# Preparative Separation of Polyphenols from Tea by High-Speed Countercurrent Chromatography

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High-speed countercurrent chromatography (HSCCC) was applied to the separation of polyphenols from tea leaves (*Camellia sinensis* L.). The capability of HSCCC to isolate pure tea polyphenols from complex mixtures on a preparative scale was demonstrated for catechins, flavonol glycosides, proanthocyanidins, and strictinin from green and black tea. The purity and identity of isolated compounds was confirmed by $^1$H NMR and HPLC-ESI-MS/MS. Gram quantities of polyphenols from tea can be isolated with the procedure described.

**Keywords:** *Green tea; black tea; Camellia sinensis; high-speed countercurrent chromatography; HPLC-ESI-MS; polyphenols; catechins; flavonol glycosides; proanthocyanidins; strictinin*

## INTRODUCTION

Tea, one of the most widely consumed beverages throughout the world, contains a wide range of polyphenols, such as, e.g., catechins, flavonol glycosides, and proanthocyanidins. Whereas in green tea, catechins are the predominant polyphenols, black tea has undergone a fermentation process which led to the formation of theaflavins from catechins and other not well characterized polyphenolic compounds which provide distinctive color to the black tea beverage. Epigallocatechin gallate (EGCG) is the most abundant catechin in green tea. Due to the antioxidative, antiinflammatory, antimutagenic, and anticarcinogenic potential of catechins (Kuroda and Hara, 1999; Wiseman et al., 1997), this class of flavonoids is of great interest in terms of health benefits of regular tea consumption. Flavonol glycosides are another class of flavonoids with a widespread occurrence in nature (Hertog et al., 1992; Boehm et al., 1998). Research indicates that there is a relationship between the consumption of flavonol-rich food and a low incidence of coronary heart disease (Hertog et al., 1993; Tijburg et al., 1997). Flavonol glycosides are present in green as well as in black tea. A range of 14 different flavonol glycosides was detected in tea (Engelhardt et al., 1992). Proanthocyanidins are colorless precursors of anthocyanidins. The presence of a wide range of proanthocyanidins in green tea was confirmed in a recent study (Kiehne et al., 1997). Contents of proanthocyanidins in green tea are roughly in the same range as the flavonol glycosides (Lakenbrink, 2000). In black tea the contents are much lower since there is a decrease during fermentation (Hashimoto et al., 1992; Lakenbrink, 2000). Due to their possible contributions to the health benefits of tea, proanthocyanidins have also attracted considerable attention. Strictinin, a member of the class of hydrolyzable tannins, was first detected in tea by Nonaka et al. (1984). However, quantitative determination of strictinin in teas is still difficult to perform due to the lack of a commercially available reference compound. The same is true for flavonol glycosides and proanthocyanidins. Catechin standards are only offered at a high price. Therefore, we tested the capability of HSCCC to isolate pure reference compounds, which could be used as standards in subsequent quantifications of tea constituents by HPLC. Pure reference compounds are also required for model fermentation studies in order to gain insight into the formation of thearubigins from various precursors. Screening for antioxidant activity or studies of metabolic pathways are further applications which can be carried out with the isolated reference components.

HSCCC is a preparative all-liquid chromatographic technique based on partitioning of compounds between two immiscible liquid phases. Details about the operation principle can be found in various monographs (Ito, 1986; Conway, 1990; Conway and Petroski, 1995). In recent years, HSCCC is emerging as a superior technique for preparative separations of natural compounds (Marston and Hostettmann, 1994; Degenhardt et al., 2000a,b). The application of HSCCC for the isolation of tea polyphenols is discussed below.

## MATERIALS AND METHODS

**Materials.** Green tea was a Chinese Lung Ching. The black tea used was a low grown Ceylon.

**Methods. Extraction of Catechins from Green Tea.** Green tea (16 g) was extracted (20 min) with 70% aqueous methanol (v/v) under stirring. The solvent was evaporated in vacuo and the residue was twice extracted with 300 mL of ethyl acetate. The aqueous phase was discarded. The pooled organic layer was evaporated in vacuo and freeze-dried. The lyophilisate was separated by HSCCC.

