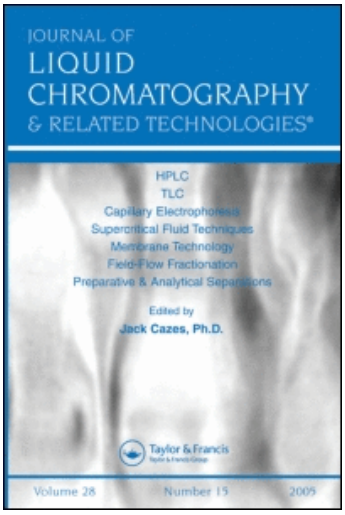

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G. C. Chen[a]
[a] Pharma-Tech Research Corporation, Clifton, New Jersey

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HIGH PERFORMANCE COUNTERCURRENT CHROMATOGRAPHY FOR QUANTITATIVE ANALYSIS OF FIVE FLAVONOIDS

G. C. CHEN*
Pharma-Tech Research Corporation
N. J. Division, P. O. Box 2743
Clifton, New Jersey 07013-2743

ABSTRACT

The stability of stationary phase in the rotating coil of modern countercurrent chromatography, and the repeatability of chromatographic process of target compounds were the focal points in this report. A PTR CCC-1000 chromatograph mounted with either column set for semipreparative (1.6mm id, total capacity of 338mL) or 3-layer analytical for rapid screen (0.85mm id, total capacity of 51mL) was used to perform quantitative analysis of five flavonoids. The same solvent system, $CHCl_3$/MeOH/H_2O (4:3:2), reported for separation of quercetin (1) apigenin and leuteolin (2) was adopted in this study. The chromatographic parameters such as k' (capacity factor), K (partition coefficient), N (number of the theoretical plate), and Rs (peak resolution) were determined from both sets of chromatograms, and compared thereafter.

*The experimental work described in this paper was performed at Dr. C.M. Liu and Ms. Li-Na Hong's laboratory, Hoffmann La Roche Inc. Nutley, NJ 07110 in conjunction with our technical support for the evaluation of their newly purchased PTR CCC-1000 Model. The author was ex-veteran employee of Roche.

The repeatability of chromatographic process was evaluated through repeated injections of respective compounds, and presented as c.v. (coefficient of variation) values. Time for analysis of first four flavones was within one and half hour, while the last isoflavone took additional one hour, when the semipreparative column set was used. The 3-layer analytical column set reduced the assay time to within one hour, but produced heavy bleeding after a large amount of flavonoids mixture was introduced. Other solvent systems such as Hexane/EtOAc/MeOH/H_2O (1:1:1:1) and 1-ButOH/H_2O were also included to monitor stationary phase retention between two column sets.

INTRODUCTION

Countercurrent chromatography (CCC) has been extensively applied to the separation of biological materials where the bioactivity needs to be preserved, and to the extraction & purification of polar components where other chromatographic techniques failed to perform (3-6). The popularity of bio-research has enhanced the usage of modern CCC, the need for standardization of commercialized units for industrial applications is thus obvious.

A commercial CCC instrument model CCC-1000 (Pharma-Tech Research Corp., Baltimore, MD) equipped with either semipreparative columns (triplet columns connected in series) or 3-layer analytical columns (triplet columns also connected in series) for rapid screening, which were

interchangeable, was used to evaluate and compare the retention & bleeding of stationary phases of selected solvent systems for stability test of the instrument; and to perform the quantitative analysis of flavonoids to determine its chromatographic parameters such as k' (capacity factor), K (partition coefficient), N (number of theoretical plate), Rs (peak resolution) as well as to obtain c.v. (coefficient of variation) values from repeated injections of same solutions for repeatability test of the chromatographic process. The studies on the gradient elution mode and automation of the foresaid unit are underway.

