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Seung Bum Hong[a]; Jung Il Kim[a]; Kyung Ho Row[a]
[a] Center for Advanced Bioseparation Technology and Department of Chemical Engineering, Inha University, Inchon, South Korea

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PREPARATIVE SEPARATION OF EGCG FROM KOREAN GREEN TEA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Seung Bum Hong, Jung Il Kim, and Kyung Ho Row*

Center for Advanced Bioseparation Technology and Department of Chemical Engineering, Inha University, 253 Yonghyun-Dong, Nam-Ku, Inchon 402-751, South Korea

ABSTRACT

Among catechin compounds abundant mainly in green tea, (−)epigallocatechin gallate (EGCG) has antioxidative and anti-carcinogenic properties. Korean green tea (Bosung, Chonnam) was used as a feed sample. The separation was done by a C_{18} reversed-phase preparative column (22 × 250 mm) with mobile phases of 0.1% acetic acid in water, acetonitrile, methanol, and ethyl acetate considered. The injection volume and the flow rate of mobile phase were fixed at 400 μL and 16 mL/min, respectively. To purify EGCG preparatively, the effluent was collected from the column outlet and concentrated, and analyzed by analytical column (3.9 × 300 mm). From the experimental results, the optimum mobile phase condition for separating EGCG from Korean green tea was 0.1% acetic acid in water/acetonitrile/methanol/ethyl acetate (80/16/1/3 vol.%). In the experimental condition, 0.6 mg of EGCG was obtained purely.

*Corresponding author.

Key Words: (−)Epigallocatechin gallate; Green tea; Preparative high performance liquid chromatography; Mobile phase composition

INTRODUCTION

Green tea included in various catechin compounds as (+)catechin [(+)C], (−)epigallocatechin gallate (EGCG), (−)epigallocatechin (EGC), (−)epicatechin gallate (ECG), and (−)epicatechin (EC). These catechin compounds have been proved to have antioxidative and anticarcinogenic properties (1–3). The antithrombotic activities and mode of action of green tea catechins (GTC) and EGCG, a major compound of GTC, were reported (4). Among these catechin compounds, EGCG has the strongest cancer preventive activities. Especially, EGCG acts by inhibiting lung tumorigenesis and acts as a chemopreventive agent for skin cancer (5,6). Epigallocatechin gallate is 25 times more effective than Vitamin E used extensively as antioxidizer and 100 times than Vitamin C (7). Recently, Morre's research team in Purdue University has proved for the first time the anticancer mechanism of green tea by the fact that EGCG restrains necessary secretion of enzyme due to increase in the cancer cells and hence kills them (8). Therefore, the research on EGCG as well as other catechin compounds and the effect of green tea is progressing rapidly. The chemical structural formula of EGCG is $C_{22}H_{18}O_{11}$ and EGCG is named as [2*R*,3*R*]-2-[3,4,5-Tri-hydroxyphenyl]-3,4-dihydro-1[2H]-benzo-pyran-3,5,7-triol3-[3,4,5-trihydroxy-benzoate].

High performance liquid chromatography hinges on speed and resolution for its effectiveness. In preparative HPLC, another factor, namely load, is important. Optimization of one separation parameter affects other separation parameters. Thus, increasing the flow rate results in a decrease in the resolution. Resolution is also impaired if the load is too high, column overloading arising from either an excessive sample volume or excessive sample mass. The loading capacity depends in turn on variables such as column radius, column length, particle diameter, and packing density of the support (9). In this work, in order to separate preparatively pure EGCG, we considered the maximum allowable load with higher purity of EGCG by changing the compositions of mobile phase at a constant injection volume.

The purpose of this study is to develop the optimum mobile phase condition of preparative chromatography at isocratic mode in order to separate EGCG from Korean green tea. The preparative column (22×250 mm) used in this experiment was packed LiChrospher 100RP-18 (15 μm, Merck Co.). The mobile phase was quaternary system like water, acetonitrile, methanol, and ethyl acetate. The composition of mobile phase at the fixed experimental conditions of injection volume and flow rates was adjusted to preparative separation.

