

Sensory-Guided Decomposition of Roasted Cocoa Nibs (*Theobroma cacao*) and Structure Determination of Taste-Active Polyphenols

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Sequential application of solvent extraction, gel permeation chromatography, and RP-HPLC in combination with taste dilution analyses, followed by LC-MS and 1D/2D-NMR experiments and thiolytic degradation, revealed that, besides theobromine and caffeine, the flavan-3-ols epicatechin, catechin, procyanidin B-2, procyanidin B-5, procyanidin C-1, [epicatechin-(4 β →8)]₃-epicatechin, and [epicatechin-(4 β →8)]₄-epicatechin were among the key compounds contributing to the bitter taste as well as the astringent mouthfeel imparted upon consumption of roasted cocoa. In addition, a series of quercetin, naringenin, luteolin, and apigenin glycopyranosides as well as a family of not previously identified amino acid amides, namely, (+)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid, (+)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid, (-)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid, (-)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid, (-)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine, (+)-*N*-[4'-hydroxy-3'-methoxy-(*E*)-cinnamoyl]-L-aspartic acid, and (+)-*N*-(*E*)-cinnamoyl-L-aspartic acid, have been identified as key astringent compounds of roasted cocoa. Furthermore, (-)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (clovamide), (-)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (deoxyclovamide), and (-)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine, reported previously as antioxidants, have been found as contributors of cocoa's astringent taste. By means of the half-tongue test, the taste thresholds of flavan-3-ols and glycosides have been determined.

KEYWORDS: Cocoa beans; astringency; bitter taste; taste dilution analysis; half-tongue test; (+)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid; (+)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid

INTRODUCTION

Due to its attractive aroma and its typical taste, the fermented and roasted seeds of the cocoa tree, *Theobroma cacao*, are enjoyed by consumers as the desirable key ingredient in beverages and chocolate confectionery. Some of the key criteria used to describe the quality of roasted cocoa are its pleasant bitterness and the slight sour taste as well as its typical astringent mouthfeel, which is perceived as a long-lasting puckering, shrinking, rough, and drying sensation in the oral cavity and can enhance the complexity and palate length of cocoa products. Although multiple attempts have been made to find a correlation between the results obtained from sensory panelists and the chemical species imparting the typical bitter taste and astringent sensation of roasted cocoa, the data reported in the literature on the key taste components are rather contradictory.

Due to its low detection threshold of 10 mg/L in water (1), the methylxanthine theobromine, which is present in cocoa beans

in concentrations between 1.8 and 3.8 g/100 g of dry weight (2), is believed to contribute to the typical bitter taste. In addition, some diketopiperazines, generated during the roasting of the fermented cocoa beans from hydrophobic amino acids, are believed to be responsible for the bitter taste of roasted cocoa (3). Among these dilactams, cyclo(Ala-Gly) and cyclo(Ala-Val) have been found as the predominating diketopiperazines in roasted cocoa samples (3). In contradiction to these findings, recent studies identified cyclo(Pro-Gly) as the major diketopiperazine in cocoa (4). Although the diketopiperazines cyclo(Val-Phe) and cyclo(Ala-Phe) have been detected in roasted cocoa only in trace amounts, model experiments demonstrated that these diketopiperazines, when mixed with theobromine, induce a bitter taste sensation similar to that perceived from an aqueous suspension of cocoa powder (3). Comparison of quantitative data of diketopiperazines and the sensorially perceived bitter intensity, however, led to the conclusion that further systematic studies are necessary to understand the typical taste of roasted cocoa on a molecular level (4).

The astringent taste sensation imparted by cocoa beans, liquor, and powder is commonly believed to be caused by (-)-epicatechin, flavan-3-ol dimers, trimers, and higher oligomers,

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called procyanidins (5, 6). Among these, in particular the dimeric procyanidins B-2 and B-5 as well as the trimeric procyanidin C-1 were established as the major low molecular weight procyanidins in nonroasted cocoa beans (7, 8). Preliminary sensory studies on crude fractions isolated from polyphenol-rich wines, ciders, and grape seeds (9–12) revealed that these compounds exhibit astringent and bitter taste qualities, but these studies have not been done with highly purified compounds, nor have recognition taste thresholds been determined so far.

To answer the puzzling question as to which nonvolatile, key taste compounds are responsible for the attractive taste of food products, we have recently developed the so-called taste dilution analysis (TDA) as a powerful screening procedure for taste-active nonvolatiles in foods (13). This approach, combining instrumental analysis and human bioresponse, recently led to the discovery of various previously unknown taste compounds such as thermally generated bitter compounds (13), cooling compounds in dark malt (14), bitter off-tastants in carrot products (15), the taste enhancer alapyridaine in beef bouillon (16), and astringent key taste compounds in black tea infusions (17).

Aimed at defining the taste of cocoa on a molecular level, the objectives of the present investigation were, therefore, to screen roasted cocoa for its key taste compounds by application of taste dilution techniques, to isolate and determine the chemical structure of the compounds inducing the most intense human taste response, and to evaluate their taste impact on the basis of their oral recognition threshold concentrations.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: caffeine and theobromine (Fluka, Neu-Ulm, Germany); (+)-catechin, (–)-epicatechin, toluene- α -thiol, and *trans*-3-indole acrylic acid (Sigma, Steinheim, Germany); hydrochloric acid (Merck, Darmstadt, Germany); naringenin-7-*O*- β -D-glucopyranoside, luteolin-7-*O*- β -D-glucopyranoside, quercetin-3-*O*- β -D-glucopyranoside, quercetin-3-*O*- β -D-galactopyranoside, apigenin-8-*C*- β -D-glucopyranoside, and apigenin-6-*C*- β -D-glucopyranoside (Roth, Karlsruhe, Germany). Solvents were of HPLC grade (Merck). Deuterated solvents were obtained from Euriso-Top (Gif-Sur-Yvette, France).

Cocoa beans, fermented in Ghana for 5 days, were roasted by the food industry. Bottled water (Vittel; low mineralization, 405 mg/L) adjusted to pH 6.0 with aqueous hydrochloric acid (0.1 mol/L) was used for the sensory experiments.

Sensory Analyses. Training of the Sensory Panel. Twelve subjects (five women and seven men, age 25–38 years) with no history of known taste disorders were trained to evaluate the taste of aqueous solutions (2 mL each) of the following standard taste compounds in bottled water (pH 6.0) by using a triangle test as described in the literature (18): sucrose (50 mmol/L) for sweet taste, lactic acid (20 mmol/L) for sour taste, NaCl (20 mmol/L) for salty taste, caffeine (1 mmol/L) for bitter taste, and sodium glutamate (3 mmol/L) for umami taste. For the puckering astringency and the velvety astringent, mouth-drying oral sensation, the panel was trained by using gallustannic acid (0.05%) and quercetin-3-*O*- β -D-glucopyranoside (0.01 mmol/L), respectively, using the half-tongue test (17). The assessors had participated earlier at regular intervals for at least two years in sensory experiments 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.

Pretreatment of Fractions. Prior to sensory analysis, the fractions or compounds isolated were suspended in water, and, after removal of 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.

Half-Tongue Test. Taste dilution factors as well as human astringency recognition thresholds were determined by means of the recently

Table 1. Yields and Sensorial Evaluation of Fractions I–V Isolated from Roasted Cocoa Nibs (RCN)

sample ^b	yield ^c (g/100 g)	intensity ^a perceived for		
		bitterness	sourness	astringency
RCN		4.1	2.8	3.0
fraction I	45.8	0	0	0
fraction II	0.3	2.5	0	0
fraction III	0.7	2.5	1.1	2.0
fraction IV	6.8	3.2	1.3	2.8
fraction V	46.4	<0.5	<0.5	<0.5

^a The taste intensity of aqueous mixtures of the individual fractions isolated from 20 g of cocoa nibs in bottled water (50 mL; pH 6.0) was rated on a scale from 0 (not detectable) to 5.0 (strongly detectable). In comparison, sensory evaluation was done with 20 g of powdered, roasted cocoa nibs suspended in bottled water (50 mL, pH 6.0). ^b Individual fractions contain the *n*-pentane solubles (I), dichloromethane extractables (II), ethyl acetate extractables (III), water solubles (IV), and the nonsoluble residue (V) isolated from RCN. ^c Yields were determined by weight.

developed half-tongue test (17) 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. 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 70 μ mol/L for [epicatechin-(4 β →8)]₄-epicatechin represents a range from 35 to 140 μ mol/L.

