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Isolation, Structure Determination, and Sensory Activity of Mouth-Drying and Astringent Nitrogen-Containing Phytochemicals Isolated from Red Currants (*Ribes rubrum*)

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Application of chromatographic separation and taste dilution analyses recently revealed, besides a series of flavon-3-ol glycosides and (*E*)/(*Z*)-aconitic acid, four nitrogen-containing phytochemicals as the key astringent and mouth-drying compounds in red currants (*Ribes rubrum*). The isolation and structure determination of the astringent indoles 3-carboxymethyl-indole-1-*N*-β-D-glucopyranoside (**1**) and 3-methylcarboxymethyl-indole-1-*N*-β-D-glucopyranoside (**2**), as well as the astringent, noncyanogenic nitriles 2-(4-hydroxybenzoyloxymethyl)-4-β-D-glucopyranosyloxy-2(*E*)-butenenitrile (**3**) and 2-(4-hydroxy-3-methoxybenzoyloxymethyl)-4-β-D-glucopyranosyloxy-2(*E*)-butenenitrile (**4**) by means of 1D/2D NMR, LC-MS/MS, and UV−vis spectroscopy are reported. The structures of compounds **1** and **2** were confirmed by synthesis. Using the recently developed half-tongue test, human recognition thresholds for the astringent and mouth-drying nitrogen compounds were determined to be between 0.0003 and 5.9 μmol/L (water). In particular, the extraordinarily low threshold of 0.0003 μmol/L evaluated for the indole **1** represents the lowest recognition threshold of any astringent phytochemical reported to date.

KEYWORDS: Red currants; astringency; taste dilution analysis; half-tongue test; 3-carboxymethyl-indole-1-*N*-β-D-glucopyranoside; 3-methylcarboxymethyl-indole-1-*N*-β-D-glucopyranoside; 2-(4-hydroxybenzoyloxymethyl)-4-β-D-glucopyranosyloxy-2(*E*)-butenenitrile; 2-(4-hydroxy-3-methoxybenzoyloxymethyl)-4-β-D-glucopyranosyloxy-2(*E*)-butenenitrile

INTRODUCTION

Besides the attractive aroma, the sensory quality of red currants (*Ribes rubrum*) as well as products made therefrom, such as juices or jams, is driven by its typical astringency as well as its sour taste. The astringency is perceived as a long-lasting puckering and mouth-drying sensation in the oral cavity and can enhance the complexity and palate length of fruit products.

To answer the question as to which nonvolatile, key taste compounds are responsible for the typical astringent taste of red currants, we recently applied the so-called taste dilution analysis (*1*) on chromatographic fractions isolated from freshly prepared red currant juice (*2*). This bioassay-guided fractionation led to the detection of the previously unknown nitrogen-containing astringent compounds 3-carboxymethyl-indole-1-*N*-β-D-glucopyranoside (**1**, **Figure 1**), 3-methylcarboxymethyl-indole-1-*N*-β-D-glucopyranoside (**2**), 2-(4-hydroxybenzoyloxymethyl)-4-β-D-glucopyranosyloxy-2(*E*)-butenenitrile (**3**), and 2-(4-hydroxy-3-methoxybenzoyloxymethyl)-4-β-D-glucopyranosyloxy-2(*E*)-butenenitrile (**4**), besides (*E*)-6-[3-hydroxy-4-(*O*-β-D-glucopyranosyl)phenyl]-5-hexen-2-one, (*3E*,*5E*)-6-[3-hydroxy-4-(*O*-β-D-glucopyranosyl)phenyl]-3,5-hexadien-2-one, (*E*)/(*Z*)-aconitic acid, and a series of flavon-3-ol mono-, di-, tri-, and tetraglycosides as the key players imparting the astringent oral taste sensation during consumption of red currant juice (*2*). Because the *N*-containing compounds **1**−**4** have as yet not been reported as key taste compounds in foods, the details on their isolation, structure determination, and sensory activity are presented.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: silver trifluoromethane sulfonate, 1,2-dichloroethane, 2,6-di-*tert*-butyl-4-methylpyridine, hydrochloric acid, sodium hydroxide, indole-3-acetic acid (Sigma, Steinheim, Germany); α-bromo-tetra-*O*-acetyl-D-glucose (Fluka, Taufkirchen, Germany). Solvents were of HPLC grade (Merck, Darmstadt, Germany). Deuterated solvents were supplied by Euriso-Top (Gif-Sur-Yvette, France). Fresh puree, made from red currant fruits harvested in 2003, was obtained from the food industry and kept frozen at −26 °C until used. Bottled water (Evian; low mineralization, 484 mg/L) adjusted to pH 4.5 with aqueous hydrochloric acid (0.1 mol/L) was used for the sensory experiments.

Sensory Analyses. Training of the sensory panel as well as the half-tongue test used for taste dilution analysis and taste threshold determination was done closely following the procedure reported recently (*2*, *3*).