**Extraction of Proanthocyanidins from Green Tea.** Extraction, removal of other tea components, and purification of the proanthocyanidin fraction was carried out according to the procedure of Lakenbrink (2000): approximately 5 g of tea was extracted with 300 mL of an aqueous acetone solution (75%, v/v) during 2 h at 50 °C. The extract was filtered with a Buchner funnel. To facilitate phase separation, sodium chloride was added. The aqueous phase was separated from the organic phase and discarded. The remaining organic phase was concentrated in vacuo. A glass column was prepared according to a procedure described by Engelhardt et al. (1992). The tea extract was applied onto the column. After washing with 200 mL of methanol and 100 mL of 2% acetic acid in methanol (v/v), the proanthocyanidins were eluted with 70%

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aqueous acetone (v/v). The eluate was evaporated and freeze-dried. The lyophilisate was separated by HSCCC.

**Extraction of Flavonol Glycosides from Black Tea.** The flavonol glycosides were extracted from black tea following a procedure described by Engelhardt et al. (1992) and cleaned-up on a polyamide column. The eluate was freeze-dried prior to HSCCC separation.

**HPLC-ESI-MS/MS.** A Bruker Esquire HPLC-MS with UV−vis detector in series (set at 280 nm) was used; MS-parameters were the following: negative mode; capillary, 2500 V; end plate, 2000 V; capillary exit, −120 V; skim 1, −40 V; skim 2, −8 V; dry gas, 325 °C; gas flow, 9 L/min; nebulizer, 40 psi; fragmentation amplitude, 1.0 V.

*Catechins:* Hypersil RP18 column 5 $\mu$m (250 mm × 2 mm) from Phenomenex (Aschaffenburg, Germany). Solvents: 9% acetonitrile in 2% aqueous acetic acid (v/v, solvent A), 80% aqueous acetonitrile (v/v, solvent B); linear gradient from 100% A, 0% B (initial) for 15 min; to 68% A, 32% B in 15 min; isocratic at 68% A, 32% B for 5 min; flow rate, 0.35 mL/min.

*Proanthocyanidins:* LUNA RP18 column 5 $\mu$m (150 mm × 4.6 mm) from Phenomenex (Aschaffenburg, Germany). Solvents: 2% aqueous acetic acid (v/v, solvent A), acetonitrile (solvent B); linear gradient from 95% A, 5% B (initial) to 74% A, 26% B in 75 min; flow rate, 0.35 mL/min.

**Flavonol Glycosides and Strictinin.** Flow injection at the Bruker Esquire ESI-MS/MS with electrospray ionization in the negative mode was used. Dry gas was nitrogen with a gas flow of 4 L/min (300 °C); the nebulizer was set at 10 psi. The MS parameters were the following: negative mode; capillary, 3500 V; end plate, 3000 V; other parameters, see HPLC-ESI-MS. MS/MS was performed by using different fragmentation amplitudes.

**HPLC-DAD.** A System Gold Solvent Module 126 from Beckman Instruments with diode array detector 168 and Beckman 502 autosampler has been used.

*Flavonol Glycosides:* Nucleosil RP18 column 5 $\mu$m (250 mm × 4.6 mm) from Techlab (Erkerode, Germany); 2% aqueous acetic acid (v/v, solvent A), acetonitrile (solvent B). Conditions: initial 94% A, 6% B, in 28 min to 83% A, 17% B, in 25 min to 80% A, 20% B, 12 min isocratic at 80% A, 20% B, after flushing with 100% B, 20 min conditioning of column; detection at 354 nm; flow rate, 1 mL/min.