EXPERIMENTS

Chemicals

The green tea used in this experiment was cultivated at Bosung (Chonnam, Korea) and purchased from a domestic market. The standard chemicals (+)C, EGCG, ECG, and EC were purchased from Janssen Chimica and Sigma Co. The chemical structure of EGCG was shown in Fig. 1. The extra-pure grade solvents of methanol and acetonitrile were purchased from J. T. Baker (Phillipsburg NJ). Ethyl acetate and chloroform were purchased from Oriental Chemical Industries (Inchon, Korea). Main mobile phase, water was distilled and deionized prior to use.

Extraction and Pretreatment

Initially, catechin compounds were extracted by distilled water at 50°C from 5 g of dry Korean green tea. The green tea was placed in a 500 mL triangle flask with 150 mL distilled water. Then, the neutral extract was filtered and concentrated to 30 mL with a rotary evaporator (Resona technics, Switzerland). The extract was partitioned with an equal volume of chloroform to eliminate impurities. Catechin compounds were extracted from the water layer with an equal volume of ethyl acetate.

Chromatography

The analytical HPLC system in this experiment was Waters Model 600 liquid chromatography equipped with the Waters 616 Multisolvent Delivery

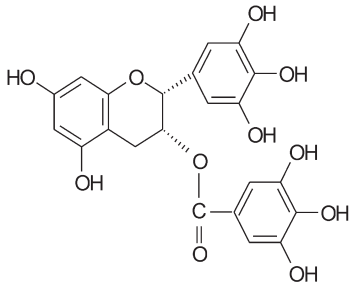

Figure 1. Chemical structure of EGCG.

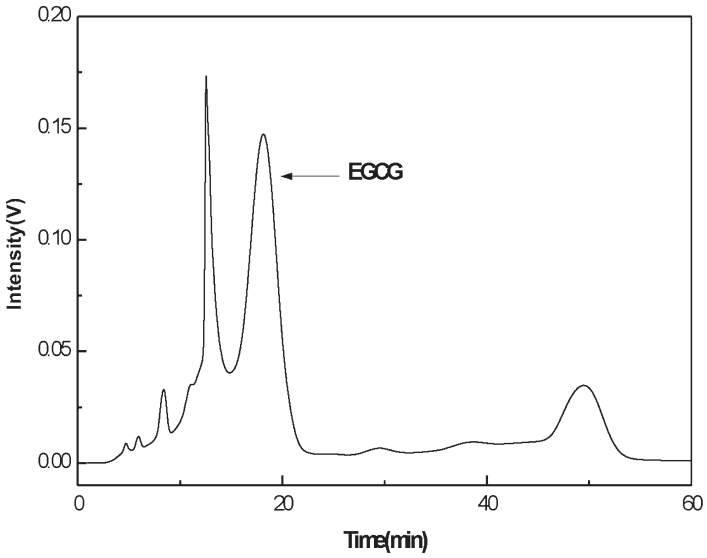

Figure 2. Chromatogram of EGCG in Korean green tea (water/acetonitrile/methanol/acetic acid = 862/130/15/5 vol., 3.9 × 300 mm column, 10 μL injection, 1 mL/min).

System and 2486 Dual λ absorbance. The data acquisition system was MILLENNIUM[32] (Waters Co.) installed in a PC.

The preparative HPLC system in this experiment was Waters Model 600S liquid chromatography (Waters Associates, Milford, MA, USA) equipped with the Waters 515 Multisolvent Delivery System with 486 Tunable Absorbance preparative Detector, and injector (5 mL sample loop) of Rheodyne. The data acquisition system was Chromate (Ver. 3.0, Interface Eng., Korea) installed in a PC.

