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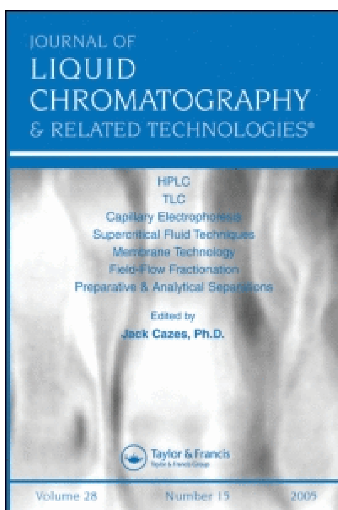

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EXTRACTIONS AND PURIFICATIONS

SEPARATION AND PURIFICATION OF THREE INDIVIDUAL CATECHINS FROM TEA POLYPHENOL MIXTURE BY CCC

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ABSTRACT

Countercurrent chromatography (CCC) was applied to the separation and purification of three catechins, i.e., epigallocatechin-3-o-gallate (EGCG), gallocatechin-3-o-gallate (GCG), and epicatechin-3-o-gallate (ECG) from a polyphenol mixture. Analytical CCC was used to select suitable two-phase solvent systems from ethyl acetate-ethanol-water and hexane-ethyl acetate-water systems by adjusting mutual volume ratios in each system. Using the optimized solvent systems, the preparative separation was successfully performed by two-step separation where 1 g crude sample was efficiently purified, yielding 275 mg of EGCG, 140 mg of GCG, and 130 mg of ECG, each at high purity about 98% by HPLC analysis. The structures of these catechins were identified by FAB-MS, 1H, and 13C NMR.

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INTRODUCTION

Among numerous types of polyphenols contained in green tea leaves, flavan-3-ols and their gallates, referred to as 'catechins' (Fig.1), are the most important major components. It has been recognized that these catechins possess many biological activities, such as antioxidant,[1] anti-mutagenic, and anti-carcinogenic properties,[2] anti- HIV activities,[3] prevention of dental caries,[4] reduced risk of cardio-vascular injury.[5] Because of these properties, catechins should be seriously considered as potent compounds for medical use. However, catechins with high purity are currently only commercially available as their standards offered at a high price. This may be mainly due to a lack of the efficient purification method of these compounds. The isolation of catechins using conventional methods, such as column chromatography, requires complicated multiple steps resulting in low recoveries of products due to irreversible adsorption and decomposition onto the solid support.

Countercurrent chromatography (CCC) is a unique liquid-liquid partition chromatography, which uses no solid matrix and, therefore, eliminates irreversible adsorption of samples. The retention and separation is purely based on the partition coefficients of solutes. Since the last decade, CCC has been successfully applied to the separation of various natural products,[6-7] including the application of separation of polyphenols.[8]

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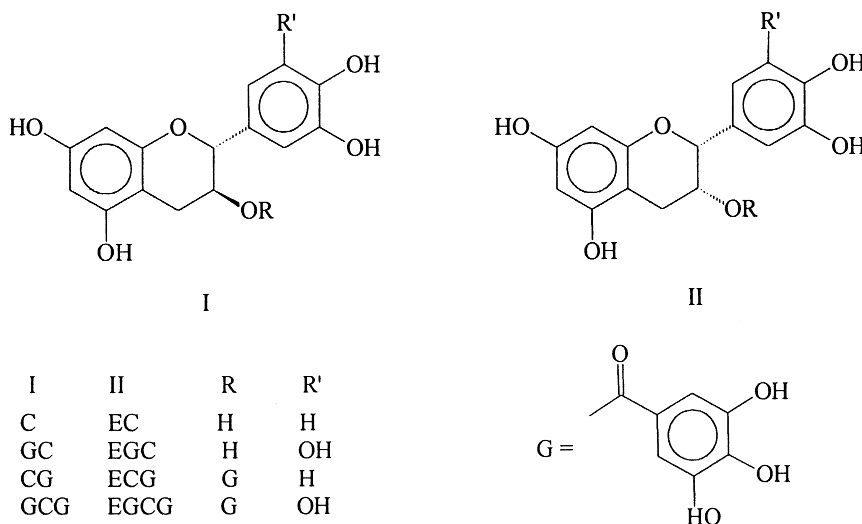

I	II	R	R'
C	EC	H	H
GC	EGC	H	OH
CG	ECG	G	H
GCG	EGCG	G	OH

Figure 1. The structure of catechins.