**Proton Nuclear Magnetic Resonance ($^1$H NMR) Spectroscopy.** All experiments were performed on a Bruker AMX 300 spectrometer (300 MHz). Spectra of catechins and strictinin were recorded in acetone-$d_6$ and methanol-$d_4$. Assignments of catechins were made on the basis of spectral data published by Davis et al. (1996).

*Epiafzelechin Gallate (**3**) (300 MHz, $CD_3OD$, in ppm):* $\delta$ 7.30 (2 H, dd, $J$ = 8.5, 2.5 Hz, H 2′/6′); 6.94 (2 H, s, H 2″/H6″); 6.71 (2 H, dd, $J$ = 8.5, 2.5 Hz, H 3′/5′); 5.96 (2 H, s, H 6/8); 5.52 (1 H, m, H 3); 5.08 (1 H, br s, H 2); 3.01 (1 H, dd, $J$ = 17.4, 4.5 Hz, H 4$\beta$); 2.86 (1 H, dd, $J$ = 17.4, 2.0 Hz, H 4$\alpha$). ESI-MS (negative mode): molecular peak 425 $[M - H]^-$, MS/MS of 425: $m/z$ 273 $[M -$ galloyl$]^-$, 169 [gallic acid $- H]^-$.

**High-Speed Countercurrent Chromatography (HSCCC).** A high-speed model CCC-1000 (HSCCC) manufactured by Pharma-Tech Research Corporation (Baltimore, MD) was equipped with three preparative coils connected in series (total volume: 850 mL). The separations were run at a revolution speed of 1000 rpm and at flow rates from 2.5 to 3 mL/min. A Biotronik HPLC pump BT 3020 was used to pump the liquids. All samples were dissolved in an 1:1 mixture of light and heavy phase and injected into the system by loop injection. The amount of sample injected varied from 0.1 to 1 g. Depending on the sample up to 50 mg of pure reference compounds could be obtained. Stationary phase retention was in the range 53−80%. Five-milliliter fractions were collected with a Pharmacia LKB Super Frac fraction collector. Elution was monitored with a Knauer UV−vis detector and chromatograms were recorded on a Knauer L 250 E plotter. Chromatograms were digitalized with a scanner. Solvent systems used are given in the text and were optimized for each application.

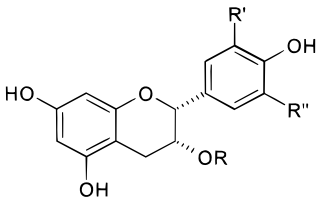


**Figure 1.** Structure of isolated catechins. **1**, epigallocatechin gallate (EGCG): R = galloyl; R′,R″ = OH. **2**, epicatechin gallate (ECG): R = galloyl; R′ = H, R″ = OH. **3**, epiafzelechin gallate (EAG): R = galloyl; R′,R″ = H.

