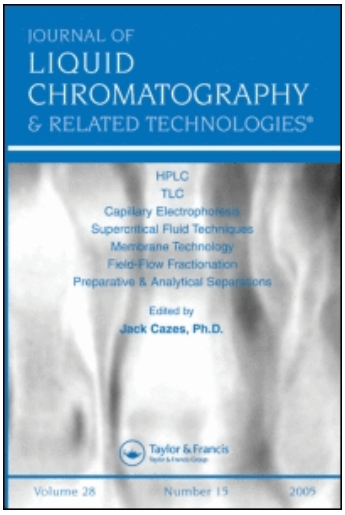

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Determination of Log $P_{o/w}$ for Catechins and Their Isomers, Oligomers, and Other Organic Compounds by Stationary Phase Controlled High-Speed Countercurrent Chromatography

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Abstract: A new technique, stationary phase volume controlled high-speed countercurrent chromatography (HSCCC), was used to determine the octanol-water partition coefficients ($P_{o/w}$) of catechins and their isomers, oligomers, and other organic compounds. The stationary phase volume in the CCC column was effectively controlled under hydrodynamic equilibrium system. The log $P_{o/w}$ values ranging from -1.35 to $+3.60$ were measured within 21 min using this new HSCCC technology. The linear relationship (correlation coefficient value, $r = 0.993$) was observed between log $P_{o/w}$ values obtained by the shake-flask method and those values by the HSCCC method. In this technique, it is possible to inject multiple samples successively

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into the CCC column at short intervals to measure their retention times without renewing the stationary phase volume.

Keywords: High-speed countercurrent chromatography (HSCCC), Stationary phase volume, Stationary phase fraction (S_F) controlled HSCCC, Log $P_{o/w}$ value, Catechins

INTRODUCTION

Logarithm of the octanol-water partition coefficient, log $P_{o/w}$, represents physicochemical properties of drugs and industrial chemicals, and is the most widely employed descriptor for quantitative structure-activity relationships between all kinds of biological, pharmaceutical, and environmental properties.[1–5] During the past two decades, the numerous experimental methods for the determination and the estimation of log $P_{o/w}$ have been reported. These methods may be classified into two categories, a direct determination or an indirect estimation. Standard procedure for the determination of octanol-water partition coefficients, $P_{o/w}$, is the shake-flask method, which can directly determine the $P_{o/w}$ using the octanol-water two-phase system. However, this process is time consuming, tedious, prone to an emulsion problem, and requires a relatively large amount of pure compounds. Chromatographic and electrophoretic methods require very little material and much shorter analysis time, and they are relatively easily automated. Hydrophobicity of the compounds is also estimated using computer calculation. However, these indirect estimation methods do not provide the accurate $P_{o/w}$ of some materials.

Countercurrent chromatography (CCC) is a powerful technique to measure the octanol-water partition coefficients. Since CCC can partition solutes between two immiscible liquids of octanol and water in the absence of a solid support matrix, as in the shake-flask method, it is classified as a reliable direct determination method of $P_{o/w}$ values. Centrifugal partition chromatography has been explored as a novel technique for measuring liquid-liquid partition coefficients.[6–13] Droplet CCC,[14] dual-mode CCC,[15–17] and high-speed CCC.[15,17] are also simple and powerful techniques to calculate the partition coefficient from the elution volume of components using the octanol-water two-phase system. The advantages of using a liquid stationary phase in CCC are: (i) either phase of the octanol-water two phase system can be used as the stationary phase; and (ii) partition coefficient $P_{o/w}$ is calculated from the retention volume of the solute. However, all these CCC techniques require a long analysis time for some components with high hydrophobicity.

Type-J high-speed CCC (HSCCC) is essentially a form of liquid-liquid chromatography in which the stationary phase is retained with the aid of a centrifugal force field and Archimedean screw effect. HSCCC has various advantages over the other CCC techniques, such as short analysis time and high peak resolution.

In the present study aiming at speedy determination of the partition coefficient, $P_{o/w}$, the volume of the upper octanol or lower aqueous

stationary phase in the HSCCC column was controlled according to the hydrophobicity of the analytes. Once the hydrodynamic equilibrium was reached in the HSCCC column, it is possible to inject multiple samples successively, as in HPLC. The retention volumes of several catechins and their isomers, oligomers, and other organic compounds were determined and the $P_{o/w}$ values were calculated. The log $P_{o/w}$ values obtained by stationary phase volume controlled HSCCC technique were very similar to those obtained by the shake-flask method. The log $P_{o/w}$ values were determined within 21 min by HSCCC using an 8 mL-capacity column at 2–8 mL/min.

