A Simple Isolation Method for the Major Catechins in Green Tea Using High-Speed Countercurrent Chromatography

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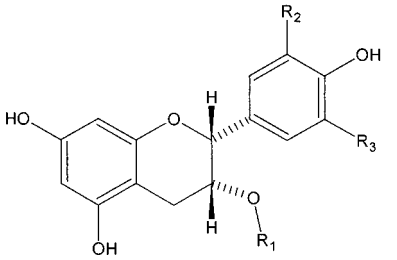
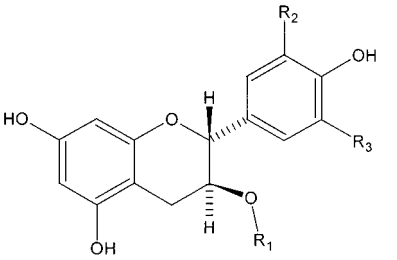
An efficient protocol for the preparative purification of the major tea catechins (−)-epicatechin gallate (**3**) and epigallocatechin gallate (**5**) has been developed, employing liquid−liquid partitioning, high-speed countercurrent chromatography, and gel chromatography for final purification. The method is suitable for scale-up to significantly larger quantities.

Considerable interest has arisen recently concerning the possible chemopreventive effects of certain secondary metabolites in medicinal, aromatic, and food plants. Among these, natural products with antioxidant and antimutagenic activity have attracted particular attention.[1−7] Green tea (*Camellia sinensis* (L.) Kuntze, Theaceae) has a longstanding reputation in Asia for its health-promoting properties. Numerous in vitro and in vivo studies on green tea preparations have demonstrated the antimutagenic, anticarcinogenic, and antioxidant properties of the phenolic compounds which form the major portion of the soluble tea constituents.[8] Among these, the catechins (about 25%) form the major fraction in unfermented tea. Typical samples contain 1−3% (−)-epicatechin (**1**) and (+)-catechin (**2**), 3−6% (−)-epicatechin gallate (ECG, **3**) and (−)-epigallocatechin (EGC, **4**), 9−13% (−)-epigallocatechin gallate (EGCG, **5**), and 3−4% (+)-gallocatechin (**6**).[9] For quality control of green tea preparations and for in-depth investigations of the biological and pharmacological properties of green tea polyphenols, reference compounds of certified purity are required in milligram to multigram quantities. Whereas simple phenolics such as **1** and **2** are readily available, the cost of major green tea catechins such as **3**−**5** from commercial suppliers is high.

We have therefore developed a simple and efficient method for the preparative isolation of flavanol gallates starting from a commercially available spray-dried tea extract, the HPLC chromatogram of which is shown in Figure 1S (Supporting Information). In a first step, liquid−liquid partitioning between EtOAc and H_2O (1:1) afforded an enriched catechin fraction in the organic layer. Highly polar tea components and excipients required for the preparation of a nonhygroscopic spray-dried powder remained in the aqueous phase. Subsequently, the catechin portion was submitted to high-speed centrifugal countercurrent chromatography (HSCCC). A broad range of HSCCC-compatible solvent systems was evaluated, in particular *n*-hexanes−ethyl acetate−*n*-butanol−methanol−water, *n*-hexanes−ethyl acetate−water, ethyl acetate−methanol−water, and chloroform−methanol−*n*-butanol−water systems of various proportions.[10−17] Partitioning behavior of the catechins was assessed by TLC analysis of the two phases. The solvent systems *n*-hexanes−ethyl acetate−water (e.g., 1:5:5) and ethyl acetate−methanol−water (5:1:5 and 5:2:5) showed favorable partitioning between the two layers. The low-density difference in the ethyl acetate−methanol−water systems, however, precluded a retention



1: R_1 = H, R_2 = H, R_3 = OH
3: R_1 = galloyl, R_2 = H, R_3 = OH
4: R_1 = H, R_2 = OH, R_3 = OH
5: R_1 = galloyl, R_2 = OH, R_3 = OH



2: R_1 = H, R_2 = H, R_3 = OH
6: R_1 = OH, R_2 = OH, R_3 = OH

of the stationary phase in the HSCCC coils when operating in the ascending mode. Satisfactory phase retention and good separation of **3** and **5** could be obtained with *n*-hexanes−ethyl acetate−water (1:5:5) (see Figure 2S in Supporting Information). A drawback was that EGCG (**5**) eluted only after more than 240 min. A significantly faster separation of the catechin gallates was achieved with a simple ethyl acetate−water (1:1) system. Using the aqueous layer as stationary phase, acceptable phase retention could be obtained in the 320 mL coils with flow rates up to 1.5 mL/min. At higher flow rates, significant displacement of the stationary phase was observed. A typical chromatogram is shown in Figure 1. Although the two compounds of interest eluted with very little retention (*K* value ≈ 0.1 for **3** and 0.35 for **5**), the TLC analysis revealed that fractions b and d consisted essentially of ECG (**3**) and EGCG (**5**), respectively. Fraction e contained pure (+)-catechin (**2**), whereas other catechins, caffeine, and polar substances remained in the stationary phase. For purification of **3** and **5**, the HSCCC run could be terminated after 90 min. Sample amounts of up to 600 mg could be