The present paper describes the application of CCC to the separation and purification of three individual catechins: epigallocatechin-3-o-gallate (EGCG), gallocatechin-3-o-gallate (GCG), and epicatechin-3-o-gallate (ECG) from a crude sample of tea polyphenol mixture.

EXPERIMENTAL

Apparatus

CCC separation was performed with the following two models of mutilayer coil planet centrifuge (MLCPC) manufactured by Beijing Institute of New technology Application, Beijing, China.

(1) Model GS-20 analytical MLCPC: The mutilayer coil was prepared by winding 70 m long, 0.8 mm I.D. PTFE (polytetrafluoroethylene) tubing coaxially onto a spool with radius from 40 mm (internal) to 80 mm (external) by about 400 turns. The β value ranges from 0.4 to 0.72, and the total capacity is 35 mL. The rotation speed is adjustable from 0 to 2000 rpm, and 1800 rpm was used in the present study.

(2) Model GS10A2 preparative MLCPC: The multilayer coil was prepared by winding 130 m long, 1.6 mm I.D. PTFE tubing coaxially onto a spool with radius from 60 mm (internal) to 120 mm (external) by about 500 turns. The β value ranges from 0.5 to 0.75 and the total capacity is 260 mL. The rotation speed is adjustable from 0 to 1000 rpm, and 800 rpm was used in the present study.

These two CCC systems are equipped with an NS-1007 constant-flow pump, a Model 8823A UV monitor operating at 254 nm, a Yokogawa Model 3057 recorder, and a sample injection valve with a 2-mL or 20-mL sample loop.

Reagents

All solvents used for CCC were of analytical grade and were purchased from Beijing Chemical Factory, Beijing, China. The tea polyphenol sample mixture was provided by Qun Li Pharmaceutical Ltd., Hainan, China.

Solvent Selection and CCC Separation

In the present study, a series of preliminary experiments was performed to determine suitable two-phase solvent systems using our analytical MLCPC. Analytical CCC, with its speedy and minimum solvent consumption, offers a very promising way to carry out method development for preparative CCC sepa-

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rations.[6] Two sets of solvent systems composed of ethyl acetate-ethanol-water and hexane-ethyl acetate-water were examined by varying mutual volume ratio. Before separation, each solvent mixture was thoroughly equilibrated in a separation funnel and the two phases were separated just before use. The sample solution was prepared by dissolving the polyphenol mixture in the mobile phase of the solvent system to be used for separation.

In the analytical separation, the coiled column was first entirely filled with the organic stationary phase, and the MLCPC was rotated at 1800 rpm, while the aqueous mobile phase was pumped into the column at a flow-rate of 1.0 mL/min. After the mobile phase front emerged and hydrodynamic equilibrium was established, 1 mL of the sample solution containing about 16 mg sample was injected through the injection valve. The effluent of the column was monitored with an UV detector at 254nm. Peak fractions were collected according to the elution profile.

The preparative separation was similarly performed on 1 g of sample (in 20 mL lower phase) at a flow-rate of 2.0 mL/min at 800 rpm. During the separation, collected fractions were protected by flushing with N2, and after separation they were freeze-dried under a dark condition.

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HPLC Analysis

The polyphenol mixture sample and CCC peak fractions were analyzed using a Shimadzu Class-VP HPLC system equipped with a Phenomenex LUNA C18 column (150 × 4.6 mm ID), a C18 precolumn (4 mm × 2.0 mm ID), and a Photodiode Array (PDA) Detector. The mobile phase, composed of A and B at a 1:1 volume ratio, was pumped at a flow-rate of 1 mL/min, where A: acetic acid: methanol: water (1:1:98, v/v/v) and B: acetic acid: methanol: water: DMF (N, N-dimethylformamide) (1:1:48:50, v/v/v/v).

MS and ^{1}H and ^{13}C NMR Identification

Each catechin component purified by CCC was identified by fast atom bombardment (FAB) MS, and ^{1}H and ^{13}C NMR. FAB-MS was taken on a Finnigan MAT711 Tabspec instrument, while ^{1}H and ^{13}C NMR spectra taken on a Bruker AM-500 spectrometer (in acetone-d_6).