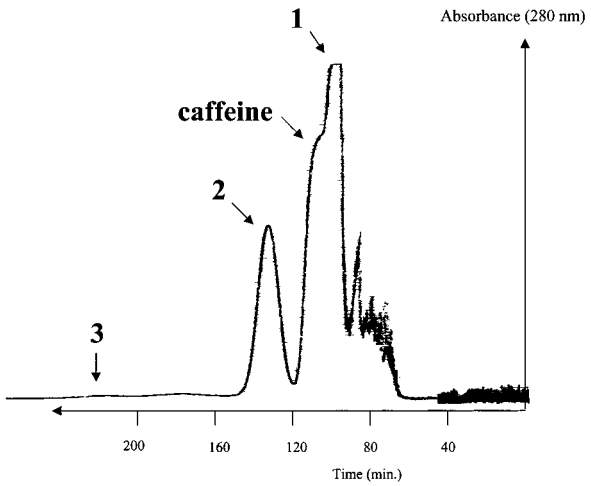


**Figure 2.** HSCCC separation of a crude catechin mixture (conditions see text); EGCG **1**; ECG **2**; EAG **3**.

## RESULTS AND DISCUSSION

**Purification of Individual Catechins.** Catechins represent about 20% of green tea dry weight. HPLC methods are routinely used for analysis of green tea catechins. Pure reference compounds are required for identification and quantification purposes. It is feasible to isolate catechins from tea by means of preparative HPLC or Sephadex gel chromatography (Davis et al., 1996; Amarowicz and Shahidi, 1996). However, these procedures are rather time-consuming. Due to the length of separation degradation of catechins may occur. Therefore, a rapid separation by HSCCC was developed, which allows isolation of gram quantities of pure compounds during a working day. The HSCCC separation of a crude catechin mixture isolated from green tea is shown in Figure 2. Separation of EGCG (**1**), ECG (**2**), and EAG (**3**) (cf. Figure 1) was achieved with a solvent system of hexanes−ethyl acetate−methanol−water (1.5/5/1.5/5 with the less dense layer acting as stationary phase; flow rate, 2.8 mL/min). The apparent shoulder on the first peak was determined to be caffeine. The sample load was 400 mg of the crude mixture, yielding 45, 7, and 4 mg of pure **1**, **2**, and **3**, respectively. Peak purity and identity of isolated compounds was checked by HPLC-ESI-MS, HPLC−UV, and $^1$H NMR. $^1$H NMR data of **1** and **2** are in good agreement with data published by Davis et al. (1996). In some green teas minor impurities were present in the isolated fractions. In those cases, crystallization from an aqueous solution resulted in pure catechins. Decaffeination of the tea by extraction with chloroform prior to HSCCC separation enables the isolation of pure **1**.

It is noteworthy that a successful separation of catechins by centrifugal partition chromatography (CPC)

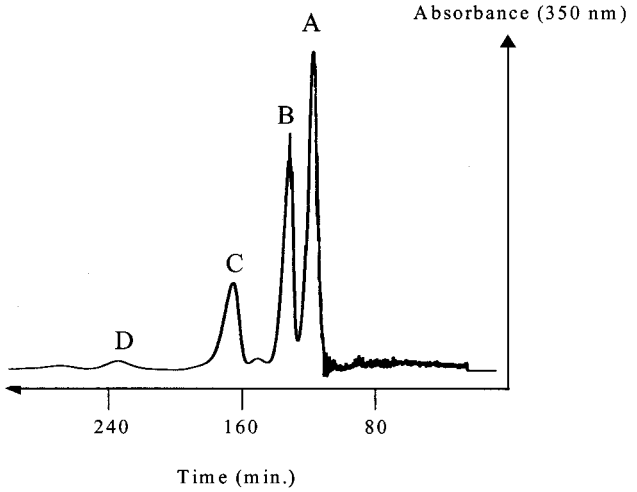


**Figure 3.** Separation of flavonol glycosides from black tea by HSCCC; solvent system: ethyl acetate−water (1/1; stationary phase, upper layer; flow rate, 2.8 mL/min). Peak A: mixture of flavone-*C*-glycosides and flavonol-*O*-trisaccharides. Peak B: quercetin 3-*O*-rutinoside, **4**. Peak C: kaempferol 3-*O*-rutinoside, **5**. Peak D: unknown.

has been described by Okuda et al. (1995). However, separation by HSCCC employing the solvent system hexanes−ethyl acetate−methanol−water (1.5/5/1.5/5) is performed more rapidly and allows isolation of flavan-3-ol gallates from tea.

**Purification of Flavonol Glycosides.** Flavonol glycosides are yellowish compounds which are important for the color of green tea. Apart from the coloring properties, flavonol glycosides are known to possess considerable antioxidative capacities (Engelhardt and Galensa, 1997). Investigation into the composition of flavonol glycosides from tea has shown that tea leaves contain a variety of different flavonol-*O*-glycosides. The aglycons myricetin, quercetin, and kaempferol are glycosylated with one, two, or three sugar moieties (Engelhardt et al., 1992). About 14 different flavonol glycosides are known to be present in tea. The complexity is further enhanced by the presence of a number of flavone-*C*-glycosides. They represent another group of eight compounds of similar polarity (Engelhardt and Galensa, 1997). By using HSCCC, two flavonol glycosides were purified in a single run on a preparative scale. Peaks were assigned by means of HPLC-DAD and ESI-MS/MS through comparison with authentic reference compounds (Engelhardt et al., 1992).