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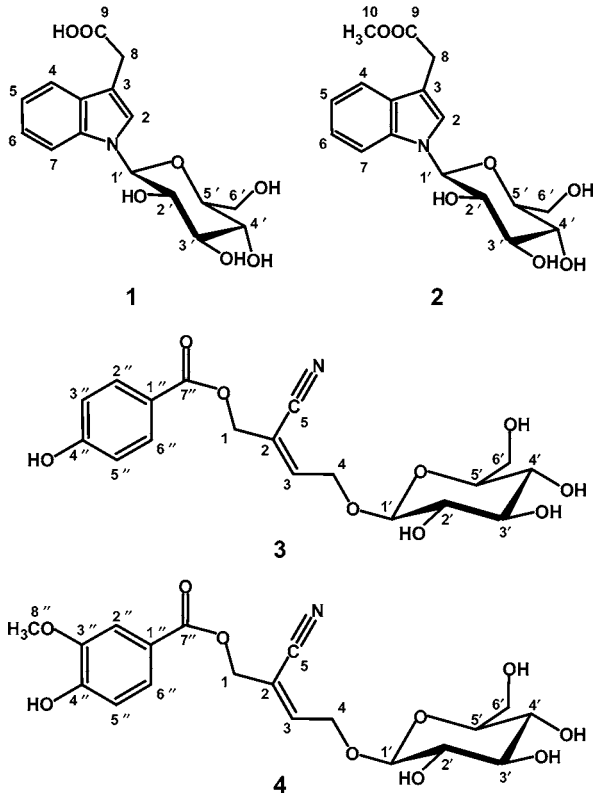

Figure 1. Chemical structures of the astringent indoles **1** and **2** and the astringent non-cyanogenic nitriles **3** and **4** isolated from red currant.

Solvent Extraction of Red Currant Puree. A mixture of red currant puree (10 kg) and methanol (40 L) was vigorously stirred for 1 h at room temperature under an atmosphere of argon. After filtration, the residual fruit material was extracted for 1 h with a mixture (7:3, v/v; 3 × 10 L) of methanol and water adjusted to pH 4.0 with aqueous formic acid (1% in water). After filtration, the liquid layers were combined and freed from methanol under vacuum to obtain the methanol extractables. After the addition of water (2 L), the methanol solubles were extracted with ethyl acetate (5 × 1.5 L), and the combined organic layers were freed from solvent under high vacuum to give the intensely astringent tasting solvent extractables after freeze-drying twice.

Gel Permeation Chromatography (GPC). An aliquot (4 g) of the ethyl acetate extractable fraction isolated from red currant puree was dissolved in a mixture (40:60, v/v; 20 mL) of methanol and water adjusted to pH 4.0 with aqueous formic acid (1% in water) and then applied onto the top of a water-cooled 400 × 50 mm glass column filled with a slurry of Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) conditioned with the same solvent mixture. Chromatography was performed with methanol/water (40:60, v/v; pH 4.0; 900 mL), followed by methanol/water (50:50, v/v; pH 4.0; 900 mL), methanol/water (60:40, v/v; pH 4.0; 2700 mL), methanol/water (80:20, v/v; pH 4.0; 900 mL), and, finally, methanol (2700 mL) with a flow rate of 3 mL/min. Monitoring the effluent by means of an L-7420 type UV−vis detector (Merck Hitachi, Darmstadt, Germany) operating at 272 nm, the seven fractions I−VII were collected as shown in **Figure 2**, and the individual fractions were freed from solvent under vacuum 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.

Taste Dilution Analysis (TDA). Aliquots of the seven GPC fractions were dissolved in 5.0 mL of bottled water (pH 4.5) in their "natural" ratios, that is, the concentration ratios present in the authentic fruit puree. These solutions were then used for the TDA following precisely the procedure reported recently (*1*, *2*). 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 plus/minus one dilution step.

Isolation of Nitrogen-Containing Astringent Compounds 1−4 from GPC Fraction II. GPC fraction II was dissolved in a mixture

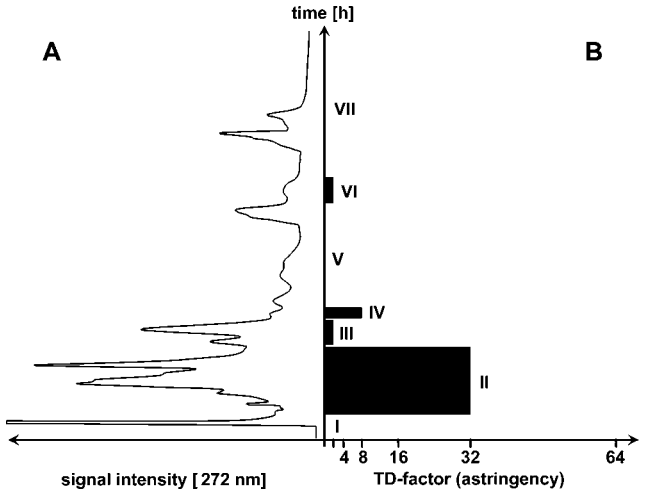

Figure 2. (**A**) GPC chromatogram and (**B**) taste dilution (TD) chromatogram of the ethyl acetate extractables isolated from red currant puree.

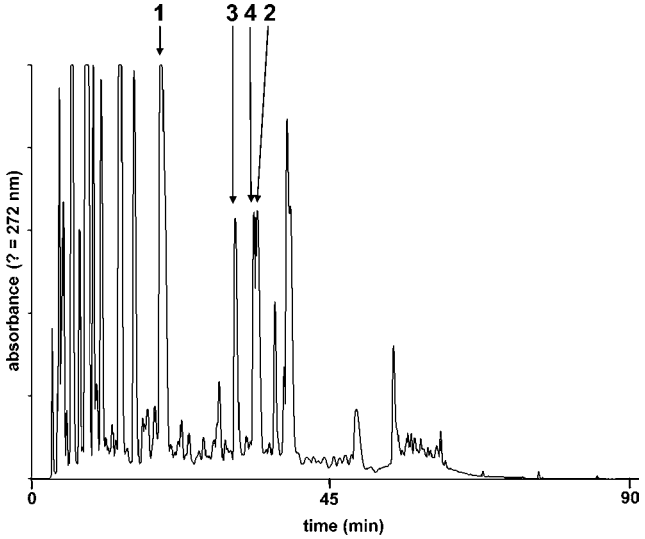

Figure 3. RP-HPLC chromatogram (272 nm) of GPC fraction II and peaks of the astringent-tasting compounds **1**–**4**.