THEORETICAL STUDY OF HSCCC

As mentioned earlier, high-speed countercurrent chromatography (HSCCC) is essentially a form of liquid-liquid partition chromatography where a solute in the column is distributed between the two immiscible liquid phases according to its partition coefficient (K), which is defined as the ratio of the solute concentration in the stationary phase (C_s) to that in the mobile phase (C_m), i.e., $K = C_s/C_m$. The retention volume (V_R) is conventionally expressed by[18,19]

$$V_R = V_m + KV_s \tag{1}$$

where V_m and V_s are the mobile phase and stationary phase volumes in the coiled column. This equation clearly indicates that the retention time of the solute (V_R), which represents the analysis time of the partition coefficient, increases proportionally with the stationary phase volume (V_s) retained in the column after the solvent front emerges.

In order to regulate the stationary phase volume, we introduce a new dimensionless parameter called the "stationary phase fraction (S_F)", which is a ratio of the stationary phase volume to the column capacity (V_C), i.e.,

$$S_F = V_s/V_C \tag{2}$$

From Equations (1) and (2), we obtain

$$V_R = V_C + S_F V_C(K - 1) \tag{3}$$

where $V_C = V_m + V_s$.

Equation (3) indicates that retention volume of the solute (V_R) is controlled by adjusting the stationary phase fraction (S_F) within a range of $0 < S_F < 1.0$, while the partition coefficient K is easily computed from the retention volume V_R of the solute and column capacity (V_C).

If we use the octanol-water two phase system for CCC, the above equation is applied to determine the octanol-water partition coefficients ($P_{o/w}$). Accordingly, Equation (3) is rewritten as the following form:

$$V_R = V_c + S_F V_c(P_{o/w} - 1) \tag{4}$$

Table 1. Estimated retention volumes and times of solutes with different $P_{O/W}$ values by stationary phase fraction (S_F) controlled HSCCC[a]

Stationary phase fraction (S_F) (amount of stationary phase, mL)	$P_{o/w}$	Log $P_{o/w}$	Retention volume (mL)	Retention time (min)[b]
0.5	2	0.30	100	20
(25)	5	0.70	150	30
	10	1.00	275	55
	100	2.00	2525	505
	1000	3.00	25025	5005
0.2	2	0.30	60	12
(10)	5	0.70	90	18
	10	1.00	140	28
	100	2.00	1040	208
	1000	3.00	10040	2008
0.02	2	0.30	51	10.2
(1)	5	0.70	54	10.8
	10	1.00	59	11.8
	100	2.00	149	29.8
	1000	3.00	1049	209.8

[a]Reversed phase HSCCC with 50 mL capacity column.
[b]Flow rate: 5 mL/min.

Using the HSCCC technique, it is possible to control the stationary phase volume and measure the $P_{o/w}$ value within a reasonable time scale. In Table 1, the estimated retention volumes (mL) and retention times (min) of solutes with different $P_{o/w}$ values were calculated from the theoretical reversed-phase HSCCC, under the stationary phase fraction (S_F) controlled at 0.02, 0.2, and 0.5. The stationary phase is the octanol-rich upper phase and the lower aqueous phase is used as a mobile phase at 5.0 mL/min. Under the stationary phase fraction (S_F) controlled at 0.5, 0.2, and 0.02, the estimated retention volumes and times are increased 20–25 folds as the $P_{o/w}$ values of solutes is increased from 2 to 1000. By controlling S_F at 0.02, the estimated retention time becomes the shortest, and $P_{o/w}$ values of 1000 are determined within 210 min.

EXPERIMENTAL

Apparatus

Figure 1 shows a photograph of the type-J high-speed CCC centrifuge (Hitachi Tokyo Electronics Inc., Tokyo, Japan). The apparatus holds a multilayer coiled separation column and a counter-weight symmetrically at a distance of 10 cm from the central axis of the centrifuge. The large separation

Figure 1. Photograph of the type J coil planet centrifuge.

column was fabricated by winding a single piece of 1.0 mm ID and 50 m long PTFE (polytetrafluoroethylene) tubing (Tokyo Rikakikai Co. Ltd., Tokyo, Japan) directly onto the holder hub making 8 coiled layers between a pair of flanges ($\beta = 0.5–0.66$). The total capacity of the column is about 40 mL. The small separation column consisting of a single layer of coil with 8 mL capacity (10 m long) was also prepared. The revolution speed of the apparatus was regulated at 1000 rpm with a speed controller. The coil rotates around its axis as it simultaneously revolves around a central axis, producing efficient mixing of the two phases while retaining the stationary phase in the column.

Reagents

1-Octanol for determination of the partition coefficient, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were obtained from Kanto Chemicals (Tokyo, Japan). Water was freshly deionized and distilled before use. Test samples, including catechins, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate were purchased from Wako (Osaka, Japan). Other catechins and their oligomers,

(−)-catechin, (+)-epicatechin, (−)-gallocatechin, (−)-catechin gallate, (−)-gallocatechin gallate, procyanidin B1, B2, and C1 were obtained from Funakoshi (Tokyo, Japan). Anthracene and potassium nitrate for the determination of the void volumes of the column for the normal-phase and the reversed-phase CCC operations were obtained from Kanto. Other chemicals were of reagent grade.