RESULTS AND DISCUSSION

As mentioned above, the starting material for CCC separation was a tea polyphenol mixture. According to the result of HPLC analysis (Fig.2), this mix-

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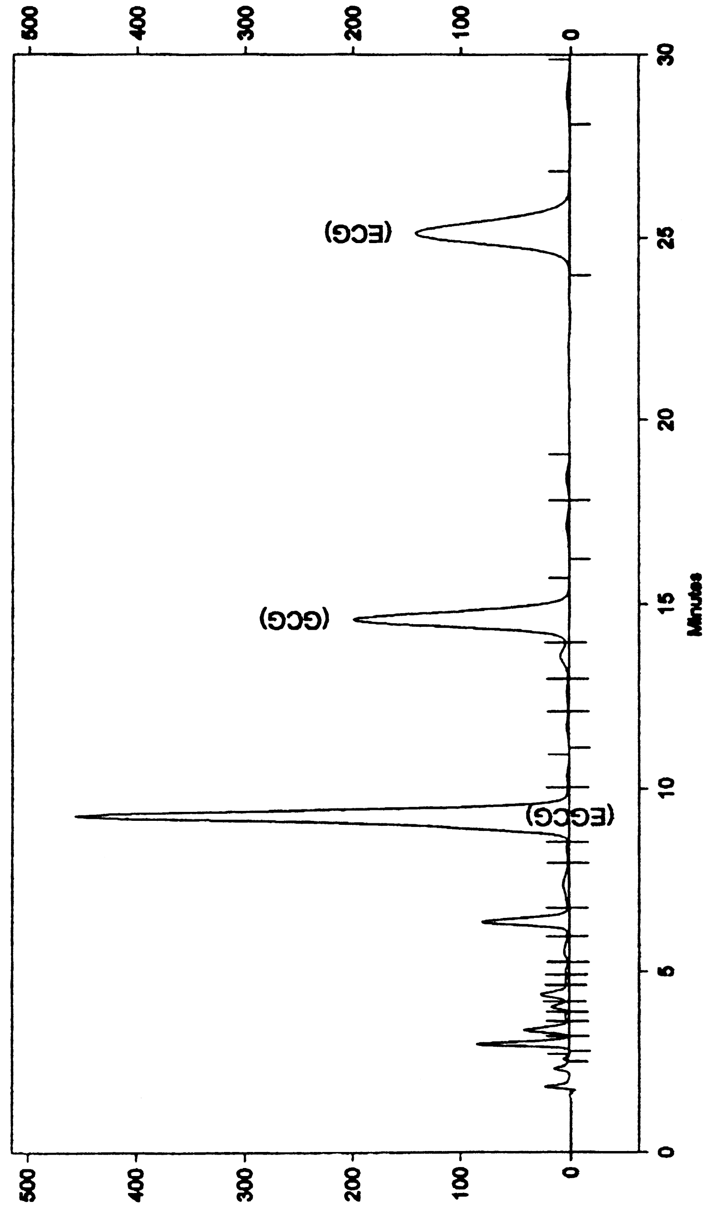

Figure 2. HPLC chromatogram of tea polyphenol mixture. Conditions: Simadzu Class-VP, Phenomenex LUNA C18 column (150 x 4.6 mm I.D.); mobile phase: A: acetic acid:methanol:water (1:1:98, v/v/v), B: acetic acid:methanol: water:DMF (1:1:48:50), A:B 1:1; flow rate: 1 mL/min; detection: 280 nm.

ture mainly contained three catechins that were later identified as EGCG, GCG, and ECG.

During our previous work aimed at obtaining a pure EGCG standard from different sources of material by CCC, it was found that the traditional quaternary solvent system composed of hexane-ethyl acetate-ethanol-water was not suitable to be used for the separation, because EGCG was eluted too quickly and contaminated with closely eluting impurities.

Our purpose of the present studies is to separate EGCG, GCG, and ECG, all at high purity by a single CCC run. In order to achieve this goal, the performance of two different solvent systems, composed of ethyl acetate-ethanol-water and hexane-ethyl acetate-water at various volume ratios, was examined by analytical CCC.

In the ethyl acetate-ethanol-water system, the retention time of three catechins can be conveniently adjusted by relative volume of ethanol in the solvent system. When the relative volume of ethanol was small, ranging from 20:1:20 to 25:1:25, 50:1:50, or even 1:0:1 v/v/v, all catechins were well resolved, whereas, the retention time of GCG and ECG became too long. As the relative volume of ethanol was increased, the retention time of all catechins was decreased, but the early eluting EGCG peak was contaminated with polar impurities. However, these problems were solved by a stepwise elution with three different compositions of the mobile phase, by increasing the relative volume of ethanol as shown in Fig.3.

