz and a singlet at 7.4 ppm integrating for the protons H−C(2′) and H−C(6′), thus confirming myricetin as the aglycon. Homo- and heteronuclear correlation experiments gave a comprehensive picture on the type of sugar linked to the myricetin as well as on the conformation at the anomeric carbon atoms and identified the mouth-coating and astringent compounds in fractions PA-I/1, PA-I/2, and PA-I/3 as myricetin-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside], myricetin-3-*O*-β-D-galactopyranoside, and myricetin-3-*O*-β-D-glucopyranoside (**Figure 4**), respectively.

UV−vis analysis and LC-MS/MS spectrometry of the five compounds isolated from the HPLC fractions PA-I/4, PA-I/6, and PA-I/8−10 all showed absorption maxima at 243 and 345 nm and a fragment ion at *m*/*z* 303, typically reported for the quercetin aglycon. The structure determination of these five quercetin-3-glycosides was achieved by means of ^{1}H and ^{13}C NMR spectroscopy including ^{1}H,^{1}H-COSY, eHSQC, and HMBC spectroscopy, which allowed the full assignment of all carbon and proton resonances. As an example, the ^{1}H NMR spectrum of the taste compound isolated from fraction PA-I/4 is given in **Figure 5**. Besides the signals expected for the quercetin aglycon, the ^{1}H NMR spectrum showed three doublets at 4.40, 4.56, and 5.04 ppm corresponding to the individual anomeric carbon atoms of three pyranose rings. On the basis of the coupling constants between the anomeric and the vicinal protons, two of the three sugars were identified as β-anomers, whereas the third sugar was α-glycosidically linked. With the help of heteronuclear experiments the hexose linked to the hydroxyl group at C(3) of quercetin was readily identified through the low-field shift of its anomeric proton H−C(1″) resonating at 5.04 ppm and was unambiguously confirmed by a long-range coupling between this proton and C(3) in the HMBC spectrum. The HMBC experiment also allowed the determination of the interconnec-

flavonol-3-monoglycosides	R_1	R_2	R_3	R_4
kaempferol-glc	H	H	OH	H
kaempferol-gal	H	H	H	OH
quercetin-glc	H	OH	OH	H
quercetin-gal	H	OH	H	OH
myricetin-glc	OH	OH	OH	H
myricetin-gal	OH	OH	H	OH

flavonol-3-diglycosides	R_1	R_2
kaempferol-glc-rha	H	H
quercetin-glc-rha	H	OH
myricetin-glc-rha	OH	OH

flavonol-3-triglycosides	R_1	R_2	R_3
kaempferol-glc-rha-glc	H	H	OH
kaempferol-gal-rha-glc	H	OH	H
quercetin-gal-rha-glc	OH	OH	H
quercetin-glc-rha-glc	OH	H	OH

Figure 4. Structures of mono-, di-, and triglycosylated flavon-3-ols identified in black tea infusion (glc, glucose; gal, galactose; rha, rhamnose).

