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Fractionation of Gallotannins from Mango (*Mangifera indica* L.) Kernels by High-Speed Counter-Current Chromatography and Determination of Their Antibacterial Activity

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High-speed counter-current chromatography was applied to the separation of gallotannins from mango (*Mangifera indica* L.) kernels. The kernels were defatted and subsequently extracted with aqueous acetone [80% (v/v)]. The crude extract was purified by being partitioned against ethyl acetate. A hexane/ethyl acetate/methanol/water solvent system [0.5:5:1:5 (v/v/v/v)] was used in the head-to-tail mode to elute tannins according to their degree of galloylation (tetra-*O*-galloylglucose to deca-*O*-galloylglucose). The compounds were characterized using liquid chromatography and mass spectrometry in the negative ionization mode. Purities ranged from 72% (tetra-*O*-galloylglucose) to 100% (octa-*O*-galloylglucose). The iron binding capacity of gallotannins was dependent on the number of galloyl groups in the molecule, with a larger capacity at lower degrees of galloylation. The minimum inhibitory concentration against *Bacillus subtilis* did not change among the different gallotannins tested and was in the range of 0.05−0.1 g/L in Luria-Bertani broth but up to 20 times higher in media containing more iron and divalent cations.

KEYWORDS: High-speed counter-current chromatography; gallotannins; *Mangifera indica* L.; antibacterial activity; iron binding capacity; chrome azurol S (CAS) test; LC−MS

INTRODUCTION

Phenolic compounds make up a very diverse class of secondary plant metabolites. Corresponding to their biological roles in plants as UV screens, as attractants for insects, or as part of the plant defense system against pathogens (*1*), they are often located in the outer layers of the plant tissue or in the seeds. Polyphenols have potential for various food applications because of their antioxidant activities (*2*), coloring properties (*3*), and antimicrobial activities (*4*). Plant parts rich in polyphenols are often removed during food processing, for example, by peeling or pressing. Therefore, byproducts originating from fruit and vegetable processing represent a rich and valuable source of bioactive compounds (*5*).

Mango peels and kernels are byproducts of the mango processing industry and contain a variety of phenolic compounds such as flavonols and xanthones (*6*, *7*), and gallotannins (*8*, *9*). The latter consist of a glucose core that is acylated with 4−12 gallic acid residues. The selective antimicrobial properties of hydrolyzable tannins are well-known (*10*, *11*), but the antibacterial activities of purified gallotannins have been elucidated only recently (*9*, *12*). Bacteria of the genera *Listeria*, *Bacillus*, *Clostridia*, and *Staphylococcus* were sensitive to gallotannins, but lactic acid bacteria exhibited strong resistance. Data on the mode of antimicrobial action of individual gallotannins are not available, and further studies are hampered by a lack of suitable methods for purifying hydrolyzable tannins in sufficient quantities.

High-speed counter-current chromatography (HSCCC) is known to be an efficient technique for the isolation of components in milligram to gram quantities and has successfully been applied, for example, to phenolic acids (*13*, *14*), xanthones (*15*), flavonoids (*16*), and isoflavones (*17*). Studies on the isolation of hydrolyzable tannins by HSCCC are scarce. Penta-*O*-galloylglucose was the only hydrolyzable tannin isolated from the leaves of *Acer truncatum* Bunge (*18*). However, to the best of our knowledge, the fractionation of several gallotannins by HSCCC has so far not been reported. Therefore, the objective of this study was the development of a method for the fractionation of gallotannins from crude mango kernel extracts according to their degree of galloylation in quantities and purities sufficient for bioassays to determine their structure−function relationship.

MATERIALS AND METHODS

Solvents and Reagents. Hexane was of technical grade; all other solvents were of analytical grade. Solvents as well as sodium hydroxide, ferric chloride, and ethylenediaminetetraacetic acid (EDTA) were purchased from Fisher Scientific (Ottawa, ON). Chrome azurol S (CAS), gallic acid, hexadecyltrimethylammonium bromide (HDTMA), Trizma hydrochloride (Tris buffer), and hydrochloric acid were purchased from Sigma-Aldrich (St. Louis, MO).