(5:95, v/v; 5 mL) of acetonitrile and water and, after membrane filtration, was fractionated by preparative HPLC on ODS-Hypersil RP-18, 250 × 21.2 mm i.d., 5 μm (ThermoHypersil, Kleinostheim, Germany) using an acetonitrile/aqueous formic acid gradient at a flow rate of 20 mL/min. Using an aqueous formic acid (1.5% in water) as solvent A and acetonitrile as solvent B, chromatography was started with a linear gradient from 5% B to 17% B within 35 min and then maintained isocratically for 15 min, thereafter increasing the amount of solvent B to 50% B within 20 min and then to 100% within 10 min. The compounds eluting as peaks 1−4 (**Figure 3**) and imparting intense astringency were collected, freed from solvent under vacuum, and freeze-dried two times to give compounds **1**−**4** as white amorphous powders. After rechromatography using the same chromatographic procedure, the taste compounds were obtained with a purity of >99% (based on HPLC analysis).

3-Carboxymethyl-indole-1-N-β-D-glucopyranoside, ***1*** *(**Figure 1**):* UV−vis (acetonitrile), λ_{max} = 215, 267 nm; LC-TOF/MS, $C_{16}H_{19}NO_7$; LC-MS (ESI^-), *m*/*z* 336 (100, $[M - H]^-$), 174 (52, $[M - H - 162]^-$); ^1H NMR (400 MHz, D_2O; COSY), δ 3.59 [dd, 1H, *J* = 9.2, 9.2 Hz, H−C(4′)], 3.67 [m, 1H, H−C(5′)], 3.69 [dd, 1H, *J* = 9.1, 9.1 Hz, H−C(3′)], 3.75 [m, 1H, H−C(6a′)], 3.81 [s, 2H, H−C(8)], 3.86 [m, 1H, H−C(6b′)], 4.01 [dd, 1H, *J* = 9.1, 9.1 Hz, H−C(2′)], 5.56 [d, 1H, *J* = 9.1 Hz, H−C(1′)], 7.19 [dd, 1H, *J* = 8.1, 8.1 Hz, H−C(6)], 7.29 [dd, 1H, *J* = 8.1, 8.1 Hz, H−C(5)], 7.39 [s, 1H, H−C(2)], 7.57 [dd, 2H, H−C(4, 7)]; ^{13}C NMR (100 MHz, MeOD; HMQC, HMBC), δ 30.9 [C(8)], 60.6 [C(6′)], 69.3 [C(4′)], 71.6 [C(2′)], 77.1 [C(3′)], 77.1

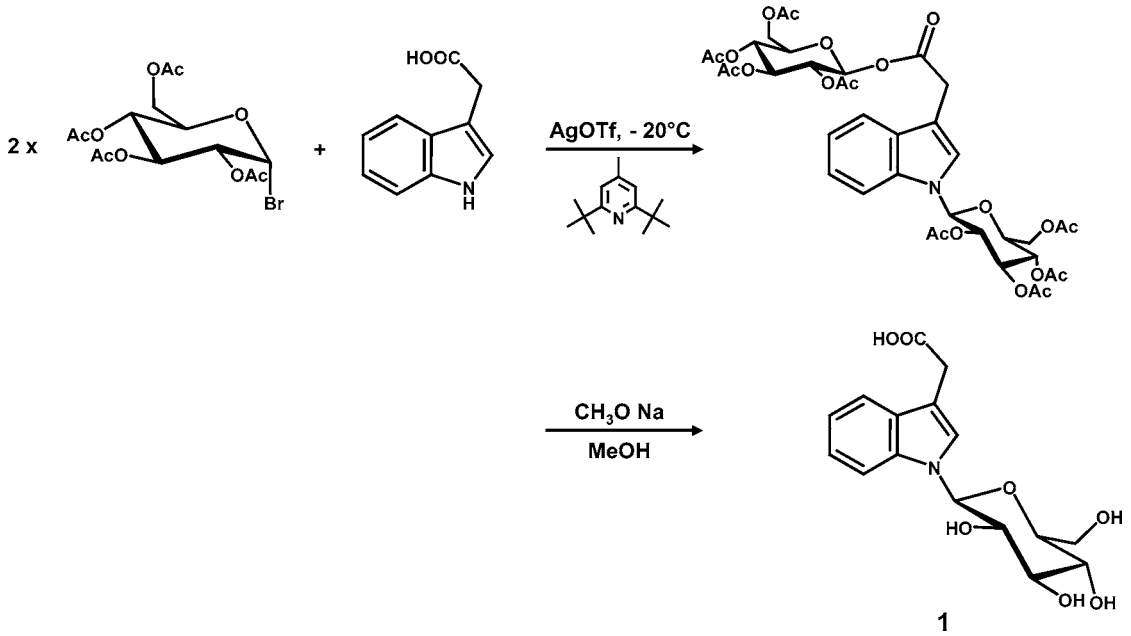

Figure 4. Synthetic sequence used for the preparation of 3-carboxymethyl-indole-1-*N*-β-D-glucopyranoside (**1**).

[C(5′)], 84.4 [C(1′)], 110.0 [C(3)], 110.2 [C(7)], 119.1 [C(4)], 120.6 [C(6)], 122.5 [C(5)], 124.5 [C(2)], 128.0 [C(3a)], 136.4 [C(7a)], 177.6 [C(9)].