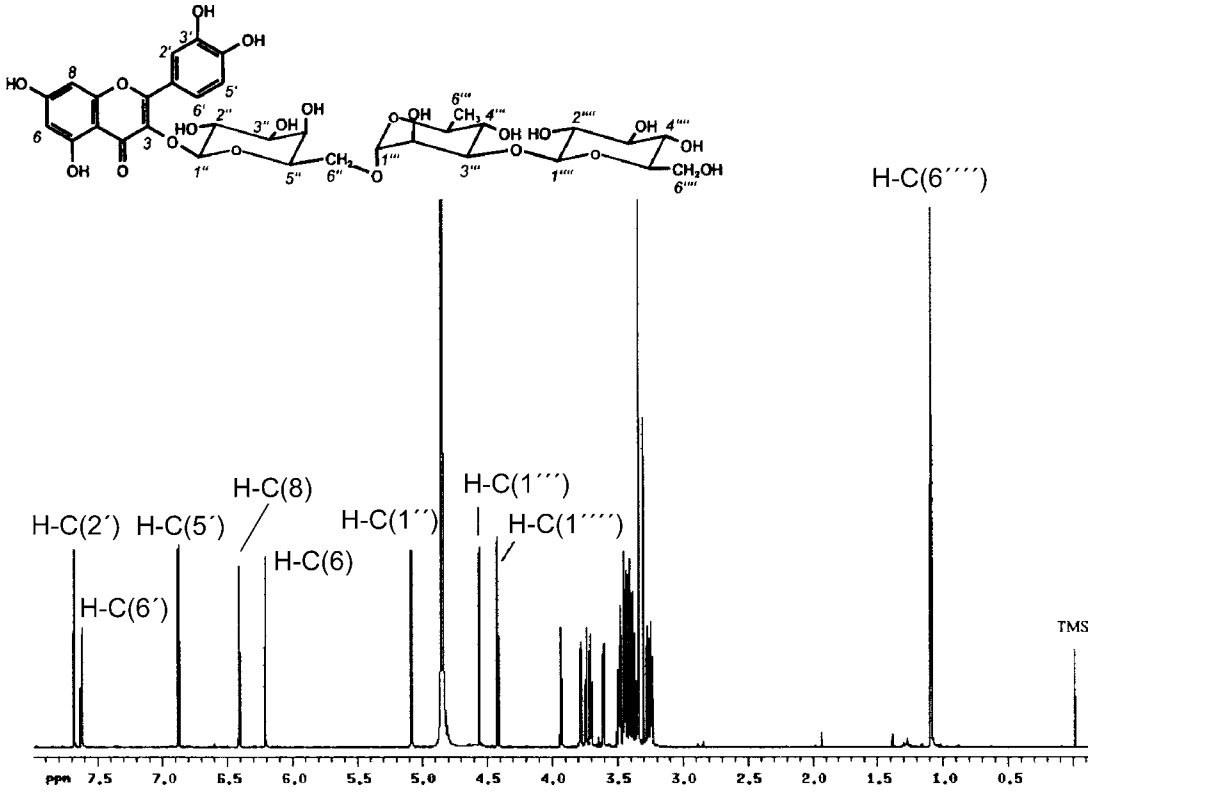

Figure 5. ^{1}H NMR spectrum (900 MHz, CD_3OD) and chemical structure of quercetin-3-*O*-[β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-galactopyranoside].

tivity of the three sugar units in the molecule; for example, the anomeric proton H−C(1‴) at 4.56 ppm showed a heteronuclear correlation with C(6″) resonating at 66.4 ppm, and H−C(4‴) resonating at 3.49 ppm showed connectivity to the carbon atom C(1⁗) detected at 104.4 ppm. Taking all spectroscopic data into consideration, the structure of the astringent, mouth-coating compound in fraction PA-I/4 was determined to be the quercetin-3-*O*-[β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-galactopyranoside] (**Figure 4**). Similar to this triglycoside, the astringent compounds eluting in fractions PA-I/6, PA-