Sequential Solvent Extraction of Roasted Cocoa Nibs. Roasted cocoa nibs (100 g) were frozen in liquid nitrogen, crushed in a grinding mill, and then extracted with *n*-pentane (5 × 300 mL) at room temperature for 30 min. After centrifugation, the organic layers were combined and freed from solvent in a vacuum to give the pentane solubles (fraction I). The residual cocoa material was then extracted five times with a mixture (7:3, v/v; 300 mL each) of acetone and water for 45 min at room temperature while stirring. After filtration, the liquid layer was freed from acetone under reduced pressure at 30 °C, the aqueous solution obtained was extracted with dichloromethane (4 × 200 mL), and the combined organic layers were freed from solvent in a vacuum to give the dichloromethane extractables (fraction II). The remaining aqueous layer was adjusted to pH 5.0 with aqueous hydrochloric acid (0.1 mmol/L) and extracted with ethyl acetate (5 × 200 mL), and the organic phase was freed from solvent in a vacuum to give the ethyl acetate extractables (fraction III) and the aqueous phase was freeze-dried to give the water solubles (fraction IV). In addition, the insoluble residue of the powdered cocoa nibs was freeze-dried to give fraction V. The individual fractions were freeze-dried three times to remove trace amounts of solvents, their yields were determined by weight, and their taste profiles were evaluated in aqueous solutions as given in Table 1.

Multistep Ultrafiltration. A solution of fraction IV (1.2 g) in water (50 mL) was separated into different molecular weight fractions by means of an ice-cooled ultrafiltration cell (Amicon, Witten, Germany) using sequentially the filters YM 30, YM 10, YM 3, and YM 1 (Millipore, Bedford, MA) with cutoffs of 30, 10, 3, and 1 kDa, respectively, at a nitrogen pressure of 3 bar. Five ultrafiltration fractions, namely, fraction IV-UF1 (<1 kDa; 910 mg, yield = 76%), IV-UF2 (1–3 kDa; 120 mg, yield = 10%), IV-UF3 (3–10 kDa; 50 mg, yield = 4%), IV-UF4 (10–30 kDa; 80 mg, yield = 7%), and IV-UF5 (>30 kDa; 40 mg, yield = 3%), were collected, freeze-dried, and stored in a desiccator at –18 °C.

Gel Permeation Chromatography (GPC). Fractions III and IV isolated from roasted cocoa nibs and ultrafiltration fraction IV-UF2 (1.0 g each), respectively, were dissolved in a mixture (50:50, v/v; pH 3.5; 10 mL) of methanol and water, filtered, and then applied onto the

top of a water-cooled glass column (100 cm × 5 cm; Amersham Pharmacia Biotech, Uppsala, Sweden), filled with a slurry of Sephadex LH-20 (Amersham Pharmacia Biotech) in the same solvent mixture. Chromatography was performed with methanol/water (50:50, v/v; pH 3.5; 0.5 L), followed by methanol/water (70:30, v/v; pH 3.5; 1 L), methanol/water (90:10, v/v; pH 3.5; 1 L), and, finally, methanol (2 L) at a flow rate of 3 mL/min. The effluent was monitored by means of a UV-1575 type UV-vis detector (Jasco, Gross-Umstadt, Germany) operating at 270 nm, and 19 fractions were collected from fraction III/IV or fraction IV-UF2, respectively, by a fraction collector; the individual fractions were freed from solvent in a vacuum at 30 °C and were then freeze-dried twice. The residue of each GPC fraction was used for the taste dilution analysis as well as for chemical analysis.

HPLC Fractionation of GPC Fractions. The individual GPC fractions III/IV-1–19 collected were dissolved in a mixture (50:50, v/v; pH 2.5; 2 mL) of methanol and aqueous formic acid (0.1% in water; pH 2.5), and, after membrane filtration, aliquots (50–200 μ L) were fractionated by semipreparative HPLC on ODS-Hypersil RP-18, 250 × 10 mm i.d., 5 μ m (ThermoHypersil, Kleinostheim, Germany), using a methanol/aqueous formic acid (0.1% in water; pH 2.5) gradient at a flow rate of 3 mL/min. The effluent was separated into subfractions, which were collected in cooled glass vials by means of a fraction collector. The corresponding fractions obtained from 20 HPLC runs were collected, combined, freed from solvent in a vacuum, and freeze-dried twice, and the residues obtained were analyzed by means of the taste dilution analysis (TDA).

TDA. Aliquots of the GPC fractions and the HPLC fractions, respectively, were dissolved in “natural” ratios in exactly 3.0 and 2.0 mL of bottled water (pH 6.0) and, then, sequentially diluted 1:2 with bottled water. The serial dilutions of each of these fractions were then presented to the sensory panel in order of ascending concentrations, and each dilution was evaluated for astringency by means of the half-tongue test. The dilution at which a taste difference between the diluted extract and the blank (control) could just be detected was defined as the taste dilution (TD) factor (13). The TD factors evaluated by three 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.

Identification of the Alkaloids. An aliquot (20 mg) of the GPC fraction III/IV-6 was dissolved in a mixture (50:50, v/v; 2 mL) of methanol and aqueous formic acid (0.1% in water; pH 2.5) and membrane filtered, and aliquots (100 μ L) were separated by semipreparative HPLC on RP-18, ODS-Hypersil, 5 μ m (ThermoHypersil), starting with a mixture (10:90, v/v) of methanol and aqueous formic acid (0.1% in water, pH 2.5) for 5 min, increasing the methanol content to 70% over 40 min and then to 100% over 0.5 min, and, thereafter, eluting with methanol for 10 min at a flow rate of 3.0 mL/min. Two main peaks were collected, and the solvents were removed in a vacuum and freeze-dried three times to give both compounds as white powders in a purity of >99%. LC-MS and NMR spectroscopy as well as cochromatography with the reference compounds led to the identification of these compounds as the alkaloids theobromine and caffeine.

Identification of Flavon- and Flavanon-3-ol Glycosides. Aliquots of the GPC fractions III/IV-9–14 (100 mg each) were dissolved in a mixture (30:70, v/v) of methanol and aqueous formic acid (0.1% in water; pH 2.5), and, after membrane filtration, aliquots (100 μ L) were fractionated by HPLC on RP-18, ODS-Hypersil, 5 μ m (ThermoHypersil). With the effluent monitored at 345 nm, chromatography was performed with a mixture (30:70, v/v) of methanol and aqueous formic acid (0.1% in water, pH 2.5) for 10 min, increasing the methanol content to 60% over 30 min and then to 100% over 0.5 min, and, thereafter, eluting with methanol for 10 min at a flow rate of 3.0 mL/min. HPLC-degustation, HPLC-DAD, and HPLC-MS/MS led to the detection of a flavon-3-ol pentoside in GPC fraction III/IV-14. After isolation by semipreparative HPLC using the conditions reported above, quercetin-3-O- α -L-arabinopyranoside could be identified by means of LC-MS and NMR experiments.

Quercetin-3-O- α -L-arabinopyranoside: UV-vis (MeOH/water; pH 2.5), λ_{\max} = 243, 345 nm; LC-MS (ESI⁺), m/z 891 (100, [2M + Na]⁺), 435 (53, [M + 1]⁺), 303 (31, [M + 1 - 132]⁺); ¹H NMR (500 MHz, CD₃OD; COSY), δ 3.44 [dd, 1H, J = 3.3, 13.4 Hz, H-C(5a'')], 3.64

[dd, 1H, J = 3.3, 8.4 Hz, H-C(3'')], 3.81 [m, 1H, H-C(4'')], 3.83 [dd, 1H, J = 13.4 Hz, H-C(5b'')], 3.90 [dd, 1H, J = 6.6, 8.4 Hz, H-C(2'')], 5.16 [d, 1H, J = 6.6 Hz, H-C(1'')], 6.20 [d, 1H, J = 2.1 Hz, H-C(6)], 6.40 [d, 1H, J = 2.1 Hz, H-C(8)], 6.87 [d, 1H, J = 8.5 Hz, H-C(5')], 7.57 [dd, 1H, J = 2.2, 8.5 Hz, H-C(6')], 7.74 [d, 1H, J = 2.2 Hz, H-C(2')]; ¹³C NMR (125 MHz, CD₃OD; HMQC, HMBC), δ 66.7 [C(5'')], 69.0 [C(4'')], 72.7 [C(2'')], 74.1 [C(3'')], 94.5 [C(8)], 99.8 [C(6)], 104.5 [C(1'')], 105.6 [C(4a'')], 116.1 [C(5')], 117.3 [C(2')], 122.8 [C(1')], 123.0 [C(6')], 135.7 [C(3)], 146.1 [C(3')], 147.7 [C(2)], 149.8 [C(4')], 158.5 [C(8a)], 163.2 [C(5)], 165.8 [C(7)], 179.1 [C(4)]. The NMR data are in agreement with those reported earlier for that compound isolated from cranberry (19).

In addition, HPLC-DAD, HPLC-MS/MS, and comparison of chromatographic, spectroscopic, and sensory data with those obtained for the corresponding reference compounds led to the unequivocal identification of naringenin-7-O- β -D-glucopyranoside, luteolin-7-O- β -D-glucopyranoside, quercetin-3-O- β -D-glucopyranoside, and quercetin-3-O- β -D-galactopyranoside in GPC fractions III/IV-12 and III/IV-13, as well as apigenin-8-C- β -D-glucopyranoside and apigenin-6-C- β -D-glucopyranoside in GPC fractions III/IV-9–13.