3-Methylcarboxymethyl-indole-1-N-β-D-glucopyranoside, ***2 (Figure 1):*** UV−vis (acetonitrile), λ_{max} = 215, 267; LC-TOF/MS, $C_{17}H_{21}NO_7$; LC-MS (ESI$^-$), *m/z* 350 (100, [M − H]$^-$), 188 (54, [M − H − 162]$^-$); ^{1}H NMR (400 MHz, D_2O; COSY), δ 3.52 [dd, 1H, *J* = 9.3, 9.3 Hz, H−C(4′)], 3.59 [m, 1H, H−C(5′)], 3.62 [dd, 1H, *J* = 9.0, 9.0 Hz, H−C(3′)], 3.71 [s, 3H, H−C(10)], 3.73 [m, 1H, H−C(6a′)], 3.80 [s, 2H, H−C(8)], 3.89 [dd, 1H, *J* = 2.0, 12.1 Hz, H−C(6b′)], 3.92 [dd, 1H, *J* = 9.0, 9.0 Hz, H−C(2′)], 5.45 [d, 1H, *J* = 9.1 Hz, H−C(1′)], 7.10 [dd, 1H, *J* = 8.0, 8.0 Hz, H−C(6)], 7.20 [dd, 1H, *J* = 8.0, 8.0 Hz, H−C(5)], 7.38 [s, 1H, H−C(2)], 7.57 [dd, 2H, *J* = 8.6, 8.6 Hz, H−C(4, 7)]; ^{13}C NMR (100 MHz, d_3-MeOD; HMQC, HMBC), δ 30.0 [C(8)], 50.9 [C(10)], 61.2 [C(6′)], 70.0 [C(4′)], 72.2 [C(2′)], 77.7 [C(3′)], 79.2 [C(5′)], 85.2 [C(1′)], 108.3 [C(3)], 110.1 [C(7)], 118.3 [C(4)], 119.6 [C(6)], 121.7 [C(5)], 124.0 [C(2)], 128.2 [C(3a)], 136.9 [C(7a)], 173.3 [C(9)].

2-(4-Hydroxybenzoyloxymethyl)-4-β-D-glucopyranosyloxy-2(E)-butenenitrile, ***3 (Figure 1):*** UV−vis (acetonitrile), λ_{max} = 211, 255 nm; LC-TOF/MS, $C_{18}H_{21}NO_9$; LC-MS (ESI$^+$), *m/z* 396 (100, [M + H]$^+$), 234 (59, [M + H − 162]$^+$); ^{1}H NMR (400 MHz, d_3-MeOD; COSY), δ 3.23 [dd, 1H, *J* = 8.4, 8.4 Hz, H−C(2′)], 3.30 [m, H−C(5′)], 3.31 [m, 1H, H−C(4′)], 3.39 [dd, 1H, *J* = 8.4, 8.4 Hz, H−C(3′)], 3.70 [dd, 1H, *J* = 4.9, 12.1 Hz, H−C(6a′)], 3.89 [dd, 1H, *J* = 1.8, 12.1 Hz, H−C(6b′)], 4.36 [d, 1H, *J* = 8.0 Hz, H−C(1′)], 4.56 [dd, 1H, *J* = 6.4, 14.6 Hz, H−C(4a)], 4.68 [dd, 1H, *J* = 6.4, 14.6 Hz, H−C(4b)], 4.93 [s, 2H, H−C(1)], 6.87 [m, 2H, H−C(3″, 5″)], 6.90 [m, 1H, H−C(3)], 7.93 [m, 2H, H−C(2″, 6″)]; ^{13}C NMR (100 MHz, d_3-MeOD; HMQC, HMBC), δ 61.1 [C(6′)], 62.9 [C(1)], 66.6 [C(4)], 70.0 [C(5′)], 73.5 [C(2′)], 76.6 [C(4′)], 76.6 [C(3′)], 102.8 [C(1′)], 111.5 [C(2)], 114.8 [C(5)], 114.8 [C(3″)], 114.8 [C(5″)], 119.8 [C(1″)], 131.4 [C(2″)], 131.4 [C(6″)], 147.7 [C(3)], 162.4 [C(4″)], 165.5 [C(7″)].

2-(4-Hydroxy-3-methoxybenzoyloxymethyl)-4-β-D-glucopyranosyloxy-2(E)-butenenitrile, ***4 (Figure 1):*** UV−vis (acetonitrile), λ_{max} = 211, 263 nm; LC-TOF/MS, $C_{19}H_{23}NO_{10}$; LC-MS (ESI$^+$), *m/z* 426 (100, [M + H]$^+$), 264 (62, [M + H − 162]$^+$); ^{1}H NMR (400 MHz, d_3-MeOD; COSY), δ 3.23 [dd, 1H, *J* = 8.4, 8.4 Hz, H−C(2′)], 3.30 [m, 1H, H−C(5′)], 3.31 [m, 1H, H−C(4′)], 3.39 [m, 1H, H−C(3′)], 3.70 [dd, 1H, *J* = 4.9, 12.1 Hz, H−C(6a′)], 3.89 [dd, 1H, *J* = 1.8, 12.1 Hz, H−C(6b′)], 3.92 [s, 3H, H−C(8″)], 4.36 [d, 1H, *J* = 8.0 Hz, H−C(1′)], 4.57 [dd, 1H, *J* = 6.4, 14.6 Hz, H−C(4a)], 4.68 [dd, 1H, *J* = 6.4, 14.6 Hz, H−C(4b)], 4.95 [s, 2H, H−C(1)], 6.89 [m, 1H, H−C(5″)], 6.90 [m, 1H, H−C(3)], 7.59 [m, 1H, H−C(2″)], 7.61 [m, 1H, H−C(6″)]; ^{13}C NMR (100 MHz, d_3-MeOD; HMQC, HMBC), δ 54.9 [C(8″)], 61.1 [C(6′)], 63.2 [C(1)], 66.7 [C(4)], 70.0 [C(5′)], 73.4 [C(2′)], 76.3 [C(4′)], 76.5 [C(3′)], 102.0 [C(1′)], 111.7 [C(2)], 112.0 [C(2″)], 114.6 [C(5″)], 114.8 [C(5)], 120.2 [C(1″)], 123.8 [C(6″)], 147.5 [C(3″)], 147.6 [C(3)], 152.0 [C(4″)], 165.5 [C(7″)].

