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ABSTRACT

A number of important parameters affecting the countercurrent chromatography (CCC) technology, including the variation of retention of stationary phase, and mixing efficiency with rotational speeds, optimum rotational speed for retention of stationary phase of different solvent systems, improvement of retention of stationary phase in the column by changing the inner structure of column tubing, and optimum of the column size for a better retention of stationary phases were studied. The results showed that the convoluted tube yielded higher retention of stationary phase than the standard tube, that most solvent systems produced a significant retention of stationary phase at an optimum

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rotational speed, and that a suitable size column is helpful for retention of stationary phase. Based on the basic studies, an apparatus equipped with a 10 L column of 8.5 mm I.D. convoluted tube was made, and successfully used, to purify 150 g of crude tea extract yielding 40 g of epigallocatechin gallate of over 92.7% purity at a recovery rate of 82.6%. The result suggests that it is possible to further scale up this slow rotary counter-current chromatograph for industrial use.

Key Words: Slow rotary countercurrent chromatography; Stationary phase retention; Rotary speed; Solvent system; Convoluted Teflon tubing; Tea extract; Epigallocatechin gallate.

INTRODUCTION

Many favorable features of countercurrent chromatography (CCC) have been demonstrated abundantly in recent literature.^[1-4] Its true strength might be in the single step fractionation of fine chemicals and pharmaceuticals from a natural and biotechnology origin. Many separations have been successfully performed at the laboratory scale; progress has been made in the systematic selection of suitable solvent systems, and scale-up appears, in principle, possible.

One way of scaling-up CCC is simply enlarging the capacity of high-speed countercurrent chromatographs (HSCCC). The gram-quantity separation of DNA-amino acid was achieved with a 750 mL capacity multilayer coil,^[5] and later multigram separation of the same sample was demonstrated using a 1.6 L capacity coil consisting of three multilayer coils connected in series.^[6] Recently, Du et al. have made a scaled-up high-speed chromatograph equipped with a set of three columns, each 820 mL in capacity, for the separation of tea extract.^[7]

The other way is to utilize the slow rotary mode of column, which was first described by Ito and Bhatnagar.^[8,9] In this system, the best result was attained by rotating the coil slowly around its horizontal axis at a critical speed that yields high retention of the stationary phase.

For scaling-up slow rotary CCC (SLCCC), it is necessary to make some basic studies such as the variation in retention of stationary phase and mixing efficiency with rotational speeds, optimum rotational speeds for retention of stationary phase of different solvent systems, improving retention of stationary phase in the column by changing the inner structure of column tubing, optimising the column size for a better retention of stationary phases, etc.





In this paper, we describe our studies on these aspects and an actual preparative separation with an SLCCC apparatus equipped with a 10-L column.

EXPERIMENTAL

Basic Studies on the Variation of Retention of Stationary Phase with Rotational Speed

The simple trial apparatus for SLCCC used in this part was manufactured in Zhejiang University, Hangzhou, China. It was equipped with a 7-layer helical column made of a piece of 98 m long 5.0 mm I.D. standard Teflon tubing. The capacity of the column was 1920 mL. Each terminal of the column was connected to a flow tube which led through a rotary seal. The column can rotate around its own horizontal axis at a desired speed by a speed controller. A binary solvent system of chloroform/water was used to test the retention of stationary phase with rotational speed.

The retention volume of the stationary phase at different rotational speeds was determined as follows: The column was first completely filled with the stationary phase. Then the mobile phase was delivered through the column as the apparatus was started at a designated rotational speed. The effluent from the outlet of the column was collected into the graduated cylinder to measure the volume of the stationary phase eluted out from the column. After the hydrodynamic equilibrium was established in the column (as indicated by no more carryover of the stationary phase), the volume of the stationary phase in the cylinder was noted. The retained volume of the stationary phase is the difference between the column capacity and the eluted stationary phase volume.

Basic Studies on Resolution and Mixing Efficiency at Three Different Rotational Speeds

The above apparatus was used for a basic study and mixing efficiency by separating a mixture of caffeine and theophylline at the column rotation of 75, 300, and 800 rpm. The experiments were performed with the binary solvent system of chloroform/water with the lower phase as stationary phase. For each separation, the column was first completely filled with the stationary phase. Then the mobile phase was delivered into the column at a flow rate of 3.2 mL/min as the apparatus was rotated at a designed speed. The sample solution was injected through a sample loop after the hydrodynamic equilibrium was established in the column.