Sample Preparation. Mature Brazilian mango fruits (*Mangifera indica* L. cv. 'Tommy Atkins'), obtained from a local supermarket, were washed, and the peels and pulp were removed from the kernels with a stainless steel knife. After the seed coat had been manually

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dehusked, the kernels were immediately lyophilized and finely ground using a knife mill.

Lyophilized, ground kernels (230 g) were defatted by extraction with hexane (3 × 1 L) for 50 min while being stirred. The extracts were filtered; the supernatants were discarded, and residual hexane was allowed to evaporate. The dried residues (200 g) were extracted with 800 mL of aqueous acetone [80% (v/v)] in a nitrogen atmosphere for 6 h while being stirred at ambient temperature. During extraction, the mixture was sonicated every hour for 3 min. The extract was filtered and the residue re-extracted with 400 mL of aqueous acetone [80% (v/v)] for 1 h. The combined supernatants were evaporated to dryness at 30 °C in vacuo and dissolved with 150 mL of water. The crude extract was extracted twice with 150 mL of dichloromethane to remove phospholipids. The samples were centrifuged at 5000 rpm for 15 min to accelerate phase separation. The remaining aqueous phase containing the polyphenol fraction was extracted three times with 150 mL of ethyl acetate. The combined ethyl acetate layers were evaporated to dryness at 30 °C in vacuo.

HSCCC Apparatus. HSCCC was performed using a model TBE-300B high-speed counter-current chromatograph (Tauto Biotech, Shanghai, China). The separation device consisted of an upright coil type-J planet centrifuge with three multilayered coils connected in series. The polytetrafluoroethylene tubes had an inner diameter of 1.6 mm (semi-preparative range) and a total volume of 305 mL. The distance r from the coil to the holder shaft ranged from 2.5 to 4 cm, and the revolution radius R, or the distance between the holder axis and the central axis of the centrifuge, was 5 cm. The β values varied from 0.5 (internal terminal) to 0.8 (external terminal). The rotation speed was adjustable, ranging from 0 to 1000 rpm. The HSCCC apparatus was equipped with a 501 PrimeLine solvent delivery module (Analytical Scientific Instruments, El Sobrante, CA), a VUV-24 Visacon UV−vis detector (Reflect Scientific Inc., Orem, UT), and a model CHF 122SC fraction collector (Avantec Toyo Kaisha, Ltd., Tokyo, Japan).

Selection of the Solvent System. The two-phase solvent system was selected according to the recommendations outlined by Ito (*19*). The stability of the prepurified mango kernel extract in the selected solvent system at ambient temperature and with the impact of light was monitored over a period of 1 week by high-performance liquid chromatography (HPLC). The partition coefficient $K_{U/L}$ was determined via addition of 0.1 g of the dried ethyl acetate extract to the mutually equilibrated solvent systems (1 mL of each phase) in a 5 mL glass vial. After the sample had undergone thorough vortex mixing and settled at ambient temperature, aliquots of 0.75 mL of both phases were dried separately under vacuum at 35 °C, and the dry weight was determined gravimetrically. The $K_{U/L}$ values were calculated as the ratio of the dry weight in the upper phase to that in the lower phase. Hexane/ethyl acetate/methanol/water systems [1:5:1:5 (v/v/v/v) and 0:5:0:5 (v/v/v/v)] were not suitable because the $K_{U/L}$ values were 0.06 and 6.06, respectively. Therefore, the ratio of hexane to methanol was further varied.

For the determination of the retention of the stationary phase, the column was first entirely loaded with the stationary phase. Then the mobile phase was pumped into the rotating column. After a hydrodynamic state had been established, the volume of the stationary phase that was replaced by the mobile phase was determined in a graduated cylinder. The retention of the stationary phase was expressed as the ratio of the stationary phase retained in the column to the total column capacity. Settling times t were determined according to Ito (*19*).

Preparation of the Two-Phase Solvent System. The selected solvent system was thoroughly equilibrated in a separatory funnel by being vigorously shaken for 2 min at ambient temperature. The solvent was left for equilibration at room temperature overnight, and the two phases were separated shortly before use. The upper phase was used as the stationary phase, while the lower phase served as the mobile phase.

Sample solutions were prepared by dissolving the dried ethyl acetate extract in a solvent mixture consisting of 5 mL of both phases. It was ensured that the sample solution forms two phases when mixed with either phase in the column. For the analytical runs performed to establish a solvent system and to optimize the separation by HSCCC, 0.25 g of crude extract was used, whereas aliquots of 1 g were applied for the preparative runs.