To develop a suitable separation system for flavonol glycosides, the polarity of the solvent system was stepwise adjusted to ideally resolve the target compounds in a single run (Degenhardt et al., 2000b). Two compounds, i.e., quercetin-3-*O*-rutinoside (**4**) and kaempferol-3-*O*-rutinoside (**5**) (cf. Figure 4) were successfully purified with a mixture of ethyl acetate−water (1/1) (cf. Figure 3). Sample loads in the gram range could be applied. HSCCC is capable of purifying several hundred milligrams of the above-mentioned flavonol glycosides within 2 h. To separate the mixture of compounds that coeluted under peak A (cf. Figure 3), the polarity of the solvent system was further increased by addition of *n*-butanol (cf. Figure 5). The added *n*-butanol dramatically increased retention of the solutes through enhanced partitioning into the organic phase. However, due to the complexity of the mixture, only partial separation of the compounds was achieved under these conditions.

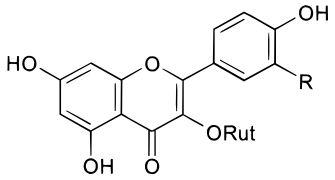


**Figure 4.** Structures of isolated compounds (cf. Figure 3). C: kaempferol 3-*O*-rutinoside, **5**, R = H (UV maxima by HPLC-DAD, 265, 346 nm; ESI-MS negative mode, *m*/*z* 593 [M − H]$^-$; ESI-MS/MS of 593, *m*/*z* 285). B: quercetin 3-*O*-rutinoside, **4**, R = OH (UV maxima by HPLC-DAD, 256, 353 nm; ESI-MS negative mode, *m*/*z* 609 [M − H]$^-$; ESI-MS/MS of 609, *m*/*z* 301).

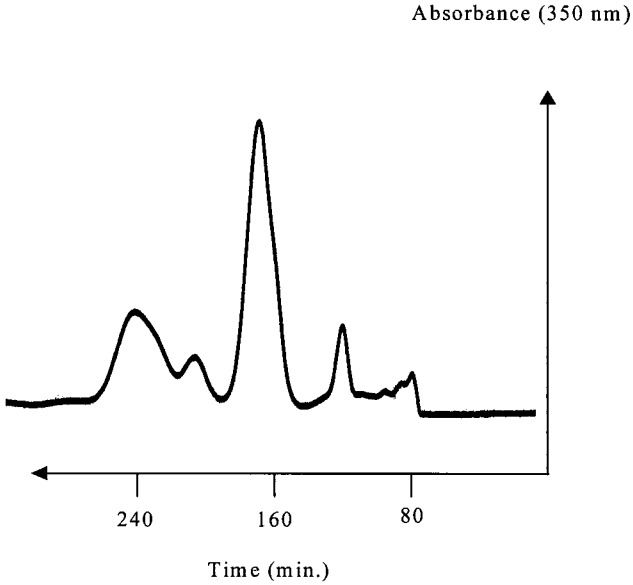


**Figure 5.** Attempted HSCCC separation of peak A (cf. Figure 3) with ethyl acetate−*n*-butanol−water (3.5/1.5/5; stationary phase, upper layer). Only partial purification of the flavonoid mixture could be achieved in this run.

