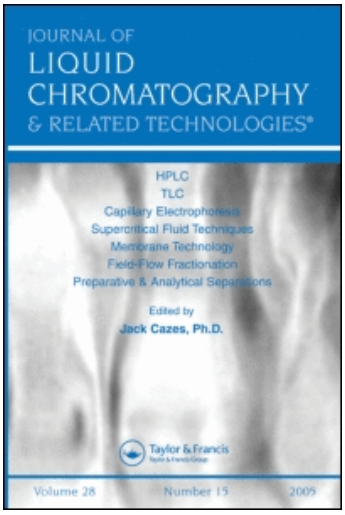

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SEPARATION OF EPIGALLOCATECHIN GALLATE FROM KOREAN GREEN TEA BY RP-HPLC

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ABSTRACT

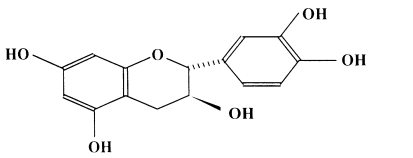
Catechin compounds, abundant mainly in green tea, have chemo-preventive effects against carcinogenesis. The green tea used in this experiment was cultivated at Bosung (Chonnam, Korea) and purchased from a domestic market. The extract at 50°C water from the powder of Korean green tea was partitioned with chloroform and ethyl acetate. The resulting solution was further purified on a preparative column (4.6×250 mm, 15μm). Finally separation was achieved on a μ-Bondapak C_{18} (3.9×300 mm, 10 μm) column.

The elution orders of the catechin compounds contained in the green tea were EGC, C, EC, EGCG, and ECG. Identification of EGCG was confirmed by the standard chemical. The content of EGCG was 121.3 mg/5 g of dry Korean green tea and the purity was higher than 98%. In preparative separation, the optimum mobile phase for isolating EGCG from the extract consisted of 0.1% acetic acid in water/ethyl acetate/acetonitrile, 87/1/12 vol.% with 1.0 mL/min of the mobile phase flow rate.

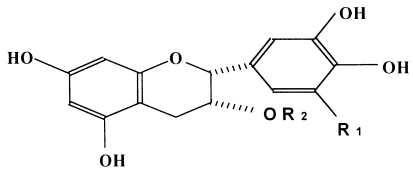
INTRODUCTION

Recently, the demand on green tea has increased due to the concern for health and because of various preferences. Although its chemical composition varies with growing conditions, season, and variety cultivated, the principal catechin compounds included in green tea are (+)catechin (C), (-)epi-gallocatechin gallate (EGCG), (-)epigallocatechin (EGC), (-)epicatechin gallate (ECG), and (-)epicatechin (EC). Figure 1 shows the chemical structures of some of the catechin compounds. These catechin compounds have been proven to have a variety of physiological functions of duodenum, colon, skin, gastric, lung, breast, esophageal, pancreatic, and prostate cancer.[1-5] Industry and governmental research institutes have been actively studying to produce highly pure catechin compounds on a commercial scale. Also, a variety of effects have been reported for these catechin compounds, e.g., dementia prevention, AIDS virus inhibition, microwaves protection, and environmental hormone inhibition, etc.

The most interesting substance in catechin compounds of green tea is EGCG (Epigallocatechin Gallate). Among these catechin compounds, EGCG has the strongest cancer preventive activities. The chemical structural formula of EGCG is $C_{22}H_{18}O_{11}$ and EGCG is named as [2R,3R]-2-[3,4,5-



(+)Catechin (C)



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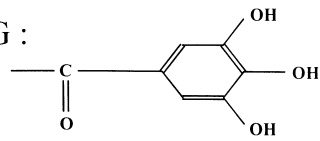

	R_1	R_2
(-)Epicatechin (EC)	H	H
(-)Epigallocatechin (EGC)	OH	H
(-)Epicatechin gallate (ECG)	H	G
(-)Epigallocatechin gallate (EGCG)	OH	G

Figure 1. Chemical structures of catechin compounds.