Naringenin-7-O- β -D-glucopyranoside: UV-vis (acetonitrile/water; pH 2.5), λ_{\max} = 223, 271, 327 nm; LC-MS (ESI⁺), m/z 435 (100; [M + 1]⁺), 847 (53; [2M + 1]⁺), 273 (7; [M - glc + 1]⁺); ¹H and ¹³C NMR data were identical with those measured for the reference compound.

Luteolin-7-O- β -D-glucopyranoside: UV-vis (acetonitrile/water; pH 2.5), λ_{\max} = 251, 347 nm; LC-MS (ESI⁺), m/z 449 (100; [M + 1]⁺), 897 (43; [2M + 1]⁺), 287 (4; [M - glc + 1]⁺); ¹H and ¹³C NMR data were identical with those measured for the reference compound.

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

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

Apigenin-8-C- β -D-glucopyranoside: UV-vis (acetonitrile/water; pH 2.5), λ_{\max} = 215, 267, 331; LC-MS (ESI⁺), m/z 433 (100; [M + 1]⁺), 887 (44; [2M + Na]⁺), 271 (3; [M - glc + 1]⁺); ¹H and ¹³C NMR data were identical with those measured for the reference compound.

Apigenin-6-C- β -D-glucopyranoside: UV-vis (acetonitrile/water; pH 2.5), λ_{\max} = 215, 267, 331; LC-MS (ESI⁺), m/z 433 (100; [M + 1]⁺), 887 (25; [2M + Na]⁺), 271 (7; [M - glc + 1]⁺); ¹H and ¹³C NMR data were identical with those measured for the reference compound.

Identification of (+)-Catechin and (-)-Epicatechin. Aliquots (20 mg) of the GPC fraction III/IV-12 were dissolved in a mixture (30:70, v/v) of methanol and aqueous formic acid (0.1% in water, pH 2.5), and, after membrane filtration, aliquots (50–100 μ L) were applied on a semipreparative RP-18 column, ODS-Hypersil, 5 μ m (ThermoHypersil). With the effluent monitored at 270 nm, chromatography was performed with a mixture (10:90, v/v) of methanol and aqueous formic acid (0.1% in water, pH 2.5) for 5 min, increasing the methanol content to 60% over 30 min and then to 100% over 0.5 min, and, thereafter, eluting with methanol for 10 min at a flow rate of 3.0 mL/min. Comparison of the chromatographic, spectroscopic, and sensory data of the most intensely astringent tasting compounds with those obtained for the corresponding reference compounds led to their unequivocal identification as (+)-catechin and (-)-epicatechin.

(+)-Catechin: UV-vis (MeOH/water; pH 2.5), λ_{\max} = 225, 267 nm; LC-MS (ESI⁺), m/z 291 (100; [M + 1]⁺), 581 (43, [2M + 1]⁺); ¹H and ¹³C NMR data of catechin were identical with those measured for the reference compound.

(-)-Epicatechin: UV-vis (MeOH/water; pH 2.5), λ_{\max} = 225, 267 nm; LC-MS (ESI⁺), m/z 291 (100; [M + 1]⁺), 581 (49, [2M + 1]⁺); ¹H and ¹³C NMR data of epicatechin were identical with those measured for the reference compound.

Isolation of Epicatechin-(4 β -8)-epicatechin (Procyanidin B-2), [Epicatechin-(4 β -8)]₂-epicatechin (Procyanidin C-1), and Epicatechin-(4 β -6)-epicatechin (Procyanidin B-5) from GPC Fractions III/IV-13, III/IV-15, and III/IV-16, Respectively. Aliquots (50 mg)

Identification of *N*-Phenylpropenoyl-L-amino Acids in GPC Fractions III/IV-4–8. The structure determination of (+)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid, (+)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid, (–)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid, (–)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid, (+)-*N*-[4'-hydroxy-3'-methoxy-(*E*)-cinnamoyl]-L-aspartic acid, and (+)-*N*-[(*E*)-cinnamoyl]-L-aspartic acid in GPC fractions III/IV-4 and III/IV-5, the identification of (–)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine, (–)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-tyrosine, (–)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine, and (–)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine in GPC fractions III/IV-7 and III/IV-8, and the details of the NMR, MS, and synthesis demonstrating the chemical identity of these compounds in roasted cocoa nibs is published elsewhere (20).

Thiolysis. Following the procedure reported in the literature (21) with some modifications, aliquots (4 mg) of the freeze-dried fraction III/IV or aliquots (2.5 mg) of purified individual flavan-3-ol oligomers were dissolved in methanol (1 mL) upon sonication. An aliquot (50 μ L) of the solution of fraction III/IV or an aliquot (200 μ L) of the solution of the isolated flavan-3-ols was placed into a plastic cap (3 mL) Safe-lock tube (Eppendorf, Germany) and mixed with a methanolic solution of hydrochloride (3.3% in MeOH; 50 μ L) and toluene- α -thiol (5% in MeOH; 100 μ L for fraction III/IV, 50 μ L for isolated flavan-3-ols). After the plastic cap had been sealed, reaction was carried out at 40 °C for 30 min in a water bath. After cooling and membrane filtration, the reaction mixture was directly analyzed by analytical HPLC on RP-18.

Isolation of Epicatechin-4(*S*)-benzylthioether. A solution of the fraction III/IV (140 mg) in methanol (1 mL) was mixed with a methanolic solution of hydrochloride (3.3% in MeOH; 2 mL) and toluene- α -thiol (100 μ L). After the plastic cap had been sealed, reaction was carried out at 40 °C for 30 min in a water bath. The reaction mixture was cooled and membrane filtered, and the target compound was isolated by semipreparative HPLC using an RP-18 column, ODS-Hypersil, 5 μ m (ThermoHypersil). With the effluent monitored at 270 nm, chromatography was performed with a mixture (35:65, v/v) of methanol and aqueous formic acid (0.1% in water, pH 2.5) for 5 min, increasing the methanol content to 70% over 40 min and then to 100% over 0.5 min, and, thereafter, eluting with methanol for 10 min at a flow rate of 3.0 mL/min. The target compound was collected, and the solvent was removed in a vacuum and freeze-dried three times to give the epicatechin-4(*S*)-benzylthioether as a white, amorphous powder in a purity of >99%.

Epicatechin-4(S)-benzylthioether: UV-vis (MeOH/water; pH 2.5), λ_{\max} = 225, 267 nm; LC-MS (ESI⁺), *m/z* 435 (100, [M + Na]⁺), 413 (30, [M + 1]⁺); ¹H NMR (400 MHz, CD₃OD; COSY), δ 3.85 [dd, 1H, *J*_{2,3} = 1.1 Hz, *J*_{3,4} = 2.3 Hz, H-C(3)], 3.96 [s, 2H, H-C(7'')], 4.05 [d, 1H, *J*_{3,4} = 2.3 Hz, H-C(4)], 5.22 [s, 1H, H-C(2)], 5.90 [d, 1H, *J* = 2.3 Hz, H-C(8)], 5.96 [d, 1H, *J* = 2.3 Hz, H-C(6)], 6.68 [dd, 1H, *J* = 2.1, 8.2 Hz, H-C(6')], 6.75 [d, 1H, *J* = 8.2 Hz, H-C(5')], 6.92 [d, 1H, *J* = 2.1 Hz, H-C(2')], 7.21 [m, 1H, H-C(4'')], 7.30 [m, 2H, H-C(3''), 5'')], 7.41 [m, 2H, H-C(2''), 6'')]; ¹³C NMR (100 MHz, CD₃OD; HMQC, HMBC), δ 38.0 [C(7'')], 44.0 [C(4)], 71.7 [C(3)], 75.7 [C(2)], 95.9 [C(8)], 96.9 [C(6)], 100.3 [C(4a)], 115.4 [C(2')], 116.0 [C(5')], 119.3 [C(6')], 128.0 [C(4'')], 129.6 [C(3''), C(5'')], 130.1 [C(2''), C(6'')], 132.2 [C(1')], 140.5 [C(1'')], 145.8 [C(3')], 146.1 [C(4')], 157.4 [C(8a)], 159.0 [C(5)], 159.2 [C(7)].

