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Effect of particle size in preparative reversed-phase high-performance liquid chromatography on the isolation of epigallocatechin gallate from Korean green tea

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Abstract

To isolate epigallocatechin gallate (EGCG) of catechin compounds from Korean green tea (Bosung, Chonnam), a C_{18} reversed-phase preparative column (250×22 mm) packed with packings of three different sizes (15, 40–63, and 150 μm) was used. The sample extracted with water was partitioned with chloroform and ethyl acetate to remove the impurities including caffeine. The mobile phases in this experiment were composed of 0.1% acetic acid in water, acetonitrile, methanol and ethyl acetate. The injection volume was fixed at 400 μl and the flow rate was increased as the particle size becomes larger. The isolation of EGCG with particle size was compared at a preparative scale and the feasibility of separation of EGCG at larger particle sizes was confirmed. The optimum mobile phase composition for separating EGCG was experimentally obtained at the particle sizes of 15 and 40–63 μm in the isocratic mode, but EGCG was not purely separated at the particle size of 150 μm. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Green tea; Preparative chromatography; Particle size; Mobile phase composition; Catechins; Epigallocatechin gallate

1. Introduction

Green tea has been receiving much attention lately, because it contains useful components. Green tea provides catechin compounds, polysaccharides, flavonoids, vitamin B complex, vitamin C, vitamin E, R-aminobutyric acid and fluoride in its natural state. Green tea protects against esophageal cancer and reduces heart disease [1,2]. Green tea catechins (GTCs) are composed of (+)catechin [(+)C], (−)epigallocatechin gallate (EGCG), (−)epigallocatechin (EGC), (−)epicatechin gallate (ECG), (+)epicatechin [(+)EC] and (−)epicatechin [(−)EC]. These catechin compounds have been proved to have anti-oxidative and anticarcinogenic properties [3–6]. The antithrombotic activities and mode of action of GTCs and EGCG, a major compound of GTCs, were reported [7]. The chemical structural formula of EGCG is $C_{22}H_{18}O_{11}$. EGCG acts as a good inhibitor of urokinase and an enzyme crucial for cancer growth [8,9]. Also, EGCG acts on inhibition of lung tumorigenesis and as a chemopreventive agent for skin cancer [10–13]. EGCG is 20 times more active than vitamin C, 30 times more than vitamin E and 2–4 times more active than butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) [14]. Therefore, the research on

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EGCG as well as other catechin compounds and the effect of green tea is being progressed actively.

Reversed-phase preparative high-performance liquid chromatography (HPLC) was applied to the purification of EGCG from green tea. Preparative chromatography is a purification process and aims at the isolation of a pure substance from a mixture. As for production-scale chromatography, the amount of a component that can be separated per unit time is an essential criterion. The mass of solute isolated in a given period of time depends on parameters such as column dimensions and eluent flow rate and is often increased at the expense of purity [15]. HPLC 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 of the resolution [16]. 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 [17]. Different eluent velocities in a chromatographic column lead to a band distortion which has a detrimental effect on the separation performance [18,19].

The mobile phase was a quaternary system of water with a small amount of acetic acid, acetonitrile, methanol and ethyl acetate. The purpose of this study is to investigate the optimum mobile phase condition to separate EGCG in accordance with particle size in an isocratic mode. In a preparative column (250×22 mm), the experiments were performed on three particle sizes of 15, 40–63, and 150 μm.

2. Experimental

2.1. Chemicals

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

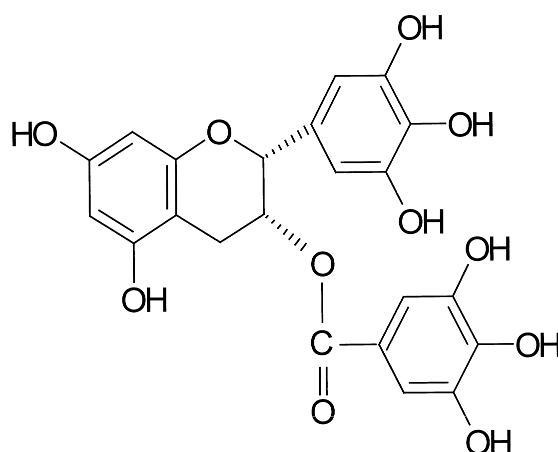

Fig. 1. Chemical structure of EGCG.