Separation of Gallotannins by HSCCC. The column was entirely filled with the stationary phase. Then the apparatus was rotated at 1000 rpm while the mobile phase was pumped through the column to establish the hydrodynamic equilibrium, as indicated by a clear mobile phase eluting at the tail outlet. The sample was injected, and the mobile phase was pumped at a flow rate of 3 mL/min in the head-to-tail mode. The pressure was in the range of 4−6 bar, for the preparative runs approximately 5 bar. Chromatographic runs were monitored at 280 nm. Fractions were collected on ice in intervals of 3 min. After a run time of 300 min, the upper phase was used as the mobile phase to facilitate the elution of higher galloylated tannins. Peak fractions were collected according to the elution profile and analyzed by liquid chromatography and mass spectrometry (LC−MS). Fractions from five repeated preparative runs were collected and freeze-dried (Labconco Freezone12, Kansas City, MO) to avoid any impact by methanolysis. The dried fractions were dissolved in water to yield concentrations of 10 g/L.

LC−MS Analysis. LC−MS analyses were performed using a HPLC series 1200 system (Agilent Technologies, Mississauga, ON) equipped with ChemStation software, a degasser, a binary gradient pump, a thermo-autosampler, a column oven, and a diode array detector. The HPLC system was connected in series with a 4000 QTrap mass spectrometer fitted with an ESI source (Applied Biosystems, Streetsville, ON). Data acquisition and processing were performed using Analyst version 1.5. The column was a 150 mm × 3.0 mm Synergi 4 μm Hydro-RP, 80 Å column (Phenomenex, Torrance, CA), with a C18 ODS guard column, operated at 21 °C. Samples were cooled at 10 °C; aliquots of 20 μL were injected, and the compounds were eluted with 2% (v/v) acetic acid in water (eluent A) and 0.5% acetic acid in a water/acetonitrile mixture [50:50 (v/v), eluent B] at a flow rate of 0.5 mL/min using the following gradient: from 20 to 35% B over 25 min, from 35 to 40% B over 25 min, from 40 to 80% B over 20 min, 80% B for 2 min, and from 80 to 20% B over 0.5 min (*8*). For a better resolution of the highly galloylated tannins (nona- and deca-*O*-galloylglucose), the gradient was adjusted as follows: from 20 to 47% B over 3 min, from 47 to 53% B over 60 min, from 53 to 80% B over 20 min, and from 80 to 20% B over 0.5 min. UV−vis spectra were recorded from 200 to 600 nm (peak width of 2 nm), and monitoring was performed at 280 nm. Mass spectrometric data were obtained in the enhanced MS scan mode. Negative ion mass spectra were recorded in the range of m/z 50−2050 at a scan speed of 1000 Da/s and at a voltage of −4000 V. Nitrogen was used as the drying gas at 60 AU (arbitrary units); the curtain gas was 20 AU. The declustering potential was −70 V. The ion source temperature was set at 500 °C.

Bacterial Strain and Culture Conditions. The susceptibility of bacteria to differently galloylated tannins and two reference compounds (gallic acid and EDTA) was assessed using *Bacillus subtilis* FAD 110. *B. subtilis* FAD 110 has previously been identified as a sensitive indicator strain for testing the antibacterial activity of gallotannins (*9*). The cultures were incubated at 37 °C in Luria-Bertani (LB) broth [5 g/L glucose (Fisher Scientific), 5 g/L yeast extract (Becton, Dickinson and Co., Sparks, MD), and 5 g/L NaCl (Fisher Scientific)] and de Man, Rogosa, Sharpe (MRS) broth (Becton, Dickinson and Co.) to determine the influence of different media on the antimicrobial activity of gallotannins. Stock cultures were maintained at −70 °C in 30% glycerol.