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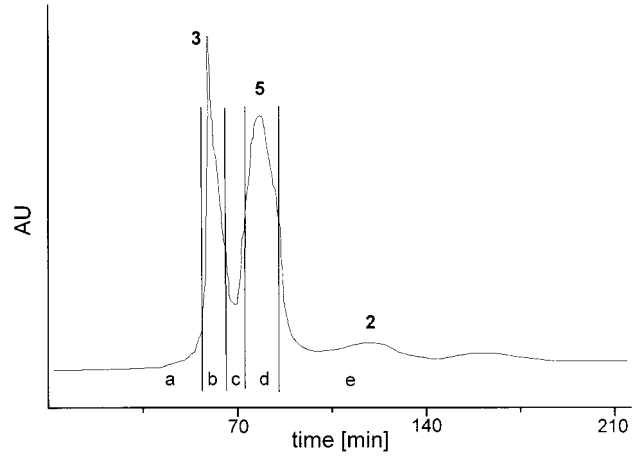

Figure 1. HSCCC chromatogram of the enriched catechin fraction of green tea extract (for details see Experimental Section). ECG (**3**) elutes first, followed by **5** and **2**; a, b, c, d, and e denote collected fractions.

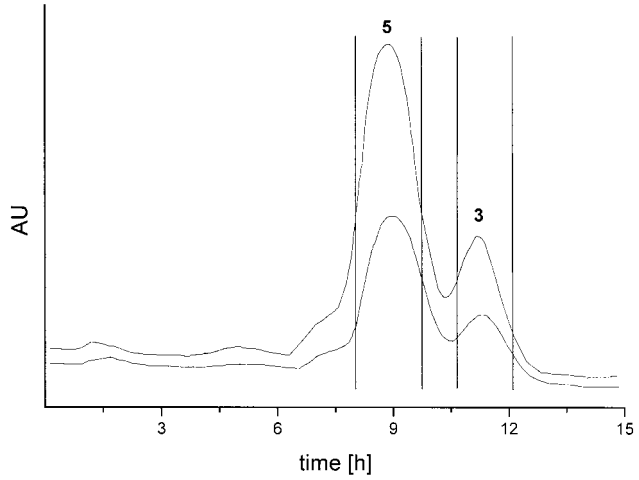

Figure 2. Gel filtration of fraction c (containing **3** and **5**) on Sephadex LH-20 (for details see Experimental Section).

separated in a single run on the 320 mL coils without significant loss of separation performance. Typical yields of fractions b, c, and d were 90−150 mg each. Final purification of **3** and **5** was achieved by gel filtration over Sephadex LH-20 with methanol as mobile phase, whereby minor accompanying polyphenols were removed. Additional amounts of catechin gallates could be obtained by gel filtration of the mixed fraction c (Figure 2). Sample amounts of up to 250 mg per run could be handled without noticeable loss of column performance. Purity and identity of the compounds was assessed by HPTLC, LC-DAD-ESIMS, and NMR spectroscopy. Purity determined by HPLC was 98%. Chromatographic mobility, DAD spectra, and ESI-MS of the minor impurities indicated that they were catechin derivatives structurally related but not identical to **1**−**6**. The overall yields for ECG (**3**) and EGCG (**5**) from the tea extract were 2.2% and 9.5% (w/w), respectively.