Measurement of Settling Time

Vigorous shaking of the octanol-water phase system frequently causes emulsification resulting in a long settling time. To avoid an emulsification of the two-phase system, several concentrations of the potassium phosphate buffers at pH 7.4 are dissolved in the aqueous phase. The settling time of the eight kinds of octanol-aqueous two-phase systems containing various concentrations of potassium phosphate was measured. A 2.0 mL volume of each phase was delivered into a 10 mL capacity graduated glass cylinder, which was then sealed with a glass stopper. The contents were vigorously mixed by inverting the cylinder, which was immediately placed on a flat table to measure the time required for the mixture to settle into two clear layers. The experiment was repeated several times to obtain the mean value.

Measurement of Partition Coefficients by Shake-Flask Method

Partition coefficient values of catechins and their related compounds in octanol-50 mM potassium phosphate two-phase system are directly determined using the conventional shake-flask method on a small scale. The 1.5 mL volume of each phase was delivered into a test tube and 1 mg of the compound was added. The contents were thoroughly mixed and allowed to settle at room temperature. After the clear two layers were formed, an aliquot (usually 0.2 mL) of each phase was diluted with 2.0 mL of methanol and the absorbance was measured at 280 nm using a Jasco V-530 spectrophotometer (Tokyo, Japan). The partition coefficient ($P_{o/w}$) was obtained by dividing the absorbance of the upper octanol phase by that of the lower aqueous phase.

Calculation of Hydrophobicity (Log P_{cal}) from Chemical Structure by Computer Software

The hydrophobicity, log P_{cal}, was calculated from the several data and the chemical structure by the fragment method. The software of Pallas 3.0 (CompuDrug International Inc., San Francisco, CA, USA) was used for the calculation of the log P_{cal}.

Controlled Stationary Phase Fraction

The column (1.0 mm I.D. × 50 m, 40 mL capacity) of the HSCCC apparatus was first entirely filled with the mixture of the octanol-rich upper phase and the potassium phosphate-rich lower phase at a desired ratio. In both the reversed-mode HSCCC (octanol-rich upper phase is stationary phase) and the normal-mode HSCCC (potassium phosphate-rich lower phase is stationary phase), the upper to lower phase volume ratios of 100 : 0, 80 : 20, 60 : 40, 40 : 60, 20 : 80, and 10 : 90 were investigated. In the reversed-phase HSCCC, the potassium phosphate buffer-rich lower mobile phase was eluted through the column at different flow rates ranging from 1.0 to 8.0 mL/min while the apparatus was rotated at 1000 rpm. In the normal-phase mode, the octanol-rich upper phase was similarly used as the mobile phase. The effluent from the outlet of the column was collected into a 100 mL graduated cylinder to measure the volume of the stationary phase eluted from the column, as well as the total elution volume of the mobile phase. Once the mobile phase eluted at the outlet of the column, the centrifuge run was stopped and the column contents emptied into a graduated cylinder by connecting the inlet of the column to a pressured nitrogen line. The volume ratio of the stationary phase to the mobile phase in the column contents was measured to calculate the stationary phase fraction (S_F).

Stationary Phase Fraction Controlled High-Speed Countercurrent Chromatography with a Large Capacity Column

The column (1.0 mm I. D. × 50 m, 40 mL capacity) of the CCC apparatus was first entirely filled with the desired volume ratio between the octanol and phosphate buffer phases. In the reversed-phase HSCCC, the potassium phosphate buffer-rich lower mobile phase was eluted through the column while the apparatus was rotated at 1000 rpm. After the upper octanol phase and the lower aqueous phase reached the hydrodynamic equilibrium in the coiled column and the base line was stabilized, the 100 μL solution containing 0.25 mg potassium nitrate as a dead volume marker and 1.0 mg sample was injected into a Reodyne 7166 injector (Reodyne, Rohnert Park, CA, USA). The effluent was continuously monitored with a type L-7455 diode array detector (Hitach, Tokyo, Japan). In the normal-phase HSCCC mode, the octanol-rich upper phase was similarly used as the mobile phase with anthracene as a dead volume marker. From the obtained retention volume of an anthracene and that of the solute, stationary phase fraction (S_F) and the total capacity from the injector to the detector including the column volume, the partition coefficient $P_{o/w}$ of the solute was calculated.

Stationary Phase Fraction Controlled High-Speed Countercurrent Chromatography with a Small Capacity Column

For the rapid determination of log $P_{o/w}$ values for a short time, we used a small capacity column (1.0 mm I. D. × 10 m, 8 mL capacity). The CCC experiment was performed under the same conditions as applied to the large capacity column as described above.