Antimicrobial Activity of Gallotannins. The antimicrobial activity of gallotannins was determined by the critical dilution assay (*20*). Serial 2-fold dilutions of the samples with LB or MRS media were prepared on 96-well cell culture cluster plates and the media inoculated with overnight cultures of the indicator strain. The microtiter plates were incubated overnight at 37 °C. The minimum inhibitory concentrations (MICs) were defined as the lowest concentrations of the substances that prevented the growth of microbial strains and were expressed in grams of dry matter per liter. After overnight incubation at 37 °C, minimum bactericidal concentrations (MBCs) in grams of dry matter per liter were determined via addition of 10 μL aliquots of each well to 100 μL of fresh medium and incubated again overnight. The MBC determined the lowest concentration that prevented the growth of bacteria on these plates. As the inoculum initially contained $\sim 10^7$ cfu/mL, it can be estimated that failure to grow upon subculture corresponds to inactivation by at least 10^4 cfu/mL by tannins.

Iron Binding Capacity of Gallotannins. We performed a miniaturized chrome azurol S (CAS) assay to determine the affinity of the hydrolyzable tannins for iron(III) (*21*). The CAS−iron(III)−hexadecyltrimethylammonium bromide (HDTMA) ternary complex, which shows a

maximum absorbance at 630 nm at pH 5.6, served as an indicator. When a strong chelator removes the iron from the dye, its color turns from blue to orange or yellow. On the basis of the original test of the CAS assay, a solution was prepared as follows: 6 mL of a 10 mM HDTMA solution was pipetted in a 100 mL volumetric flask and diluted with Tris-HCl [tris-(hydroxymethyl)aminomethane] buffer (0.2 M, adjusted to pH 5.6 with NaOH). A mixture of 1.5 mL of an iron(III) solution (1 mM $FeCl_3 \cdot 6H_2O$, 10 mM HCl in Tris buffer) and 7.5 mL of a 2 mM CAS solution in Tris buffer was slowly added under stirring and made up to 100 mL with Tris buffer. A fresh CAS solution was prepared before every test. All glassware was cleaned with 6 M HCl.

We measured the iron binding capacity by preparing serial 2-fold dilutions of the samples (1 g/L) with a CAS solution on 96-well cell microtiter plates, and the samples were then further diluted with a CAS solution. For comparison, an aqueous ethylenediaminetetraacetic acid (EDTA) solution (10 g/L) and a gallic acid solution (1 g/L) were tested in the same way. Water was used as a control. The absorbance was measured in black-colored microtiter plates at 630 min after equilibrium was reached at 10 min. Because in the experimental setup the CAS concentration exceeded the iron concentration, semimaximal absorbance is caused by equal concentrations of the CAS−Fe−HDTMA complex and the gallotannin−iron complex.

RESULTS AND DISCUSSION

HSCCC Solvent System. The selected solvent system yields approximately equal volumes of each phase and provides retention of > 50%, leading to a satisfactory peak resolution. HPLC analysis revealed that components were stable in the solvent system chosen.

The $K_{U/L}$ value is one of the most important parameters in determining peak resolution because the retention and separation in HSCCC are exclusively based on solute partitioning between mobile and stationary phases (*19*). Acceptable K values were found for the hexane/ethyl acetate/methanol/water system at ratios of 0.5:5:1:5 (v/v/v/v) and 0.75:5:1:5 (v/v/v/v), with K values being 1.43 and 1.31, respectively. Settling times of 22 and 23 s, respectively, indicated an acceptable retention of the stationary phase in the column and satisfactory separation efficiency. The solvent systems thus met the criteria outlined by Ito (*19*). A similar solvent system was selected for the isolation of penta-*O*-galloylglucose from an ethyl acetate extract of the leaves of *A. truncatum* Bunge (*18*).

The hexane/ethyl acetate/methanol/water solvent system [0.5:5:1:5 (v/v/v/v)] led to a good separation of the compounds using the head-to-tail mode (**Figure 1a**). Switching the phases to the tail-to-head mode resulted in a poor separation of the components and was not considered. When the hexane proportion was increased [0.75:5:1:5 (v/v/v/v) hexane/ethyl acetate/methanol/water], gallotannins eluted faster at the cost of separation efficiency. Therefore, the 0.5:5:1:5 (v/v/v/v) hexane/ethyl acetate/methanol/water solvent system was chosen for the preparative runs. To reduce the total run time, the mobile phase was switched from the lower to the upper phase after 300 min, which led to square-shaped peaks toward the end of the run. Higher sample loads impaired separation of the peaks. As a consequence, fraction collection during the preparative runs started in the peak slope (**Figure 1b**).

