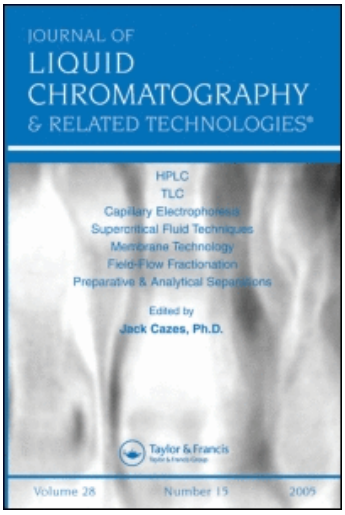

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Separation of Catechin Compounds by Retention Theory in RP-HPLC

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Abstract: An efficient optimization method was used to separate catechin compounds by RP-HPLC. In this work, the binary mobile phase of water and acetonitrile was used with the buffer of acetic acid (AA). The elution profiles were calculated by the plate theory based on the linear and quadratic equations of retention factor, $\ln k = \mathrm{In}k_w + SF$, $\ln k = L + MF + NF^2$, $k = A + B/F$, where F was the vol.% of acetonitrile. We modified the retention theory to calculate the elution profile in both isocratic and gradient modes. The final calculated results showed that the first mobile phase composition was water in 0.1% AA/acetonitrile in 0.1% AA, 90/10 vol.%, followed 30 min later by the second composition of mobile phase, which was linearly changed to 70/30 vol.%. In the experimental conditions, the agreement between the experimental data and the calculated values was relatively good. Catechin compounds from Korean and Chinese green tea as potential powers of anticancer and antioxidant components were target materials in this work. The full content of the catechin compounds of EGC, (+) C, EC, EGCG, and ECG in Chinese green tea extracted was 249.92 mg/g, so it was 2.15 times of that from Korean tea.

Keywords: Mobile phase composition, Retention theory, Retention factor, RP-HPLC, HCI program, Catechin compounds

INTRODUCTION

HPLC is used for the separation, purification, and collection of the single component, the qualitative and quantitative analysis by adsorption effect, division effect, ion exchange effect, and an exclusion effect when the mobile phase passes through the solid phase. Normally, reversed-phase,

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high performance liquid chromatography (RP-HPLC) was used to separate useful compounds from crude plants.

Recent studies have shown that tea confers great beneficial effects on the health of consumers, including reduced cholesterol level and hypertension control, as well as anti-oxidation and anti-microbial effects, and protection against cardiovascular disease and cancer.[1] The main components of green tea are polysaccharides, flavonoids, vitamins B, C, E, R-amino butyric acid, catechin compounds, and fluoride. Among them, catechin compounds have been the focus of investigations into the strong sulfated effect and anti-cancer function. The pharmaceutical activities of the components have been studied.[2–4] The main catechin compounds found in various teas are (−) epigallocatechin (EGC), (+) catechin (+C), (−) epicatechin (EC), (−) epigallocatechin gallate (EGCG), and (−) epicatechin gallate (ECG). These catechin compounds have been proven to have a variety of physiological functions affecting the duodenum, colon, skin, lung, and breast, as well as an effect against esophageal, pancreatic, and prostate cancer.[5–8] EGCG exhibits stronger sulfated effects 20 of 30, and 2 to 4 times higher than vitamins C, E, and BHA or BHT, respectively.[9] Sulfated agents protect the vital cells by combining with and, thereby, rendering harmLess the free radicals before they react with the other vital cells. Therefore, the high sulfated effect of catechin compounds will become more important in the future of the cosmetic industries. Sulfated agents protect the vital cells by combining the free radicals before they react with the other vital cells. Basini et al.[10] demonstrate that EGCG from green tea can negatively affect reproductive performances in swine by inhibiting granulosa cell proliferation. EGCG did not bind and intercalate with the dsDNA, suggesting that it must inhibit the enzymes by interacting with them directly. The same results were obtained with other tea catechin derivatives.[11] Therefore, we advise that the use of EGCG as an alternative for antibiotics in feeding supplementation should be carefully considered.[10]

