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# RAPID SEPARATION OF FLAVONOIDS BY ANALYTICAL HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

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## SUMMARY

A commercial model of the analytical high-speed counter-current chromatography instrument was used for separation of flavonoids from a crude ethanol extract of dried fruits of sea buckthorn (*Hippophae rhamnoides*). Using a two-phase solvent system of chloroform–methanol–water (4:3:2), a five-fold increase in flow-rate of the mobile phase from 60 to 300 ml/h resulted in a rapid separation of five components in less than 15 min without significant loss in peak resolution. Major flavonoid component, isorhamnetin, was identified in its pure state by mass spectrometric analysis.

## INTRODUCTION

Counter-current chromatography (CCC) is a recently developed partition method that totally eliminates the use of solid supports[1,2]. Being a support-free chromatography, the method enables efficient separations of a broad spectrum of samples ranging from small ions and molecules to macromolecules and particles without adsorptive loss or deactivation of samples, contamination, and tailing of solute peaks. Most of the centrifugal CCC systems eliminate the use of rotating seals which would become a potential source of complications in the conventional flow-through centrifuge system. During the past twenty years, a series of flow-through coil planet centrifuge schemes has been developed for performing CCC. These instruments are

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suitable for separating various natural and synthetic products in both analytical and preparative purposes.

Recently, an epoch-making advance in the CCC technology has been brought forth by a discovery of a unique hydrodynamic phenomenon which led to the development of high-speed CCC[3,4]. This new CCC method is characterized by high partition efficiency and large retention capacity of the stationary phase under a high flow-rate of the mobile phase, thus yielding highly efficient separations in a few hours. Several types of high-speed CCC instruments have been introduced for analytical and preparative separations. We have used these schemes to separate several kinds of medicinal herbs and obtained excellent results in terms of partition efficiency, separation time and sample loading capacity[5,6].

In order to further improve the performance of high-speed CCC, a question may be raised whether analytical or semi-preparative separations in this technique can be completed within half an hour as in high-performance liquid chromatography (HPLC), while retaining all the special advantages inherent to the support-free partition chromatography. As reported earlier, an analytical high-speed CCC instrument is a miniature multilayer coil planet centrifuge equipped with an analytical column prepared from a long single piece of narrow-bore tubing[7]. Efficient separations can be effected by decreasing both the revolutional radius and weight of the column holder so that the system permits application of higher revolutional speeds which will promote counter-current flow of the two solvent phases through small-diameter tubing. In this case, retention of the stationary phase in the separation column will become more stable while the flow-rate of the mobile phase can be increased to shorten the separation time.

In the present study, a Pharma-Tech Model CCC-2000 analytical high-speed CCC instrument was used to separate a crude flavonoid mixture extracted from dried fruit of sea buckthorn (*Hippophae rhamnoides*). In order to evaluate the analytical capability of the method, a small amount of the sample, 3 mg of the mixture, was loaded in each separation. A series of experiment was performed to study the effects of flow-rates on partition efficiency. At the maximum flow-rate of 300 ml/h, the separation was completed within 15 min without significant loss in peak resolution.

## EXPERIMENTAL

### *Apparatus*

An overall view of the Pharma-Tech Model CCC-2000 instrument (Pharma-Tech Research Co., Baltimore, MD, U.S.A.) is illustrated in Fig. 1, where the cut-out on the left shows the main part of the centrifuge. The rotary frame is driven by a motor around the central axis of the centrifuge. This frame consists of a pair of aluminum plates rigidly bridged by links and holds a column holder and the counterweight holder in the symmetrical positions at a distance of 2.5 in. from the central axis of the centrifuge. The holder shaft is equipped with a plastic planetary gear which is coupled to an identical stationary sun gear rigidly mounted on the central axis of the centrifuge. This gear arrangement produces the desired synchronous planetary motion of the column holder: the holder revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity in the same direction. This motion prevents twisting of the flow tubes and,