Analysis of Glycosidically Bound Carbohydrates. An aliquot (2 mg) of the target compound, dissolved in aqueous hydrochloric acid (2 mol/L; 1 mL), was heated at 110 °C for 120 min. After cooling, an aqueous solution of Na_2CO_3 (4 mol/L; 300 μL), followed by pyridine (50 μL), was added, and the solution was freeze-dried. The water-free residue was dissolved in a solution (100 μL) of 1% hydroxylamine hydrochloride in water-free pyridine and was then heated at 70 °C for 30 min. After cooling, 1-(trimethylsilyl) imidazole (100 μL) was added, and the solution was heated for an additional 30 min at 70 °C and, after cooling, was directly injected into the HRGC-MS system.

Synthesis of 3-Carboxymethyl-indole-1-*N*-β-D-glucopyranoside. By adoption of the modified Koenigs−Knorr methodology reported for *O*-glycoside synthesis (*4*) (**Figure 4**), an activated molecular sieve (4 Å; 1.5 g) was added to a suspension of silver trifluoromethane sulfonate (2.0 mmol) in anhydrous 1,2-dichloroethane (10 mL) under an atmosphere of argon in the dark with stirring at room temperature. The mixture was then cooled to −20 °C, α-bromotetra-*O*-acetyl-D-glucose (2.0 mmol) and indole-3-acetic acid (1.0 mmol) were added, and, after 15 min of stirring at −20 °C, 2,6-di-*tert*(butyl)-4-methylpyridine (2.0 mmol) was added. After removal of the cooling bath, the mixture was stirred at room temperature for 48 h and then filtered, water (10 mL) was added to the filtrate, the pH value was adjusted to 5.0 with sulfuric acid (1 mol/L), and the organic solvent was removed under vacuum. The aqueous layer was freeze-dried, and the residue was dissolved in chloroform (30 mL) and cooled to 0 °C in an ice bath. After the addition of a methanolic solution of sodium methylate (1 mol/L, 3 mL), the mixture was stirred for 2 h at 0 °C, and then an equivalent amount of sulfuric acid (1 mol/L) was added and the pH value was adjusted to 5.0 with aqueous sulfuric acid (1 mol/L). The organic solvent was removed under vacuum and, after membrane filtration (0.45 μm), the title compound was isolated from the aqueous layer by preparative HPLC on ODS-Hypersil RP-18, 250 × 21.2 mm i.d., 5 μm (ThermoHypersil, Kleinostheim, Germany), using an acetonitrile/aqueous formic acid gradient at a flow rate of 20 mL/min and monitoring the effluent at 272 nm. Using an aqueous formic acid (1.5% in water) as solvent A and acetonitrile as solvent B, chromatography started isocratically with 5% B for 10 min, and then solvent B was increased to 20% within 10 min and then to 70% within 5 min. After freeze-drying, the title compound (0.5 mmol; 50% yield) was obtained as a white, amorphous powder in high purity of >99%. Spectroscopic (NMR, LC-MS, UV−vis) and chromatographic data (t_R)

as well as the taste threshold concentration of the synthetic title compound were identical to the data obtained for the 3-carboxymethyl-indole-1-*N*-β-D-glucopyranoside (**1**) isolated from red currants.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of an MD-2010 plus photodiode array detector and two PU 2087 pumps. Chromatographic separations were performed on stainless steel columns packed with ODS-Hypersil, 5 μm, RP-18 material (ThermoHypersil, Kleinostheim, Germany) either in analytical (250 × 4.6 mm i.d., flow rate = 1.0 mL/min) or preparative scale (250 × 21.2 mm i.d., flow rate = 20 mL/min).

LC−Time-of-Flight/Mass Spectrometry (LC-TOF/MS). High-resolution mass spectra of the compounds were measured on a Bruker Micro-TOF (Bruker Daltronics, Bremen, Germany) mass spectrometer and referenced to sodium formate. The deviation of the measured from the calculated molecular mass was <1.5 ppm.

LC-MS/MS. Electrospray ionization (ESI) mass and product ion spectra were acquired on an API 4000 QTRAP mass spectrometer (Applied Biosystems, Darmstadt, Germany) with direct flow infusion. For ESI, the ion spray voltage was set at −4500 V in the negative mode and at 5500 V in the positive mode. The mass spectrometer was operated in the full-scan mode detecting positive or negative ions. The MS/MS parameters were set to induce fragmentation of the $[M - H]^-$ or $[M + H]^+$ molecular ions into specific product ions after collision with nitrogen as collision gas (4×10^{-5} Torr).