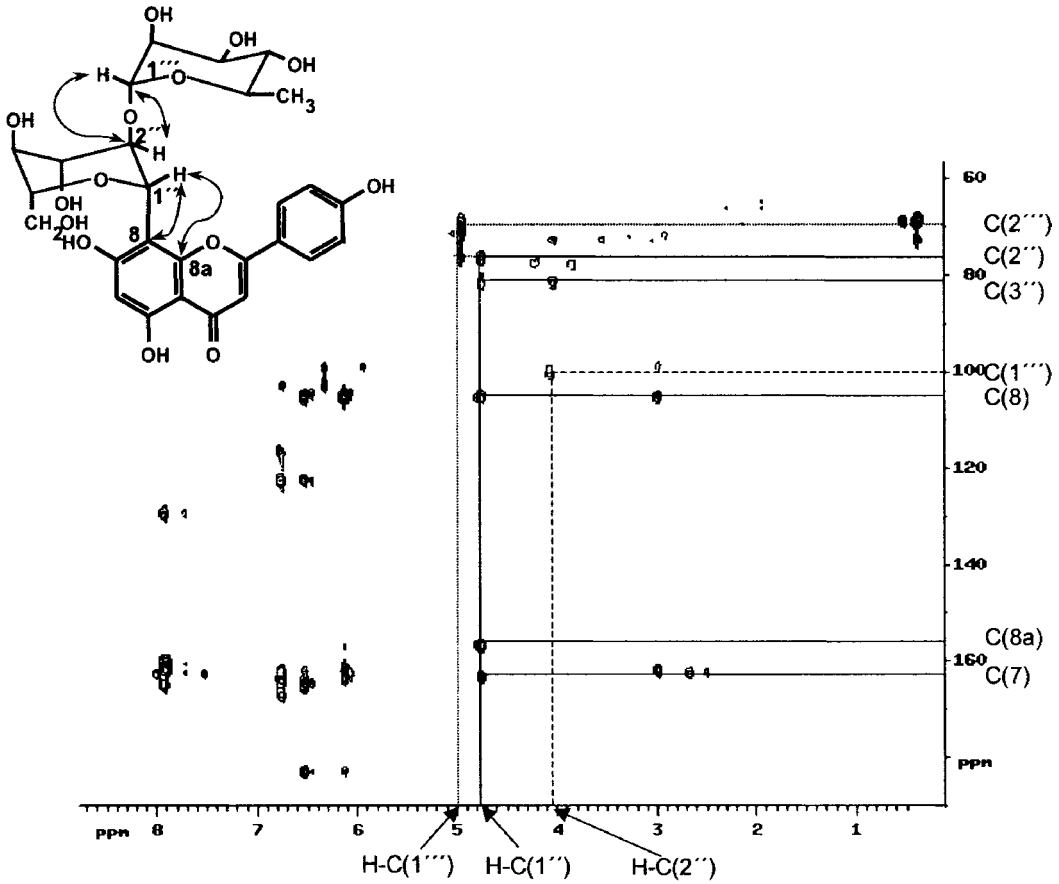

Figure 6. Section of HMBC spectrum (900 MHz, DMSO-d_6) and structure of apigenin-8-*C*-[α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside].

I/8, PA-I/9, and PA-I/10 were identified as quercetin-3-*O*-[β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside], quercetin-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside], quercetin-3-*O*-β-D-glucopyranoside, and quercetin-3-*O*-β-D-galactopyranoside, respectively (**Figure 4**).

UV−vis spectroscopy revealing absorption maxima at 255 and 339 nm as well as LC-MS analysis pointed out that the astringent compounds eluting in fractions PA-I/12−14 and PA-I/16 were kaempferol-3-glycosides. 1D- and 2D-NMR experiments revealed a comprehensive picture of the chemical structures of the taste compounds and led to the identification of kaempferol-3-*O*-[β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-galactopyranoside] in fraction PA-I/11, kaempferol-3-*O*-[β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside] in fraction PA-I/12, kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside] in fraction PA-I/13, kaempferol-3-*O*-β-D-glucopyranoside in fraction PA-I/14, and kaempferol-3-*O*-β-D-galactopyranoside in fraction PA-I/16, the structures of which are given in **Figure 4**.

In contrast to the substances identified above, the absorption maxima at 219, 255, and 327 nm detected for the astringent compound isolated from fraction PA-I/7 did not correspond to any flavon-3-ol glycoside. LC-MS studies revealed a $[M + 1]^+$ ion at *m*/*z* 579 and demonstrated the cleavage of 146 Da, most likely corresponding to the loss of one molecule of rhamnose. To get a closer look at the structure of this molecule, 1D- and 2D-NMR experiments were performed. The protons at the aglycon showed a singlet at 6.79 ppm for H−C(3) and demonstrated the compound to be a flavone glycoside. The B-ring showed an AA′BB′ spin system of two doublets resonating at 8.05 ppm for H−C(2′) and H−C(6′) and at 6.93 ppm for H−C(3′) and H−C(5′), each with an intensity of two protons and a coupling constant of 8.8 Hz. In addition, a singlet was detected at 6.27 ppm corresponding to the proton in position C(6), but no proton signal was detectable for the position C(8). These data clearly indicate that the sugar moiety is linked via C(8) to the flavone. This assumption was confirmed by heteronuclear correlation spectroscopy (HMBC), which showed the coupling of the sugar proton H−C(1″) with the carbon atoms C(8) and C(8a) resonating at 104.8 and 156.5 ppm (**Figure 6**). In addition, the downfield shift of the protons demonstrates that the carbohydrate is linked as a C-glycoside to position C(8) of apigenin. LC-MS analysis as well as the detection of 15 carbon-linked sugar protons indicated the presence of a second sugar moiety in the molecule. The resonance signal at 4.99 ppm corresponding to an anomeric proton showed a coupling constant of 1.1 Hz, thus indicating that this second sugar was present as an α-anomer. Heteronuclear correlation experiments revealed this second sugar to be an α-L-rhamnopyranose and indicated a C(1‴)→C(2″) linked disaccharide (**Figure 6**). To the best of our knowledge, the astringent apigenin-8-*C*-[α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside] has not been previously reported in tea.

Sensory Activity of Tea Phenols. Prior to sensory analysis, the purity of all the compounds was checked by LC-MS as well as ^{1}H NMR spectroscopy. To study the sensory activity of the astringent compounds identified, the human sensory recognition thresholds were determined in water using the half-tongue test described above (**Table 1**). The oral sensation imparted by the catechins was described as astringent with relatively high threshold concentrations ranging from 190 to 930 μmol/L, thus confirming data reported in the literature (*10*). In particular, the