Trihydroxyphenyl] -3,4-dihydro-1 [2H] -benzopyran-3,5,7-triol 3-[3,4,5-trihydroxybenzoate]. EGCG is twenty-five times more effective than Vitamin E, extensively used as an anti-oxidant, and one hundred times than Vitamin C. ECGC is identified as a non-toxic anti-cancer agent, killing only various cancer cells but not normal cells.[6]

Recently, Morre's research team at Purdue University have proven the fact that EGCG inhibits the necessary secretion of enzymes resulting from growth of the cancer cells, thereby killing the cancer cells and the anti-cancer mechanism of green tea for the first time.[7] Therefore, the research for EGCG as well as other catechin compounds, and the effect of green tea is being undertaken actively.

Okushio et al. analyzed it by using HPLC with a mobile phase of acetonitrile-ethyl acetate-0.05% phosphoric acid aqueous solution (12:2:86) with a Capcell-pak C-18 AG column.[8] Bronner and Beecher partitioned and quantified catechin compounds contained in green tea, jasmine tea, and red tea with a mobile phase of acetonitrile-acetate, acetonitrile-ascorbate, methanol-acetate, in a C_{18} column.[9]

In Korea, Lee et al. concentrated it after extracting green tea at 80°C hot water and separated it by HPLC with 20% methanol and 80% acetonitrile as the mobile phase after partitioning it into ethyl acetate.[10]

The purpose of this research is to establish the extraction, purification, and analysis condition of catechin compounds contained in Korean green tea and to obtain pure EGCG after refining it with preparative column. To investigate the extraction condition, the operating variables were temperature, agitating rate, and dipping time. Under HPLC conditions, the composition was varied through using mobile phases of acetonitrile, water, methanol, ethyl acetate, and acetic acid.

EXPERIMENTAL

Chemicals

The green tea used in this experiment was cultivated at Bosung (Chonnam, Korea, 1997) and purchased from a domestic market. The standard chemicals of (+)catechin (C), (-)epigallocatechin gallate (EGCG), (-)epigallocatechin (EGC), (-)epicatechin gallate (ECG), (-)epicatechin (EC) were purchased from Janssen Chimica and Sigma Co.

The extra-pure grade solvents of methanol, acetonitrile were purchased from J. T. Baker (Phillipsburg NJ, U. S. A.). The water was distilled and deionized prior to use.

Instrumentation and Method

The HPLC systems used in this experiment were: Waters Model 600S liquid chromatograph (Waters Associates, Milford, MA, U. S. A.), Waters 616 Multi-solvent Delivery System, and 2486 Dual λ absorbance. The data acquisition system was Millennium[32] (Waters Co.) installed in a PC. The mobile phases of water, methanol, acetonitrile, ethyl acetate, and acetic acid were experimented with. The chromatographic column packed with Lichrospher 100RP-18 (15μm, Merck Co.) was packed in-house by a vacuum pump and a commercial analytical column was μ-Bondapak C_{18} (3.9×300 mm, 10 μm, Waters Co.).

Initially, catechin compounds from Korean green tea were extracted by distilled water. 5g of dry Korean green tea was weighed and placed in a 500 mL triangle flask with 150 mL distilled water. The operating temperature, agitating rate, and dipping time of extraction were changed. Then, the extract was filtered and concentrated to 30 mL with a rotary evaporator (Resona Technics, Switzerland). The extract were 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.

To concentrate EGCG of ethyl acetate layer in the partition step, a preparative column with Lichrospher 100RP-18 packings (15 μm) with the mobile phase of 0.1% acetic acid in water/acetonitrile, 87/13 vol. % was used. The injection volume and the flow rate of mobile phase were 15 μL/min and 1 mL/min, respectively. The effluent was collected from the column outlet and concentrated to 1 mL for HPLC-analysis. The injection volume was 20 μL and the experimental conditions of mobile phase composition and flow rate were equal to those in the preparative column. In preparative HPLC, the composition of mobile phase was changed in order to improve resolution by adjusting retention times of EGCG.