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The Optimum Rotational Speed and Stationary Phase Retention for 20 Different Solvent Systems

The retention of stationary phase of 19 solvent systems including chloroform, ethyl acetate, *n*-butanol, and *n*-hexane systems, were measured at various rotary speeds at room temperature to determine the optimum rpm for each solvent system. The apparatus used was equipped with a horizontal rotary shaft with 13 cm O.D. around which 30.4 m long, 5.7 mm average I.D. convoluted Teflon tube (Zeus Industrial Products, Raritan, NJ) was wound forming two coiled layers, 32 loops per layer, with a total capacity of 780 mL. The rotation speeds of the apparatus can be regulated at 0–200 rpm. All experiments were performed at a fixed flow rate of 5 mL/min.

The Stationary Phase Retention Characteristics of Two Different Phase Systems in Four Different Types of Tubing

In order to select efficient tubing for SLCCC, the retention of the stationary phase of two different phase systems was measured at room temperature, each in the following four different types of tubing: Teflon tubing (SW-10, 2.6 mm I.D., 38 m long, and 200 mL capacity), standard Teflon tubing (SW-8, 5.5 mm I.D., 8.5 m long, and 202 mL capacity), convoluted Teflon tubing (TS-12, 5.7 mm average I.D., 8 m long, and 204 mL capacity) and convoluted Teflon tubing (TS-018, 8.5 mm average I.D., 4 m long, and 207 mL capacity) (Fig. 1). Each tube was wound around a holder hub of 17 cm diameter making a one-layer or two-layer (SW-10) coil column. The two solvent systems used were *n*-hexane/methanol/water (6:5:3) with a hydrophobic organic phase, and *n*-butanol/water with a hydrophilic organic phase. The measurement was performed under a given set of test conditions described in Table 2.

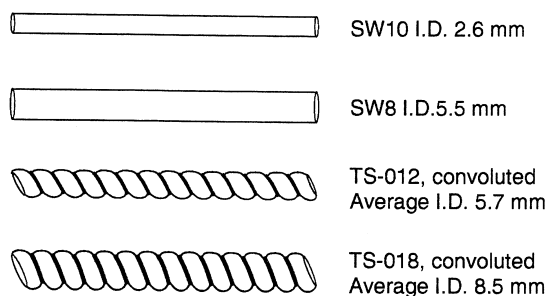


Figure 1. Four types of Teflon tubing used for the present study.





How Resolution or Mixing Efficiency Varies for Three Different Types of Tubing

In order to compare the resolution or mixing efficiency of different types of tubing, a mixture of progesterone and 11-hydroxylprogesterone, each 5 mg, was separated with three different types of columns made of 5.5 mm standard tubing, 5.7 mm convoluted tubing, and 8.5 mm convoluted tubing. The separation was performed with a two-phase solvent system composed of *n*-hexane/methanol/water (6 : 5 : 3) using the upper phase as stationary phase at a flow rate of 5 mL/min at a rotary speed of 50 rpm.

How Stationary Phase Retention Changes with Hub Diameter

In order to study the relationship between stationary phase retention and hub diameter, a piece of 5.7 mm convoluted tubing was wound onto six different holder hubs of 5, 9, 14.5, 19.5, 22, and 24.5 cm O.D. with a 25 cm length of single layer column. The tested solvent systems used were hexane/methanol/water (6 : 5 : 3) and *n*-butanol/water, that is the same as used in the previous studies.

An Example of a Preparative Run, Which Isolates 40 g of Epigallocatechin Gallate

Based on the results of studies on the stationary phase retention vs. hub diameter, a seven coiled layer, 10-L capacity column (9 cm helical diameter for

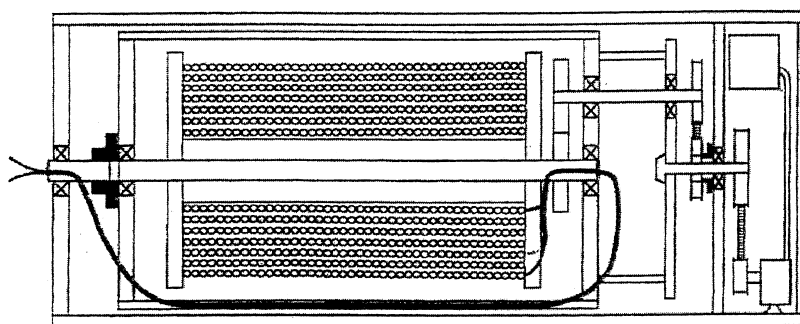


Figure 2. Cross-sectional view of seal-free SLCCC instrument equipped with a large convoluted multilayer coil. This apparatus was used in experiments described in Figs. 3–6.