RESULTS AND DISCUSSION

Settling Time

In HSCCC, the retention of the stationary phase is influenced by the viscosity and the interfacial tension of the two-phase systems. The octanol-water system is highly viscous and required a long settling time due to a high tendency of emulsification. This problem is largely solved by adding a small amount of salt in the aqueous phase, which minimizes the emulsification of the two phases in the column and increases the retention of the stationary phase. The use of a phosphate buffer solution at pH 7.4 further provides important advantages, such as preventing formation of the skewed peaks by ionized analytes and providing a physiological environment. For the determination of a suitable buffer concentration in the aqueous phase, the settling times of the several octanol-potassium phosphate two-phase systems are measured. As shown in Figure 2, when the concentrations of the potassium phosphate buffer are increased from 5 mM to 50 mM, the settling time of the two phases decreased from 150 s to 37 s. Further increase of the buffer concentration to 100–200 mM, however, failed to improve the settling times. In general, a settling

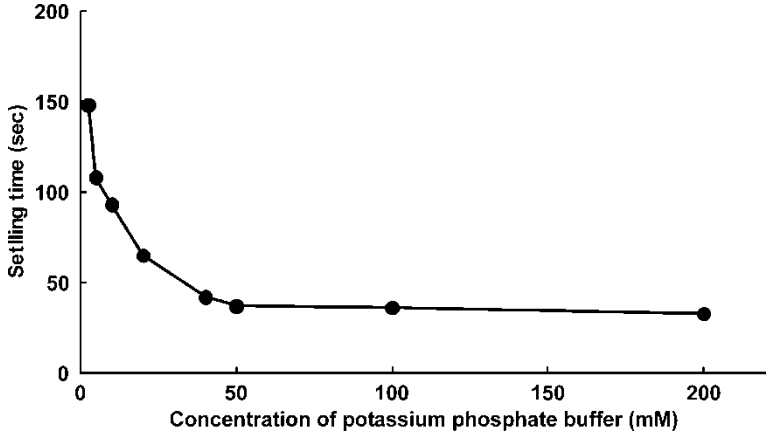


Figure 2. Influence of concentration of potassium phosphate buffer at pH 7.4 on the settling time of octanol-potassium phosphate buffer two-phase system.

time of around 30 s show low-viscosity behavior and may be run in the multilayer-coil CCC using the low-viscosity flow mode.[18] Then we used the octanol-50 mM potassium phosphate buffer at a neutral pH of 7.4 for the determination of the partition coefficient of the solute in the present study.

The log $P_{o/w}$ values of hydrophobic aromatic compounds using the octanol-50 mM potassium phosphate buffer at pH 7.0 are very similar to those obtained in the octanol-water system by the shake-flask method.[20] As described below, the HSCCC method is found to be very useful for the determination of log $P_{o/w}$ values from -1.35 to $+3.60$.

Control of Stationary Phase Fraction

In the conventional HSCCC technique, it requires a long time to elute the analytes with a partition coefficient of over 5 under the reversed-phase mode, if the retention of the stationary phase is in a normal range. However, if the amount of the stationary phase in the column is reduced, the analysis of such analytes will be carried out within a reasonable time scale. In the conventional HSCCC technique, the column is first entirely filled with the stationary phase, followed by elution with the mobile phase, to maximize stationary phase volume retained in the column. In the present studies, however, the column is first filled with a mixture of the two phases at various volume ratios according to the partition coefficient of the analytes, and this limits the amount of the stationary phase retained in the column.

Table 2 shows the relationship between the volume of the two phases initially introduced into the separation column and the stationary phase fractions measured after the hydrodynamic equilibrium is established in the column. In the normal-phase CCC mode, increasing the volume ratio of the upper mobile phase resulted in a significant decrease of the stationary phase fraction. When the volume ratio of the mobile phase was increased from 60 to 98, the stationary phase fraction was decreased from 0.36 to 0.004. In the reversed-phase CCC mode, the stationary phase fraction, after the equilibrium, greatly depends on the initial ratio between the upper phase (stationary phase) and the lower phase (mobile phase) in the column. When the volume ratio of the stationary phase was decreased below 20, the stationary phase fraction sharply decreased, so that it was possible to control the stationary phase fraction between 0.281 and 0.015. At every UP/LP ratio, the stationary phase fraction in the reversed-phase CCC is higher than those in the normal-phase CCC.

Determination of $P_{O/W}$ of Catechins and Related Compounds Obtained by Stationary Phase Fraction Controlled CCC

Chemical structures of catechin and its related compounds are shown in Figure 3. The partition coefficient values of these compounds were determined

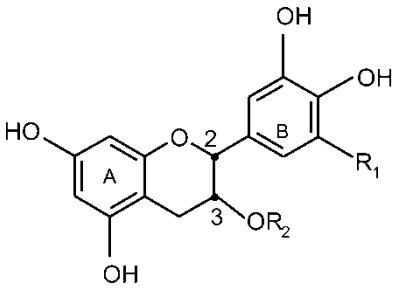
Table 2. Influence of ratio of packed upper and lower phase in CCC column on stationary phase fraction (S_F)