MATERIALS AND METHODS

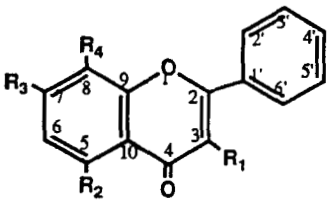
Apparatus

High performance model CCC-1000 was evaluated for its performance. Two column sets (A and B) were employed: A set consists of three 3-layer identical units wound from 0.85mm I.D. PTFE tubing, and connected in series, which had a total capacity of about 50mL; B set consists of three identical units wound from 1.6mm I.D. PTFE tubing, and also connected in series, which had a total capacity of about 340mL. Both sets can be interchanged and mounted on the foresaid chromatograph.The A set had a β value of 0.74-0.75,

and the B set, 0.5-0.75. The direction of coil winding was opposite to that of coil rotation, the flow of mobile phase was from the peripheral end to center end, and its rate was regulated by a Waters Prep LC 3000/System Controller 600 E Unit. The detector was an LKB 2238 Uvicord S11 (time constant 2, ABS range 2, and lamp 2 for 254nm filter). The recorder was an LKB/Bromma 2210 operating at 0.1V, having a chart speed of 1mm/min.

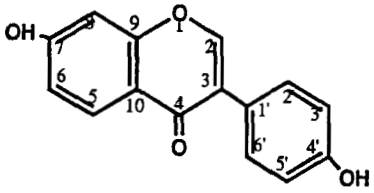
Solvent Systems and Test Compounds

All solvents were HPLC grade, the solvent system for the separation of flavonoids was $CHCl_3/MeOH/H_2O$ (4:3:2); the other two solvent systems, Hexane/EtOAc/MeOH/H_2O (1:1:1:1) and 1-BuOH/H_2O, were also included for comparison of the retention of stationary phases and the effect of their density differences between the upper and lower phases for two column sets. All test compounds except for Daidzein were from Aldrich Chemical Co., and used without further purifications. Daidzein, a gift of Dr. Liu and Ms. Hong, was also used without further purification (for structures, refer to Figure 1). All test compounds were dissolved in the mobile phase; if there appeared a solubility problem, appropriate solvents such as MeOH or $CHCl_3$ or 1-2 drops



FLAVONES

1. Flavone: R1=R2=R3=R4=H
2. 7,8-dihydroxyflavone: R1=R2=H, R3=R4=OH
3. Chrysin: R1=R4=H, R2=R3=OH
4. Galangin: R4=H, R1=R2=R3=OH



ISOFLAVONE

5. Daidzein: 4',7-dihydroxyisoflavone

Figure 1 Structures of flavonoids

of 0.1N KOH were added. The range of initial concentration was 1-2 mg/mL, which were diluted with mobile phase to make working solutions in the range of 50-365ug/mL for the respective compounds as well as for the mixture of five flavonoids.

Experimental Design

The selected solvent systems were pre-equilibrated in a separatory funnel, then the volumes of each phase were

measured, followed by the determination of density difference. The stationary (lighter) phase was first pumped in, without the rotation of the apparatus, at a rate of 5 or 10 mL/min for smaller or larger coil set, respectively. After the columns were completely filled, the unit was rotated to reach its desired speed (i.e., 1000rpm for the 1.6mm I.D. column, and 1200rpm for the 0.85mm I.D. column), the mobile (heavier) phase was pumped in at a fixed rate (i.e.,1.5mL/min for the 1.6mm I.D. column, and 0.8mL/min for the 0.85mm I.D. column), the displaced stationary phase being measured after the hydrodynamic equilibrium between two phases in the rotating coils had been established (pre-run measurement). Working solutions were then injected in triplicate or more through the sample injection port of Rheodyne Teflon Valves with a loop size of 0.5mL for the 1.6mm I.D. column, and of 0.2mL for the 0.85mm I.D. column . At the end of the run, the entire column was emptied via N_2 pushing to measure the volume of the stationary phase retained in the column as well as the total volume of the solvent collected (post-run measurement). Chromatograms so obtained were analyzed for k', K, N, R_s values of respective flavonoids.