Characterization of Gallotannins by LC−MS. Fractions obtained from analytical HSCCC runs were subjected to LC−MS in the negative ionization mode. Gallotannins were characterized on the basis of retention time and fragmentation pattern (data not shown, **Figure 2**, and **Table 1**). As shown in **Figure 1a**, tannins elute according to their degree of galloylation, starting with tetra-*O*-galloylglucose. Both peaks D_1 and D_2 were found to consist of hepta-*O*-galloylglucose.

Retention times increased with the degree of galloylation (**Figure 2**). Sharp peaks were observed for tetra- to hepta-*O*-galloylglucose, whereas octa- to deca-*O*-galloylglucose yielded

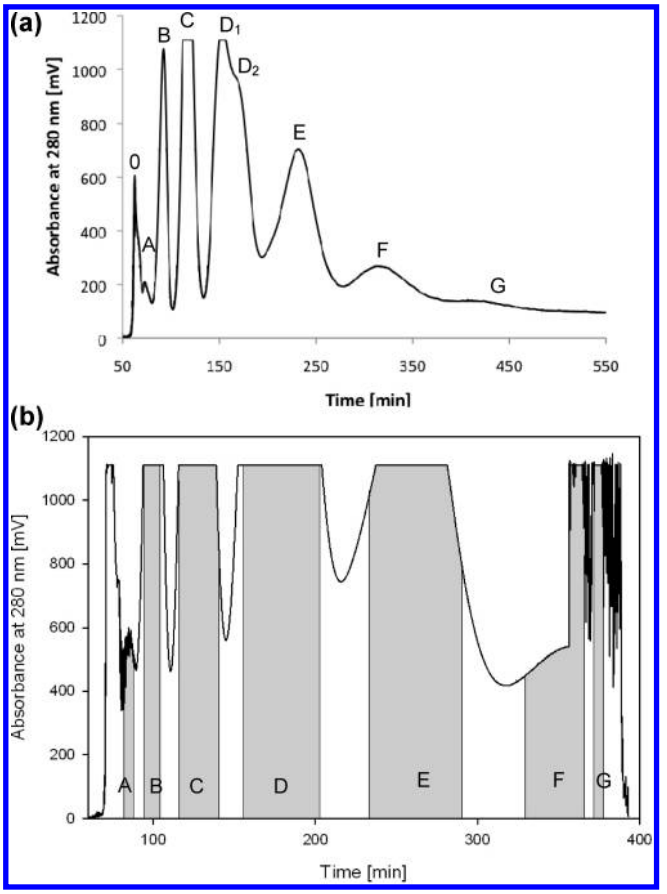

Figure 1. (**a**) Separation of gallotannins from mango kernels according to their degree of galloylation by HSCCC [head-to-tail elution mode; 0.5:5:1:5 (v/v/v/v) hexane/ethyl acetate/methanol/water solvent system; 0.25 g of prepurified extract]: (0) mangiferin and tri-*O*-galloylglucose, (A) tetra-*O*-galloylglucose, (B) penta-*O*-galloylglucose, (C) hexa-*O*-galloylglucose, (D_1) hepta-*O*-galloylglucose, (D_2) hepta-*O*-galloylglucose, (E) octa-*O*-galloylglucose, (F) nona-*O*-galloylglucose, and (G) deca-*O*-galloylglucose. (**b**) Fraction collection from the HSCCC run [head-to-tail elution mode; 0.5:5:1:5 (v/v/v/v) hexane/ethyl acetate/methanol/water solvent systeml; 1 g of prepurified extract]. Peak assignment: tetra-*O*-galloylglucose (fraction A), penta-*O*-galloylglucose (fraction B), hexa-*O*-galloylglucose (fraction C), hepta-*O*-galloylglucose (fraction D), octa-*O*-galloylglucose (fraction E), nona-*O*-galloylglucose (fraction F), and deca-*O*-galloylglucose (fraction G).

flatter peak shapes. Within each fraction except for penta-*O*-galloylglucose, several compounds with identical mass-to-charge ratios were obtained. Depending on the position to which the galloyl residues are attached, several structural isomers exist, which results in the appearance of several peaks for one specific degree of galloylation in the HPLC chromatogram. These findings are in accordance with those obtained by Salminen et al. (*22*).