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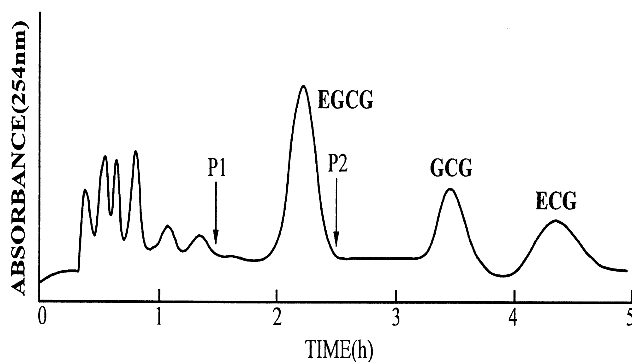

Figure 3. Analytical CCC separation of EGCG, GCG, and ECG from tea polyphenol mixture by ethyl acetate-ethanol-water solvent system. Conditions: apparatus: Model GS20 analytical MLCPC; column: multilayer coil of 0.8 mm I.D. PTFE tubing with a total capacity of 35 mL; rotation speed: 1800 rpm; solvent system: ethyl acetate-ethanol-water (25:1:25, v/v/v) to 10:1:10 (at P1) to 5:1:5 (at P2); mobile phase: lower aqueous phase; flow rate: 1 mL/min; detection: 254 nm; sample size: 16 mg; retention of stationary phase: 51%.

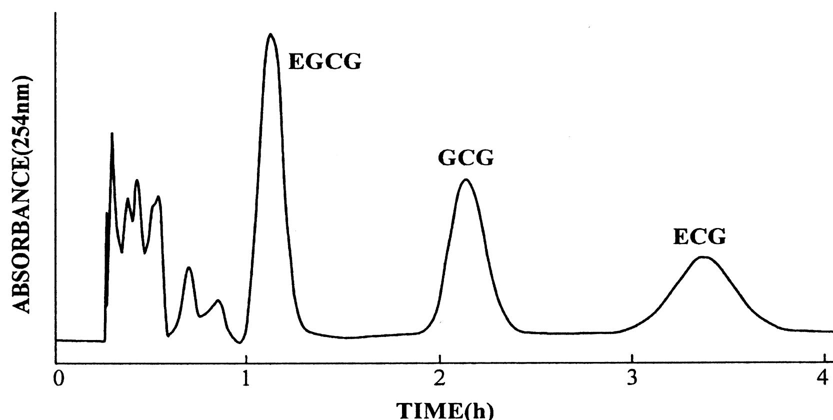

Figure 4. Analytical CCC separation of EGCG, GCG, and ECG from tea polyphenol mixture by hexane-ethyl acetate-water solvent system. Conditions: solvent system: hexane-ethyl acetate-water (1:10:10, v/v/v); sample size: 16 mg; retention of stationary phase: 62%.

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In this separation, the column was first eluted with the first mobile phase (ethyl acetate-ethanol-water, 25:1:25) until all polar impurities were eluted. Then, the mobile phase was switched to the second mobile phase (10:1:10) at P1 to elute the EGCG peak. Finally, the column was eluted with the third mobile phase (5:1:5) at P2 to elute the remaining two catechins. During the whole procedure, no bleeding of stationary phase was observed.

In the hexane-ethyl acetate-water system, the polarity of the solvent system is conveniently adjusted by changing the volume ratio between hexane and ethyl acetate. For example, all catechins including EGCG can be well resolved with polar phase compositions of 1:10:10 (Fig.4), 1:20:20, or 1:30:30 v/v/v.

As shown in Fig. 3 and Fig. 4, three catechins can be successfully separated with two different solvent systems by analytical CCC. However, if these solvent systems were directly applied to preparative CCC, the elution time for GCG and ECG would become excessively long. Therefore, the following two-step separation was performed: In the first step, EGCG was purified from the mixture using the first solvent system composed of ethyl acetate-ethanol-water by stepwise elution from 25:1:25 to 10:1:10, v/v/v, as shown in Fig. 5. Then, the residues in the column were pushed out, concentrated, and subjected to the second step separation using the second solvent system composed of hexane-ethyl acetate-water at 1:4:5, v/v/v, which resulted in elution of pure fractions of GCG and ECG (Fig. 6). This preparative CCC was applied to 1g of a polyphenol mixture yielding 275 mg of EGCG, 140 mg of GCG, and 130 mg of ECG.