Optimization in RP-HPLC involves the selection of the optimum experimental condition for adequate separation and acceptable retention time for each individual sample. Nevertheless, obtaining a balance between resolution and analysis time is not always easy in chemical and pharmaceutical laboratories. An efficient optimization method should be employed during the method development process in order to deal with these optimization problems. Computers have been used as an aid in HPLC method development since the late 1970s.[9,12,13] Such software developments have been frequently demonstrated, with an increasing range of applications.[14–18] Especially, an optimization scheme was designed and programmed, with the resulting HCI software being developed by High-Purity Separation Lab., Inha University, to optimize chromatographic separation. The scope of the HCI program is limited to the analytical condition, but it can be utilized in normal-phase as well as reversed-phase liquid chromatography, for both the isocratic and gradient modes. The basic function enables the prediction of sample

retention times with given mobile phase composition, as well as column efficiency and resolution, sample elution profile in specific and optimized operating conditions.

In isocratic elution, the mobile phase composition is unchanged during the separation. The various sample components have a wide range of k values. However, the disadvantages of the isocratic mode are poor resolution of early eluting bands, broadening of late eluting bands making detection difficult, tailing peaks, and unnecessarily long separation time. These disadvantages are often overcome by changing the solvent strength during the operation. Gradient elution is usually performed by changing the mobile phase compositions. The changes in the solvent strength can be made stepwise or continuously. Gradient elution offers several advantages: total analysis time can be significantly reduced, overall resolution of a mixture is increased, peak shape is improved (less tailing), and effective sensitivity is increased since there is little variation in peak shape. More importantly, it provides the maximum resolution per unit time. Optimization of gradient elution is very important for analytical HPLC and scale-up column chromatography. The theory of the gradient elution process contains two general problems. The first one is connected with a total theory of solute migration under stepwise gradient conditions. Under the assumption that the relationships between the capacity factor and composition of the mobile phase are known, this problem was considered. To calculate the retention of solutes in the gradient program having five steps, Markowski and Golkiewicz obtained the analytical expressions.[19] The second problem is to predict the value of the retention factor for any composition of the multi component mobile phase by empirically determined equations. More often the correlations are based on a linear dependence of log k via the content of one or more of the components in the mobile phase for binary and ternary mixtures.[19]

In this work, the mobile phase condition for five biologically active catechin compounds was optimized by the HCI program software. Naturally occurring catechin compounds show a wide variety of biological effects. HPLC is the conventional means of analyzing catechins in tea and additional biological component constituents. A modified equation was suggested to calculate the distances migrated by the solutes in step and gradient modes. The optimum composition of mobile phase for the separation of the five catechin compounds was obtained on the basis of the optimum resolutions and appropriate separation times. The elution profiles in the optimal mobile phase condition and operating mode were calculated by retention theory for comparison with the experimental data. Then, the second goal of this research is to extract and purify the catechin compounds from the Korean and Chinese green tea with the new mobile phase composition on a gradient mode and compare the amounts of five catechin compounds (3,3′,4′,5,7-pentahydroxyflavan and its derivatives) by RP-HPLC. The five catechin compounds chosen were the EGC, +C, EC, EGCG, ECG. Figure 1 shows the chemical structure of five catechin compounds.

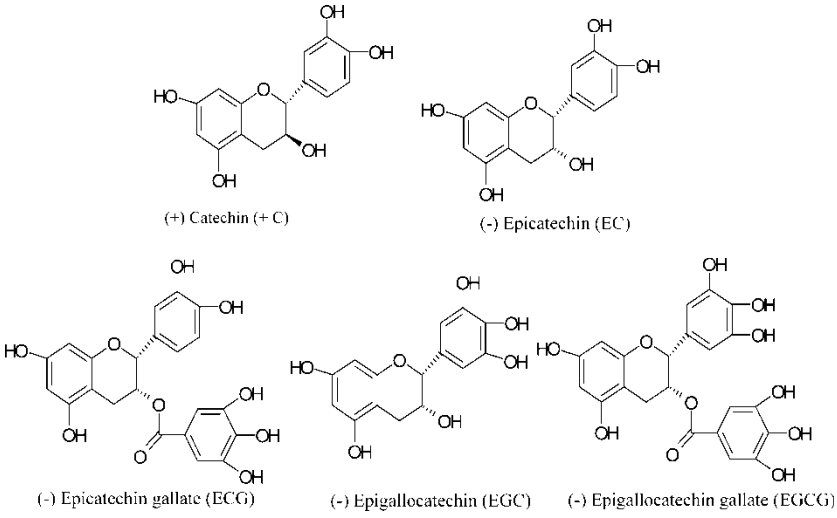

Figure 1. Chemical structure of catechin compounds.