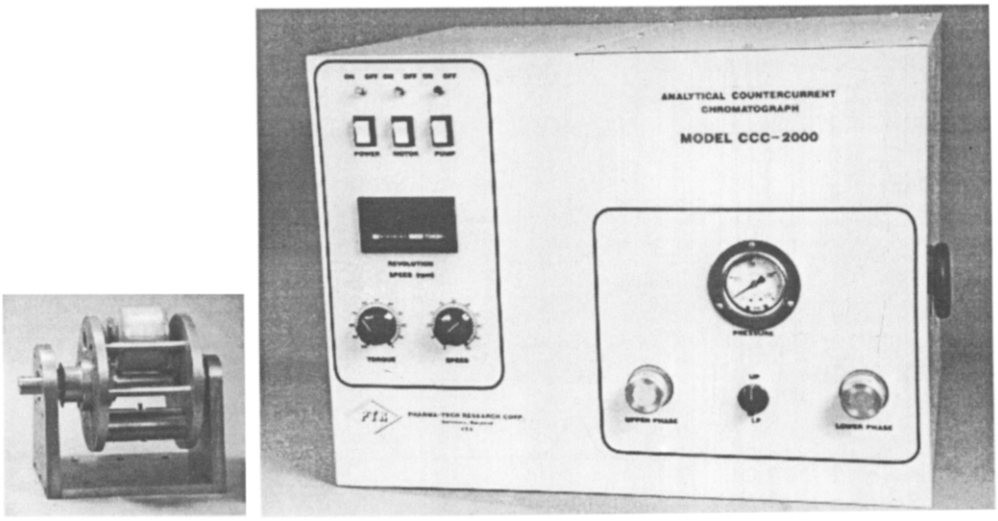


Fig. 1. An overall view of the analytical CCC instrument used in the present experiments.

therefore, permits continuous elution of the mobile phase through the rotating column without the use of rotary seals. The revolutional speed of the centrifuge can be continuously adjusted up to 2000 rpm with a speed controller. A multilayer coiled column was prepared from a single piece of 0.85 mm I.D. heavy wall PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.). The $\beta$ value (ratio of the radius of rotation to the radius of revolution) measures 0.4 at the internal terminal to 0.75 at the external terminal. The total capacity of the column is 43 ml including 3 ml in the flow tubes.

The present model is equipped with an LDC/Milton Roy Pump, a revolution speed controller with digital rpm display, a pressure gauge, etc. (Fig. 1).

*Preparations of two-phase solvent system and sample*

In the present experiments, a two-phase solvent system of chloroform–methanol–water (4:3:2, v/v/v) was selected. The lower non-aqueous phase of the above solvent system was used as the mobile phase in the head-to-tail elution mode. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and separated into two phases before use.

The sample of crude flavonoid mixture used in the present experiments was prepared from dried fruits of sea buckthorn (*H. rhamnoides*) by ethanol extraction. The chromatogram obtained from 100 mg of this sample with the above solvent system by using the Ito multilayer coil separator and extractor (P.C. Inc., Potomac, MD, U.S.A.) is shown in Fig. 2[6]. Five main compounds were found in the chromatogram including isorhamnetin at peak 2 and quercetin at peak 4. In the present study, 3 mg of this sample were dissolved in 0.5 ml of the solvent mixture and loaded for each experiment.

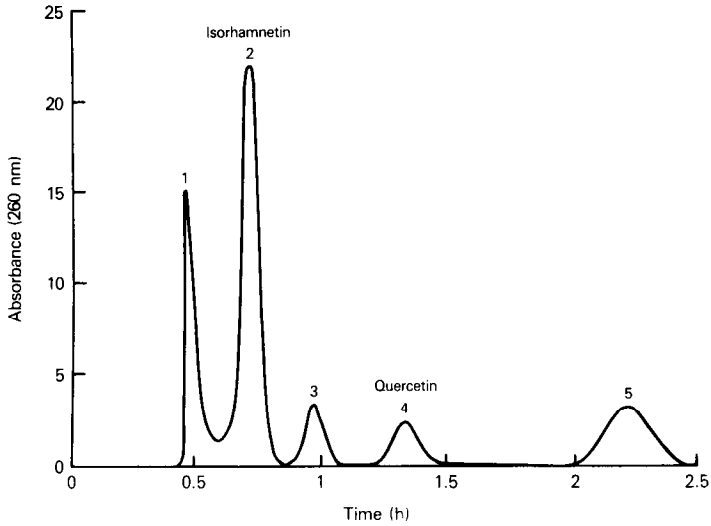


Fig. 2. Chromatogram obtained from 100 mg extract by the Ito multilayer coil separator and extractor.

*Experimental procedures*

In each experiment, the multilayer coiled column of the analytical high-speed CCC centrifuge was entirely filled with the stationary upper phase. Then, 0.5 ml of sample solution containing 3 mg of the sample mixture was injected into the column through the sample port. The centrifuge was rotated at the optimum revolutional speed of 1800 rpm while the mobile lower phase was pumped into the column in a head-to-tail elution mode. The effluent from the outlet of the column was continuously monitored with an LKB Uvicord S at 278 nm and then fractionated into test tubes for 1 ml each tube with an LKB fraction collector. Each fraction was diluted with 2 ml of methanol and the absorbance was determined at 260 nm with a Zeiss spectrophotometer (Model PM6).

A series of experiments was performed by applying a wide range of flow-rates from 60 to 300 ml/h. After each separation, the retention volume of the stationary phase was measured by emptying the column contents into a graduated cylinder by connecting the column inlet to a pressured nitrogen line. The maximum pressure at the outlet of the pump was also recorded in each run.