RESULTS AND DISCUSSION

The retention of the stationary phase for selected solvent systems was compared (TABLE 1). It clearly showed that the B set retained more volume of the stationary phase in the rotating coil than that of A set, the order of retention (in %) among the selected solvent systems was N0.1(88)>NO.2(81) >NO.3(64) for the B set VS NO.2(70)>NO.1(69)>NO.3(31) for the A set. No significant background fluctuations were observed from the routine run of both column sets during a two months test period. With repeated injections of target compounds, the bleedings of stationary phases (NOs 1 & 2 solvent systems) were only 1-2% during 6-9 hr. run on B set; while the same solvent systems produced 4-6% loss of the stationary phase in 2-4 hour run on the A set. Heavy bleeding of 8-33% and up was observed when using NO.3 solvent system & injection of fermentation product into the B set , but no data were available for the A set of the same study. Heavy bleeding was also observed when applying NO.1 solvent system, and making single injection of flavonoid mixture or daidzein itself (TABLE 2). Although the cause of such bleeding was probably the overloading, the impurities present in the daidzein sample may have emulsified the solvent to upset the initial hydrodynamic balance in the

TABLE 1

A Comparison Among the Retention of the Stationary Phase, Bi-phasic Volume Ratio after Mixing, and Density Differences between Two Phases of Selected Solvent Systems

Solvent System	Stationary Phase Retained in %*				Density, g/mL		Volume ratio
	Pre-run		Post-run		LP/UP	=LP-UP	LP/UP
	A	B	A	B			
1. C/M/W (4:3:2)	71	87	67	88	1.38/0.94	0.44	1.00
2. H/EA/M/W (1:1:1:1)	72	82	68	80	0.91/0.75	0.16	1.33
3. 1-BuOH/W	30	69	31	58	0.99/0.85	0.14	0.80

* Operational Conditions: Upper phase, stationary; rotational speed:1200rpm for A, and 1000rpm for B; flow rate: 0.8mL/min for A, and 1.5mL/min for B-1 & 1.0mL/min for B-2 ans B-3; A run on 0.85mm I.D. column set; and B, on 1.6mm I.D. column set.

Abbreviations: =density difference; C=chloroform; EA=ethyl acetate; M=methanol; W=water; 1-BuOH=1-butanol; LP=lower phase; Up=upper phase.

TABLE 2

Comparison of Bleedings of Stationary Phases for Selected Solvent Systems

Solvent System	Bleeding of Stationary Phase in %	
	A	B
1. C/M/W (4:3:2)	4(7 inj. 2-h run) 18-24*	2(12 inj. 9-h run)
2. H/EA/M/W (1:1:1:1)	6(3 inj. 4-h run)	1(6 inj. 6-h run)
3. 1-BuOH/W		8-33 & up**

A: run on 0.85mm I.D. column set at a flow rate of 0.8mL/min & rotational speed of 1200rpm; and B: run on 1.6mm I.D. column set at a flow rate of 1.5mL/min for B-1 & B-2, of 1.0mL/min for B-3, B has rotational speed of 1000rpm.
* Multiple injections of daidzein(200ug/mL) or single injection of flavonoids mixture(about 800ug/mL).
**Single injection of fermentation product with concentrations of 5 and 52 mg/mL, respectively, showed that bleedings were proportional to the concentrations.
All abbreviations were same as those used in TABLE 1.

rotating coil, causing significant amount of stationary phase to be pushed out.