**Purification of Proanthocyanidins.** Proanthocyanidins, the colorless precursors of anthocyanidins, are responsible for the astringent taste of many fruits. Heat treatment in acidic solution, cleavage of the C−C bond as well as oxidation results in the formation of colored anthocyanidins. Proanthocyanidins are of importance due to their antioxidative properties. They also influence technological processes, i.e., the brewing of beer (Engelhardt and Galensa, 1997). Recent research (Kiehne et al., 1997) has shown that a number of proanthocyanidins is present in green tea. Isolation of pure compounds was achieved by preparative HPLC (Lakenbrink, 2000). Most recently, Lakenbrink (2000) reported the purification of EGC-(4$\beta$→8)-EGCG, strictinin, EC-trimers, and EGCG-(4$\beta$→8)-EGCG by multilayer coil countercurrent chromatography (MLCCC) using the relatively polar solvent system of TBME−*n*-butanol−acetonitrile−water (2/2/1/5). Hence, the potential of HSCCC to isolate and purify proanthocyanidins on a preparative scale within a short time was evaluated. After cleanup of a tea extract on a polyamide column, proanthocyanidins were separated with hexanes−ethyl acetate−methanol−water (1/5/1/5, mobile phase: more dense layer) by HSCCC. Figure 6 shows the HSCCC separation which was monitored at 280 nm. Peak assignments using HPLC-MS are based on the retention time and characteristic fragmentation pattern previ-

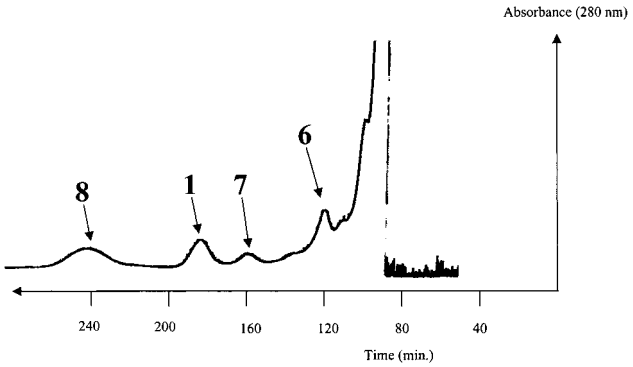


**Figure 6.** HSCCC separation of a crude proanthocyanidin mixture from green tea; conditions, see text; ECG-(4$\beta$→8)-ECG, **6** (molecular peak 881, most prominent daughter ions, *m/z* 729, 577, 559, 441, 407, 289); EAG-(4$\beta$→8)-ECG, **7** (molecular peak 865, most prominent daughter ions, *m/z* 713, 561, 543, 441, 423, 407, 289), EGCG **1**, mixture of three epigallocatechin digallates, **8**.

ously described through comparison with authentic reference compounds (Lakenbrink, 2000). The following compounds were obtained in pure form: ECG-(4$\beta$→8)-ECG (**6**), EAG-(4$\beta$→8)-ECG (**7**). The compound eluting after 190 min (cf. Figure 6) was found to be EGCG **1**, whereas a later fraction (240 min) contained three peaks with the molecular weight of 609 (HPLC-ESI-MS, negative mode) and *m/z* 457 as the most prominent daughter ion (cf. Figure 7). The compounds were tentatively identified as a mixture of three epigallocatechin digallate isomers (**8**) (Nonaka et al., 1983). The use of the solvent system ethyl acetate−ethanol−water (10/1/10; less dense layer as mobile phase; flow rate, 2.9 mL/min) resulted in a purification of EGCG-(4$\beta$→8)-ECG (**9**) (cf. Figure 8).

**Purification of Strictinin (10).** Polyphenol **10** (cf. Figure 10) was identified in green and black tea by Nonaka et al. (1984) and Hashimoto et al. (1992). Although the hydrolyzable tannin **10** is present in some teas in significant amounts, still no exact quantitative data are available. Moreover, it is discussed that strictinin **10** could take part in reactions which may lead to the formation of thearubigins. Isolated **10** could therefore be useful as a model compound for fermentation studies.