The base peak in the mass spectra of tetra- to hepta-*O*-galloylglucose was the deprotonated molecule $[M - H]^-$, while for the larger gallotannins the doubly charged ions $[M - 2H]^{2-}$ predominated. As described previously for di- to hexa-*O*-galloylglucose (*22*), even gentle ionization produced some fragments in addition to the molecular ion. In all fractions except tetra-*O*-galloylglucose, the ion at m/z 787, corresponding to tetra-*O*-galloylglucose, and its doubly charged ion at m/z 393 were abundant. This confirms previous reports according to which galloyl residues linked to the penta-*O*-galloylglucose core via *meta*-depside bonds are more susceptible to cleavage than those directly attached to the glucose core (*8*, *22*).

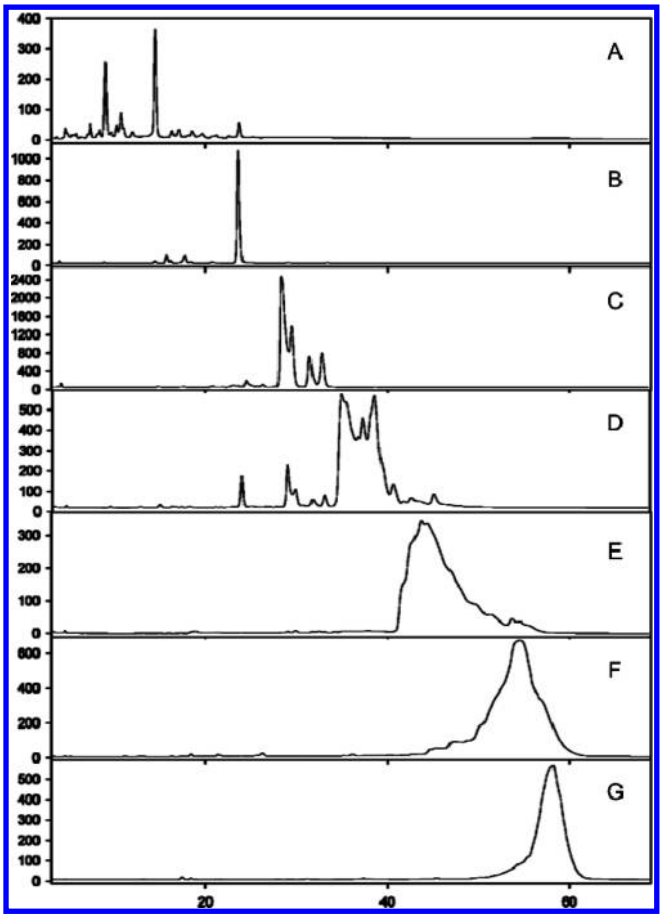

Figure 2. HPLC chromatograms of tetra-*O*-galloylglucose (**A**), penta-*O*-galloylglucose (**B**), hexa-*O*-galloylglucose (**C**), hepta-*O*-galloylglucose (**D**), octa-*O*-galloylglucose (**E**), nona-*O*-galloylglucose (**F**), and deca-*O*-galloylglucose (**G**). Panels **A**−**G** refer to fractions shown in **Figure 1b**.

Separation of Gallotannins by HSCCC. Fractions were collected from preparative chromatographic runs as shown in **Figure 1b** and analyzed by LC−MS as described above. Fraction A contained tetra-*O*-galloylglucose with a purity of 72%, as judged by LC−MS analysis; impurities included mangiferin and unidentified substances, which eluted at the beginning of the run.

Purities of 90% were obtained for penta- and hepta-*O*-galloylglucose, while hexa-*O*-galloylglucose was 94% pure. Octa-*O*-galloylglucose was recovered with a purity of 100%. Nona- and deca-*O*-galloylglucose were obtained with purities of 87 and 86%, respectively. On the basis of the initial weight of 5.0 g of dried ethyl acetate phase, the yield of the isolated compounds was 14%. In comparison to the previously described methods for separating tannins by their degree of galloylation (*9*, *12*), the amounts of purified compounds were considerably higher, leading to significant time and solvent savings. Purified gallotannins were characterized for their iron binding capacities and antibacterial activities.