The separation of five flavonoids was performed on both column sets. The chromatograms (FIGURE 2) were compared for Rs. In the B set (TABLE 3): Rs values greater than 1.5 (a complete resolution) were obtained for all pairs with exception of chrysin, galangin pair which had about 2% overlapping (Rs=0.95). Although total analysis time was 160 minutes for all five components, ninety minutes would be

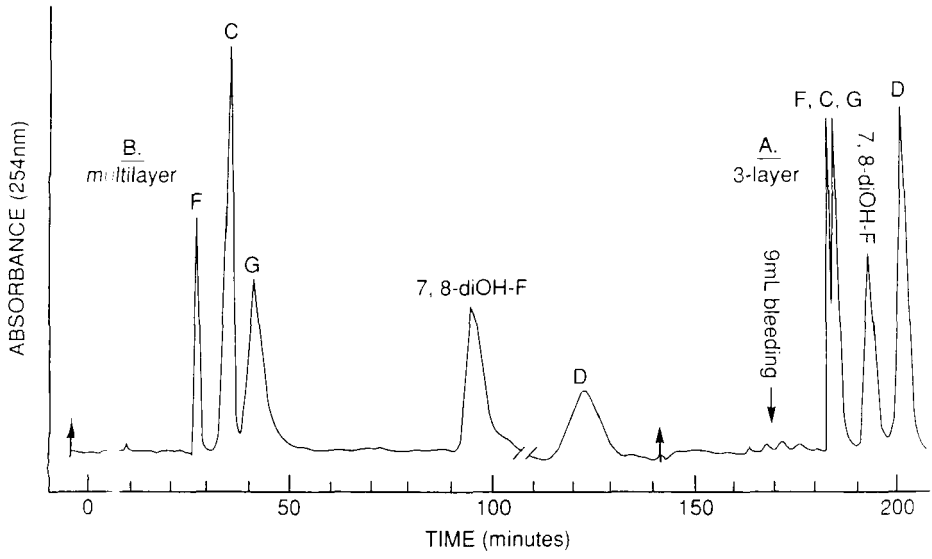


Figure 2 Comparison of separation of five flavonoids between A. 3-layer 0.85mm I.D. column set and B. multilayer 1.6mm I.D. column set. Abbreviations: F=flavone, C= chrysin, G=galangin, 7,8-diOH-F=7,8-dihydroxy-flavone, and D=daidzein.

enough if only flavones (first four) needed to be separated; the elution of isoflavone (daidzein) itself took additional one hour. When the flavonoid mixture was injected into A set (TABLE 4), analysis time was reduced to within one hour. However, due to heavy bleeding of the stationary phase, all retention times were shifted toward the longer end considerably as compared to that of the individual injections. The order of shift increased as follow: daidzein → 7,8-diOH-flavone → galangin → chrysin → flavone with the flavone shifted most. Loss of stationary phase had caused more

TABLE 3

Repeatability of Chromatographic Process* and k',K,N, & R_s Values of 5-Flavonoids

Flavonoids	RT(min.)^		H(mm)^			N	k'	K=C_s/C_m	R_s
	$\bar{x}$	s	$\bar{x}$	s	c.v.				
1. Flavone	32.7(3)	0.2	62.0	1.3	2.1	1901	0.21	0.029	1,2 pair=1.9
2. Chrysin	41.3(4)	0.6	90.5	1.7	1.9	710	0.53	0.072	2,3 pair=0.95
3. Galangin	44.5(4)	0.3	43.6	1.9	4.4	880	0.65	0.088	3,4 and 4,5 pairs are
4. 7,8-dihydroxy -Flavone	95.8(4)	0.5	39.1	2.0	5.0	1332	2.55	0.347	completely resolved
5. Daidzein	160.0(1)		16.0			1504	4.92	0.670	
5a.Daidzein**	79.8(3)	0.3	72.0	1.0	1.4	708	0.75	0.190	

* Operational conditions: this study was performed on 1.6mm I.D. column set at a flow rate of 1.5mL/min & rotational speed of 1000rpm; sample sizes(ug/mL) were 1-100, 2-364.5, 3-360, 4-350, 5-200, and 5a-123; solvent system: C/M/W (4:3:2).

^ Data were obtained from multi-injections of respective compounds.