THEORETICAL BACKGROUND

The important parameter for quantification in HPLC is retention factor (k). Retention volume of a sample compound (V_R) can be expressed in terms of the elution volume of an unretained material (V_0). This factor k is given as the ratio of ($V_R - V_0$) to V_0 and is proportional first to the free energy change associated with the chromatographic distribution process, and second to the partition coefficient. Thus, solute retention is affected by the thermodynamics of distribution between the two phases.

In this work, the logarithmic retention factor, k, is correlated by a linear relationship and quadratic relationship involving the vol.% (F) of organic modifier:[20,21]

$$\ln k = \ln(k_w) + SF \tag{1}$$

$$k = A + B/F \tag{2}$$

$$\ln k = L + MF + NF^2 \tag{3}$$

where A, B, L, M, N, $\ln(k_w)$, and S are empirical constants, which should be experimentally determined. Equations (1), (2), and (3) were applied to the binary mobile phase in RP-HPLC. In this work, the above three equations are called Snyder, Langmuir, and Binary-poly equations, respectively. Retention volume in isocratic mode is expressed by the retention factor as follows:

$$Vr, n = V_0(1 + k_n) \tag{4}$$

where $V_{r,n}$, and k_n are the retention volume and retention factor in the nth mobile phase composition, respectively, and V_0 is the dead volume of unretained compound. The prediction of retention time under gradient conditions has been described by assuming that a gradient step is similar to a sequence of short isocratic steps. The modified equation is proposed for predicting the retention volume of step-gradient elution:

$$V_{R,g} = V_0(1 + k_2) + (k_1 - k_2)\frac{V_{g,1}}{k_1} \tag{5}$$

where k_1 and k_2 are the retention factors in the first and second mobile phase compositions, respectively, and are obtained by Eqs. (1), (2), and (3). $V_{g,1}$ is the volume of the first mobile phase in the step gradient elution passing through an inlet of the chromatographic column until the second mobile phase is introduced to the column inlet. It can be calculated by the summation of the HPLC mixer volume and gradient volume. In the case of linear gradient mode, the mobile phase composition changes gradually and continuously. As the linear gradient mode may be envisaged as the infinite small segments of a step gradient, Eq. (5) is modified and extended to a linear gradient mode. The retention volume in the linear gradient mode can be calculated:

$$V_{R,g} = V_{r,\infty} + (V_{r,\infty} - V_0)\sum_{i=1}^{\infty}\frac{V_{r,i} - V_{r,i+1}}{(V_{r,i} - V_0)^2} \tag{6}$$

The number of theoretical plates, N, is calculated in isocratic mode,

$$N = 16\left(\frac{t_R}{\omega}\right)^2 \tag{7}$$

The number of theoretical plates is assumed to be independent of the mobile phase composition throughout this work. It was obtained by the average value from several runs. In gradient mode, the number of theoretical plates was calculated by substituting t_R into t_{Rg} in Eq. (7), w was calculated by substituting t_R into t_{Rg} or $t_{R,n}$ in Eq. (6) according to mobile phase shape and inserting the average value of N. The resolution between components 1 and 2 is given by:

$$R_{12} = \frac{2(t_{R1} - t_{R2})}{\omega_1 - \omega_2} \tag{8}$$

The optimum resolution was obtained by calculating the retention time and peak width from Eqs. (5–7). According to the plate theory, the chromatographic column is mathematically equivalent to a plate column where the total length is divided into N. It is assumed that instantaneous equilibrium is