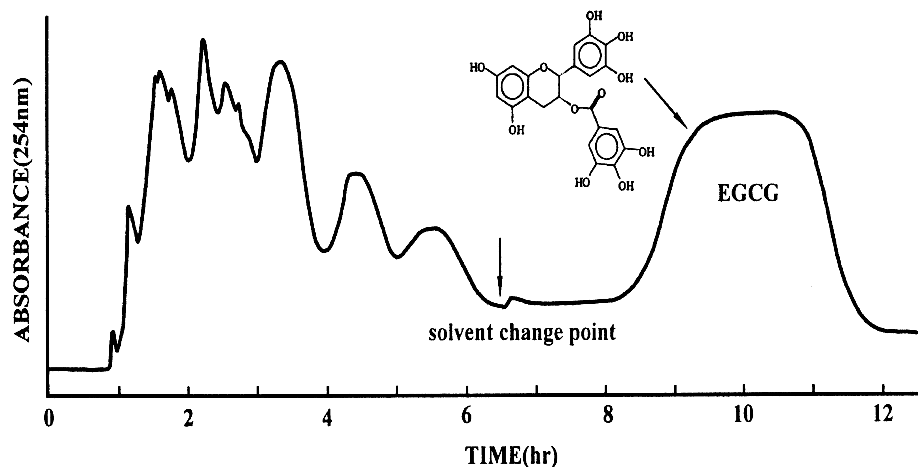

Figure 5. Preparative CCC separation of EGCG from tea polyphenol mixture by ethyl acetate-ethanol-water solvent system. Conditions: apparatus: Model GS10A2 preparative MLCPC; column: Multilayer coil of 1.6 mm I.D. PTFE tubing with a total capacity of 260 mL; rotation speed: 800 rpm; solvent system: ethyl acetate-ethanol-water (25:1:25, v/v/v) to 10:1:10; mobile phase: lower aqueous phase; flow rate: 2 mL/min; detection: 254 nm; sample size: 1 g; retention of stationary phase: 70%.

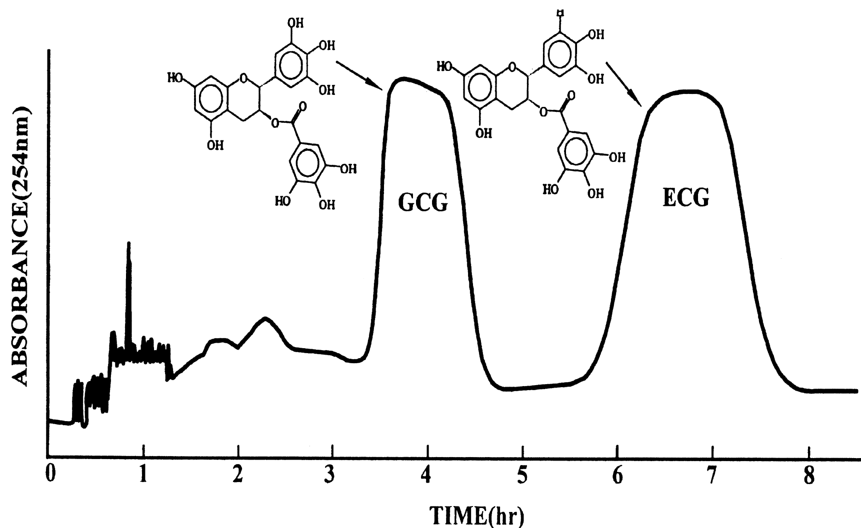

Figure 6. Preparative CCC separation of GCG and ECG from the residual in the column of the first separation by hexane-ethyl acetate-water solvent system. Conditions: solvent system: hexane-ethyl acetate-water (1:4:5,v/v/v); retention of stationary phase: 83%.