The wavelength was fixed at 280 nm and the experiment was performed at room temperature. The size of the analytical column was 3.9 × 300 mm, while that of the preparative column was 22 × 250 mm. To purify EGCG among catechin compounds, the packing of LiChrospher 100RP-18 (15 μm, Merck Co.) was in-house packed by pump with solvent. The mobile phases of 0.1% acetic acid in water, methanol, acetonitrile, and ethyl acetate were experimented.

RESULTS AND DISCUSSION

Recently, a study of stationary phases and elution conditions for the analytical HPLC determination of six catechins has been reported (10). The

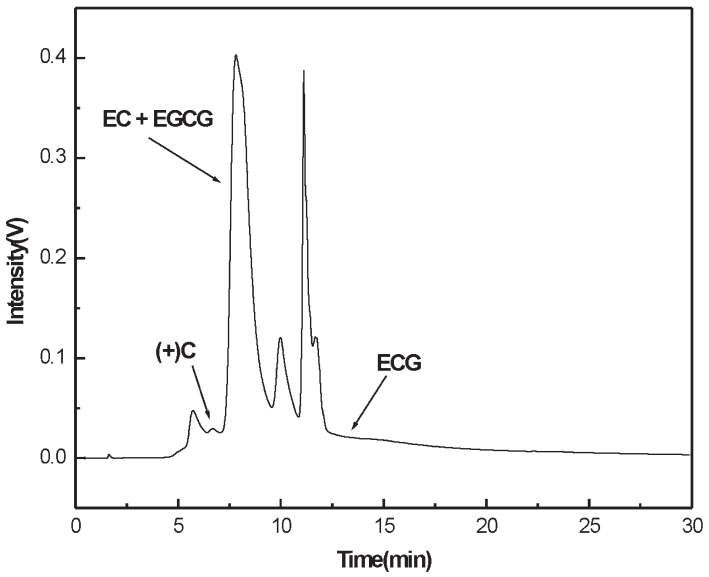

Figure 3. Preparative separation of EGCG from Korean green tea (0.1% acetic acid in water/acetonitrile/methanol = 70/14/16 vol.%, 22 × 250 mm column, 400 μL injection, 16 mL/min).

mobile phase composition was water/methanol/orthophosphoric acid, 79.9/20/0.1 (vol.%), with Kingsorb column (5 μm, 4.6 × 150 mm). They reported that nine catechin compounds including EGCG were well resolved. Resolution is better at gradient mode than at isocratic mode, but operating gradient mode in preparative scale is relatively not simple, compared to isocratic mode with a single pump. Therefore, it is better to use isocratic mode than gradient mode in larger scale. In this work, EGCG was sufficiently separated in the preparative column (15 μm, 22 × 250 mm) at isocratic mode, and the mobile phase composition was 0.1% acetic acid in water/acetonitrile/methanol/ethyl acetate, 80/16/1/3 (vol.%). The injection volume and the flow rate of mobile phase were 400 μL and 16 mL/min, respectively. And the pressure drop through the chromatographic column was ca. 1800 psi.

Figure 2 shows EGCG contained in the water-extract of powder of Korean green tea. The mobile phase composition was water/acetonitrile/methanol/acetic acid 862/130/15/5 (vol.) with analytical column (15 μm, 3.9 × 300 mm). To isolate EGCG efficiently, prior to chromatographic separation, such steps as solvent extraction and partition stage contacting with other solvents were done. Catechin compounds contained in Korean green tea were extracted by dipping for