Purification of **10** (cf. Figure 9) from green tea was achieved by HSCCC using ethyl acetate−ethanol−water (10/1/10; more dense layer as mobile phase; flow rate, 2.7 mL/min). Structural elucidation was done by $^1$H NMR and ESI-MS/MS. $^1$H NMR data are in line with spectral data published by Nonaka et al. (1984).

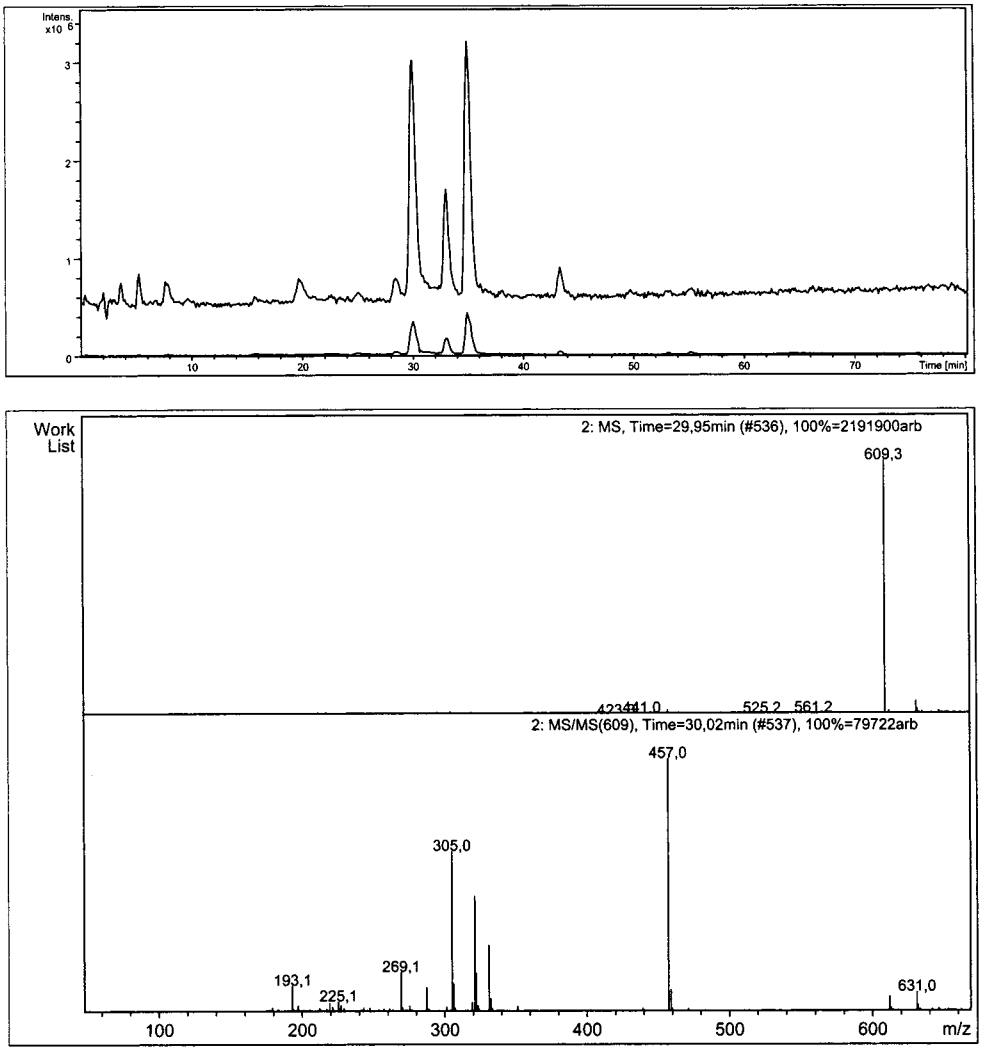


**Figure 7.** HPLC-ESI-MS/MS of epigallocatechin digallates **8**; the mass spectra of all three compounds show identical fragmentation patterns (molecular peak *m/z* 609; most prominent daughter ions, *m/z* 457, 331, 321, 305, 269, 193).