## RESULTS

Fig. 3 shows the result of separation obtained at a flow-rate of 60 ml/h. Five main components were completely separated and eluted out in about 90 min. High partition efficiency of the present method is evidenced by a minor peak present between the first and the second major peaks, which are not detected in the semi-preparative separation shown in Fig. 2. The peak fraction collected from peak 2 was analyzed by a Finnigan-MAT mass spectrometer, the results being illustrated in Fig. 4. It clearly shows that peak 2 (Fig. 3) consists of isorhamnetin in a highly purified state. Retention value of the stationary phase was 86% and the maximum pressure at the outlet of the pump was 170 p.s.i.

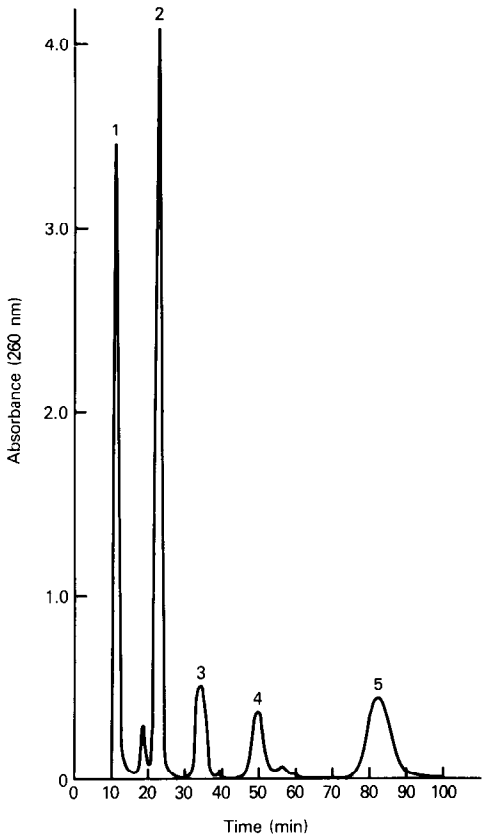


Fig. 3. Chromatogram obtained from 3 mg extract by analytical counter-current chromatography at a flow-rate of 60 ml/ml.

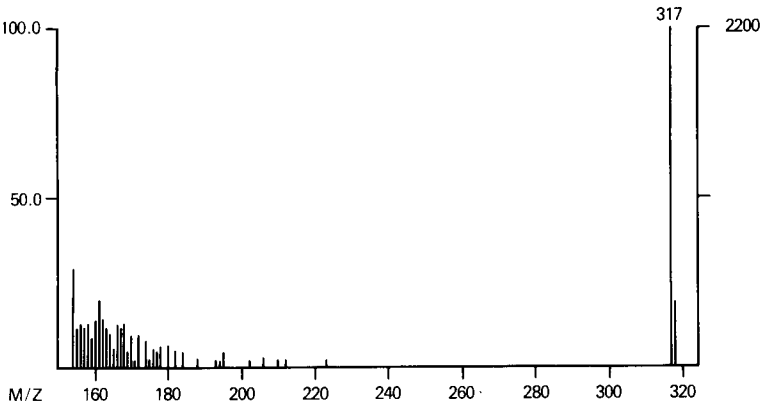


Fig. 4. Mass spectrum of the fraction from peak 2 in Fig. 3. Sample: sea buckthorn second peak. Conditions: Finnigan 4500 mass spectrometer scanned from 150 to 500 a.m.u. in 0.5 s with sample applied to the "Direct Exposure Probe" using CI/ammonia ionisation conditions.