established for the solute between mobile and stationary phases. A material balance on solute around the plate N leads to the following equation:

$$C_N = C_0 \sum_{i=N-r}^{N-1} \frac{(av)^i}{i!} e^{-av} \tag{9}$$

where c_N is the outlet concentration of solute, c_0 the initial concentration, and a is the following equilibrium constant,

$$a = \frac{1}{v_m + K^{v_s}} \tag{10}$$

where v_m and v_s are the volume of mobile and stationary phases in a theoretical plate, respectively. Equation (9) enables the prediction of the concentration elution profile for each component. The equilibrium constant (K) is correlated in terms of partition coefficient as:

$$K = k\left(\frac{\varepsilon}{1-\varepsilon}\right) \tag{11}$$

where ε is the total porosity of the chromatographic column, and it is assumed to be 0.75.

EXPERIMENTAL

Reagents and Chemicals

Five standard catechin compounds, (−) Epicatechin (EC), (−) Epigallocatechin Gallate (EGCG), (−) Epigallocatechin (EGC), (−) Epicatechin gallate (ECG), and (+) Catechin (+C), were purchased from Sigma (St. Louis, MO, USA). HPLC grade solvent, acetonitrile was from Ducksan Pure Chemical (Kyungki-Do, Korea). Acetic acid (AA) was purchased from Duksan Chemical Co. Ltd. (Ansan, Korea). Twice distilled water was filtered by a decompression pump (Division of Millipore, Waters, USA) and filter (FH-0.45 μm). The Korean green tea used in this experiment was cultivated at Bosung (Chongnam, Korea). The Chinese green tea was purchased from Yanji market (Jilin, China).

Sample Preparation

Five 0.5 mg, standard catechin compounds were dissolved in 1 mL of water, and the concentration of the solutions was adjusted to 500 μg/mL. The constant 20 μL injection volume of mixture solution was used throughout.

Apparatus and Method

The HPLC experiments were performed with Waters 600E pump (Waters, Milford, MA, USA) and 486 UV absorbance detector (Waters, Milford, MA, USA), with Chromate 3.0 data acquisition system (Interface Eng.). Sufficient times were allowed for the stabilization of the column and detector signal after each injection, and the solvents in the reservoirs were continuously stripped with helium to degas the mobile phase. The flow rate of the mobile phase was 1 mL/min and was monitored at the fixed wavelength of 280 nm. The column was purchased from RS-tech Co (Daejeon, Korea). The column size was 0.46 $\times$ 25 cm and packed by C_{18}, 100 Å, 5 μm. Chromate (ver. 3.0 Interface Eng., Korea) connected to a PC was used as a data acquisition system. The extraction was concentrated with a rotary evaporator (Resona Technics, Switzerland). All the experimental runs were carried out at ambient temperature.

Extraction of Components from Tea

The operating temperature, agitating rate, and stirring time extraction, were cited in the referenced paper of Kang et al.[22] and Hong et al.[23] Initially, 2 g of ground leaves of various teas were extracted with 100 mL of pure water at temperatures of 50°C for 4 hours, respectively, under continuous stirring at 775 rpm. Each extraction was filtered by filter paper (pore size: 5 μm). The filtered samples were concentrated to 10 mL by rotary evaporator. The solution was initially partitioned with water/chloroform(1:1 vol.%). Then the water phase was collected and the impurities associated with the chloroform phase were discarded. As a second partition, water/ethyl acetate(1:1 vol.%) was used. Catechin compounds moved into the ethyl acetate layer and were collected for analysis. The solution collected by this method was then injected into the analytical column. All solutions of extracts and standards were filtered by using a filter (MFS-25, 0.2 μm TF, WHATMAN, U.S.A.) before injection into the HPLC system. The 0.5 mg of standard chemicals of catechin compounds were dissolved in 1 mL water and were used for calculation of calibration curves.

Calibration Curve

The calibration curves were constructed to measure the amount of five catechin compounds and the linearly was shown in Table 1. y is the volume of five catechin compounds in the water (μL) of injected samples, while x is the peak area (mV $*$ sec). The 5 μL, 10 μL, 15 μL, and 20 μL volumes of catechin compounds were injected into the HPLC system to calculate the regression coefficient. The regression coefficients of the straight lines in the five catechin compounds were above 0.98.