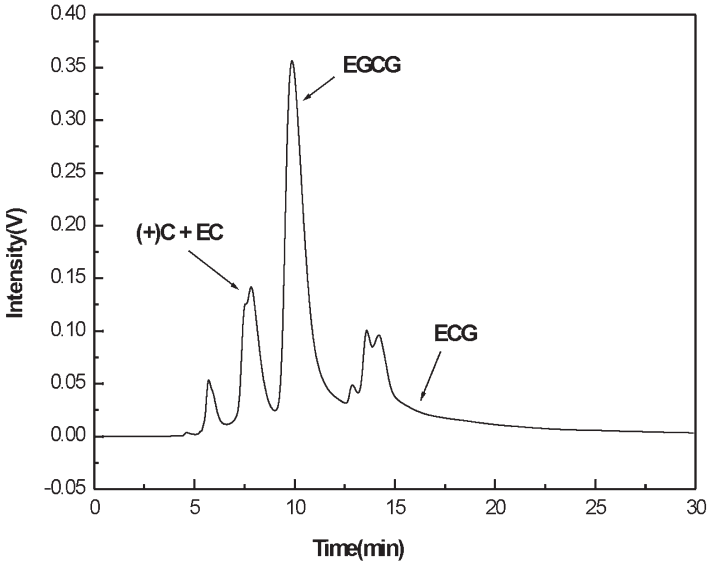

Figure 4. Preparative separation of EGCG from Korean green tea (0.1% acetic acid in water/acetonitrile/ethyl acetate = 80/18/2 vol.%, 22 × 250 mm column, 400 μL injection, 16 mL/min).

4 hr in 50°C water with 300 rpm of agitation speed. To remove unnecessary components, the extract was purified by mixing with chloroform, followed by ethyl acetate (11). The extract through partition process needed the purification process, which used chromatographic column in order to concentrate sample and increase the degree of purity. In reversed-phase, organic modifiers of methanol and acetonitrile were commonly used. The contents of the modifiers were changed in the ternary mobile phase. The mobile phase was composed of 0.1% acetic acid in water/acetonitrile/methanol, 70/14/16 (vol.%), and isocratic mode was applied, but catechin compounds were not well resolved as shown in Fig. 3. In addition, the components of EC and EGCG were almost coeluted around 8 min. In Fig. 4, the mobile phase composition was 0.1% acetic acid in water/acetonitrile/ethyl acetate, 80/18/2 (vol.%). The retention times of the three catechin compounds [(+)C, EC and EGCG] were longer. The retention times of EGCG in Figs. 3 and 4 were 7.817 and 9.867 min, respectively. In addition, it was observed that the components of (+)C and EC were coeluted. But the resolution was improved and, as shown in Fig. 4, adding ethyl acetate in the mobile phase of acetic acid-containing solution resolved EGCG from the neighboring peaks. The resolution (defined by the ratio of the difference of two retention times to average

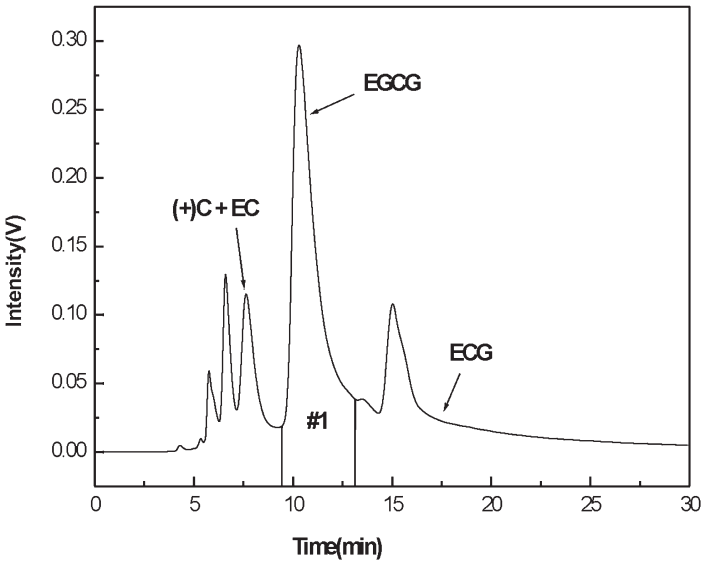

Figure 5. Preparative separation of EGCG from Korean green tea (0.1% acetic acid in water/acetonitrile/methanol/ethyl acetate = 80/16/1/3 vol.%, 22 × 250 mm column, 400 μL injection, 16 mL/min).