Iron Binding Capacity of Gallotannins. Tannins exhibited ~10 times higher iron binding capacities than EDTA (data not shown). Gallotannins were able to remove iron from the CAS−iron(III)−HDTMA ternary complex, which resulted in a decrease in absorbance at 630 nm. **Figure 3** shows the concentrations of gallotannins that caused semimaximal absorbance, corresponding to approximately equal concentrations of the CAS−Fe−HDTMA complex and the gallotannin−iron complex. A decrease in the level of galloylation (deca- to penta-*O*-galloylglucose) caused semimaximal absorbance at lower iron concentrations, correlating with an increased affinity for iron. Concentrations of >0.0625 g/L led to precipitations.

Table 1. Main *m*/*z* Ratios of Gallotannins Observed from Negative Ion Experiments (% Base Peak)

fraction	compound	*m*/*z* ratio
A	tetra-*O*-galloylglucose	$[M-H]^-$ = 787 (100); $[M-2H]^{2-}$ = 393 (21) $[M-170-H]^-$ = 617 (5)
B	penta-*O*-galloylglucose	$[M-H]^-$ = 939 (100); $[M-2H]^{2-}$ = 469 (27) $[M-170-H]^-$ = 769 (2)
C	hexa-*O*-galloylglucose	$[M-H]^-$ = 1091 (100); $[M-2H]^{2-}$ = 545 (18) $[M-152-H]^-$ = 939 (8); $[M-152-2H]^{2-}$ = 469 (22) $[M-152-170-H]^-$ = 769 (2)
D_1/D_2	hepta-*O*-galloylglucose	$[M-H]^-$ = 1243 (100); $[M-2H]^{2-}$ = 621 (33) $[M-152-H]^-$ = 1091 (18); $[M-152-2H]^{2-}$ = 545 (20) $[M-2\times152-H]^-$ = 939 (8); $[M-2\times152-2H]^{2-}$ = 496 (14) $[M-2\times152-170-H]^-$ = 769 (2)
E	octa-*O*-galloylglucose	$[M-H]^-$ = 1395 (65); $[M-2H]^{2-}$ = 697 (100) $[M-152-H]^-$ = 1243 (12); $[M-152-2H]^{2-}$ = 621 (47) $[M-2\times152-H]^-$ = 1091 (6); $[M-2\times152-2H]^{2-}$ = 545 (18) $[M-3\times152-H]^-$ = 939 (3); $[M-3\times152-2H]^{2-}$ = 469 (12)
F	nona-*O*-galloylglucose	$[M-H]^-$ = 1547 (74); $[M-2H]^{2-}$ = 773 (100) $[M-152-H]^-$ = 1395 (11); $[M-152-2H]^{2-}$ = 697 (40) $[M-2\times152-H]^-$ = 1243 (3); $[M-2\times152-2H]^{2-}$ = 621 (19) $[M-3\times152-H]^-$ = 1091 (2); $[M-3\times152-2H]^{2-}$ = 545 (10) $[M-4\times152-H]^-$ = 939 (1); $[M-4\times152-2H]^{2-}$ = 469 (11)
G	deca-*O*-galloylglucose	$[M-H]^-$ = 1699 (30); $[M-2H]^{2-}$ = 849 (100) $[M-152-H]^-$ = 1547 (23); $[M-152-2H]^{2-}$ = 773 (41) $[M-2\times152-H]^-$ = 1395 (2); $[M-2\times152-2H]^{2-}$ = 697 (23) $[M-3\times152-H]^-$ = 1243 (<1); $[M-3\times152-2H]^{2-}$ = 621 (11) $[M-4\times152-H]^-$ = 1091 (<1); $[M-4\times152-2H]^{2-}$ = 545 (10) $[M-5\times152-H]^-$ = 939 (<1); $[M-5\times152-2H]^{2-}$ = 469 (<1)

o-Dihydroxyphenyl groups are thought to be responsible for the chelation of iron(III) ions (*23*). In this study, gallic acid (3,4,5-trihydroxybenzoic acid) did not exhibit any iron binding capacity. Within the tannin molecule, *o*-dihydroxyphenyl groups can bind several iron(III) ions and each iron(III) can itself coordinate with up to three *o*-dihydroxyphenyl moieties belonging to different polyphenol molecules. This leads to the formation of a lattice, which eventually results in coprecipitation of iron and polyphenols and removal of iron(III) from the solution (*23*). The increase in the level of galloylation involves an addition of *o*-dihydroxyphenyl groups in the molecule. The decrease in the Fe binding capacity at higher concentrations might be explained by steric effects within the larger molecules.