RESULTS AND DISCUSSION

The operating temperature, agitating rate, and dipping time extraction were changed in the ranges of 30-90°C, 100-700 rpm, 2-8 hours in a stirrer, respectively. From the experimental results, the most of catechin compounds in Korean green tea were extracted with the experimental conditions of, 50°C water for four hours dipping with 300 rpm of agitation speed. The experimental results are listed in Table 1. The resulting chromatogram of the extract in the extraction condition is shown in Figure 2. A preparative column was used in order to remove impurities. The preparative column was packed Lichrospher 100RP-18 (15 μm) and the composition of mobile phase was water/acetonitrile/methanol/acetic acid, 862/130/15/5 (vol.).[11]

Table 1

Amount of EGCG with Operating Conditions

Agitation Rate	Dipping Time	Operating Temperature	Peak Area (Containing EGCG, μV·sec)
300 rpm	4 hour	30°C	58,139,650
300 rpm	4 hour	50°C	87,102,022
300 rpm	4 hour	70°C	46,452,004
300 rpm	4 hour	90°C	37,277,074

Operating Temperature	Dipping Time	Agitation Rate	Peak Area (Containing EGCG, μV·sec
50°C	4 hour	100 rpm	74,261,006
50°C	4 hour	300 rpm	87,102,022
50°C	4 hour	500 rpm	77,321,113
50°C	4 hour	700 rpm	73,092,168

Operating Temperature	Agitation Rate	Dipping Time	Peak Area (Containing EGCG, μV·sec)
50°C	300 rpm	2 hour	80,476,529
50°C	300 rpm	4 hour	87,102,022
50°C	300 rpm	6 hour	78,430,650
50°C	300 rpm	8 hour	69,869,236

In the figure, it was also observed that catechin compounds were not well separated due to many impurities contained in the range of 10 and 23 min. Unnecessary high polar components still remained in the extract from Korean green tea using pure water as an extraction solvent. To remove the unnecessary components, a partition step was included in this work. In the partition process, several solvents such as methylene chloride, ethyl acetate, hexane, ethyl ether, and chloroform were tested.

The volume ratio of the solvent and water extract containing catechin compounds was 1:1 and the experimental result was listed in Table 2. Chloroform was a potential solvent in the partition step because it removed caffeine and pigments from the extract compared with other solvents. The remaining water

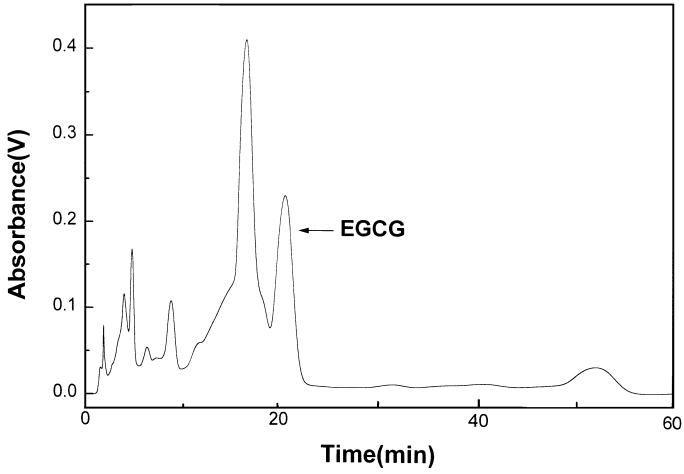

Figure 2. Chromatogram of extract of Korean green tea (preparative column, water/acetonitrile/methanol/acetic acid=862/130/15/5 vol., 10 μL injection, 1.0 mL/min).

Table 2

(+)Catechin and EGCG+EC in the Partition Step with Solvents

Partition Solvent	Layer	(+)Catechin (mV·Sec)	EGCG+EC (mV·Sec)
Methylene chloride	Methylene chloride	×	×
	Water	3400	18708
Ethyl acetate	Ethyl acetate	857	19429
	Water	2192	1319
Hexane	Hexane	×	×
	Water	3879	14311
Ethyl ether	Ethyl ether	299	3885
	Water	4703	19385
Chloroform	Chloroform	×	×
	Water	3320	19269

x = not detected.