High-Resolution Gas Chromatography−Mass Spectrometry (HRGC-MS). Electron impact (EI) GC-MS data were acquired on an HP 6890 series gas chromatograph and an HP 5973 mass spectrometer (Hewlett-Packard/Agilent, Böblingen, Germany). Chromatographic separation was performed on a 60 m × 0.25 mm fused-silica capillary with a 0.25 μm methyl silicone coating (J&W Scientific DB-1 column, Agilent) using 0.6 mL/min helium as carrier gas. The injector temperature was set at 250 °C, and the injection volume was 1 μL with split injection (1:50). The initial oven temperature was set to 140 °C and then raised at a rate of 4 °C/min to 210 °C, thereafter at a rate of 8 °C/min to 300 °C, and, finally, held isothermally for 10 min at 300 °C. Heating the transfer line at 300 °C, the mass spectrometer was operated in the electron impact mode (EI; 70 eV electron energy) with a source temperature of 230 °C and the quadrupole heated at 150 °C. Mass spectra were acquired in the full-scan mode ranging from *m*/*z* 40 to 800 with a scan rate of 2.0 scans/s.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, and 2D NMR data were acquired on a Bruker DPX-400 (Bruker BioSpin, Rheinstetten, Germany). D_2O and d_3-MeOD were used as solvents, and chemical shifts were referenced to the solvent signal. For structural elucidation and NMR signal assignment, COSY, HMQC, HMBC, and ROESY experiments were carried out using the pulse sequences taken from the Bruker software library. Data processing was performed by using XWin-NMR software (version 3.5; Bruker, Rheinstetten, Germany) as well as Mestre-C (Mestrelab Research, A Coruña, Spain).

RESULTS AND DISCUSSION

With the aim of isolating the astringent nitrogen-containing phytochemicals, red currant puree was extracted with methanol/water to obtain the methanol solubles, which were further separated into a highly polar fraction and a semipolar fraction by means of ethyl acetate fractionation as reported recently (*2*). After removal of trace amounts of solvents under vacuum, the ethyl acetate extractables, exhibiting an intense astringent and mouth-drying oral sensation, were further separated by means of GPC.

GPC. To locate the key astringent compounds in the ethyl acetate extractables, this fraction was further separated by GPC using Sephadex LH-20 as the stationary phase and a methanol/water gradient as the mobile phase. Monitoring the effluent at 272 nm, seven fractions I−VII (**Figure 2**) were collected, individually freeze-dried, then dissolved in the same amount of water, and, finally, used for the TDA. To achieve this, each solution was stepwise diluted 1:1 with water, and the dilutions were then presented in order of increasing concentrations to trained sensory panelists, who were asked to judge the astringent oral sensation by application of the recently developed half-tongue test (*3*) until the threshold was reached. As this so-called TD factor, obtained for each fraction, is related to its taste activity in water, the GPC fractions I−VII were rated according to their relative astringency impact (**Figure 2**). Due to its high TD factor of 32, GPC fraction II was evaluated with the highest taste impact for astringency. In comparison, the other fractions were evaluated with a significantly lower astringent taste impact.

To isolate the taste compounds exhibiting the astringent oral sensation, GPC fraction II was further fractionated by HPLC on RP-18 material (**Figure 3**). By monitoring the effluent at 272 nm, the effluents of the individual peaks were collected and freed from solvent under vacuum, and aliquots of the isolates were taken up in water in their "natural" concentrations and were then sensorially evaluated for astringency. Finally, the compounds isolated from peaks 1−4 (**Figure 3**) were purified by RP-18 rechromatography, thus affording the pure astringent stimulants, which were analyzed by means of UV−vis, LC-MS/MS, and NMR spectroscopy.

Identification of the Astringent Indoles 1 and 2. LC-MS analysis of the astringent compound **1** (**Figure 1**), exhibiting a UV−vis absorption maximum at 267 nm, showed the pseudomolecular ion $[M + H]^+$ with *m*/*z* 338, indicating the presence of one nitrogen atom in the target molecule. Further LC-MS/MS studies performed in the ESI^+ mode revealed a loss of 162 amu to give the daughter ion *m*/*z* 176 and an additional loss of 46 amu to give the daughter ion *m*/*z* 130, thus indicating the cleavage of a hexose moiety and one molecule of CO_2. Acidic hydrolysis of an aliquot of the isolated compound, derivatization of the liberated sugar to give the persilylated aldoxime, followed by HRGC-MS analysis, unequivocally identified glucose as the carbohydrate moiety present in the tastant.

1D and 2D NMR spectroscopic experiments revealed the anomeric proton signal H−C(1′) resonating at 5.56 ppm, the large coupling constant (9.1 Hz) of which indicated a β-configuration of the hexoside. Furthermore, heteronuclear multiple bond correlation experiments (HMBC) demonstrated a long-range correlation between the anomeric proton H−C(1′) of the β-D-glucose moiety and the carbon atoms C(2) and C(7a) of the aglycone. All of the protons and carbons in the molecule were undoubtedly assigned by means of ^{1}H/^{1}H (COSY) and ^{1}H/^{13}C correlation spectroscopy (HMQC and HMBC).

To further confirm the structure of compound **1**, 3-carboxymethyl-indole-1-*N*-β-D-glucopyranoside was synthesized using a modified Koenigs−Knorr methodology as outlined in **Figure 4**. Two equivalents of α-bromotetra-*O*-acetyl-D-glucose were reacted with 1 equiv of indole-3-acetic acid in the presence of silver trifluoromethane sulfonate and 2,6-di-*tert*-butyl-4-methylpyridine in anhydrous 1,2-dichloroethane. Treatment of the intermediary diglycoside with a methanolic solution of sodium methylate afforded 3-carboxymethyl-indole-1-*N*-β-D-glucopyranoside after HPLC purification in yields of about 50%. The LC-MS, NMR, and chromatographic data, as well as the threshold concentration of that compound, were identical to those determined for the authentic compound **1** isolated from red currants.