** Solvent system: H/EA/M/W (1:1:1:1), and t_o (retention time of solvent front) =45.5min.

Abbreviations: $\bar{x}$=average of x; s=the standard deviation; c.v.=the coefficient of variation, c.v.=s/$\bar{x}$ x100; RT=retention time; N=the number of theoretical plates; H=peak height; k'=capacity factor; K=partition coefficient; R_s=resolution; C_s=concentration of solute in the stationary phase; and C_m=concentration of solute in the mobile phase.

TABLE 4

Repeatability of Chromatographic Process* and k', K, N, & R_s Values of Five Flavonoids

Flavonoids	RT(min.)^		H(mm)^			N	k'	K	R_s
	$\bar{x}$	s	$\bar{x}$	s	c.v.				
1. Flavone	19.6(3)	0.3	79.0	3.9	4.9	1537	0.067	0.029	1,2 &2,3 pairs were
2. Chrysin	22.3(3)	0.3	58.5	0.5	0.9	884	0.166	0.072	not resolved**
3. Galangin	24.0(3)	0.6	64.8	4.0	6.2	752	0.203	0.088	3,4 & 4,5 pairs were
4. 7,8-dihydroxy -Flavone	37.5(3)	0	75.0	1.3	1.8	743	0.803	0.347	completely resolved
5. Daidzein	56.1(3)	0.1	81.5	1.8	2.2	842	0.982	0.670	

* Operational conditions: This study was performed on 0.85mm I.D. column set at flow rate of 0.8mL/min & rotational speed of 1200rpm. Solvent system: C/M/W (4:3:2). Sample sizes(ug/mL) were 1-50, 2-106, 3-200, 4-200, & 5-200.

^ Data were obtained from multi-injections of respective compounds.

** Due to heavy bleeding after injecting the flavonoid mixture, all retention times were shifted toward longer side with the first three compounds shifted from 20-24 min. to 42-45 min. No 4 compound shifted to 52 min. and the last one, about 4-5 min.

All abbreviations were same as those used in TABLE 3.

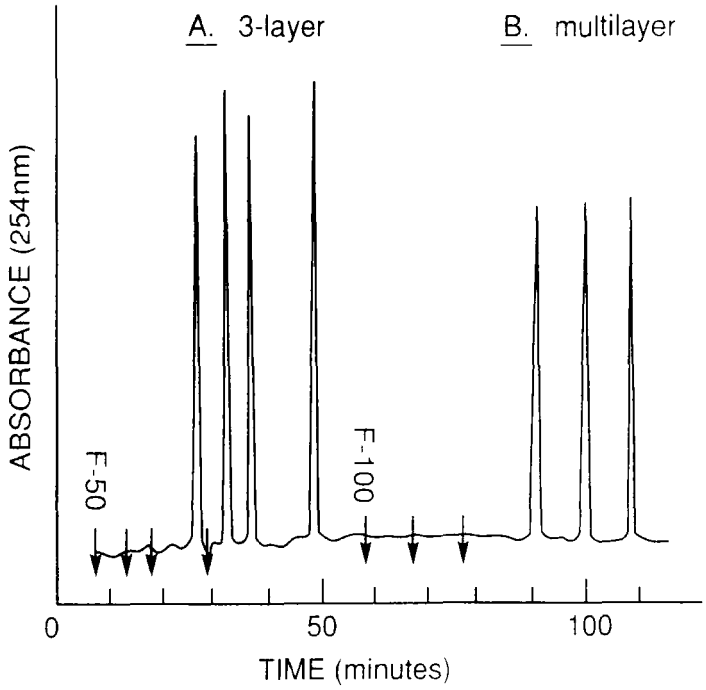


Figure 3 Countercurrent chromatograms of flavone.
A. Repeated injections of 50 ug/mL flavone standard into a 0.2mL loop size sample port using 3-layer 0.85mm ID column set; and B. Repeated injections of 100 ug/mL flavone standard into a 0.5mL loop size sample port using multilayer 1.6mm I.D. column set.

mobile phase to occupy the column space, and hence prolonged the migration of solvent front and of solutes. The loss of stationary phase also means the reduced peak resolution of solutes. Therefore compounds having only small difference in partition coefficient will overlap; indeed, flavone, chrysin, and galangin were only partially resolved.