2.2. Extraction and pretreatment

Initially, catechin compounds from green tea were extracted by distilled water at 50°C at 300 rpm for 4 h. A 5 g amount of dry Korean green tea was weighed and placed in a 500 ml triangle flask with 150 ml distilled water. Then, the 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. Then, the ethyl acetate layer was filtered with syringe filters (Waters, USA), and injected into the HPLC system without further treatment.

2.3. Chromatography

The analytical HPLC system in this experiment was a Waters Model 600S Controller (Waters Associates, Milford, MA, USA) equipped with a Waters 616 Multisolvent Delivery System and a 2487 dual-

wavelength absorbance detector, and injector (20 μl sample loop) from Rheodyne. The data acquisition system was Millenium[32] (Waters).

The preparative HPLC system in this experiment was a Waters Model 600S liquid chromatography equipped with a Waters 515 Multisolvent Delivery System and a 486 tunable absorbance preparative detector, and injector (5 μl sample loop) from Rheodyne. The data acquisition system was Chromate (Ver. 3.0, Interface Eng., South Korea) installed on a personal computer.

The wavelength was fixed at 280 nm and the experiment was performed at room temperature. The size of the analytical column was 300×3.9 mm (15 μm), while that of the preparative column was 250×22 mm. To purify EGCG from catechin compounds, the packings of ODS (15, 40–63, and 150 μm; YMC) were packed in the laboratory by a pump with solvent. Mobile phases of 0.1% acetic acid in water, methanol, acetonitrile and ethyl acetate were used in this experiment. The flow rates of the mobile phase were fixed at 15, 25 or 30 ml/min with 15, 40–63 or 150 μm ODS, respectively. This experiment was implemented at room temperature.

3. Results and discussion

Recently, as the GTCs contained in green tea have been regarded as anticancer agents, many researchers focused on the separation of the GTCs in the isocratic or gradient mode [20–22]. On an analytical scale, the analysis is normally done in the gradient mode, but the isocratic mode has the great advantage of simpler apparatus, so it is particularly useful in a preparative system. In this work, the mobile phase compositions were varied with the particle sizes to separate EGCG preparatively. The mobile phases used were 0.1% acetic acid in water, acetonitrile, methanol, and ethyl acetate. C_{18} packings of 15, 40–63 and 150 μm were packed in the same preparative column (250×22 mm). At the fixed injection volume of 400 μl, the mobile phase flow rates of the three preparative columns were 16, 25, and 30 ml/min, respectively. The resulting pressure drop through the preparative column ranged from 1600 to 1800 p.s.i. (1 p.s.i.=6894.76 Pa).

Prior to HPLC injections, the water-extract samples were partitioned with chloroform and ethyl acetate in sequence to remove the impurities including caffeine [23]. The partitioned sample was analyzed with an analytical column (15 μm, 300×3.9 mm) to measure the amount of EGCG in the feed sample. Fig. 2 shows the resulting chromatogram. It confirms that a large amount of EGCG was well resolved from the other GTCs. The mobile phase composition was 0.1% acetic acid in water–acetonitrile–ethyl acetate (87:10:3, v/v). In Table 1, the retention times of EGCG were compared in dependence on the content of water and acetonitrile. As the amount of water is smaller and that of acetonitrile is larger, the retention time of EGCG is shorter in reversed-phase HPLC. According to the experimen-

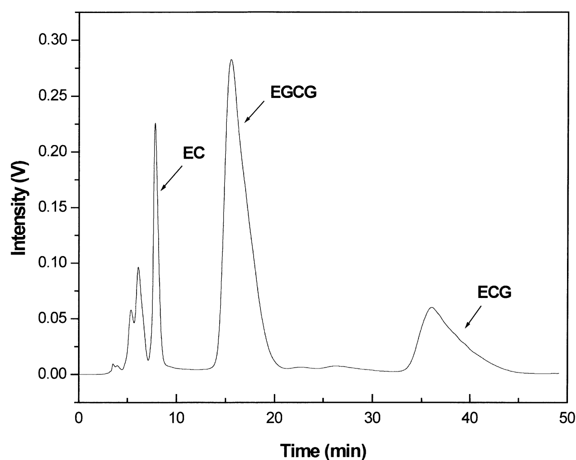

Fig. 2. Analytical separation of EGCG from Korean green tea (0.1% acetic acid in water–acetonitrile–ethyl acetate, 87:10:3, v/v, 300×3.9 mm, 15 μm, 20 μl injection, 1 ml/min).