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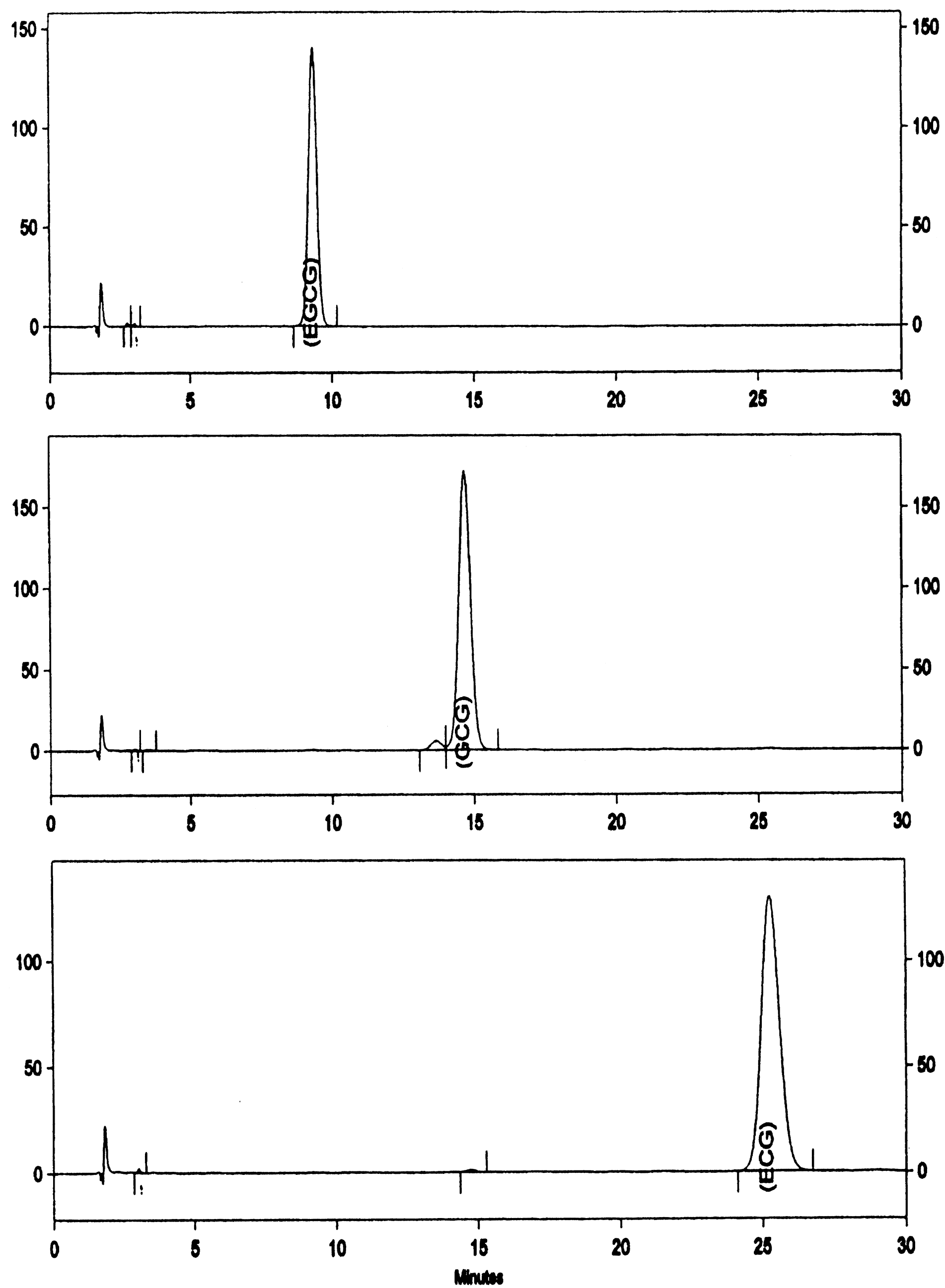

Figure 7. HPLC chromatograms of EGCG (top), GCG (middle) and ECG (bottom) purified by preparative CCC. The condition is described in Figure 2 caption.

Each fraction was analyzed by HPLC with a PDA detector at 280 nm, as shown in Fig. 7 where the first peak in each chromatogram was a solvent peak. From the relative peak area, the purity of each fraction was computed as about 98%. The chromatograms showed no measurable impurity peaks at wavelengths between 200 to 500 nm.

The structure of each catechin obtained by preparative CCC was identified by FAB-MS, 1HNMR, and 13CNMR. The fragments [MH+] in MS spectrum indicate two of the compounds have the identical molecular mass of 458, which corresponds to EGCG and GCG, whereas, the other one has a molecular mass of 442, which corresponds to ECG. The chemical shifts and coupling constants obtained in 1H and 13C NMR spectra, confirmed the assignments of each peak fraction against the reference data published elsewhere.[9]

The overall results of the present studies, demonstrate that CCC is a powerful tool for preparative isolation of individual catechins with high purity from a polyphenol mixture. The sample loading capacity in this preparative separation is greater than that of the standard CCC separation.

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