UP[a]	LP[b]	S_F
Normal-phase CCC[c]		
0	100	0.400
20	80	0.363
40	60	0.370
60	40	0.360
70	30	0.266
80	20	0.186
90	10	0.090
95	5	0.044
98	2	0.004
Reversed-phase CCC[c]		
100	0	0.630
80	20	0.613
60	40	0.612
50	50	0.591
40	60	0.490
30	70	0.406
20	80	0.281
10	90	0.141
5	95	0.061
2	98	0.030
1	99	0.015

[a]UP: Upper phase, [b]LP: Lower phase, [c]flow-rate: 1.0 mL/min.

by the stationary phase controlled CCC technique using a large capacity column. In this experiment, the hydrophilic compounds with log $P_{o/w}$ values below zero were determined by the normal-phase CCC mode where the lower aqueous phase was used as a stationary phase, whereas the hydrophobic compounds having log $P_{o/w}$ over zero were obtained by the reversed-phase CCC mode using octanol-rich upper phase as a stationary phase.

Figure 4A shows the chromatograms of the reversed-phase stationary phase fraction controlled CCC of epicatechin (EC) and epicatechin gallate (EC_g). The exact void volume (V_0) was measured by the elution volume of potassium phosphate phase, and the stationary phase volume (V_s) was calculated from column capacity (V_c). The retention time of EC was 45.2 min under the stationary phase fraction of 0.418. ECg with higher hydrophobicity than EC was also eluted under a small amount of stationary phase fraction, $S_F = 0.033$ with the retention time of 57.8 min. The log $P_{o/w}$ values of 0.1



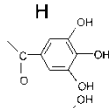
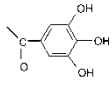
		cis	trans
R_1	R_2	(2R, 3R) (2S, 3S)	(2R, 3S) (2S, 3R)
H	H	epicatechin (EC)	catechin (C)
OH	H	epigallocatechin (EGC)	gallocatechin (GC)
OH		epigallocatechin gallate(EGCg)	gallocatechin gallate (GCg)
H		epicatechin gallate (ECg)	catechin gallate (Cg)

Figure 3. Chemical structures of catechins and related compounds.

for EC, 1.10 for ECg, were calculated from the retention time and the stationary phase fraction. The hydrophobicity of ECg is greater than that of EC. It shows that the gallate ester formation of epicatechin increases the hydrophobicity of the molecule.

Figure 4B shows the chromatogram of the normal-phase HSCCC mode of epigallocatechin (EGC). A void volume was estimated from the elution volume of anthracene. The retention time of 52.3 min was obtained under the stationary phase fraction of 0.101. The log $P_{o/w}$ value of EGC, which has tri-hydroxyl groups on the B ring, was measured by the normal-phase HSCCC mode. The log $P_{o/w}$ value of EGC was -0.53. This showed that the increase of one hydroxyl group on the B-ring of epicatechin decreased log $P_{o/w}$ value of the molecule, indicating that EGC is more polar than EC.

Table 3 shows the log $P_{o/w}$ values of catechin isomers determined by HSCCC together with the calculated values by personal computer software named PALLAS ver. 3. Since catechin has two asymmetric carbon atoms at 2 and 3 positions of A ring, each monomer molecule has a set of cis-trans diastereomers (EC, C) and a set of enantiomers ((−)-EC, (+)-EC). It was found that the enantiomers, such as (−)-EC and (+)-EC have identical log $P_{o/w}$ values of 0.10. On the other hand, the log $P_{o/w}$ values of four sets of cis-trans diastereomers, (EC, C), (EGC, GC), (EGCg, GCg), and (ECg, Cg), were significantly different. It was very interesting that the trans-form catechin molecules