Table 1. The calibration equations of five catechin compounds

Catechin compounds	Equations	r^2
EGC	$y = 6 \times 10^{-6}x + 1.0831$	0.9950
(+) C	$y = 2 \times 10^{-6}x + 0.9397$	0.9998
EC	$y = 3 \times 10^{-6}x + 1.829$	0.9887
EGCG	$y = 2 \times 10^{-6}x + 0.4047$	0.9940
ECG	$y = 2 \times 10^{-6}x + 1.3117$	0.9985

x: Peak area (mV * Sec).
y: Volume of catechin compounds in the water (μL).

RESULTS AND DISCUSSION

Determination of the Optimum Mobile Phase

To determine the optimum separation condition of the five catechin compounds, several experimental runs were performed in isocratic mode. The required input data for the HCI program included the retention factors, column dead volume, column specification (diameter and length), packing diameter, and mobile phase flow rate to predict elution profiles through a chromatographic column. The retention times of the five catechin compounds were measured with different organic modifier contents. If the retention factor is expressed as a function of mobile phase composition, the elution profile of a solute can be estimated for any change in mobile phase composition by the HCI program.

The linear correlation between ln k and mobile phase composition was assumed as in Eqs. (1) and (2), and the quadratic Equation as in (3). Their regression analyses are presented in Table 2. The regression coefficients of all five catechin compounds approached closely to 1.00 in Eq. (3). In isocratic mode, the binary system of 0.1% AA in water and 0.1% AA in acetonitrile was used. The elution order of the five catechin compounds was EGC, + C, EC, EGCG, and ECG, and it was not changed with mobile phase composition.

In Figure 2, the retention factors of EGCG with content of the acetonitrile in mobile phase were calculated by Binary-poly, Snyder, and Langmuir equations. The calculated retention factor of EGCG by the Binary-poly equation showed close agreement with the experimental data. The agreement of calculated and experimental retention factor was higher than that of the other two equations. In this work, the Binary-poly equation was used to calculate the optimum separation condition. Three other catechin compounds showed a very similar trend to that of EGCG.

In Figure 3, the resolution of catechin compounds with acetonitrile contents in mobile phase were calculated by Snyder (a), Langmuir (b), and Binary-poly (c) equations, respectively. These figures show that the resolution of catechin

Table 2. Parameters of the Snyder, Langmuir and Binary-Poly equations on content of acetonitrile mobile phases

Mobile phase: H_2O/ACN				
Snyder				
Catechin	Ln(kw)	S	r^2	
EGC	2.825	−0.167	0.984	
+C	3.159	−0.160	0.988	
EC	4.367	−0.207	0.989	
EGCG	4.456	−0.208	0.989	
ECG	6.239	−0.253	0.994	
Langmuir				
Catechin	A	B	r^2	
EGC	−2.316	56.103	0.983	
+C	−3.251	81.734	0.987	
EC	−1.973	71.054	0.970	
EGCG	−9.555	207.748	0.9769	
ECG	−40.489	824.194	0.9710	
Binary-Poly				
Catechin	L	M	$N(\times 10^{-2})$	r^2
EGC	4.374	−0.390	0.743	0.999
+C	4.443	−0.345	0.617	0.999
EC	5.916	−0.430	0.743	0.999
EGCG	6.054	−0.438	0.767	0.999
ECG	7.636	−0.454	0.670	0.999

compounds was decreased with increasing acetonitrile contents. At acetonitrile contents above 20%, the resolution of these catechin compounds was very low, below 1.0 and similar. These catechin compounds exhibited good resolutions only in water mobile phase, but, simultaneously, the retention time was very long. Therefore, the optimum separation condition of the five catechin compounds was determined to be gradient mode.

Figure 4 compared the experimental results and calculated resolutions by Snyder, Langmuir, and Binary-poly equations for acetonitrile modifier. The calculated resolutions were slightly larger than the experimental resolutions, but, these results had little influence on the calculated optimum separation condition.