peak width) is better in the quaternary mobile phase of water, acetonitrile, methanol, and ethyl acetate in Fig. 5. The resolution between EC and EGCG was 1.69. In terms of column efficiency, the number of theoretical plate of EGCG was 410 compared to 372 in Fig. 4. The mobile phase composition was 0.1% acetic acid in water/acetonitrile/methanol/ethyl acetate, 80/16/1/3 (vol.%) at isocratic mode. It is more advantageous to use this mobile phase composition than that in Fig. 4 to collect EGCG with high purity. Consequently, in this work, this experimental condition (80/16/1/3 vol.%) is the optimal mobile phase composition to separate EGCG preparatively by HPLC. The effluents were collected in the range 9.5–13 min as shown in Fig. 5 (marked as #1), and were concentrated by vaporization. The concentrated EGCG was analyzed with analytical column (15 μm, 3.9 × 300 mm) as shown in Fig. 6. The mobile phase was the same as in Fig. 5. Other catechin compounds [(+)C, EC] were almost resolved and pure EGCG was obtained in this experimental condition. In a single run, the amount of EGCG was 0.6 mg with 98% of purity. In this work, at the constant injection volume of 400 μL and the flow rate of 16 mL/min, the mobile phase compositions were varied to resolve EGCG to the highest purity. If the amount is increased and/or the flow rate of mobile phase is changed, the purity is

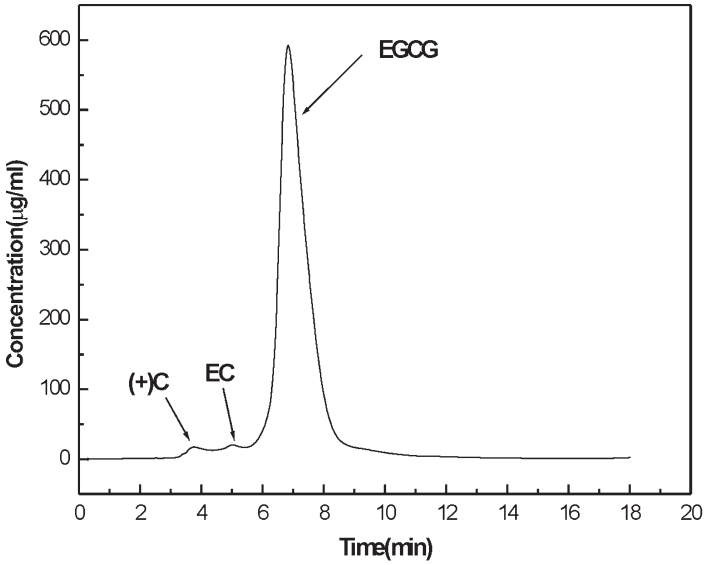

Figure 6. Analytical separation of EGCG from Korean green tea (0.1% acetic acid in water/acetonitrile/methanol/ethyl acetate = 80/16/1/3 vol.%, 3.9 × 300 mm column, 20 μL injection, 1 mL/min).

worse. The amount of EGCG except #1 in Fig. 5 was less than 2%, so the yield was more than 98%.

The larger the packing size is, generally the column efficiency and resolution deteriorate because of smaller contact area of sample with the surface of packing, larger diffusivity, and longer flow paths (12). From the point of economical view, the cost of packings is rarely a topic for commercial separations; however, the pressure drop involved for small particles is the real component, which makes preparative HPLC so expensive. We are working on the effect of particle sizes on the resolution of EGCG with mathematical modeling, and plan to publish the result in the near future to obtain a basic design of the process for scale-up.

CONCLUSION

In this work, the chromatographic column (15 μm, 22 × 250 mm) was used for separating EGCG on preparative scale. The research focused on the separation of large amount of feed injection changing the mobile phase composition, and from the experimental results, the optimal phase condition was

0.1% acetic acid in water/acetonitrile/methanol/ethyl acetate, 80/16/1/3 (vol.%) in isocratic mode. The collected effluent was analyzed by analytical chromatography, and the purity of EGCG was 98%.

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