A simple and efficient three-step procedure for the isolation of ECG (**3**) and EGCG (**5**) has been developed. The first two purification steps involve liquid−liquid partitioning only.[18] The method is easily amenable to scale-up, since preparative HSCCC instrumentation with coil volumes of several liters is available. Even though the ethyl acetate−water system was selected for fast throughput, the separation achieved was sufficient to afford satisfactory purity after gel chromatography. The procedure is economically attractive. Inexpensive solvents such as methanol, ethyl acetate, and water are employed throughout, and these can be easily recycled via distillation. The cost of the dextran gel column is offset by the durability of the sorbent. Multiple separations can be carried out without any noticeable loss of column performance.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were measured with a Bruker DRX 400 spectrometer operating at 400.13 and 100.61 MHz, respectively. HPLC was carried out with a Hewlett-Packard Series 1100 instrument equipped with a DAD and a HP ChemStation. HPLC-ESIMS were obtained on a PE Sciex API 165 mass spectrometer with Turbo Ionspray interface coupled to a HP Series 1100 HPLC system. Gel chromatography was carried out on a Pharmacia Biotech column (6 cm × 90 cm i.d.) packed with Sephadex LH-20 (Amersham Pharmacia Biotech), connected to a 10 mL sample loop, a P1 pump, a Uvicord II detector (278 nm), a Rec2 chart recorder, and a SuperFrac fraction collector (all Pharmacia LKB). The instrumentation for HSCCC consisted of a CCC-1000 instrument (PharmaTech, Baltimore, MD) equipped with three semipreparative coils of a total volume of 320 mL, a 20 mL sample loop, a PharmaTech HPLC pump, a Uvicord II detector (278 nm), Rec2 chart recorder, and a RediFrac fraction collector (all Pharmacia LKB). TLC was carried out on Al GF_{254} silica gel sheets (Merck) with ethyl acetate−butan-2-one-formic acid−water (3:6:1) as mobile phase.

Detection was with Godin's reagent (vanillin (0.5%), perchloric acid (1.5%) in aqueous ethanol, and sulfuric acid (10%) in ethanol), followed by heating of the TLC plate at 105 °C for 5 min. HPLC grade acetonitrile was purchased from Roth (Karlsruhe, Germany). HPLC grade water was purified over a Millipore Milli-RO 3 Plus module coupled to a Milli-Q RG system. Methanol was distilled from technical grade solvent. All other chemicals were of analytical grade.

Plant Material. The spray-dried green tea extract (Lot. Nr. V98189) was obtained from Dragoco Co. (Holzminden, Germany). The material used for preparation of the extract was *Camellia sinensis* (L.) (Kuntze), "China Green Fannings" quality, and was authenticated at Dragoco Co.

Isolation. An aliquot (20 g) of the green tea extract was partitioned between ethyl acetate−water (1:1) (1000 mL). After the separation of the phases, the organic layer was evaporated to dryness to afford an enriched catechin fraction (4.1 g, 21%). Solvent systems for HSCCC were tested by small-scale partitioning of the catechin fraction (ca. 10 mg in 10 mL). After phase settling, the two layers were separately analyzed by TLC for estimation of partitioning behavior. HSCCC of the enriched catechin fraction was carried out with the binary system ethyl acetate−water (1:1), with the aqueous layer as stationary phase. The separation was carried out with a rotational speed of 800 rpm and a flow rate of 1.5 mL/min (tail to head elution). Stationary phase retention was typically around 70%. After reaching phase equilibrium in the coils, an aliquot of the catechin fraction (600 mg) dissolved in 20 mL of the binary solvent system was injected. Fractions of 4.5 mL were collected and combined on the basis of the UV and TLC analysis to afford 5 fractions (a−e). The HSCCC separation step was carried out repeatedly. Typical yields for the ECG-containing fraction b, the EGCG-containing fraction d, and the mixed ECG/EGCG fraction c were 102, 145, and 90 mg, respectively. Final cleanup of the fractions b and d and separation of additional amounts of **3** and **5** from fraction c were achieved by gel chromatography on Sephadex LH-20 with methanol as eluent. Typically, sample amounts of 100−250 mg were dissolved in 10 mL of mobile phase. Flow rate was 7 mL/min; fractions of 4 min were collected and combined on the basis of the UV chromatogram.

HPLC-DAD-MS. Separations were carried out on a Hypersil ODS RP 18 column (5 μm, 250 mm × 4.6 mm) (Supelco); eluent A, acetic acid 2%; eluent B, acetonitrile; gradient profile, B 8% (0 min) → B 8% (8 min) → B 22.6% (20 min) → B 40% (25 min); flow rate, 1 mL/min; column temperature, 20 °C;

DAD-detection at 330 and 250 nm; ESIMS, positive ion mode; nebulizer temperature, 350 °C; ion spray voltage, 5400 kV; declustering potential, 60 V.

(−)-Epicatechin gallate (3): ^{1}H NMR and ^{13}C NMR spectral data in agreement with literature;[19] ESIMS *m*/*z* 443 $[MH]^+$.

(−)-Epigallocatechin gallate (5): ^{1}H NMR and ^{13}C NMR spectral data in agreement with literature;[19] ESIMS *m*/*z* 459 $[MH]^+$.

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Supporting Information Available: HPLC chromatogram of the tea extract (Figure 1S) and HSCCC chromatogram of the extract using a *n*-hexanes−ethyl acetate−water (1:5:5) system (Figure 2S). This material is available free of charge via the Internet at http://pubs.acs.org.

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