Table 1
Retention times of EGCG with the content of water and acetonitrile

Particle size (μm)	Retention time of EGCG (min)		
15	79:17:1:3*	80:16:1:3	81:15:1:3
	9.03	10.30	11.51
40~63	84:12:1:3	85:11:1:3	86:10:1:3
	8.28	8.80	9.53
150	87:9:1:3	88:8:1:3	89:7:1:3
	13.78	15.17	20.08

*0.1% acetic acid in water–acetonitrile–methanol–ethyl acetate.

tal results, in the larger particles with the small surface area per unit packed volume, the separation of EGCG was relatively well performed by increasing the water content, as it was retained longer on the C_{18} surface of the packings. Besides, the resolution of EGCG was gradually improved from the neighboring peaks by adjusting the content of methanol and ethyl acetate. Fig. 3 shows the chromatogram at the particle size of 15 μm in a preparative column (250×22 mm). EGCG was separated from other catechin compounds. The mobile phase composition was 0.1% acetic acid in water–acetonitrile–methanol–ethyl acetate (80:16:1:3, v/v). Figs. 4 and 5 show the chromatograms at the particle sizes of 40–63 and 150 μm, respectively. As the particle size becomes larger, generally the column efficiency and resolution deteriorate because of the smaller contact area of the sample with the surface of the solid packings, larger diffusivity, and longer flow paths. However, the resolution of EGCG was not lower. These are interesting results, and this may be attributed to the slightly different mobile phase compositions and flow rates. The mobile phase compositions and the flow rate of mobile phase were 0.1% acetic acid in water–acetonitrile–methanol–ethyl acetate (85:11:1:3, v/v) and 25 ml/min for 40–63 μm, and (88:8:1:3, v/v) and 30 ml/min for 150 μm. To confirm the purity of EGCG in Figs. 3–5, the effluents (marked as 1, 2, and 3, respectively) were collected and vaporized to concentrate for analysis. The samples containing EGCG were analyzed with the analytical column (15 μm, 300×3.9 mm) and the results are shown in Fig. 6. The mobile phase was 0.1% acetic acid in water–acetonitrile–methanol–ethyl acetate (80:16:1:3, v/v). The purity of EGCG was relatively good at 15 and 40–63 μm as seen from Fig. 6(a), (b), but the effluent marked as 3

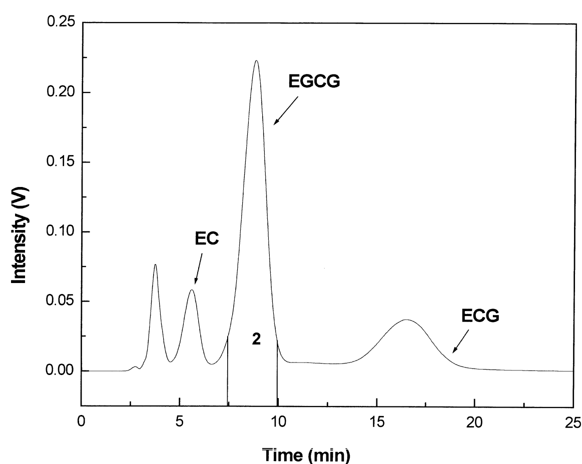

Fig. 4. Preparative separation of EGCG from Korean green tea (0.1% acetic acid in water–acetonitrile–methanol–ethyl acetate, 85:11:1:3, v/v, 250×22 mm, 40–63 μm, 25 ml/min).