Figure 5 shows the calculated and experimental chromatograms of catechin compounds at acetonitrile mobile phase contents. In Figure 5, the experimental data showed the mobile phase composition of 0.1% AA in water/0.1% AA in acetonitrile, 90/10 vol.%, followed at 30 min later by the second composition of mobile phase, which was linearly changed to 70/30 vol.%. The calculated values were obtained by the HCI program with Eq. (3). In the above optimum separation condition for the acetonitrile

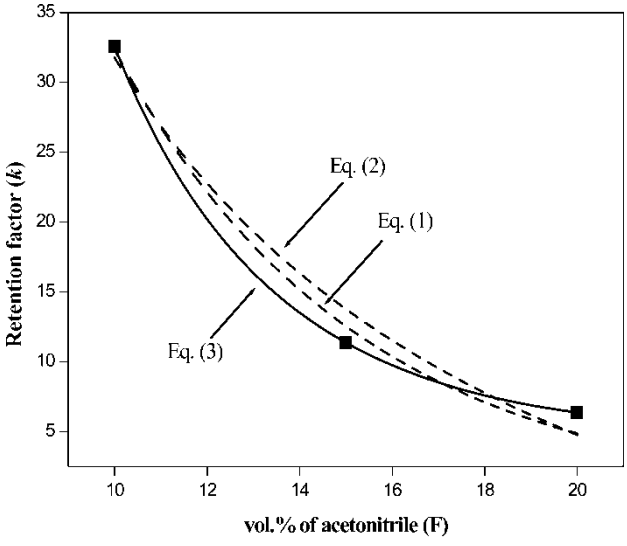

Figure 2. Calculation of the retention factor of EGCG with acetonitrile contents in mobile phase by equation of Snyder (Eq. 1), Langmuir (Eq. 2), and Binary-poly (Eq. 3).

mobile phase, the resolution of EC and EGCG remained the smallest at 0.971, with the other resolutions being more than 2.0. However, in the gradient condition, the quadratic equation of Eq. (3) gave a better fit with the experimental data. Some of the deviation from the experimental data might be attributed to the curvature of the gradient (gradient dispersion or mixing) at the junction of a segmented gradient. In the experimental conditions, the agreement between the experimental data and the calculated values was relatively good.

Separation of Catechin Compounds

The retention times of ECG, + C, EC, EGCG, and EGC were 11.54 min, 13.44 min, 18.16 min, 18.81 min and 25.78 min, respectively. Figure 6 shows the chromatogram of the extract of catechin compounds from Chinese and Korean green tea. Also, the resolutions of the five catechin compounds were fairly good. The content of all the catechin compounds were decreased except EGCG, compared to those.

The amounts of the investigated catechin compounds in Chinese and Korean green teas were calculated by calibration curve equation. The catechins in green tea make up a large percentage of the total amount of polyphenols. Certain catechins, especially epigallocatechin gallate (EGCG) are believed to provide the most protection. Normally, the amounts of