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the inner layer and 22 cm helical diameter for the outer layer) was prepared by winding 200 m long, 8.5 mm I.D. convoluted tubing (TS-018) onto a 9 cm O.D. column holder hub making seven coiled layers each consisting of 60 loops (Fig. 2). To demonstrate the preparative capability of the 10-L apparatus, tea leaves extract, which was successfully separated by HSCCC,^[7] was used for a preparative run with a solvent system composed of *n*-hexane/ethyl acetate/*n*-butanol/water/acetic acid (0.5:1:2:6:0.2, v/v). In each separation, the column was first completely filled with the organic stationary phase followed by sample charge. Then the aqueous mobile phase was eluted through the column at a flow rate of 5 mL/min in a head to tail elution mode while the column was rotated at an optimum rotary speed (21 rpm). The effluent was collected into test tubes, and each fraction was analysed by either TLC or HPLC to construct the chromatogram.

RESULTS AND DISCUSSION

Figure 3 shows percent retention of the stationary phase plotted against the applied rotary speeds. There was a maximum retention of 64% at 75 rpm in a narrow range between 0 and 100 rpm, while over 44% retention was

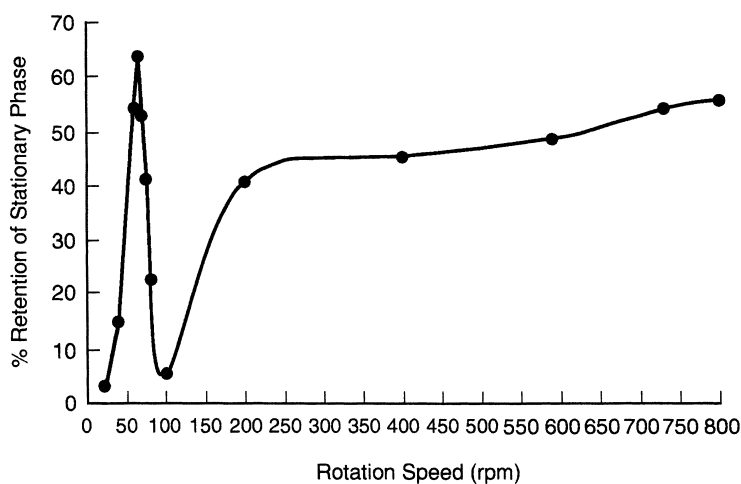


Figure 3. Percent retention of stationary phase at various rotary speeds of SLCCC. (published in Ref.^[11]). Apparatus: SLCCC instrument with rotary seal; column: 98 m long 5.0 mm I.D. standard Teflon tubing with 1920 mL capacity; solvent system: chloroform/water; stationary phase: aqueous phase; flow-rate: 5 mL/min.





obtained at the rotary speed above 250 rpm. As reported earlier,^[10] this strange retention curve may be produced by an interplay between the two forces: Archimedean screw forces, which drive both phases toward the head of the coil and the radial centrifugal force acting across the diameter of the tubing, which affects the movement of each phase through the coil. At a slow flow rate, the lighter phase mostly stays in the upper portion of coil and the heavier phase in the lower portion of the coil, both moving competitively toward the head of the coil. This results in near 50% retention of either phase at the head side of the coil. When the rotational speed is increased, the radially acting centrifugal force field tends to cancel the gravity on the upper portion of the coil, thus interfering with the movement of the upper phase. This favors the retention for the lower phase. As the rotational speed is further increased, the centrifugal force on the lower portion of the coil becomes strong enough to interfere with the motion of the lower phase. This condition favors the retention of the upper phase in the coil. Thus, a sharp peak of retention curve is produced in the low range of rotational speeds between 0 and 100 rpm. Further increase of the rotational speed creates a strong radial centrifugal force which interferes with the movement of both phases along the tube, where the two phases are evenly distributed in such a way that the upper phase occupies along the inner portion and the lower phase along the outer portion of the coil. In this situation, both phases will show near 50% retention, but the partition efficiency is decreased due to a lack of mixing and interfacial area between the two phases.