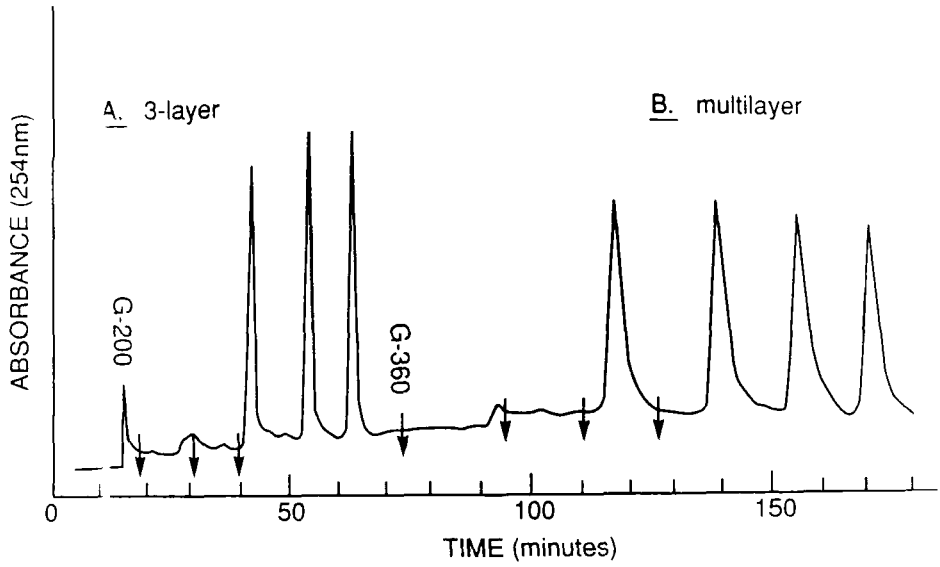


Figure 1 Countercurrent chromatograms of galangin.
A. Repeated injections of 200 ug/mL galangin standard into a 0.2 mL loop size sample port using 3-layer 0.85mm I.D. column set; and B. Repeated injections of 360 ug/mL galangin standard into a 0.5 mL loop size sample port using multilayer 1.6mm I.D. column set.

By injecting the working solutions of individual flavonoids in replicate, the retention time (RT), peak height (H), and peak width at base (W_b) from chromatograms of target compounds (FIGURES 3 & 4 as examples) were analyzed and chromatographic parameters such as k', K, N were then determined. The c.v. values were used to evaluate the repeatability of the chromatographic process, and provide a general guide for standardization of the commercial units. In the B set, values for N were in the range of 710-1901; k',

0.21-4.92; and K, 0.029-0.670 (if neglect the bleeding). In comparison, the values for A set were N, 743-1537; k', 0.067-0.982; K values in TABLE 3 were carried to TABLE 4. When one uses $k_1'/k_2' = K_1/K_2$ to calculate K for daidzein in the A set, certain discrepancy revealed. Such discrepancy likely arose from the loss of stationary phase due to the bleeding during run. The c.v. values for peak height of 5-flavonoids when run on the B set was in the range of 1.4-5.0 with an average of 3.0 %; while on the A set, the c.v. values were 0.9-6.2 with an average of 3.2%. The shift of retention time for 5-flavonoids was less than 1% for both column sets.

This report concluded that the 3-layer 0.85mm I.D. analytical column set (A set), could be applied to quick screening of solvent systems for scale-up applications using known pure compound as a marker; while the semi-preparative column set (B set), could provide the stability and the repeatability of the chromatographic process for routine operations.

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