HPLC. The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a HPLC pump system PU 1580 with an in-line degasser (DG-1580-53), a low-pressure gradient unit (LG-1580-02), and a diode array detector (DAD) type MD 1515. Chromatographic separations were performed on stainless steel columns packed with RP-18 material (ODS-Hypersil, 5 μ m, ThermoHypersil) either in analytical (250 \times 4.6 mm i.d., flow rate = 0.8 mL/min) or in semipreparative scale (250 \times 10 mm i.d., flow rate = 3.0 mL/min).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) of GPC Fraction III/IV-19. MALDI-TOF mass spectra were recorded on a Biflex III instrument (Bruker Daltonics, Bremen, Germany), in which samples were irradiated with a nitrogen laser (wavelength, 337 nm; 2 ns pulse) under high vacuum. The linear mode of operation used 19 kV ion acceleration

without postacceleration, and the reflector mode was performed with 20 kV ion acceleration and 19 kV postacceleration. All spectra were recorded with a detector voltage of 1.6 kV and were the averaged result of at least 120 laser shots. *trans*-3-Indole acrylic acid (10 mg/mL acetone) was used as the matrix. A solution of the GPC fraction III/IV-19 in acetone (1 mg/mL) was mixed with the matrix solution (1:1, v/v). The ions detected for the individual procyanidin oligomers are the following: *m/z* 1731 for [epicatechin-(4 β →8)]₅-epicatechin, *m/z* 2019 for [epicatechin-(4 β →8)]₆-epicatechin, *m/z* 2307 for [epicatechin-(4 β →8)]₇-epicatechin, *m/z* 2595 for [epicatechin-(4 β →8)]₈-epicatechin, and *m/z* 2883 for [epicatechin-(4 β →8)]₉-epicatechin.

LC-MS. An analytical HPLC column (ODS-Hypersil, 5 μ m) (Phenomenex Aschaffenburg, Germany) or Luna Phenylhexyl (5 μ m, self-packed) was coupled to an LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) using positive (ESI⁺) and negative (ESI[−]) electrospray ionization. The samples were separated using varying gradients with aqueous formic acid (0.1%, pH 2.5) and methanol as the mobile phase.

NMR. ¹H, ¹³C, and DEPT-135 NMR experiments were performed on a Bruker AV-360 spectrometer (Bruker, Rheinstetten, Germany). ¹H, COSY, HMQC, and HMBC measurements were performed on a Bruker AMX 400-III or Bruker Advance-500 spectrometer, respectively. Data processing was performed by using 1D- and 2D-WIN-NMR as well as XWin-NMR software (version 3.5; Bruker, Rheinstetten). DMSO-*d*₆ and MeOH-*d*₄ were used as solvents, and tetramethylsilane was the internal standard.

RESULTS AND DISCUSSION

A freshly prepared aqueous suspension of powdered roasted cocoa nibs imparted the typical complex and attractive cocoa taste and was used for taste profile analysis. To achieve this, a trained sensory panel was asked to rate the intensity of the taste qualities bitter, sour, sweet, salty, umami, and astringency on a scale from 0 (not detectable) to 5 (intensely detectable). High scores were found for the intensity of the bitter taste (4.1), followed by sourness (2.8), as well as the astringent, mouth-coating taste sensation (3.0). In contrast, saltiness, sweet, and umami tastes were not detectable at all (data not shown). To gain first insight into the hydrophobicity of the compounds imparting the typical taste sensation perceived in the oral cavity, roasted cocoa nibs were extracted sequentially with solvents of different polarity.

Solvent Fractionation of Roasted Cocoa Nibs. Powdered roasted cocoa nibs were extracted with *n*-pentane to give the pentane solubles (fraction I) after evaporation of the solvent in a vacuum. The residual cocoa material was then extracted with aqueous acetone, and, after removal of the acetone from the aqueous phase in a vacuum, the solution obtained was extracted with dichloromethane. The combined organic layers were freed from solvent in a vacuum to give the dichloromethane extractables (fraction II), whereas the remaining aqueous layer was extracted with ethyl acetate. The organic phase was freed from solvent in a vacuum to give the ethyl acetate extractables (fraction III), and the aqueous phase was freeze-dried to give the water solubles (fraction IV). In addition, the insoluble residue of the powdered roasted cocoa nibs was freeze-dried to give fraction V. The highest yields were obtained for fractions I and V, accounting for >92% of the dry mass of the roasted cocoa nibs (Table 1). A comparatively low yield of 6.8% was found for fraction IV, whereas fractions II and III were isolated in trace amounts with yields of <1%.

Sensory evaluation of aqueous mixtures of the individual solvent fractions by means of taste profile analysis demonstrated that the nonsoluble cocoa residue (fraction V) was nearly tasteless, thus indicating that the taste compounds had been fully isolated by the solvent extraction. Also, the pentane solubles

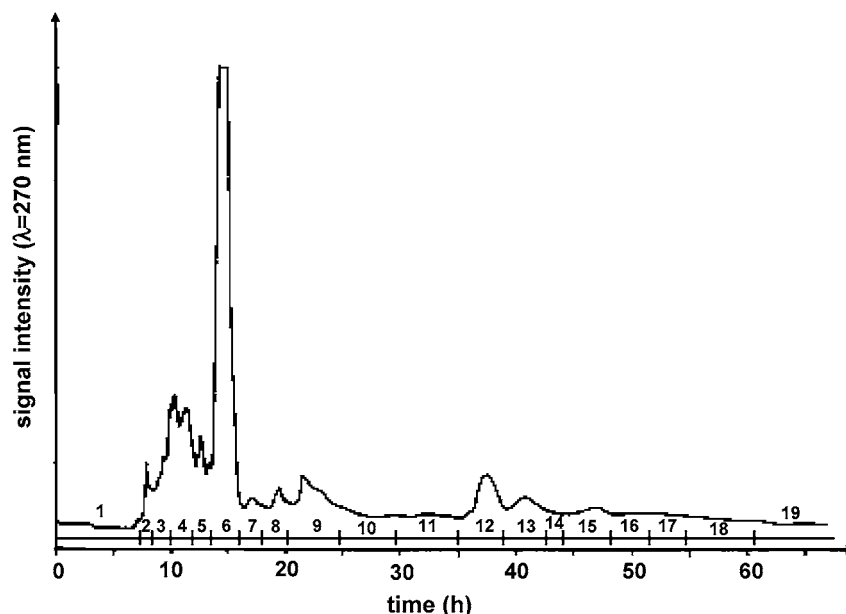


Figure 1. GPC chromatogram of the solvent fraction III/IV isolated from roasted cocoa nibs.

(fraction I) did not exhibit any significant taste quality, but imparted just the fatty mouthfeel as expected for the triglycerides of the cocoa butter. The highest scores for bitter taste (3.2), astringency (2.8), and sour taste (1.3) were found for the water solubles (fraction IV), followed by the ethyl acetate extractables (fraction III) evaluated with somewhat lower taste intensities for bitter taste (2.5), astringency (2.0), and sour taste (1.1). In contrast, the dichloromethane extractables (fraction II) exhibited exclusively an intense bitter taste (2.5) accompanied by a significant metallic aftertaste. The identification of taste compounds was focused on the intensely bitter and astringent fractions III and IV, whereas the characterization of the bitter and metallic compounds in fraction II is currently under investigation and will be published separately.

Sensory-Guided Separation of Fractions III and IV. To sort out the strongly taste-active compounds from the bulk of less taste-active or tasteless substances, first, fractions III and IV were separated by means of GPC using Sephadex LH-20 as the stationary phase and methanol/water mixtures (pH 3.5) as the mobile phase. Sensory analysis as well as LC-MS experiments indicated that both fractions III and IV contained the same compounds, just differing in their concentrations (data not shown). To speed the localization of the key taste compounds, both fractions were therefore combined, and the fraction III/IV was then separated by GPC as reported above. With the effluent monitored at 270 nm, the GPC chromatogram of fraction III/IV was recorded (Figure 1), and the effluent was separated into 19 fractions, which were collected separately. To evaluate their taste impact, these 19 fractions were freeze-dried, taken up in water, and then analyzed by TDA using the recently developed half-tongue test (17).