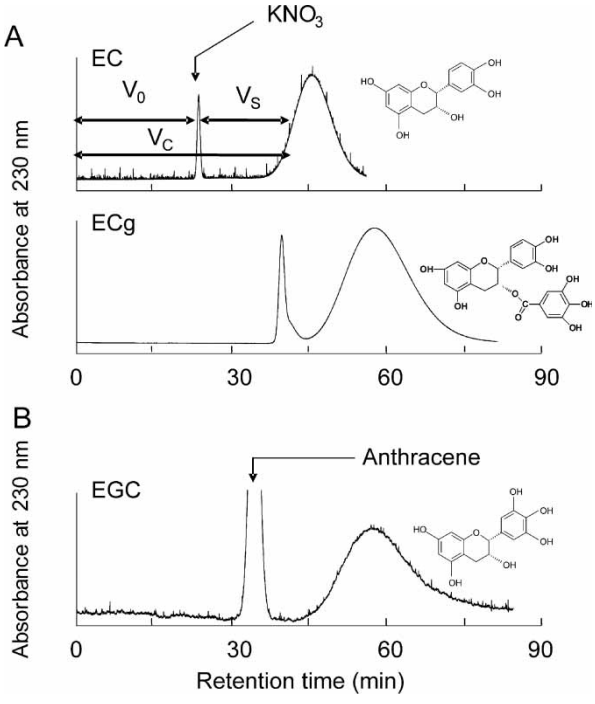


Figure 4. Stationary phase fraction controlled HSCCC chromatograms for $P_{o/w}$ measurement. (A) Reversed-phase mode HSCCC chromatograms of epicatechin (EC) and epicatechin gallate (ECg): revolution speed 1000 rpm; column: 1.0 mm × 50 m; mobile phase: aqueous with 50 mM potassium phosphate buffer at pH 7.4, flow rate 1.0 mL/min; injection 100 μL (5 mg potassium nitrate + 1 mg sample + 50 μL UP + 50 μL LP); UV detection at 230 nm; stationary phase fraction 0.418 for EC, 0.033 for ECg. (B) Normal-phase mode HSCCC chromatogram of epigallocatechin (EGC): revolution speed 1000 rpm; mobile phase: octanol, flow rate 1 mL/min; injection 100 μL (5 mg anthracene + 1 mg sample + 50 μL UP + 50 μL LP); UV detection at 230 nm; stationary phase fraction 0.101 for EGC.

have larger log $P_{o/w}$ values than the cis-form. It may be assumed that the substituent group ($-OR_2$) at the 3 position of the cis-form molecule is always close to the hydrophobic B-ring, whereas on the trans-form,the hydrophobic B-ring is far from the substituent group. Then the hydrophobicity of the B-ring reflects the hydrophobicity of the trans-form molecule.

The computer calculation values obtained by the fragment calculation method are quite different from the log $P_{o/w}$ values obtained from the HSCCC technique. The computer calculation method could not distinguish the log $P_{o/w}$ values of diastereomers, cis, and trans-form, because it uses the fragment calculation method of the molecules.

Table 4 shows the $P_{o/w}$ values of epicatechin monomer (EC), dimer (EC-EC), and trimer (EC-EC-EC). The log $P_{o/w}$ values of catechin oligomers

Table 3. Log $P_{o/w}$ values of catechins and related compounds obtained by HSCCC

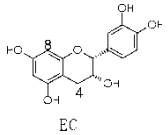
Catechins and related compounds	Log $P_{o/w}$[a]	Calculated log $P_{o/w}$ by Pallas[b]
Enantiomers		
(−)-EC (2R, 3R)	0.10	0.86
(+)-EC (2S, 3S)	0.10	0.86
Diastereomers		
EC (cis)	0.10	0.86
C (trans)	0.31	0.86
Diastereomers		
EGC (cis)	−0.53	0.43
GC (trans)	−0.31	0.43
Diastereomers		
EGCg (cis)	0.50	1.67
GCg (trans)	1.15	1.67
Diastereomers		
ECg (cis)	1.10	2.10
Cg (trans)	1.64	2.10

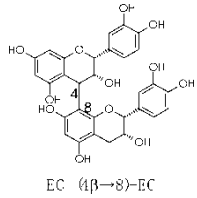
[a]Obtained by HSCCC.
[b]Computer software.

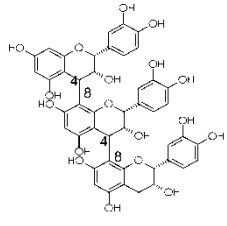
determined by the HSCCC technique decreased as the degree of polymerization increased. This indicates that the condensation of catechin monomer with C4–C8 bond leads to a formation of unique three dimensional structures of the catechin dimer and trimer molecules. We assume that the hydrophilicity of

Table 4. Log $P_{o/w}$ values of catechin oligomers

Catechin oligomers	Log $P_{o/w}$[a]	Calculated log $P_{o/w}$[b]
EC	0.10	0.86
EC-(4$\beta \rightarrow$ 8)-EC	−0.90	1.04
EC-(4$\beta \rightarrow$ 8)-EC-(4$\beta \rightarrow$ 8)-EC	−1.32	1.22


EC


EC (4β→8)-EC


EC (4β →8) EC (4β →8) EC

[a]Obtained by HSCCC.
[b]Calculated by computer software.

catechin oligomers is due to its spiral structure constructed with a hydrophobic core region containing some stacked rings, and a hydrophilic surface covered with a large number of phenolic hydroxyl moiety. However, the log $P_{o/w}$ values calculated by the computer as the fragment method are not consistent with the values obtained by the HSCCC technique. This calculation method does not take into consideration the structural conformations of oligomers.