Taking all of these data into consideration, LC-MS/MS and 1D and 2D NMR experiments, as well as synthesis, led to the unequivocal identification of the astringent compound isolated from HPLC fraction 1 of the GPC fraction II as 3-carboxymethyl-indole-1-*N*-β-D-glucopyranoside (**1**, **Figure 1**).

The astringent compound isolated from HPLC fraction 2 (**Figure 3**) showed a UV−vis absorption maximum at 267 nm being well in line with the data obtained for compound **1**. LC-MS and LC-MS/MS (ESI^+) analysis of compound **2** (**Figure 1**) showed a pseudomolecular ion $[M + H]^+$ with *m*/*z* 352 and a daughter ion with *m*/*z* 190. 1D and 2D NMR spectroscopic experiments revealed an anomeric proton signal at 5.45 ppm showing a large coupling constant of 9.1 Hz and indicating the existence of a β-glucoside as already found for compound **1**. In addition, the quaternary carbon resonating at 173.3 ppm as well as the proton signal observed at 3.71 ppm and integrating for three protons indicated the presence of a methyl ester. Careful consideration of all the data obtained from COSY, HMQC, and HMBC experiments clearly demonstrated the structure of taste compound **2** as the 3-methylcarboxymethyl-indole-1-*N*-β-D-glucopyranoside (**Figure 1**). To check that compound **2** is not formed as an artifact upon esterification of compound **1** with methanol during workup, the extraction of red currant puree was repeated by using ethanol instead of methanol as solvent. As HPLC-DAD and HPLC-MS analysis demonstrated the presence of compound **2** also when using ethanol as solvent (data not shown), the indole ester **2** has to be considered as a naturally occurring phytochemical in red currants.

Following precisely the method reported in the literature (*5*), 3-methylcarboxymethyl-indole-1-*N*-β-D-glucopyranoside was synthesized (data not shown). The LC-MS/MS and NMR as well as sensory data of the synthetic compound were identical with those obtained for the taste compound **2** (**Figure 3**) isolated from red currants. Taking all of these data into consideration, the astringent compound isolated from HPLC fraction 2 of the GPC fraction II was unequivocally identified as 3-methylcarboxymethyl-indole-1-*N*-β-D-glucopyranoside (**2**, **Figure 1**). Although indole-3-acetic acid has long been known as a plant hormone of the auxin family, neither its *N*-glucoside **1** nor its *N*-glucoside methyl ester **2** was reported earlier as an astringent taste compound in foods.

Identification of the Astringent Nitriles 3 and 4. After HPLC purification of the astringent compound **3** isolated from GPC fraction II, UV−vis and LC-MS analysis showed an absorption maximum at 255 nm and a pseudomolecular ion with *m*/*z* 396 indicating the presence of one nitrogen atom in the molecule. 1D and 2D NMR spectroscopic experiments led to the identification of an anomeric proton resonating at 4.36 ppm. This proton signal showed a large coupling constant of 8.0 Hz, thus indicating a β-configuration of a glycoside. Acidic hydrolysis of an aliquot of the isolated compound and derivatization of the liberated sugar to give the persilylated aldoxime, followed by HRGC-MS analysis, unequivocally identified glucose as the carbohydrate moiety present in the tastant. The assignment of all the protons and carbons in compound **3** was successfully achieved by means of COSY, HMQC, and HMBC experiments. For example, the HMBC experiment showed a heteronuclear coupling between the proton signals observed at 6.87 and 7.93 ppm and the quaternary carbon atoms resonating at 165.5 and 162.4 ppm, thus indicating 4-hydroxybenzoic acid as part of the molecular structure. In addition, the HMBC spectrum displayed a long-range correlation between H−C(1) and C(7″) and between the anomeric proton resonating at 4.36 and carbon atom C(4) of the aglycone, being well in line with the structure of 2-(4-hydroxybenzoyloxymethyl)-4-*O*-β-D-glucopyranosyloxy-2(*E*)-butenenitrile given in **Figure 1**. The *E*-configuration of the 2-butenenitrile moiety was confirmed by a ROESY experiment that clearly showed a NOE effect between the methylene protons H−C(1) and the olefinic proton H−C(3), but not between the methylene protons H−C(4) and proton H−C(3). In addition, the NMR data of the 2-butanenitrile substructure were consistent with literature data reported for 2-[(*E*)-*p*-coumaroyloxymethyl]-4-*O*-β-D-glucopyranosyloxy-2(*E*)-butenenitrile, coined nigrumin-5-*p*-coumarate, and the corresponding nigrumin-5-ferulate isolated from black currant seed (*6*). Therefore, the structure of astringent compound **3** was unequivocally identified as the previously not reported noncyanogenic nitrile 2-(4-hydroxybenzoyloxymethyl)-4-*O*-β-D-glucopyranosyloxy-2(*E*)-butenenitrile, named nigrumin-4-hydroxybenzoate (**Figure 1**).