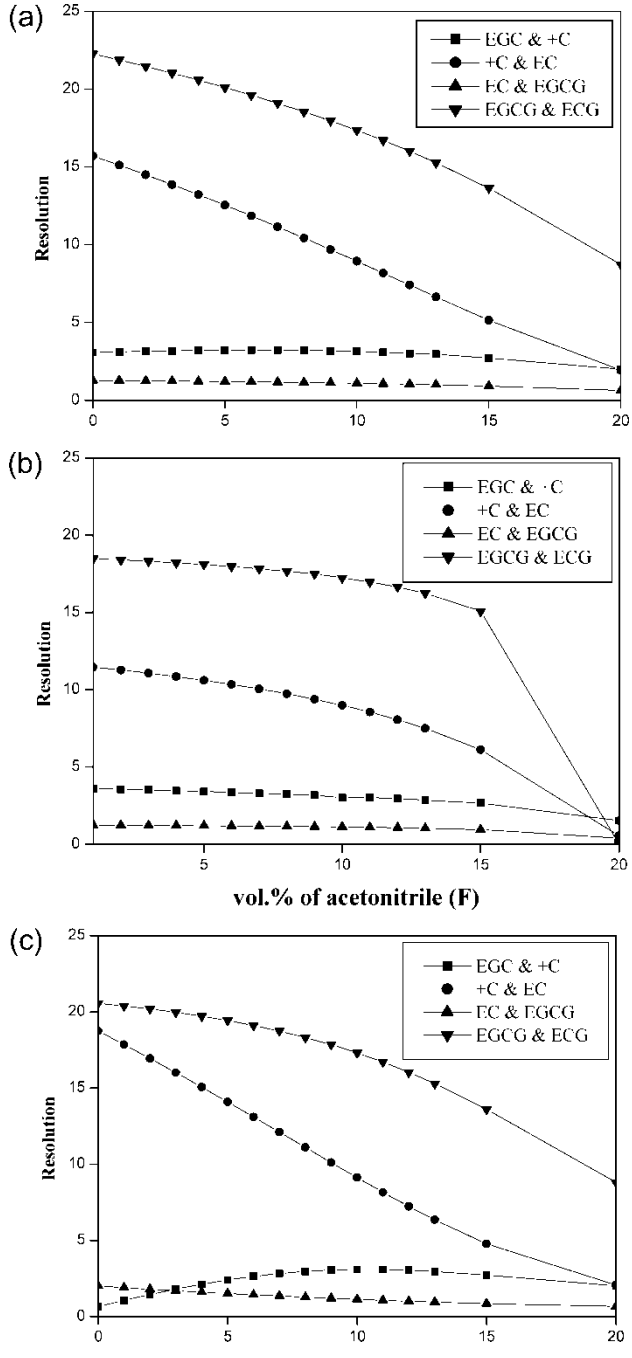

Figure 3. Calculation of the resolution of catechin compounds with acetonitrile contents in mobile phase by equation of Snyder (a), Langmuir (b), and Binary-poly (c).

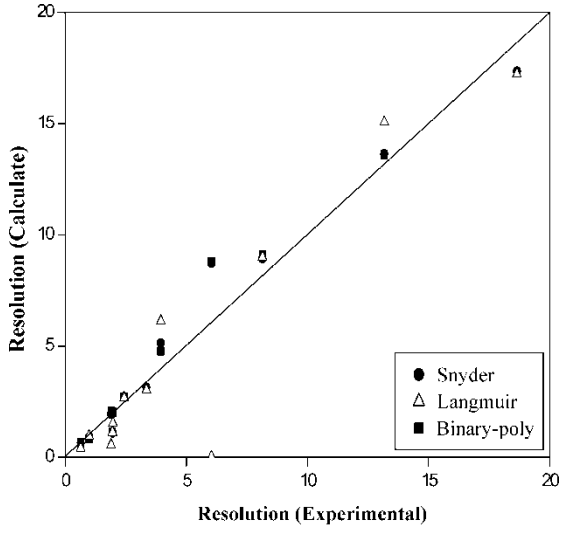

Figure 4. Comparisons of the experimental and calculated resolutions by Snyder, Langmuir, and Binary-poly equations at acetonitrile modifier.

polyphenols, catechins, and EGCG has included 37∼56%, 30∼42%, and 10∼13% of green tea solids, respectively.[24] In this study, the total amounts of five catechin compounds and EGCG from Chinese green tea were 24.99% and 14.95%, respectively. The total amounts of five catechin