Table 1. Human Astringency Thresholds of Tea Compounds

compound	taste threshold[a] (μmol/L)
flavan-3-ols (astringent)	
catechin	410
catechin gallate	250
epicatechin	930
epicatechin gallate	260
epigallocatechin	520
epigallocatechin gallate	190
gallocatechin	540
gallocatechin gallate	390
benzotropolones (mouth-drying, rough)	
theaflavin	16.0
theaflavic acid	26.0
theaflavin-3-gallate	15.0
theaflavin-3′-gallate	15.0
theaflavin-3,3′-digallate	13.0
flavon-3-ol glycosides (velvety, silky-astringent, mouth coating)	
kaempferol-3-*O*-β-D-glucopyranoside	0.67
kaempferol-3-*O*-β-D-galactopyranoside	6.7
kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside]	0.25
kaempferol-3-*O*-[β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside]	19.8
kaempferol-3-*O*-[β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-galactopyranoside]	5.8
quercetin-3-*O*-β-D-glucopyranoside	0.65
quercetin-3-*O*-β-D-galactopyranoside	0.43
quercetin-3-*O*-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside]	0.001
quercetin-3-*O*-[β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→ 6)-*O*-β-D-glucopyranoside]	18.4
quercetin-3-*O*-[β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-galactopyranoside]	1.36
myricetin-3-*O*-β-D-glucopyranoside	2.1
myricetin-3-*O*-β-D-galactopyranoside	2.7
myricetin-3-*O*-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside]	10.5
apigenin-8-*C*-[α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside]	2.8

[a] Taste threshold concentrations were determined by means of a comparative duo test in bottled water.

galloyl esters showed somewhat lower thresholds than the nongalloylated catechin derivatives; for example, the threshold concentration of 190 mmol/L found for epigallocatechin-3-gallate is 2.5 times lower than the threshold evaluated for the corresponding epigallocatechin. In comparison, the theaflavins induced a mouth-drying and rough-astringent oral sensation at significantly lower threshold concentrations between 13 and 26 μmol/L; for example, the threshold concentration of 16 μmol/L determined for theaflavin is by a factor of 33 and 58 lower than the thresholds of its precursors epigallocatechin (520 μmol/L) and epicatechin (930 μmol/L), respectively (**Table 1**). Among the group of benzotropolones, theaflavin as well as the three mono- and digallates showed similar threshold concentrations between 13 and 16 μmol/L, whereas a 2-fold higher concentration of theaflavic acid was necessary to impart the same sensory sensation.

Compared to the catechins and theaflavins, the flavon-3-ol glycosides were found to induce a silky, mouth-drying, and mouth-coating sensation at very low threshold concentrations spanning from 0.001 to 19.8 μmol/L (**Table 1**). In particular, the oral threshold of 0.001 μmol/L determined for the diglycoside quercetin-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside], also known as quercetin-3-*O*-rutinoside or rutin, is extraordinarily low, and therefore this substance is believed to have a major influence on the tea taste. Comparing this quercetin-3-*O*-rutinoside with the corresponding 3-*O*-rutinosides of the other flavon-3-ol aglycons showed that the aglycon is strongly influencing the taste intensity of these compounds; for example, the kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside] (kaempferol-3-rutinoside) and myricetin-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside] (myricetin-3-rutinoside) possess rather low threshold concentrations of 0.25 and 10.5 μmol/L, respectively, which are 250 and 10500 times above the threshold concentration determined for the quercetin analogue.

Besides the structure of the aglycon, the sugar moiety also seems to have an influence on the perception of astringency; for example, attachment of a rhamnose to the monoglycoside quercetin-3-*O*-β-D-glucopyranoside (0.65 μmol/L) decreased the threshold by a factor of 650 to give 0.001 μmol/L for quercetin-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside] (**Table 1**). It is interesting to note that additional glucosylation of this diglycoside, however, induced a strong increase of the sensory threshold from 0.001 to 18.4 μmol/L as found for the quercetin-3-*O*-[β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside].

In addition to the sugar species, the sequence of the individual monosaccharides in the glycosidic chain also seems to have an influence on the sensory activity of the flavon-3-ol glycosides; for example, the triglycosides quercetin-3-*O*-[β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside] and kaempferol-3-*O*-[β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside], both bearing the glucose directly linked to the aglycon, were evaluated with relatively high thresholds of 18.4 and 19.8 μmol/L, respectively, whereas quercetin-3-*O*-[β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-galactopyranoside] and kaempferol-3-*O*-[β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-galactopyranoside], respectively, both bearing galactose next to the aglycon, showed significantly lower thresholds of 1.38 and 5.8 μmol/L (**Table 1**).

The data obtained for catechins, theaflavins, and flavon-3-ol glycosides clearly show that the sensory activity changes with variations in the phenolic moiety as well as in the glycosylation pattern, thereby illustrating that oral thresholds of astringent compounds cannot be predicted from chemical structures but

have to be investigated on the basis of systematic sensory studies with purified reference compounds. Aimed at demonstrating their contribution to the taste of tea infusions, taste recombination as well as omission experiments using all of these compounds in their natural concentrations are currently in progress and will be published elsewhere.

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