Figure 4 shows the chromatograms of a mixture of caffeine (1 g) and theophylline (200 mg) separated at three different rotational speeds, i.e., 75, 300, and 800 rpm. The best peak resolution was obtained at 75 rpm. As expected, the peak resolution at 800 rpm was the poorest, apparently due to poor mixing of the two phases in the column. The results of the above experiments suggest that it is feasible to develop an efficient CCC apparatus using a multilayer column that rotates at a relatively low speed.

Table 1 lists the optimum rotary speeds of 20 solvent systems and the retention of stationary phases. The optimum rotary speed of polar solvent systems including *n*-butanol was below 30 rpm while those of less polar solvent systems, such as chloroform/water, were about 100 rpm. Since all the optimum rotary speeds were at or below 100 rpm, a speed range from 0 to 150 rpm is enough for the SLCCC system.

Table 2 shows percent retention of stationary phase for two solvent systems composed of hexane/methanol/water (6:5:3) and *n*-butanol/water in four different coiled tubes. The 2.6 mm I.D. tube shows almost no retention of the stationary phase, probably due to a plug flow in a narrow opening. On the other hand, both large diameter standard and convoluted tubes produced



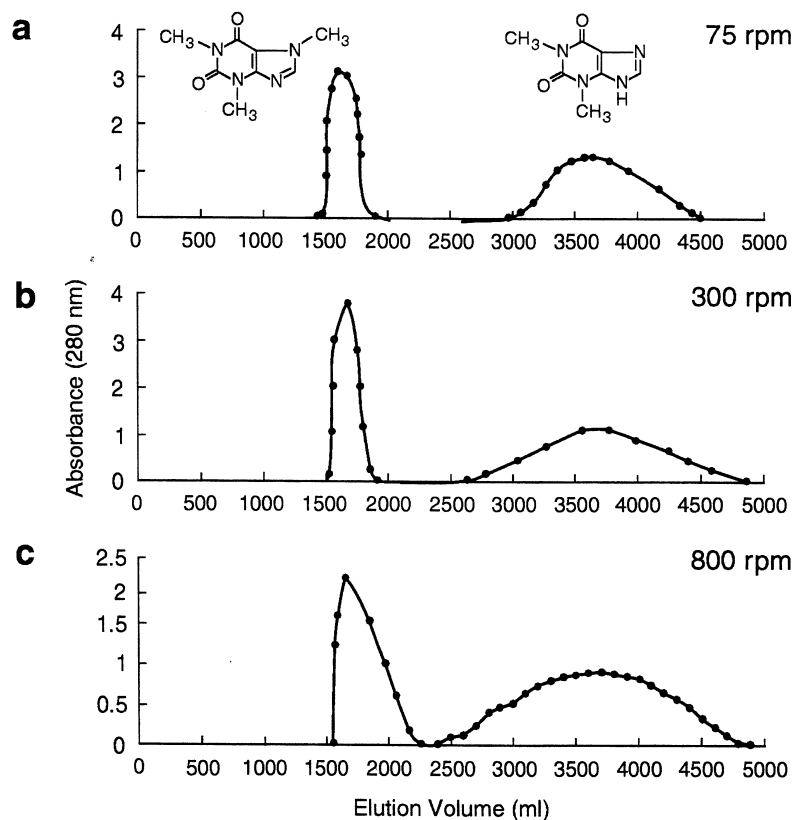


Figure 4. Chromatogram of caffeine and theophylline separated by SLCCC apparatus with rotary seal. (published in Ref.^[11]). Column capacity: 1920 mL; sample size: caffeine 1 g and theophylline 200 mg; solvent system: chloroform/water; Stationary phase: aqueous upper phase; flow-rate: 3.2 mL/min.

satisfactory levels of retention, where the convoluted tube showed substantially higher retention of the stationary phase than the standard tube of a comparable diameter. From these data, we may conclude that larger the inner diameter, the higher the retention of stationary phase. It was also clear that the convoluted tubing gives better retention than the standard tube.