Rapid Determination of Log $P_{o/w}$ Values of Catechins and Related Compounds Using Small Capacity Column

It was found that the log $P_{o/w}$ values ranging from -1.35 to $+1.64$ of catechins and their oligomers were easily determined by the HSCCC

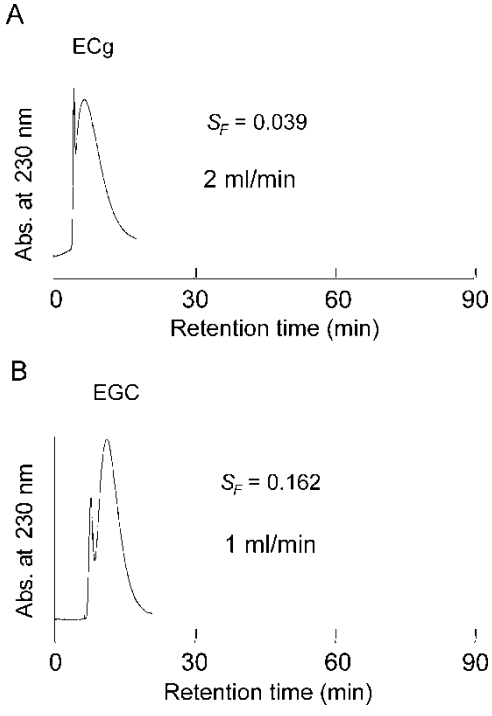


Figure 5. Rapid determination of $P_{o/w}$ using small capacity column. (A) Reversed-phase mode HSCCC chromatogram of epicatechin gallate (ECg): revolution speed 1000 rpm; column: 1.0 mm × 10 m; mobile phase aqueous with 50 mM potassium phosphate buffer at pH 7.4, flow rate 2.0 mL/min; injection 100 μL (5 mg potassium nitrate + 1 mg sample + 50 μL UP + 50 μL LP); UV detection at 230 nm; stationary phase fraction 0.039 for ECg. (B) Normal-phase mode HSCCC chromatogram of epigallocatechin (EGC): revolution speed 1000 rpm; mobile phase octanol, flow rate 1 mL/min; injection 100 μL (5 mg anthracene + 1 mg sample + 50 μL UP + 50 μL LP); UV detection at 230 nm; stationary phase fraction 0.162 for EGC.

technique within one hour, using a 50 m coiled column (40 mL capacity). In order to shorten the analysis time of log $P_{o/w}$ values, a small column (10 m long and 8 mL capacity) was used. Figure 5 shows the chromatograms of epicatechin gallate (ECg) and epigallocatechin (EGC) under the reversed-phase and normal-phase modes of HSCCC using both large and small capacity columns. In the reversed-phase mode HSCCC (Figure 5A), the flow-rate was also increased from 1.0 mL/min to 2.0 mL/min. As a result, the retention times of ECg decreased from 57.8 min (the 50 mL column at the stationary phase fraction of 0.033) to 6.0 min (the 10 mL small column at the stationary phase fraction of 0.039). The retention time of EGC under the normal-phase mode HSCCC (Fig. 5B) was also shortened from 52.3 min to 11.2 min using a 10 m column, and the obtained log $P_{o/w}$ value was quite consistent with the log $P_{o/w}$ value obtained from the 50 m column.

Rapid Determination of log $P_{o/w}$ Values of Hydrophobic Aromatic Compounds Using Small Capacity Column

The log $P_{o/w}$ values of five different hydrophobic aromatic compounds were also determined by stationary phase fraction controlled normal-phase mode HSCCC using a small column of 10 m. Table 5 shows log $P_{o/w}$ values of aromatic compounds together with their retention times. Under the stationary phase fraction of 0.0132 at a flow-rate of 4.0 mL/min, log $P_{o/w}$ values of p-cresol and benzene were around 2.0, which were obtained within 7 min. The log $P_{o/w}$ values of 2.80 and 2.96 for toluene and 1-naphthol were obtained at a high flow-rate of 8.0 mL/min within 8 min under the low stationary phase fraction of 0.0044. Under the same condition, the log $P_{o/w}$ value of naphthalene, the most hydrophobic compound used in this study, was 3.60 obtained in about 21 min.

Relationship Between Log $P_{o/w}$ Values Obtained by HSCCC and Those Values by Shake-Flask Method

Table 6 lists the log $P_{o/w}$ values of several compounds including some hydrophobic aromatics obtained by HSCCC and the shake-flask method,

Table 5. Log $P_{o/w}$ values of hydrophobic aromatic compounds

Compounds	S_F	Flow rate (mL/min)	Retention time (min)	Log $P_{o/w}$
p-Cresol	0.0132	4.0	4.8	1.88
Benzene	0.0132	4.0	6.6	2.04
Toluene	0.0044	8.0	6.0	2.80
1-Naphthol	0.0044	8.0	7.9	2.96
Naphthalene	0.0044	8.0	21.0	3.60

Table 6. Log $P_{o/w}$ values of several compounds obtained by HSCCC and shake-flask method