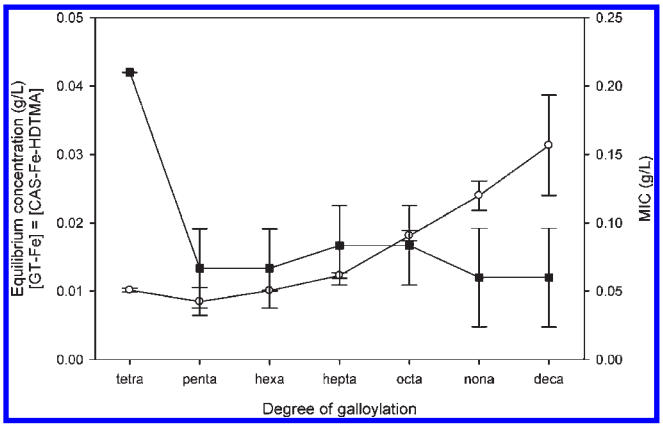

Figure 3. Equilibrium concentration when the concentration of the gallotannin (GT)−iron complex equals the concentration of the CAS−Fe−HDTMA complex (○) and minimum inhibitory concentrations (MICs) (■) toward *B. subtilis* of differently galloylated tannins. Shown are means ± standard deviations in grams of dry matter per liter of duplicate and triplicate, respectively.

Antimicrobial Activity. Antibacterial activity was previously demonstrated for penta-, hexa-, and hepta-*O*-galloylglucose (*9*, *12*). This study reports their minimal inhibitory concentrations as well as the MICs of three new purified substances, octa-, nona-, and deca-*O*-galloylglucose (**Figure 3**). The MICs of gallotannins were in the range of 0.05−0.1 g/L and not dependent on the degree of galloylation. Tetra-*O*-galloylglucose had a higher MIC; however, this fraction was contaminated with substantial amounts of unidentified components eluting from the HSCCC column at the beginning of the run. Tannins possessed bactericidal effects, with minimum bactericidal concentrations between 0.4 and 0.9 g/L in LB medium. MICs were ∼20 times higher in MRS than in LB medium (1.1 g/L for deca-*O*-galloylglucose to 2.22 g/L for tetra-*O*-galloylglucose). MBCs in MRS medium were ≥3.3 g/L. The MICs were in the range of those of commercially applied preservatives like benzoic acid (0.01−0.02%), sorbic acid (0.05−0.3%), and propionate (0.1−5%) (*24*) but higher than those of other natural components with antimicrobial activity (*20*).

The fact that the MICs and MBCs were higher in MRS medium, which contains more iron and divalent cations, demonstrates the importance of the matrix for the antibacterial activity. Iron mitigates the antibacterial activity of gallotannins. The effect of iron on the antimicrobial activity of gallotannins is consistent with their selective properties: lactic acid bacteria, which do not require iron for their growth, are not inhibited by tannins (*9*, and references cited therein). Inhibitory effects can also be reduced by an enhanced ability to sequester iron, as Mila et al. (*25*) proved by examining strains of the plant pathogen *Erwinia chrysanthemi* with alterations in the siderophore-mediated iron transport pathway.

Chelation of metal ions could result in iron deprivation, a decrease in activity of metalloenzymes, and inhibition of oxidative phosphorylation (*26*). Our study demonstrates that the degree of galloylation strongly influences the iron binding capacity but not the antibacterial activity. This indicates that the iron complexing capacity of gallotannins contributes to their antibacterial activity but is not exclusively responsible for it. Further modes of action might include their ability to interact with proteins, to inhibit enzyme activities (*27*, *28*), and to cause damage to the lipid bilayer membrane and cell wall (*29*).

In conclusion, the results of this study demonstrate that HSCCC is a powerful technique for the separation of tannins according to their degree of galloylation in quantities and purities sufficient for subsequent bioactivity studies. Understanding the correlation of certain bioactive properties and the degree of galloylation will contribute to an improved knowledge of the antibacterial modes of action.

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