The highest TD factors of 1024 and 512 were found for the puckering astringent taste of III/IV fractions 4, 12–14, and 19, followed by fractions 7 and 15–18, which still showed astringent taste after a dilution of 1:256 (Table 2). In contrast, fractions 9–11 exhibited a velvety, silky type of astringent sensation evaluated with TD factors of 64 and 16, respectively. In addition, fractions 6, 12, 13, and 15 were evaluated as bitter, and fraction 3 was judged with a TD factor of 64 for sour taste and with a TD factor of 4 for sweet taste. To gain more detailed insight into the compounds imparting the astringent and bitter taste sensations perceived for GPC fractions III/IV-3–19

Table 2. Yields, Taste Qualities, and Taste Dilution (TD) Factors of GPC Fractions Isolated from RCN Fraction III/IV

fraction ^a	yield ^b (mg)	taste quality ^c	TD factor ^c	bitter and astringent compounds identified ^d
1	14.2	nd	<1	
2	80.8	astringent	8	
3	324.6	sour	64	
		sweet	4	
4	56.0	puckering astringent	512	15–20
5	35.1	puckering astringent	128	15–20
6	93.7	bitter	16	theobromine, caffeine
7	11.3	puckering astringent	256	21–24
8	13.1	puckering astringent	128	21–24
9	35.2	velvety astringent	16	13, 14
10	26.8	velvety astringent	16	13, 14
11	18.9	velvety astringent	64	13, 14
12	46.3	puckering astringent	1024	1–6
		bitter	2	
13	38.8	puckering astringent	512	3, 4, 7
		bitter	2	
14	14.7	puckering astringent	512	7, 8, 9
15	27.7	puckering astringent	256	9
		bitter	2	
16	24.1	puckering astringent	256	9, 10
17	23.8	puckering astringent	256	11, 12
18	32.3	puckering astringent	256	11, 12, hexameric procyanidin ^e
19	15.5	puckering astringent	512	hexameric–decameric procyanidins ^e

^a Number of GPC fraction referring to Figure 1. ^b Yields were determined by weight. ^c The taste quality and the TD factor were determined by using a triangle test. ^d The structures of the compounds given as numbers are displayed in Figures 3, 4, and 8. ^e These oligomeric procyanidins have been tentatively identified by means of MALDI-TOF-MS.

evaluated with high TD factors, each individual GPC fraction was further separated by semipreparative RP-HPLC and evaluated by means of the TDA approach.

HPLC-DAD and HPLC-MS analysis of the GPC fraction III/IV-6 as well as cochromatography with reference materials led to the identification of the two alkaloids theobromine and caffeine as the key bitter compounds in that fraction.

Due to its high TD factor (Table 2), GPC fraction III/IV-12 was investigated next. The effluent of the HPLC analysis was collected in seven fractions, which were freeze-dried, taken up

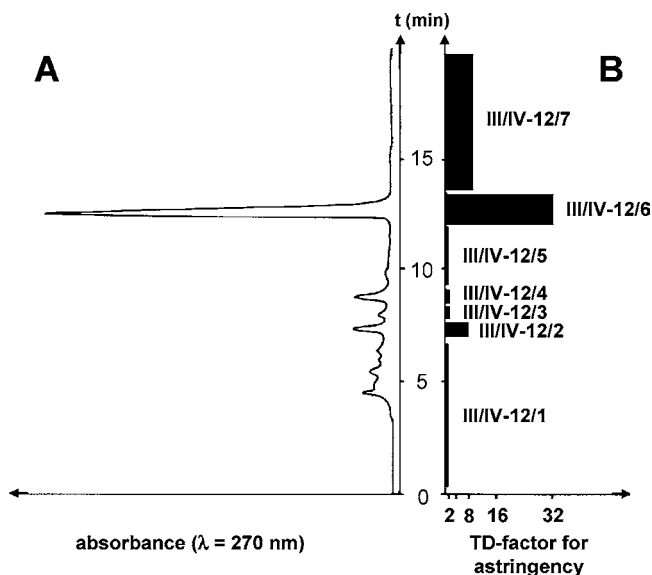


Figure 2. (A) HPLC chromatogram and (B) taste dilution (TD) chromatogram of GPC fraction III/IV-12.

in the same amount of water, and then rated in their taste impact by means of the TDA using the half-tongue test (**Figure 2**). Fraction III/IV-12/6 was judged with the highest TD factor of 32, followed by fractions III/IV-12/2 and III/IV-12/7 exhibiting an astringent taste sensation at a TD factor of 8. All of the other fractions showed significantly lower impact for astringency. Isolation of the compounds eluting in fractions III/IV-12/2 and III/IV-12/6, followed by LC-MS/MS and ^1H NMR spectroscopy as well as cochromatography with the corresponding reference compounds, led to the identification of these taste compounds as (–)-epicatechin (**1**) and (+)-catechin (**2**), respectively (**Figure 3**). In contrast, the astringent fraction III/IV-12/7 showed the typical absorption maxima expected for flavon-3-ol glycosides (17). HPLC-degustation and LC-MS analysis revealed the presence of four astringent taste compounds, two of them exhibited a molecular mass of 464 Da (compounds **3** and **4**), one a mass of 434 Da (compound **5**), and another one a mass of 448 Da (compound **6**). For all four compounds, MS/MS experiments revealed the loss of 162 amu, collaborating well with the cleavage of one molecule of a hexose to generate quercetin (m/z 302) as the aglycon of compounds **3** and **4**, naringenin (m/z 272) as the aglycon of compound **5**, and luteolin (m/z 286) as the aglycon of compound **6**. On the basis of the comparison of chromatographic (RP-HPLC), spectroscopic (LC-MS/MS, UV-vis), and sensory data as well as cochromatography with the corresponding reference compounds, quercetin-3-*O*- β -D-glucopyranoside (**3**), quercetin-3-*O*- β -D-galactopyranoside (**4**), naringenin-7-*O*- β -D-glucopyranoside (**5**), and luteolin-7-*O*- β -D-glucopyranoside (**6**) (**Figure 4**) were undoubtedly identified as the most intense astringent compounds in fraction III/IV-12/7.

Application of the HPLC–TDA approach to GPC fraction III/IV-13 showed one predominant peak, which was evaluated with the highest TD factor of 32, whereas the other fractions collected showed TD factors of <8 (data not shown). This intensely astringent-tasting peak showed absorption maxima at 225 and 267 nm as found for (–)-epicatechin and (+)-catechin derivatives. LC-MS analysis indicated a pseudo molecular ion $[\text{M} + \text{H}^+]$ with m/z 579, thus pinpointing a procyanidin dimer as the structure of the taste compound. One- and two-dimensional homo- and heteronuclear NMR experiments and comparison of the spectroscopic with those reported in the

literature (7, 22) unequivocally led to the identification of the taste compound as procyanidin B-2 (**7**) (**Figure 3**). In addition, lower amounts of quercetin-3-*O*- β -D-glucopyranoside (**3**) and quercetin-3-*O*- β -D-galactopyranoside (**4**), which have been already identified in fraction III/IV-12, were present in GPC fraction III/IV-13.

HPLC–TDA of fraction III/IV-14 revealed three astringent main fractions. Comparison of LC-MS/MS and HPLC-DAD data demonstrated one of these compounds to be procyanidin B-2 (**7**), already identified in fraction III/IV-13. The second taste compound showed the molecular mass of 434 Da and the typical absorption maxima expected for quercetin glycosides. After isolation of this glycoside from GPC fraction III/IV-14 by semipreparative HPLC, LC-MS/MS and 1D- and 2D-NMR measurements were performed. MS/MS analysis of the ion at m/z 434 revealed the loss of 132 amu, collaborating well with the cleavage of a pentose moiety, thus generating the ion m/z 302 of the aglycon quercetin. By considering all the coupling constants of the sugar moiety in the molecule, and, in particular, the coupling constant of $J = 6.5$ Hz observed for the protons H–C(1'') and H–C(2'') and comparing these values with the H–C(1)/H–C(2) coupling constants reported for β -L-arabinopyranosides, α -L-arabinopyranosides, β -L-arabinofuranosides, and α -L-arabinofuranoside (23, 24), the chemical structure of the taste compound could be identified as quercetin-3-*O*- α -L-arabinopyranoside (**8**) (**Figure 4**). The third compound evaluated with high impact for astringency showed absorption maxima at 225 and 265 nm and an $[\text{M} + 1]^+$ ion with m/z 867, thus indicating its structure as a procyanidin trimer. The amount of that compound in fraction III/IV-14 was, however, too low to enable a more precise structure determination.

By application of the HPLC–TDA, the compound (m/z 866) already detected in fraction III/IV-14 was found to be the major compound in fraction III/IV-15 and was judged with the highest TD factor of 64. None of the other HPLC fractions collected from GPC fraction III/IV showed a significant taste impact (data not shown). After isolation of the target compound by means of semipreparative HPLC, 1D- and 2D-NMR spectroscopy and comparison of the spectroscopic data with those reported earlier in the literature (7, 22) led to the unequivocal identification of the isolated trimer as procyanidin C-1 (**9**) (**Figure 3**).

For the analysis of GPC fraction III/IV-16 by means of HPLC–TDA two predominant peaks and five subfractions were collected (data not shown). The two major fractions were evaluated with the highest TD factors of 128 and 16, respectively, whereas all of the other minor compounds were judged with TD factors of ≤ 4 . Comparison of chromatographic, spectroscopic, and sensory data revealed the structure of one of the predominant compounds as the trimer procyanidin C-1 (**9**) which has been already identified in GPC fraction III/IV-15. In contrast, the second major compound detected with an intense astringent taste in fraction III/IV-16 showed the typical absorption maxima of a procyanidin and the molecular mass of 578 Da, thus suggesting a procyanidin dimer. One- and two-dimensional homo- and heteronuclear NMR experiments as well as comparison of the ^1H and ^{13}C NMR data with those reported in the literature (7) led to the identification of that taste compound as the (4 β →6)-linked epicatechin dimer procyanidin B-5 (**10**), the structure of which is given in **Figure 3**.

