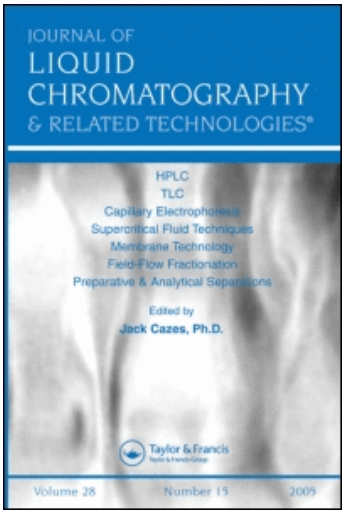


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# SEPARATION OF EPIGALLOCATECHIN GALLATE AND GALLOCATECHIN GALLATE USING MULTIPLE INSTRUMENTS CONNECTED IN SERIES

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## ABSTRACT

A synthetic mixture of epigallocatechin gallate and gallocatechin gallate was separated using multiple countercurrent chromatographic instruments by connecting the separation columns in series. Peak resolution increased according to the formula $R_{s\text{-}n} = n^{1/2}R_{s\text{-}1}$, where n is the number of columns connected; $R_{s\text{-}n}$ and $R_{s\text{-}1}$, the peak resolution obtained from n columns and a single column, respectively. Various sample sizes and concentrations were applied to four columns connected in series. The results indicated that the sample loading capacity is increased 11 times that of the single instrument for the same peak resolution.

## INTRODUCTION

The column capacity of the high-speed CCC instrument is limited; the complete separation or increasing the peak resolution for some components requires the longer length of the separation column. The simplest way of achieving this goal is to use multiple instruments and to connect their separation columns in series. Although it is also possible to recycle the effluent in a single column, this requires a tedious manipulation to store the effluent in a tube which should be inserted between the pump and the inlet of the column.[1]

In this paper, we separated a synthetic mixture of epigallocatechin gallate (EGCG) and gallocatechin gallate (GCG) (see Figure 1) using two to four high-speed countercurrent chromatographic instruments and serially connecting their multilayer coils.



## EXPERIMENTAL

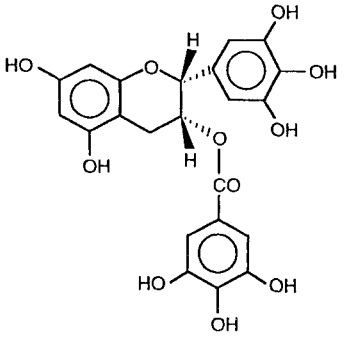
### Apparatus

Four high-speed CCC instruments were used in the present study, each equipped with a multilayer coil separation column. They were designed and fabricated at the Beijing Institute of New Technology Application, Beijing, China. The multilayer coil separation column was prepared by winding a 1.6 mm ID PTFE (polytetrafluoroethylene) tube coaxially onto the column holder hub. The total column capacity was 230 mL.

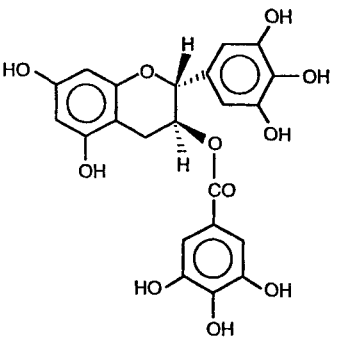
The high-speed CCC centrifuge was rotated at 800 rpm with an 8 cm revolution radius, providing about 56 x g on the axis of the rotating holder. The system was equipped with an FMI pump (Zhejiang Instrument Factory, Hangzhou, China), an injection valve, a UV detector (UV-752, made in Shanghai, China), and a recorder.

### Reagents

Hexane, ethylacetate and methanol were of an analytical grade and were purchased from Shanghai Chemical Factory, Shanghai, China. Epigallocatechin gallate (EGCG) and gallocatechin gallate (GCG), each with over 99% purity, were prepared in our laboratory by high-speed CCC.[2] They were mixed at an appropriate ratio (5:4, w/w) and used for the test sample in the present study.




EGCG




GCG

**Figure 1**. Structures of epigallocatechin gallate (EGCG) and gallocatechin gallate (GCG).



## High-Speed CCC Procedure

The high-speed CCC experiments were performed with a two-phase solvent system composed of hexane-ethyl acetate-water (1:2:3, v/v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use.

In each experiment, the multilayer coil separation column was first entirely filled with the upper, organic stationary phase. Then the column was rotated at 800 rpm followed by injection of the sample dissolved in the aqueous phase. Then the lower aqueous mobile phase was eluted through the column. Both sample injection and the mobile phase elution was performed at a flow rate of 2.5 mL/min. The effluent was continuously monitored with a UV monitor at 280 nm.

## RESULTS AND DISCUSSION

Figure 2 shows the chromatograms of a mixture containing 100 mg of EGCG and 80 mg of GCG obtained from a single column (A) and series of two (B), three (C) and four (D) columns of high-speed CCC instruments. From each chromatogram, the peak resolution ($R_s$) of the two components is computed according to the following formula:

$$R_s = 2(R_2 - R_1)/(W_1 + W_2) \quad (1)$$

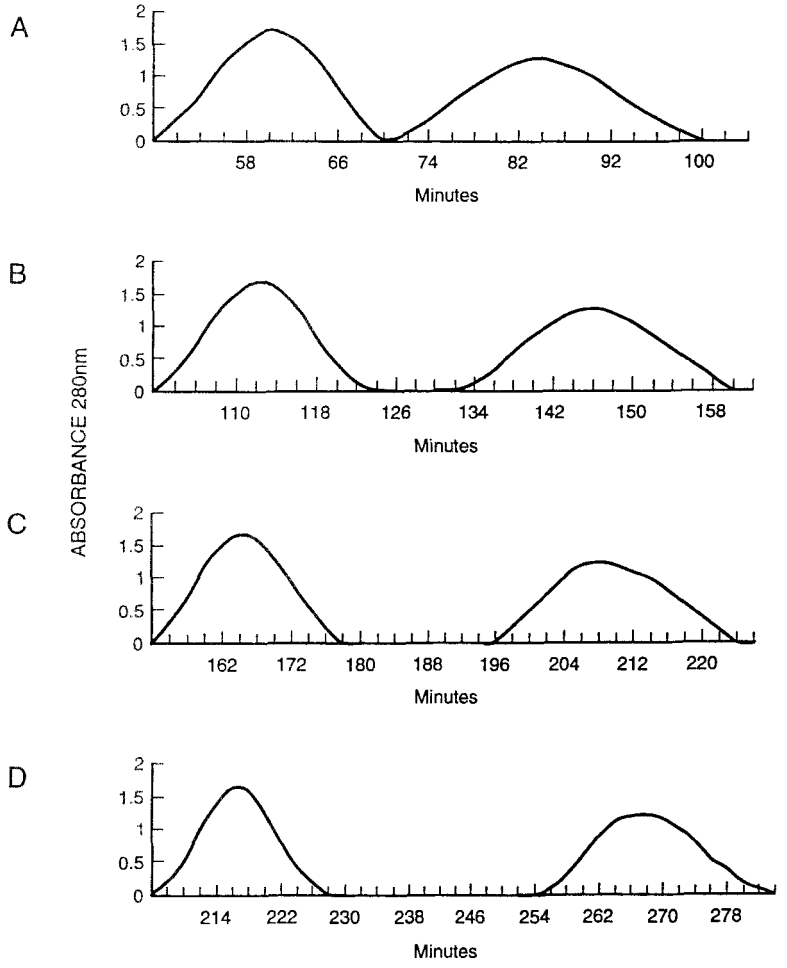



**Figure 2**. High-speed CCC separations of EGCG and GCG. A: Separation of a sample mixture containing 100 mg of EGCG and 80 mg of GCG in 30 mL mobile phase by a single column; B: Separation of the same sample mixture by two serially connected columns; C: Separation of the same sample mixture by three serially connected columns; D: Separation of the same sample mixture by four serially connected columns.

where $W_1$ and $W_2$ are peak widths and $R_1$ and $R_2$, the retention times of the respective peaks. The $R_s$ values of the two peaks for the above four separations are 1.00 (A), 1.40 (B), 1.71 (C) and 1.98 (D). These values are very close to the theoretical figures - 1.00 (A), 1.40 (B), 1.71 (C) and 2.00 (D) - obtained from Equation (2).

**Table 1**

**Peak Resolution Between EGCG and GCG by High-Speed CCC Along With the Retention of the Stationary Phase for Each of Four Columns Connected in Series.**

| Sample Wt.* (mg) & Sample Vol. (mL) | $R_s$ | Retention Volume of SP*** (mL) Column 1 | Column 2 | Column 3 | Column 4 |
|---|---|---|---|---|---|
| 180 (30)** | 1.00 | 160 | 160 | 160 | 160 |
| 180 (30) | 1.98 | 160 | 160 | 160 | 160 |
| 360 (30) | 1.95 | 160 | 160 | 160 | 160 |
| 540 (30) | 1.88 | 152 | 160 | 160 | 160 |
| 720 (30) | 1.73 | 135 | 160 | 160 | 160 |
| 900 (60) | 1.55 | 143 | 160 | 160 | 160 |
| 1080 (60) | 1.45 | 137 | 160 | 160 | 160 |
| 1260 (60) | 1.36 | 133 | 160 | 160 | 160 |
| 1440 (60) | 1.27 | 110 | 152 | 160 | 160 |
| 1620 (90) | 1.14 | 115 | 143 | 160 | 160 |
| 1800 (90) | 1.07 | 93 | 136 | 160 | 160 |
| 1980 (90) | 0.98 | 81 | 133 | 160 | 160 |
| 2160 (90) | 0.91 | 67 | 117 | 156 | 160 |

* The sample consists of EGCG and GCG at 5:4, w/w; ** Separated by a single high-speed CCC instrument; *** SP: stationary phase.

$$R_{s\text{-}n} = n^{1/2} R_{s\text{-}1} \qquad (2)$$

where n indicates the number of columns coupled, and $R_{s\text{-}1}$ & $R_{s\text{-}n}$, the peak resolution obtained from a single column and n-coupled columns, respectively. Thus, the desired $R_s$ values can be obtained by increasing n and the elution time for the separation of closely related compounds. The result indicates that the peak resolution obtainable from n-coupled columns may be predicted from that obtained for a single column.

Table 1 shows a set of data obtained from four serially connected columns, which includes the peak resolution between EGCG and GCG, together with the retention volume of the stationary phase in each of the four columns. From the table, one can observe that the peak resolution decreases with the increased sample size. This problem may be caused not only by the sample overloading, but also by the loss of the stationary phase gradually starting from the first column due to alteration of the physical properties of the solvent system by the high concentration

of the sample. When the mixture of 1100 mg of EGCG and 880 mg of GCG is separated, the peak resolution is 0.98; that is close to the peak resolution (1.0) for 100 mg of EGCG and 80 mg of GCG separated by a single high-speed CCC instrument. This indicates that sample loading capacity for four serially connected columns is equivalent to eleven times that obtained from a single column for a given peak resolution.

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