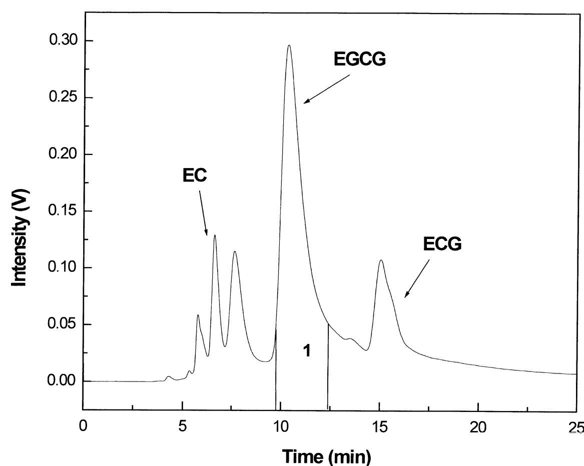

Fig. 3. Preparative separation of EGCG from Korean green tea (0.1% acetic acid in water–acetonitrile–methanol–ethyl acetate, 80:16:1:3, v/v, 250×22 mm, 15 μm, 16 ml/min).

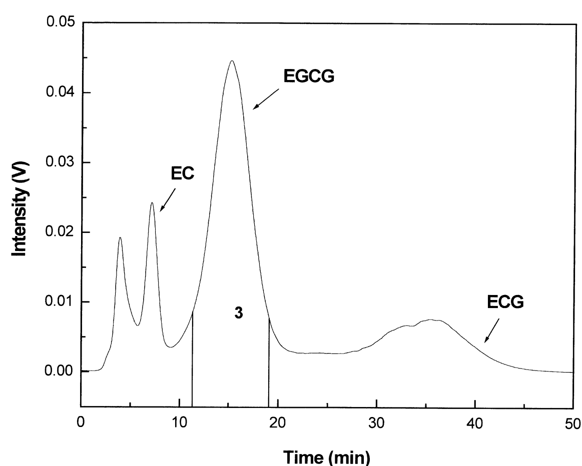

Fig. 5. Preparative separation of EGCG from Korean green tea. (0.1% acetic acid in water–acetonitrile–methanol–ethyl acetate, 88:8:1:3, v/v, 250×22 mm, 150 μm, 30 ml/min).

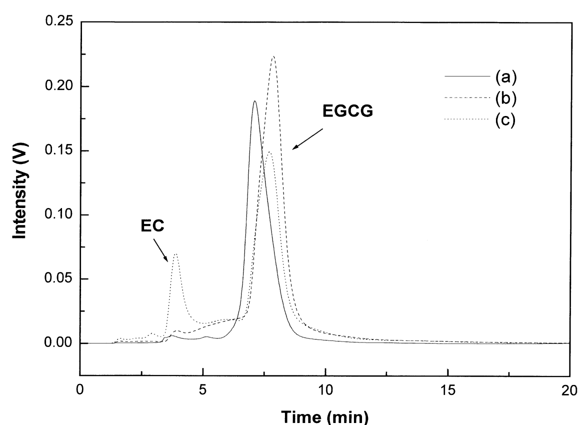

Fig. 6. Chromatogram of the collected effluents. Curves: (a)=1 (Fig. 3), (b)=2 (Fig. 4), (c)=3 (Fig. 5); 0.1% acetic acid in water–acetonitrile–methanol–ethyl acetate (80:16:1:3, v/v), 300× 3.9 mm, 15 μm, 20 μl injection, 1 ml/min.

contained more EC at 150 μm as shown in Fig. 6(c). In Fig. 6, from the unstable baseline between EC and EGCG, we surmised that (−)-gallocatechin gallate [(−)-GCG] was included. Tea catechins undergo many chemical changes, such as oxidation and epimerisation, during the course of the manufacturing and brewing processes. The epimerisation of catechins occurred in green tea infusions using both purified water and tap water at different temperatures [24]. Though EGCG was not separated at the packing size of 150 μm, also EC coeluted with EGCG has strong anti-oxidative and anticarcinogenic properties. Therefore, at the preparative scale, the packing size of 150 μm might be used for producing anti-oxidant and anticancer agents as a mixture of EC and EGCG.

4. Conclusion

In this work, we investigated the optimum mobile phase condition with a preparative column (250×22 mm) to separate EGCG in accordance with the particle sizes in the isocratic mode. The collected effluents were analyzed by analytical chromatography to measure the impurity. EGCG was relatively well separated at the particle sizes of 15 and 40–63 μm, but at 150 μm the purity of EGCG was lower because of the presence of EC. It was confirmed that as the particle size became larger, generally the column efficiency and resolution deteriorated. It was recommended that at the larger packing of 150 μm, EGCG and EC could be collected at a preparative scale.

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