Figure 5 shows the comparison of the three different tubes for the separation of a mixture of progesterone and 11-hydroxyl-progesterone. The result indicates that the small convoluted tubing produces better peak resolution than the standard wall tubing, while the large convoluted tubing yielded the best resolution despite its relatively short length. The overall results



**Table 1.** Optimum rotational speeds and corresponding retention of stationary phase of 20 solvent systems.

Solvent system	Optimum rotary speed (rpm)	Percent retention of stationary phase
<i>n</i> -BuOH/H ₂ O (1 : 1)	5	56.4
<i>n</i> -BuOH/ <i>n</i> -hexane/EtOAc/H ₂ O (2 : 2 : 1 : 6)	22	39.2
<i>n</i> -BuOH/ <i>n</i> -hexane/EtOAc/H ₂ O/acetic acid (2 : 1 : 0.5 : 6 : 0.2)	21	35.2
<i>n</i> -BuOH/ <i>n</i> -hexane/EtOAc/H ₂ O (2 : 0.25 : 1 : 6)	2	47.7
<i>n</i> -BuOH/AcOH/H ₂ O (10 : 1 : 10)	5	55.0
<i>n</i> -BuOH/AcOH/H ₂ O (4 : 1 : 5)	5	62.7
<i>n</i> -BuOH/tBME/CH ₃ CN/H ₂ O (2 : 2 : 1 : 5)	12	63.3
EtOAc/H ₂ O (1 : 1)	40	25.9
EtOAc/ <i>n</i> -BuOH/H ₂ O (1 : 1 : 2)	20	51.4
EtOAc/AcOH/H ₂ O (3 : 1 : 3)	20	56.8
EtOAc/ <i>n</i> -hexane/AcOH/H ₂ O (3 : 1 : 1 : 3)	20	60.0
<i>n</i> -Hexane/H ₂ O (1 : 1)	30	22.7
<i>n</i> -Hexane/MeOH (1 : 1)	30	50.0
<i>n</i> -Hexane/MeOH/H ₂ O (6 : 5 : 3)	60	75.5
<i>n</i> -Hexane/MeOH/EtOAc/H ₂ O (1 : 1 : 1 : 1)	40	60.0
CHCl ₃ /H ₂ O (1 : 1)	100	83.0
CHCl ₃ /MeOH/H ₂ O (4 : 3 : 2)	10	60.5
CHCl ₃ /MeOH/AcOH/H ₂ O (5 : 3 : 4 : 1)	100	85.0
CHCl ₃ /AcOH/H ₂ O (2 : 2 : 1)	100	79.5
CHCl ₃ /CH ₃ CN/H ₂ O (1 : 2 : 1)	20	33.6

Note: tBME, tert-butyl methyl ester.

suggest that the present system may be further scaled up using a larger I.D. convoluted tube.

Figure 6 indicates the effect of coil diameter on the retention of stationary phase. The optimum range is observed from 10 to 22 cm. The result indicates that an efficient multilayer coil can be prepared by winding the tube onto a 9 cm diameter hub up to a maximum of 24 cm outer diameter. With these coil dimensions, the column capacity of 10 L can be attained with a hub length of 60 cm.

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Table 2. Percent retention of two solvent systems in the coil made of three types of Teflon tube.

Teflon tubing size and type	Stationary phase	Hexane/methanol/water (6 : 5 : 3) (50 rpm)		<i>n</i> -Butanol/water (30 rpm)	
		2 mL/min	5 mL/min	2 mL/min	5 mL/min
2.6 mm I.D. standard	Upper	7.1	5.1	0	0
	Lower	6.8	4.5	0	0
5.5 mm I.D. standard	Upper	50.1	41.1	36.3	30.7
	Lower	49.7	42.7	34.3	29.1
5.7 mm I.D. convoluted	Upper	63.3	53.2	47.7	35.0
	Lower	63.0	51.0	41.5	34.5
8.5 mm I.D. convoluted	Upper	77.0	66.1	56.2	49.0
	Lower	74.8	63.7	54.3	47.5

Figure 7 shows a chromatogram of 150 g of tea extract containing epigallocatechin gallate at 30% isolated with the 10-L apparatus. The separation was performed with a solvent system composed of *n*-hexane/ethyl acetate/*n*-butanol/water/acetic acid (0.5 : 1 : 2 : 6 : 0.2, v/v) at a flow rate of 5 mL/min. A 40.05 g amount of epigallocatechin gallate was obtained with