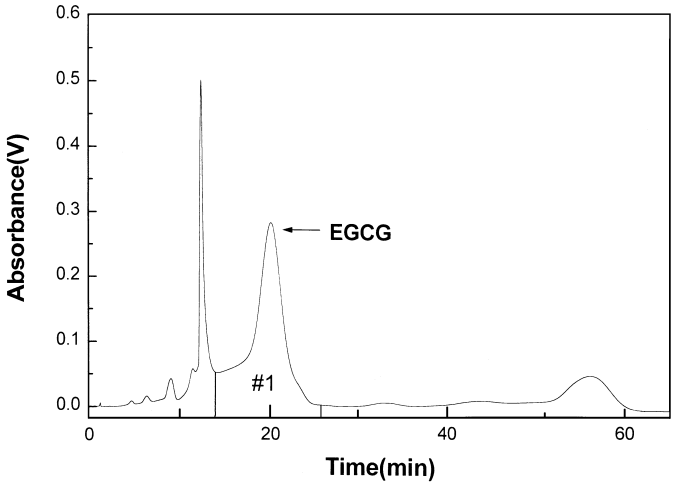

Figure 3. Purification of EGCG in ethyl acetate layer (preparative column, 0.1% acetic acid in water/acetonitrile=87/13 vol.%, 15 μL injection, 1.0 mL/min).

layer was then extracted with ethyl acetate to collect EGCG.[12] As shown in Figure 3, considerable amounts of impurities were removed in the ethyl acetate layer, so more EGCG was obtained.

Separation of the standard chemical of catechin compounds with analytical μ-Bondapak column were performed. The mobile phase was composed of 0.1% acetic acid in water/acetonitrile, 87/13 vol.% in isocratic mode, similar to the protocol previously reported.[13] The catechin compounds were eluted in the order of EGC, (+)C, EC, EGCG, and ECG. To separate EGCG from the extract in Figure 2, two different columns were used. One was a preparative column (4.6×250 mm, 15 μm, Lichrospher 100RP-18) and the other was the μ-Bondapak column.

It was reported that the extract from the partition process requires a purification process which used a Sephadex LH-20 column or a preparative column to significantly increase the degree of purity.[14,15] With the preparative column and the same mobile phase similar to the protocol previously reported,[13] the effluent in the range between 14 and 26 min marked as #1 in Figure 3 was collected and concentrated about one hundred-sixty times by a rotary evaporator. Separation of the concentrate by a μ-Bondapak column was shown in Figure 4. Impurities and other catechin compounds were almost eliminated and only pure EGCG was obtained. The figure shows the resolution of EC and EGCG, increasing the quantity of sample with 20, 100, 250, and 480 μL.

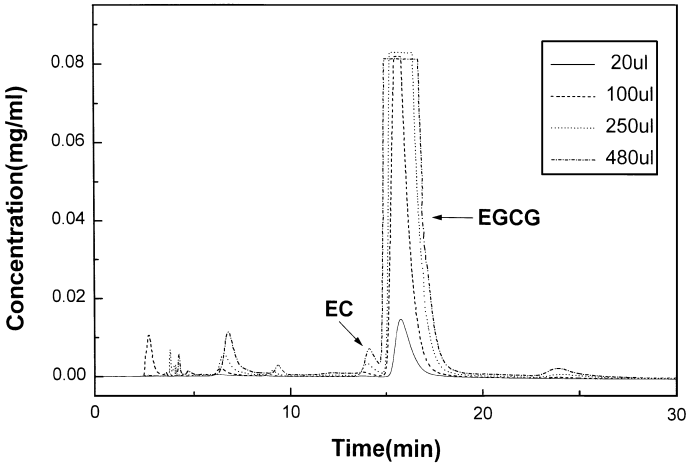

Figure 4. Increase in the injection volume of sample by μ-Bondapak column (the same mobile phase in Fig. 3, 1.0 mL/min).