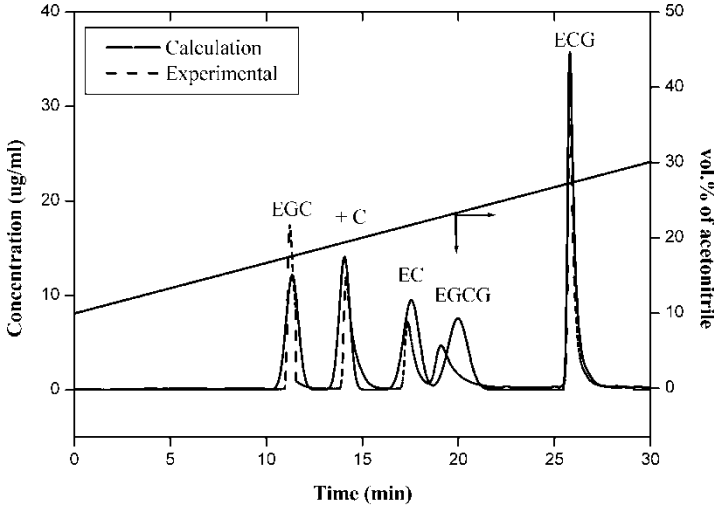

Figure 5. Comparisons of the experimental data and calculated concentration profiles of the catechin compounds in acetonitrile modifier.

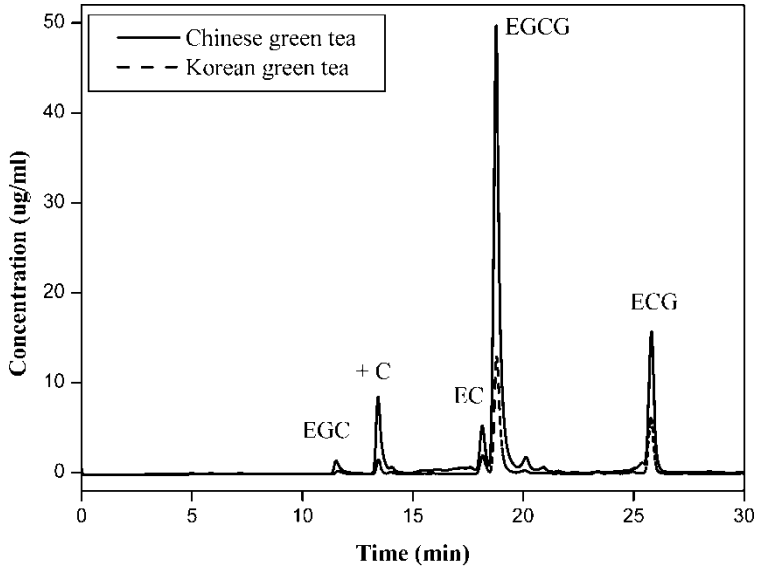

Figure 6. Chromatograms of the catechin compounds extracted from Chinese and Korean green tea.

compounds and EGCG from Korean green tea were 11.64% and 6.14%, respectively. The full content of the five catechin compounds from Chinese green tea was 249.92 mg/g, so it was 2.15 times of that from Korean tea. The amount of the EGCG from Chinese green tea was as 149.54 mg/g, so it was 2.44 times of that from Korean green tea. The amounts of the +C, EC, ECG, and EGC from Chinese green tea were as 22.15, 15.40, 45.73, and 17.10 mg/g, respectively. The amounts of the +C, EC, ECG, and EGC from Korean green tea were as 8.03, 6.78, 30.66, and 9.61 mg/g, respectively.

The amounts of catechin compounds extracted from Chinese tea were more than that from Korean tea. The EGCG were contained in significantly in higher amounts than others. The small amounts of EGC, +C, and EC were obtained. The environmental conditions of a particular crop year might affect catechin compounds content and variety very much. Also, the extracted contents of catechin compounds are very affected by the extraction methods and conditions.

CONCLUSION

The five catechin compounds were separated by changing the mobile phase compositions. Based on the retention theory, elution profiles were predicted by introducing the concept of solute migration in the mobile phase with the linear and quadratic dependency of ln$k$ in terms of the organic modifier

content. Using the HCI program, the recommended experimental conditions of mobile phase composition and gradient step were suggested, and the elution profiles calculated by the quadratic relationship of ln*k* showed better coincidence with the experimental data than the linear correlations did. From the final calculated results, the first mobile phase composition was water in 0.1% AA/acetonitrile in 0.1% AA, 90/10 vol.%, followed 30 min later by the second composition of mobile phase, which was linearly changed to 70/30 vol.%. The amount of catechin compounds in Chinese green tea extracted 114.65% was more than that from the Korean green tea. The EGCG were contained in significantly higher amounts than others. The small amounts of EGC, + C, and EC were obtained.

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