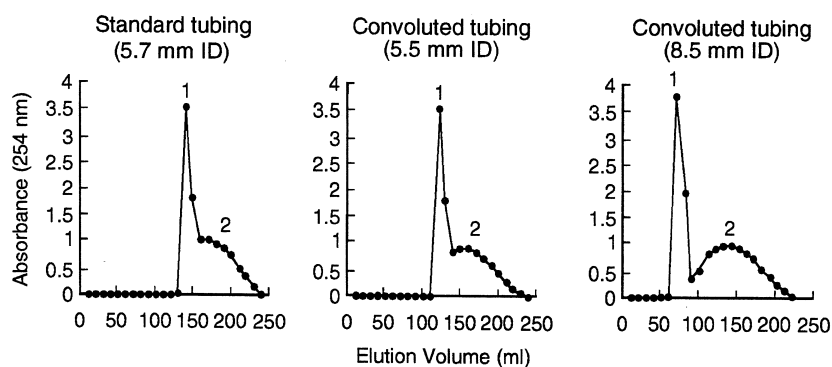


Figure 5. Chromatograms of a mixture of progesterone and 11-hydroxyprogesterone using three types of coiled columns. (published in Ref.^[12]). Experimental conditions—sample: a mixture of 5 mg of each of progesterone and 11-hydroxyprogesterone in 10 mL of lower phase; solvent system: *n*-hexane/methanol/water (6 : 5 : 3); mobile phase: upper phase; flow rate: 5 mL/min; column rotation: 50 rpm.



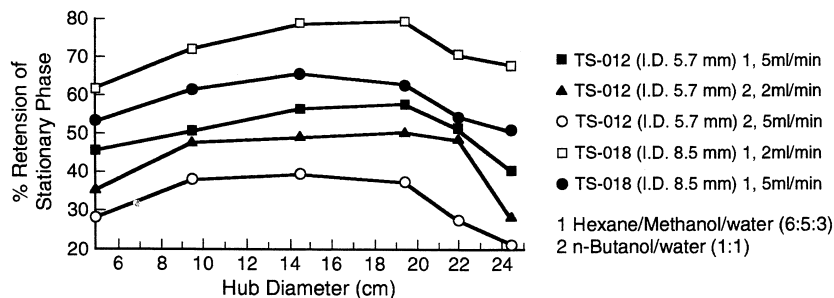


Figure 6. Change of the retention of stationary phase with different hub diameters. Apparatus: seal-free SLCCC; coil column: one layer; length of hub: 25 cm.

92.7% purity at an 82.6% recovery rate in one-step separation. The production rate was 0.52 g EGCG/hr, which was 15% higher than that obtained from the HSCCC centrifuge equipped with three serial 820 mL columns, which can separate 40 g of the same tea extract in 22 hrs.^[7] Although, the separation required 71.5 hr, which seems to be too long, the separation was performed under a minimum risk of accident because of the slow rotation of the column. The safety, together with the simplicity of the system, permits the operation to be left unattended during the separation. This is the major advantage of the SLCCC technique over the scaled-up HSCCC. After finishing the separation, the retention of stationary phase in the column was 33%, indicating that improvement of the separation is not feasible by application of a higher flow

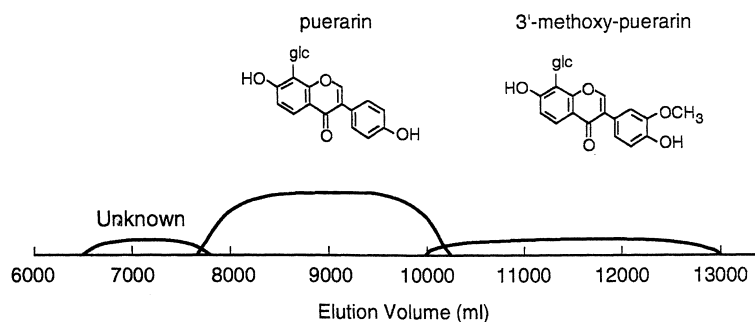


Figure 7. Preparative chromatogram of tea extract obtained by SLCCC apparatus with 10-L column. (published in Ref.^[12]). Experimental conditions—sample: 150 g of tea extract dissolved in 1.2 L of solvent consisting of equal volumes of each phase; solvent system: *n*-hexane/ethyl acetate/*n*-butanol/water/acetic acid (0.5 : 1 : 2 : 6 : 0.2); mobile phase: lower phase; flow-rate: 5 mL/min; column rotation: 21 rpm.





rate. However, it may be possible to use a higher flow rate to improve separation efficiency using a greater diameter of the column, because the larger the diameter of the tubing, the better the retention of stationary phase.

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