Table 1. Human Recognition Taste Thresholds of Nitrogen-Containing Astringent Compounds

taste compound (no.)	threshold concn for astringency[a] (mmol/L)
2-(4-hydroxybenzoyloxymethyl)-4-β-D-glucopyranosyloxy-2(*E*)-butenenitrile (**3**)	5.9
2-(4-hydroxy-3-methoxybenzoyloxymethyl)-4-β-D-glucopyranosyloxy-2(*E*)-butenenitrile (**4**)	1.2
3-methylcarboxymethyl-indole-1-*N*-β-D-glucopyranoside (**2**)	0.001
3-carboxymethyl-indole-1-*N*-β-D-glucopyranoside (**1**)	0.0003
L-tryptophan	0.5[b]
indole-3-acetic acid	0.01
3-β-D-glucopyranosyloxyindole	0.001
5-hydroxyindole-3-acetic acid	0.005
3-(2-aminoethyl)-5-hydroxyindole (serotonin)	0.005
5-hydroxyindole	0.001

[a] Taste threshold concentrations were determined by means of the half-tongue test (*3*). [b] In addition to astringency, L-tryptophan showed bitter taste above 6000 μmol/L.

LC-MS and LC-MS/MS analysis of taste compound **4** in the ESI^+ mode showed a pseudomolecular ion with *m*/*z* 426 and a loss of 162 amu to give the daughter ion *m*/*z* 264, thus indicating the presence of a hexose moiety in the molecule. In addition, the ESI-TOF/MS spectrum of compound **4** gave a $[M + Na]^+$ ion with *m*/*z* 448.1228 consistent with the molecular formula of $C_{19}H_{23}NO_{10}$. 1D/2D NMR spectroscopic experiments were performed and showed wide similarity to the data found for compound **3**. As the major difference, the protons of a methoxy group resonating at 3.92 ppm were detectable and the aromatic proton H−C(3″) was lacking. Considering all of the spectroscopic data obtained, the structure of taste compound **4** was identified as the previously unreported noncyanogenic nitrile 2-(4-hydroxy-3-methoxybenzoyloxymethyl)-4-*O*-β-D-glucopyranosyloxy-2(*E*)-butene nitrile, named nigrumin-4-hydroxy-3-methoxybenzoate (**Figure 1**).

Sensory Activity of Astringent Nitrogen Compounds. Prior to sensory analysis, the purity of all compounds was checked by HPLC-MS as well as ^1H NMR spectroscopy. To study the sensory activity of these nitrogen-containing compounds, the human recognition thresholds for astringency were determined in bottled water (pH 4.5) using the half-tongue test (**Table 1**).

The oral sensation imparted by the nitrogen-containing compounds was described as mouth-drying and astringent with threshold concentrations ranging from 0.0003 to 5.9 μmol/L. The nitriles **3** and **4** showed threshold concentrations of 5.9 and 1.2 μmol/L, which is rather similar to the values found for most of the naturally occurring flavon-3-ol glycosides (*3*). Surprisingly, two indole derivatives, **1** and **2**, were found to induce a mouth-drying astringency at extraordinarily low threshold concentrations of 0.0003 and 0.001 μmol/L (**Table 1**). On the

basis of these data, the *N*-glucoside **1** was found with the lowest astringency threshold concentration reported for any astringent phytochemical so far.

To gain some insights into the structural requirements for the low taste threshold of **1**, indole-3-acetic acid lacking the glucose moiety was analyzed by the sensory panel. As shown in **Table 1**, the threshold of 0.01 μmol/L found for indole-3-acetic acid was also rather low, but was 33 times higher compared to that of the *N*-glucoside **1**. These data clearly confirm earlier observations made for flavon-3-ol *O*-glycosides (*3*), ellagitannin *C*-glycosides (*7*), and flavan-3-ol *C*-glycosides (*8*), showing that the astringency threshold of the corresponding phenolic aglycone is significantly lowered when glycosidically linked to a carbohydrate moiety. To confirm that assumption with an indole-*O*-glycoside, 3-β-D-glucopyranosyloxyindole was evaluated, too, and, indeed, this *O*-glucoside showed a rather low threshold of 0.001 μmol/L (**Table 1**).

Differing from the indole-3-acetic acid by just the presence of the additional amino function, the amino acid L-tryptophan was also found to induce a mouth-drying and astringent sensation above a threshold concentration of 0.5 μmol/L, whereas bitter taste was perceived at concentration levels above 6000 μmol/L (**Table 1**). However the astringency threshold of L-tryptophan was 50 times above the value found for the indole-3-acetic acid.

Because the astringent activity of indoles were reported in the present investigation for the first time and to gain some insight into the influence of hydroxylation on the taste activity of indoles, dilute aqueous solutions of 5-hydroxyindole-3-acetic acid, 3-(2-aminoethyl)-5-hydroxyindole (serotonin), and 5-hydroxyindole were evaluated sensorially. These indoles were also found to exhibit astringency at rather low threshold concentrations between 0.001 and 0.005 μmol/L, thus indicating that a hydroxyl group at the 5-position might be beneficial for the astringency activity of indoles.

In summary, nitrogen-containing phytochemicals and, in particular, those with indole structure were reported for the first time as powerful astringent and mouth-drying compounds exhibiting much lower threshold concentrations as compared to polyphenols such as flavan-3-ols and flavon-3-ols. As serotonin also showed mouth-drying and astringent activity, we might hypothesize that certain serotonin receptor candidates, which are known to be present in the oral cavity (*9*), are involved in the human sensation of mouth-drying and astringency. The involvement of receptor proteins in the human astringency sensation is further corroborated by the recent finding that the neurotransmitter γ-aminobutyric acid also induces a mouth-drying and velvety astringent sensation (*10*) and also for this amino acid multiple receptors are known to be present in the oral cavity (*11*). Understanding the role of such receptors in the perception of astringent-type oral sensations will be a challenging, but important, task for future investigations.

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