Application of the HPLC–TDA on GPC fraction III/IV-17 revealed two quantitatively predominating peaks, III/IV-17/2 and III/IV-17/4, evaluated with the highest TD factors of 128 and 64, respectively (**Figure 5**). In comparison, all of the other fractions were judged with TD factors of <2 and should

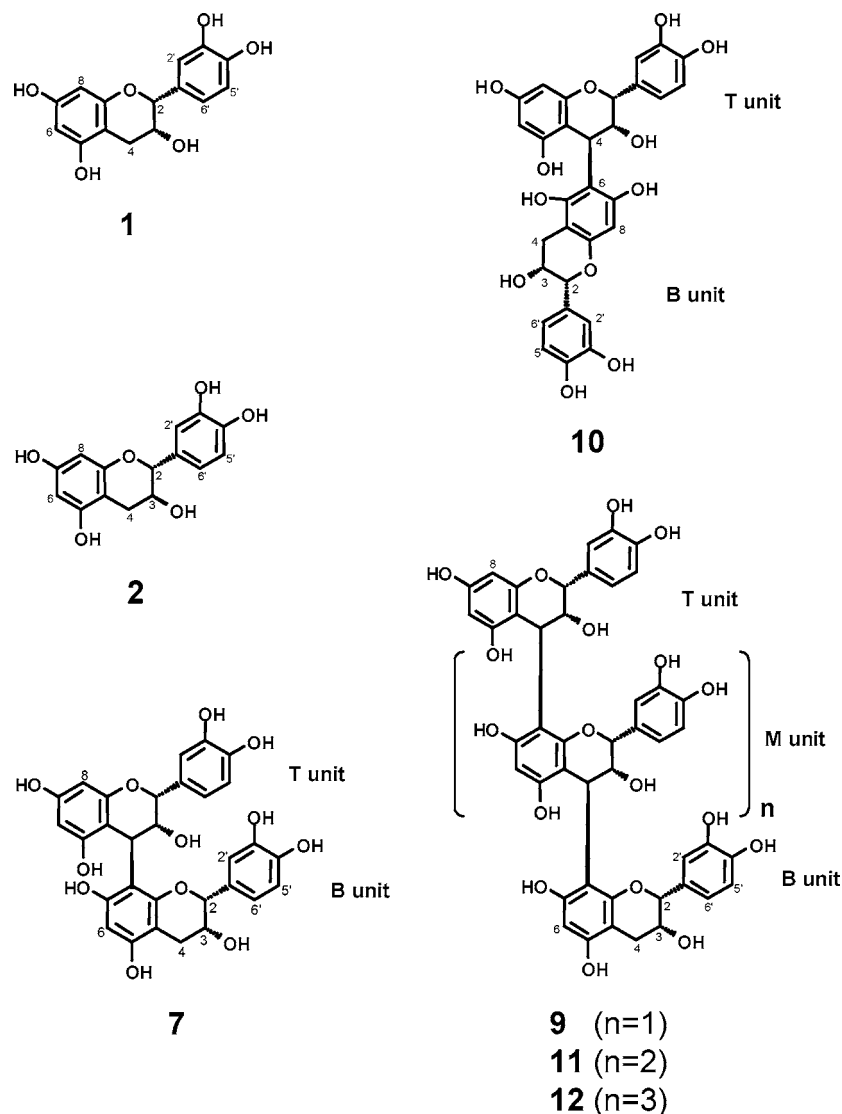


Figure 3. Chemical structures of (-)-epicatechin (**1**), (+)-catechin (**2**), procyanidin B-2 (**7**), procyanidin C-1 (**9**), procyanidin B-5 (**10**), [epicatechin-(4 β →8)]₃-epicatechin (**11**), and [epicatechin-(4 β →8)]₄-epicatechin (**12**).

therefore not be important as cocoa key taste compounds. The compounds isolated from fractions III/IV-17/2 and III/IV-17/4 showed the typical absorption maxima of oligomeric procyanidins and molecular masses of 1154 and 1442 Da, respectively, thus suggesting a procyanidin tetramer and a pentamer. To enable an unequivocal structure determination of these astringent compounds by means of LC-MS and NMR studies, these compounds needed to be isolated in higher amounts. To achieve this, fraction III/IV was separated by means of multistep ultrafiltration using cutoffs of 30, 10, 3, and 1 kDa in sequence. The ultrafiltration fraction containing compounds with molecular masses between 1 and 3 kDa was collected and separated by means of GPC to give 19 subfractions (**Figure 6**). From subfractions 14 and 15, the target compounds **11** and **12** were isolated and purified by means of semipreparative HPLC and, then, analyzed by means of UV-vis spectroscopy, LC-MS, and NMR spectroscopy at ambient temperature, followed by thiolysis (21, 25).

After thiolytic cleavage of the isolated procyanidins using toluene- α -thiol in acidic methanol, HPLC analysis of the reaction mixture led to the detection of two reaction products. These peaks could be identified as (-)-epicatechin and epicatechin-4(*S*)-benzylthioether as exemplified for the thiolytic depolymerization of pentamer, **12** (**Figure 7**). For structure

elucidation of the thioether formed, UV-vis spectroscopy, LC-MS, and 1D- and 2D-NMR spectroscopic measurements were performed. Comparison of the full NMR signal assignment achieved by means of homo- and heteronuclear δ , δ -correlation experiments (COSY, HMQC, HMBC) with the data reported for the catechin- as well as the epicatechin-4-benzylthioether (26, 27) revealed that exclusively the epicatechin-4-benzylthioether has been formed upon thiolytic cleavage of the taste compounds isolated from fractions 14 and 15. In addition, the coupling constants $J_{2,3} = 1.1$ Hz and $J_{3,4} = 2.3$ Hz observed between the protons H-C(3) and H-C(2) or H-C(4) led to the unequivocal identification of the absolute stereochemistry at C(4) of the thioether as the (*S*) configuration, thus being well in agreement with data reported in the literature (25, 28). The fact that besides the (-)-epicatechin, exclusively the epicatechin-4(*S*)-benzylthioether was formed during thiolysis demonstrated that these oligomeric procyanidins were made up just by (-)-epicatechin units. Because the absolute stereochemistry of the (-)-epicatechin units is known to remain intact during the acidic cleavage of the procyanidin oligomers (26, 29) and the internal (-)-epicatechin units is reported to give exclusively the epicatechin-4(*S*)-benzylthioether upon thiolysis (26, 28), the (*R*) configuration of the interflavanoid linkages of the epicatechin units in the tetramer **11** and the pentamer **12** could be assigned.