Compounds	HSCCC (50 m column) log $P_{o/w}$	HSCCC (50 m column) SD (n = 4)	HSCCC (10 m column) log $P_{o/w}$	HSCCC (10 m column) SD (n = 4)	Shake-flask log $P_{o/w}$	Shake-flask SD (n = 4)
Catechins						
(+)EC	0.10	0.004	0.03	0.005	0.13	0.049
(−)EC	0.10	0.001	0.03	0.005	0.11	0.013
(+)C	0.31	0.006	0.27	0.010	0.32	0.017
(−)C	0.31	0.005	0.27	0.010	0.31	0.019
(−)ECg	1.10	0.025	1.10	0.059	1.06	0.160
(−)Cg	1.64	0.085	1.44	0.075	1.55	0.127
(−)EGCg	0.50	0.038	0.53	0.011	0.39	0.024
(−)GCg	1.15	0.066	0.91	0.018	0.92	0.071
(−)EGC	−0.53	0.002	−0.40	0.011	−0.50	0.024
(−)GC	−0.31	0.007	−0.29	0.044	−0.32	0.018
Procyanidins						
PB1(EC-C)	−1.35	0.125	−1.24	0.055	−1.04	0.017
PB2(EC-EC)	−0.90	0.037	−0.73	0.086	−0.79	0.007
PC1(EC-EC-EC)	−1.32	0.023	−1.19	0.045	−1.08	0.033
Hydrophobic aromatics						
p-Cresol			1.88	0.070	1.94[a]	
Benzene			2.04	0.004	2.13[a]	
Toluene			2.80	0.040	2.71[a]	
1-Napthtol			2.96	0.147	2.98[a]	
Naphthalene			3.60	0.448	3.3[a]	

[a]Data from Ref. 21.

together with the standard deviation values. The log $P_{o/w}$ values of catechins and procyanidins (catechin, dimers, and trimers) obtained by HSCCC technique with a 50 m column are similar to those values obtained by using a 10 m column. These log $P_{o/w}$ values obtained by the HSCCC technique using the stationary phase fraction controlled method are quite consistent with the values obtained by the traditional shake-flask method. The standard deviations of the log $P_{o/w}$ values obtained by HSCCC with a small capacity column are very small compared with those obtained by shake-flask method. These results indicate that the HSCCC technique for the determination of log $P_{o/w}$ values of catechins and related compounds are very reliable and timesaving.

Figure 6A shows the relationship between log $P_{o/w}$ values of all compounds determined in the present studies obtained by the HSCCC

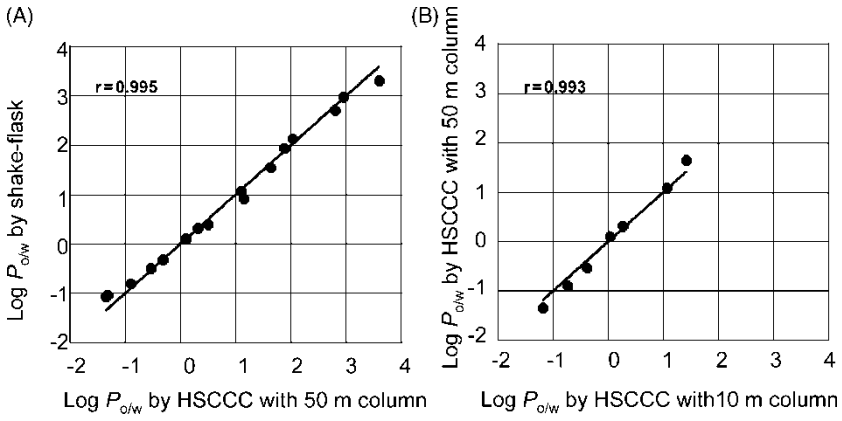


Figure 6. Correlation between log $P_{o/w}$ values of catechins, their related compounds, and hydrophobic aromatic compounds. (A) Relationship between log $P_{o/w}$ values obtained by HSCCC of catechins, their related compounds and hydrophobic aromatic compounds and those values obtained by the shake-flask method. (B) Relationship between log $P_{o/w}$ values obtained by HSCCC with 50 m column and those obtained by using 10 m column.

technique with a large capacity column and the values by the traditional shake-flask method. We have found an excellent linear relationship between these values with 0.995 of the correlation coefficient value (r), and it is clear that the stationary phase fraction controlled HSCCC is very useful for the determination of the log $P_{o/w}$ values of catechins, catechin oligomers, and various hydrophobic aromatic compounds in a short time, compared with the traditional shake-flask method.

Figure 6B shows the relationship between log $P_{o/w}$ values obtained by HSCCC using a large capacity and a small capacity columns. Good linear relationship, with 0.993 of correlation coefficient, between those log $P_{o/w}$ values was observed. It is considered that the stationary phase fraction controlled HSCCC technique using a short column is a very powerful method to determine the log $P_{o/w}$ values between -1.35 to 3.60.

In conclusion, the stationary phase fraction was easily and accurately controlled by pumping a desired volume ratio of the stationary and the mobile phases into the column. The stationary phase fraction was controlled at the minimum value of 0.0044 in the small capacity column at a high-flow rate of 8.0 mL/min. Under this condition, it was possible to determine the maximum log $P_{o/w}$ value of 3.60 in about 21 min. It was found that the stationary phase fraction control HSCCC technique was very powerful for the determination of log $P_{o/w}$ values of catechins and the related compounds in a short time.

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