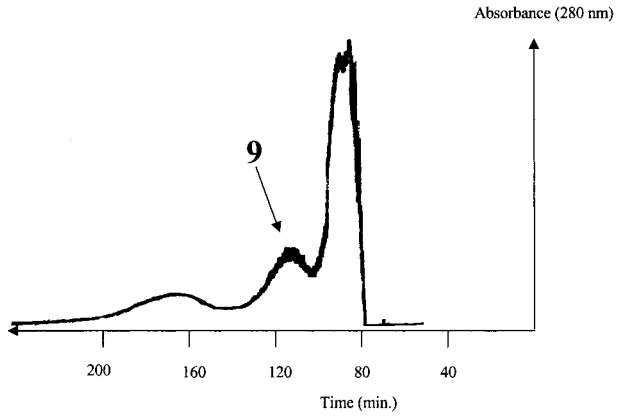


**Figure 8.** HSCCC separation of crude proanthocyanidin mixture from green tea; conditions, see text; EGCG-(4$\beta$→8)-ECG, **9** (molecular peak, 897; most prominent daughter ions, *m*/*z* 745, 619, 593, 575, 559, 441, 407, 289).

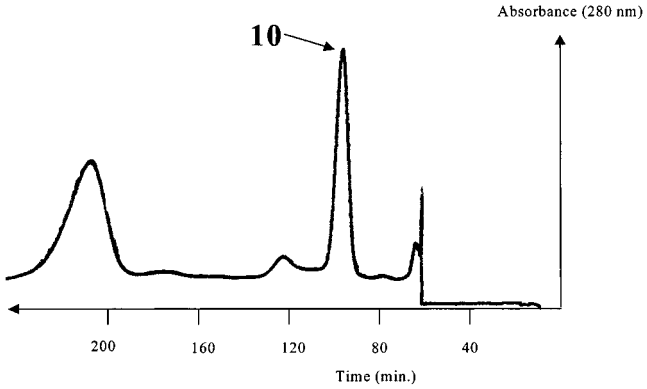


**Figure 9.** HSCCC separation of strictinin **10** from green tea using a crude proanthocyanidin mixture; conditions, see text (molecular peak, 633; most prominent daughter ion, *m*/*z* 301).

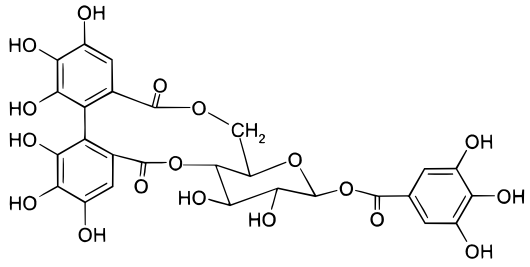


**Figure 10.** Structure of strictinin **10**.

## CONCLUSIONS

It was demonstrated that CCC has a great potential for the preparative isolation of polyphenols from tea. In combination with a suitable extraction and cleanup procedure prior to HSCCC separation, pure reference compounds are obtained on a preparative scale. CCC offers several advantages compared to preparative HPLC. Since CCC uses no solid stationary phase, artifact formation is minimized. Moreover, CCC uses gentle operation conditions. The separation column consists of an inert system of Teflon tubing and the separations are run at ambient temperature. The sample load capacity in CCC is superior to that of preparative HPLC. Isolation of several hundred milligrams of pure compounds can be obtained during a working day.

## ABBREVIATIONS USED

High-speed countercurrent chromatography, HSCCC; multilayer coil countercurrent chromatography, ML-CCC; high performance liquid chromatography, HPLC; proton nuclear magnetic resonance spectroscopy, $^1$H NMR; electrospray ionization ion trap multiple mass spectrometry, ESI-MS/MS; epigallocatechin gallate, EGCG; epicatechin gallate, ECG; epicatechin, EC; epiafzelechin gallate, EAG; epigallocatechin, EGC; *tert*-butyl methyl ether, TBME.

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