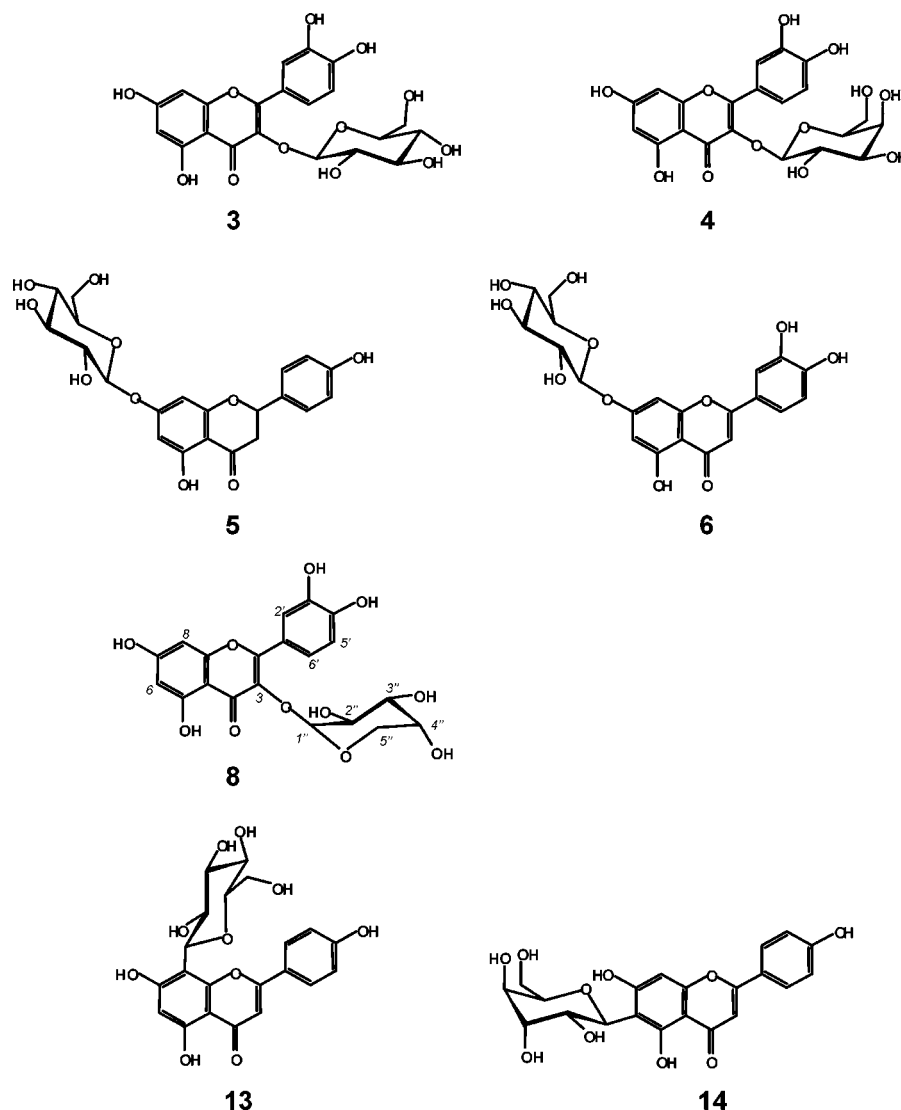


Figure 4. Chemical structures of quercetin-3-*O*-β-D-glucopyranoside (**3**), quercetin-3-*O*-β-D-galactopyranoside (**4**), naringenin-7-*O*-β-D-glucopyranoside (**5**), luteolin-7-*O*-β-D-glucopyranoside (**6**), quercetin-3-*O*-α-L-arabino-pyranoside (**8**), apigenin-8-*C*-β-D-glucopyranoside (**13**), and apigenin-6-*C*-β-D-glucopyranoside (**14**).

To unequivocally identify the structures of these procyanidin oligomers, it was necessary to distinguish between the two possible $4\beta\rightarrow 8$ and $4\beta\rightarrow 6$ interflavanoid linkages by means of NMR spectroscopy. The ^1H NMR spectra of the isolated compounds showed a methylene proton signal at 2.47 and 2.76 ppm, respectively, which was assigned as the H-C(4) of the B unit (–)epicatechin. By means of $^1\text{H}, ^1\text{H}$ correlation spectroscopy (COSY) besides the proton signals at the 4-position of the B unit, the signals of the protons H-C_B(2) and H-C_B(3) of the B unit were found to resonate at 4.95 and 4.24 ppm, respectively. Using the information obtained from COSY, HMQC, and HMBC experiments, each signal of the B unit was assigned on the basis of the long-range correlations observed from the proton H-C_B(2) to the carbon signals C_B(2'), C_B(5'), and C_B(1') and from the protons H-C_B(4) to the carbon signals C_B(4a) and C_B(5). The carbon signal C_B(8a) resonating at 153.3 ppm was identified by the observation of a heteronuclear 3J coupling with the proton H-C_B(2). Furthermore, the proton and carbon signals of the M and T units were successfully assigned. Finally, the observation of the long-range correlation of the C(8a) of the B as well as the M_{1–3} units with the corresponding

proton H-C(4) of the M_{1–3} and the T unit confirmed the proposed $4\beta\rightarrow 8$ linkage in structures **11** and **12** as given in **Figure 3**.

Finally, the determination of the absolute stereochemistry at C(4) of the epicatechin extension units was confirmed by NMR spectroscopy. Substitution of the carbon atom C(4) in the flavan by an aryl group is known to influence the ^{13}C chemical shift of carbon atom C(2) depending on the orientation of the aryl group. When the 4-aryl substituent in flavans is in the quasi-axial or trans configuration as present in procyanidins B-1 and B-2, the ^{13}C signal of C(2) is upfield shifted by 4–5 ppm, a phenomenon coined the γ -effect (8, 30). In contrast, C(4)-substituted flavans such as procyanidins B-3 and B-4, bearing the aryl group in a quasi-equatorial or cis configuration relative to the proton at C(2), show a minimal γ -effect. In the present study, the NMR studies on the isolated [epicatechin-($4\beta\rightarrow 8$)]₃-epicatechin (**11**) and [epicatechin-($4\beta\rightarrow 8$)]₄-epicatechin (**12**) showed a characteristic upfield shift of up to 2.5 ppm, thus strengthening the proposed 4(*R*) configuration of the epicatechin extension units in these procyanidin oligomers.

HPLC–TDA of GPC fractions III/IV-18 and III/IV-19 revealed several astringent-tasting peaks exhibiting molecular

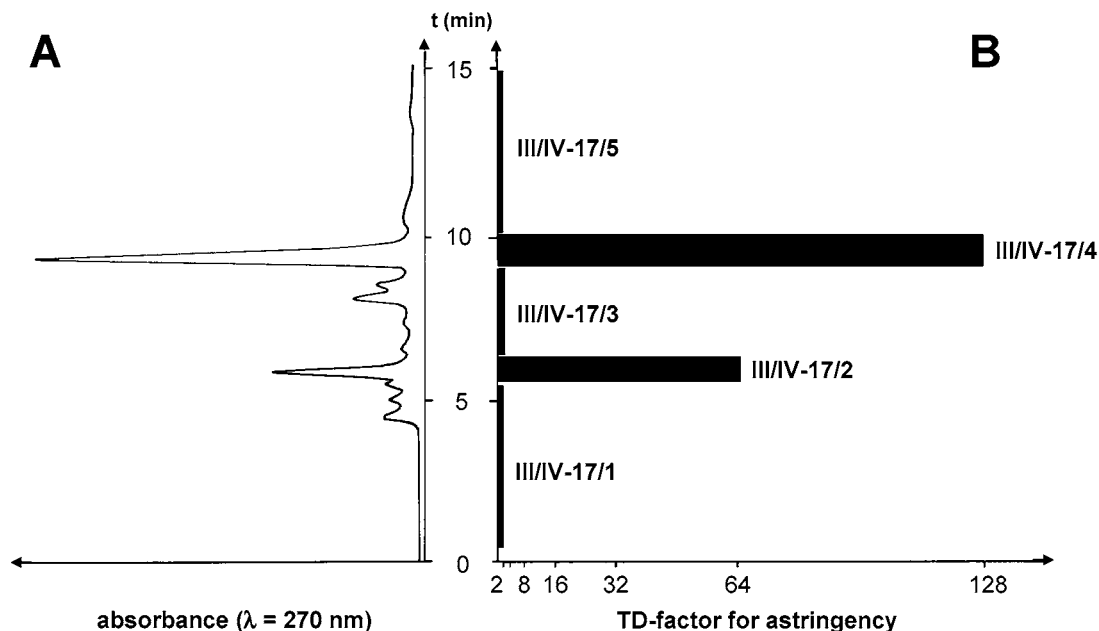


Figure 5. (A) HPLC chromatogram and (B) TD chromatogram of GPC fraction III/IV-17.

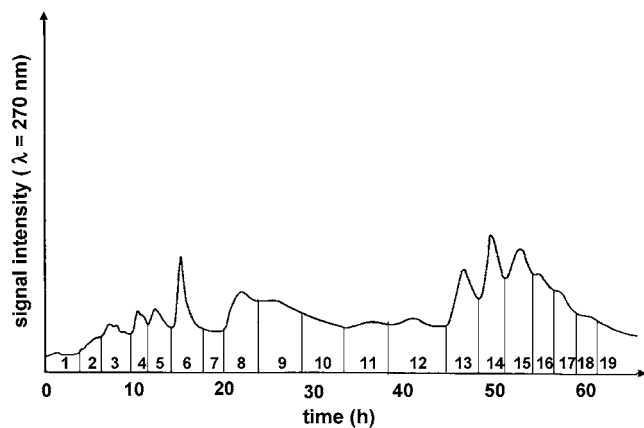


Figure 6. GPC chromatogram of the ultrafiltration fraction IV-UF2 isolated from roasted cocoa nibs.

masses of 1442 and 1730 Da being well in line with the data expected for the procyanidin pentamer and hexamer. In addition, MALDI-TOF-MS measurements of fraction III/IV-19 revealed oligomeric procyanidins up to the decameric [epicatechin-(4 β →8)]₉-epicatechin as reported earlier for procyanidins isolated from apples (31). Thiolytic degradation of GPC fraction III/IV-19, followed by HPLC analysis, revealed epicatechin-4(*S*)-benzylthioether as the only thioether formed, thus demonstrating that these procyanidin oligomers consist exclusively of (–)-epicatechin units as demonstrated for the taste compounds 9–12.

Besides the astringent fractions III/IV-12–19, fraction III/IV-3 was evaluated as tasting sweet (Table 1). To study the sugar composition of that fraction, ion chromatography coupled to a pulse amperometric detector (HPLC-PAD) was performed, thus leading to the detection of sucrose inducing the sweet taste of that GPC fraction. To screen for the presence of additional soluble carbohydrates, the GPC fractions III/IV-1–8 were analyzed by means of HPLC-PAD. Raffinose and stachyose were identified in GPC fraction III/IV-2; glucose, fructose, and sucrose in fraction III/IV-4; and fructose in fraction 5.