We changed mobile phases to purify EGCG in the extract after the partition step only with the preparative column. The reverse liquid chromatography used a high polarity index substance as a mobile phase. This research dealt with the variation of the volume ratio of water, acetonitrile, methanol, ethyl acetate, and acetic acid, and their polarity indexes were 10.2, 5.8, 5.1, and 4.4, respectively. It was experimented with the variation of the mobile phase, water/acetonitrile/methanol, water/acetonitrile/methanol/acetic acid, acetic acid in water/acetonitrile, acetic acid in water/ethyl acetate/acetonitrile and water/ethyl acetate/acetonitrile.

Table 3 shows the effect of type and the composition of mobile phase on the retention time and resolution. In the ternary system of water/acetonitrile/methanol without acetic acid, the resolution of EC/EGCG was smaller than 1. In the quaternary system of water/acetonitrile/methanol/acetic acid, 862/130/20/5 vol., adding acetic acid by Yeo's method,[11] improved the resolution of EC/EGCG. Even when adding ethyl acetate in the acetic acid containing solution, the resolution of EC/EGCG was higher than 1.5. The optimum condition of mobile phase in the preparative column was found to be 0.1% acetic acid in water/ethyl acetate/ acetonitrile, 87/1/12 vol.%. Figure 5 shows the resulting chromatogram of the condition of the mobile phase. Compared with Figure 4, only pure EGCG was isolated.

Table 3

Effect of Type and Composition of Mobile Phase on Resolution

Mobile Phase (Vol.%)	Retention Time (Min.) EC	EGCG	Resolution EC/EGCG
Water/acetonitrile/methanol=85/10/5	15.33	20.22	0.78
Water/acetonitrile/methanol=85/13/2	14.40	24.83	0.47
Water/acetonitrile/methanol=85/7/8	33.53	50.68	0.65
Water/acetonitrile/methanol=85/8/7	27.20	48.00	0.55
Water/acetonitrile/methanol/acetic acid=862/130/20/5	17.80	20.08	1.20
0.05% acetic acid in water/acetonitrile=90/10	30.69	37.99	1.59
0.05% acetic acid in water/acetonitrile=88/12	18.87	22.80	1.34
0.1% acetic acid in water/acetonitrile=90/10	27.77	33.49	1.50
0.1% acetic acid in water/acetonitrile=88/12	17.59	20.94	1.03
0.1% acetic acid in water/acetonitrile=87/13	14.50	16.83	1.24
0.2% acetic acid in water/acetonitrile=90/10	28.19	33.89	1.28
0.2% acetic acid in water/acetonitrile=88/12	18.61	22.07	1.24
0.1% acetic acid in water/ethyl acetate/acetonitrile =87/1/12	12.43	17.80	1.92

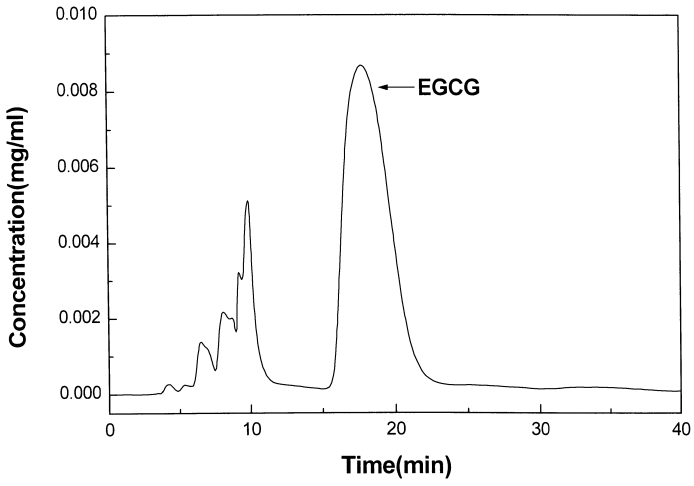

Figure 5. Preparative separation of EGCG in ethyl acetate layer (preparative column, 0.1% acetic acid in water/ethyl acetate /acetonitrile=87/1/12 vol.%, 10 μL injection, 1.0 mL/min).

In summary, to extract the pure EGCG from Korean green tea, the steps of extraction, purification, and separation were performed in this work. Consequently, based on 5 g of dry Korean green tea, 121.3 mg of EGCG was obtained with higher than 98% of purity.

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