Application of the HPLC–TDA on the combined GPC fractions III/IV-9–11, which have been evaluated with some-

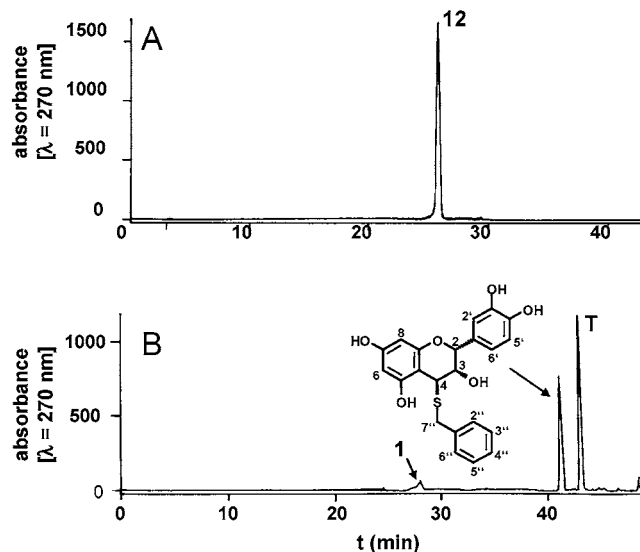


Figure 7. HPLC chromatogram of purified [epicatechin-(4 β →8)]₄-epicatechin (12) prior to (A) and after (B) thiolytic cleavage to give epicatechin-4(*S*)-benzylthioether and (–)-epicatechin (1) besides residual amounts of toluene- α -thiol (T).

what lower TD factors for astringency, revealed besides some minor amounts of (–)-epicatechin two velvety astringent compounds exhibiting the typical absorption maxima of flavon glycosides and a molecular mass of 432 Da (data not shown). MS/MS experiments indicated the loss of 162 Da, most likely corresponding to a hexose moiety, to generate the ion *m/z* 270 of the aglycon apigenin. Comparison of spectroscopic (UV–vis, LC-MS/MS), chromatographic (retention time), and sensory data (taste threshold) with those of reference compounds led to the unequivocal identification of apigenin-8-*C*- β -D-glucopyranoside (13) and apigenin-6-*C*- β -D-glucopyranoside (14) (Figure 4) as the key astringent compounds in GPC fractions III/IV-9–11.

In addition to fractions III/IV-12–19, an intense puckering astringent taste was detectable in fractions III/IV-4–8 (Table 1). Application of the HPLC–TDA to these fractions, HPLC isolation of the most active taste compounds, and comparison

Table 3. Human Taste Recognition Thresholds of Compounds Isolated from Cocoa

compound (no. ^a)	taste threshold ($\mu\text{mol/L}$) for	
	bitterness ^b	astringency ^c
flavan-3-ols (bitter, puckering astringency)		
(+)-catechin (2)	1000	600
(-)-epicatechin (1)	800	800
epicatechin-(4 β →8)-epicatechin (7)	500	200
epicatechin-(4 β →6)-epicatechin (10)	900	400
[epicatechin-(4 β →8)] ₂ -epicatechin (9)	400	300
[epicatechin-(4 β →8)] ₃ -epicatechin (11)	300	150
[epicatechin-(4 β →8)] ₄ -epicatechin (12)	200	70
[epicatechin-(4 β →8)] ₅₋₉ -epicatechin ^d	>600	30
glycosides (velvety, silky astringent, mouth coating)		
quercetin-3-O- α -L-arabinopyranoside (8)	nd ^e	22.0
naringenin-7-O- β -D-glucopyranoside (5)	nd	13.0
apigenin-6-C- β -D-glucopyranoside (14)	nd	10.8
apigenin-8-C- β -D-glucopyranoside (13)	nd	8.7
luteolin-7-O- β -D-glucopyranoside (6)	nd	5.2
quercetin-3-O- β -D-glucopyranoside (3)	nd	0.7
quercetin-3-O- β -D-galactopyranoside (4)	nd	0.4

^a The structures of the numbered compounds are given in Figures 3, 4, and 8.

^b Taste threshold concentrations were determined by means of a triangle test in bottled water. ^c Taste recognition threshold concentrations were determined by means of a half-tongue test in bottled water. ^d A mixture of procyanidins containing the oligomers from the hexamer to the decamer in their natural ratio was used for the sensory experiments. ^e Not detectable.

of spectroscopic (LC-MS, NMR, UV-vis), chromatographic, and sensory data with those of reference compounds isolated from nonfermented, nonroasted cocoa beans led to the identification of (+)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**15**), (+)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**16**), (-)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid (**17**), (-)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (**18**), (+)-*N*-[4'-hydroxy-3'-methoxy-(*E*)-cinnamoyl]-L-aspartic acid (**19**), and (+)-*N*-[(*E*)-cinnamoyl]-L-aspartic acid (**20**) in GPC fractions III/IV-4 and III/IV-5 and (-)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (**21**), (-)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (**22**), (-)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine (**23**), and (-)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (**24**) as key taste compounds in GPC fractions III/IV-7 and III/IV-8. The details of the isolation and structure determination demonstrating the chemical identity of these compounds are published separately (20).

Sensory Activity of Taste-Active Compounds. Prior to sensory analysis, the purity of all compounds was checked by HPLC-MS as well as ¹H NMR spectroscopy. To determine the human threshold concentrations for bitter taste and the astringent oral sensation, aqueous solutions of the target compound were evaluated by means of the triangle test (18) and half-tongue test (17), respectively (Table 3). The flavan-3-ols imparted a puckering astringency to the oral cavity and, at higher concentrations, also significant bitter taste. The human threshold concentrations for the bitter taste of these flavan-3-ols ranged from 200 to 1000 $\mu\text{mol/L}$ and, with the exception of (-)-epicatechin, were always above the recognition thresholds determined for the astringent sensation (Table 3). This is in contradiction to literature data reporting that monomeric flavan-3-ols are more intensely bitter than astringent (9, 12) and that these compounds taste only bitter and are not astringent (32).

The sensory data also showed that the interflavanoid linkage has a significant influence on the taste threshold concentration of procyanidins; for example, the threshold concentration of procyanidin B-5 exhibiting a 4 β →6-linkage was found to be

twice as high as the threshold evaluated for the isomeric procyanidin B-2 possessing a 4 β →8 linkage. It is interesting to note that the threshold concentration for the bitter as well as astringent taste impression decreased significantly with increasing molecular weight from the monomer to the procyanidin pentamer; for example, the threshold for bitterness or astringency of [epicatechin-(4 β →8)]₄-epicatechin is 4 or 11 times lower than that determined for the monomeric (-)-epicatechin (Table 3). Therefore, these results confirm earlier studies reporting that the taste qualities of procyanidin oligomers are strongly dependent on their degree of oligomerization (29, 33). This could be further demonstrated by the sensory analysis of a mixture of procyanidin hexamers to decamer that were isolated from cocoa. For example, the astringent taste threshold of these oligomers was found to be very low, with a value of 30 $\mu\text{mol/L}$, whereas the taste threshold for bitterness was >600 $\mu\text{mol/L}$ (Table 3).

Compared to the flavan-3-ols, the flavan-3-ol glycosides were found to induce a silky astringent, mouth-drying, and mouth-coating sensation at very low threshold concentrations ranging from 0.43 to 22.0 $\mu\text{mol/L}$ (Table 3). In particular, the threshold concentrations of 0.4 and 0.7 $\mu\text{mol/L}$ determined for quercetin-3-O- β -D-galactopyranoside and quercetin-3-O- β -D-glucopyranoside are very low and fit very well to the data reported recently (17). In comparison, naringenin-7-O- β -D-glucopyranoside and luteolin-7-O- β -D-glucopyranoside showed significantly higher threshold concentrations of 13.0 and 5.2 $\mu\text{mol/L}$, respectively, thus confirming recent findings that the structure of the aglycon is one of the drivers for the sensory activity of an astringent compound. Besides the structure of the aglycon, the sugar moiety has an influence on the perception of astringency. Whereas the replacement of the glucose moiety in quercetin-3-O-glycoside with galactose does not have much influence on the threshold concentration, the substitution with arabinose resulted in a 34-fold increase of the threshold from 0.65 to 22.0 $\mu\text{mol/L}$ (Table 3).

In summary, the data obtained for procyanidins, flavan-3-ol glycosides, flavon-7-ol glycosides, and flavanon-7-ol glycosides clearly showed that the sensory activity is strongly influenced by the molecular structure, molecular mass, and variations in the polyphenol moiety as well as 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 roasted cocoa nibs, quantitative studies, followed by taste reconstruction as well as omission experiments using these compounds in their "natural" concentrations, are currently in progress and will be published elsewhere.

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