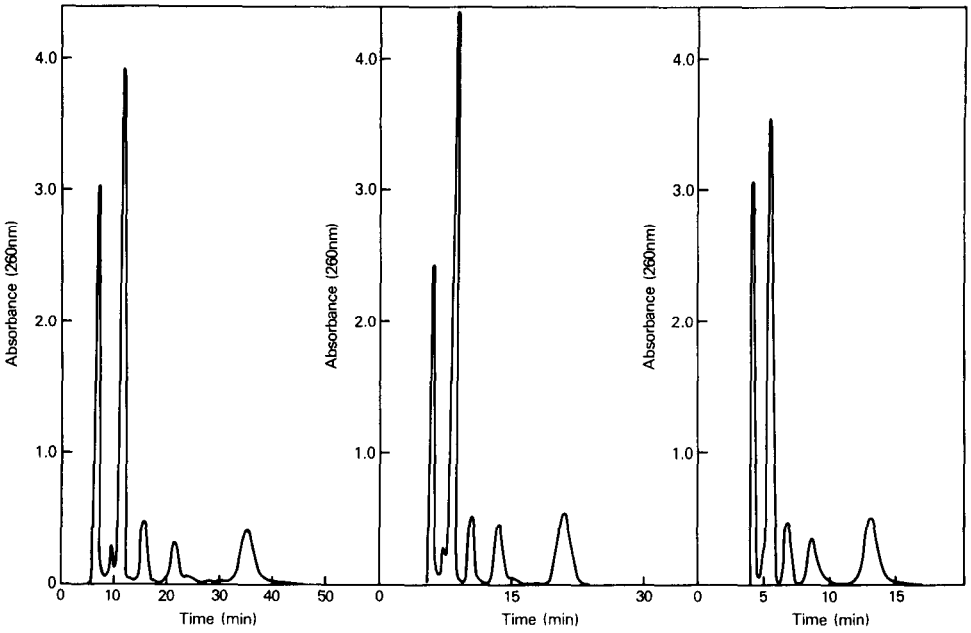


Fig. 5. Chromatograms obtained from 3 mg extract by analytical counter-current chromatography at flow-rates of 120 ml/h (left), 200 ml/h (middle) and 300 ml/h (right).

A set of chromatograms obtained at higher flow-rates of 120, 200 and 300 ml/h are shown in Fig. 5. Separation times of these experiments were 40, 25 and 15 min, respectively. The retention values were decreased from 77.5% at the 120 ml/h to 68% at the maximum flow-rate of 300 ml/h, while the maximum pressure was increased from 250 to 330 p.s.i. All of these data are summarized in Table I.

## DISCUSSION

Analytical high-speed CCC can yield highly efficient chromatographic separations of solutes in a short period of time. As demonstrated in the chromatograms

TABLE I

EFFECTS OF FLOW-RATE OF THE MOBILE PHASE ON VARIOUS PARAMETERS

Revolutional speed: 1800 rpm.

| *Flow-rate (ml/h)* | *Retention of stationary phase (%)* | *Maximum pressure (p.s.i.)* | *Separation time (min)* |
|---|---|---|---|
| 60 | 86 | 170 | 90 |
| 120 | 77.5 | 250 | 40 |
| 200 | 72.8 | 270 | 25 |
| 300 | 68 | 330 | 15 |

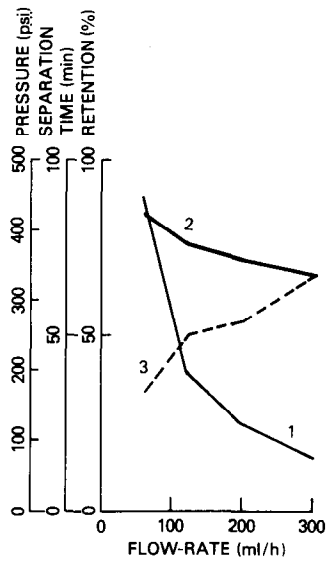


Fig. 6. Composite effects of the flow-rate on separation time (1), retention of the stationary phase (2), and the maximum column pressure (3) at the outlet of the pump.

obtained in the present studies, five major compounds present in the crude mixture were completely resolved in 90–15 min. Several small peaks of minor components can also be detected among the major peaks with high reproducibility. High purity of the separated fractions has been confirmed by mass spectrometric analysis.

By increasing the flow-rate of the mobile phase from 60 to 300 ml/h, the separation time of the crude sample mixture was shortened from 90 to within 15 min which is quite comparable with that of analytical HPLC. Even in the chromatogram obtained from the fastest elution shown in Fig. 5 (right) integrity of the five major peaks is well preserved, although resulting in loss of peak resolution among several minor components.

The sample size used in each separation was as large as 3 mg (0.5 ml) which is much greater than that applied in other analytical separation techniques. This provides an advantage in that effluent of the analytical CCC can be fractionated into test tubes to recover the purified materials by evaporating the solvent.

Fig. 6 summarizes effects of the flow-rate on various other factors such as separation time, retention of the stationary phase and the maximum column pressure measured at the outlet of the pump. The separation time of the five components decreases approximately in a reversed proportion to the flow-rate of the mobile phase from 90 min at 60 ml/h to 15 min at 300 ml/h. The retention of the stationary phase also tends to decline with the increased flow-rate but in a much slower rate. On the other hand, the maximum column pressure increases somewhat linearly from 170 to 330 p.s.i. in accordance with Poiseuille's law.

In the past, hydrodynamic motion of the two solvent phases in the rotating column was observed under stroboscopic illumination[8]. The results revealed an extremely interesting hydrodynamic phenomenon in which about 25% of the segment

in each helical turn located near the center of the centrifuge showed an evidence of vigorous agitation while in the rest of the segment the two solvent phases were separated into two distinct layers with the heavier phase along the outer portion and the lighter phase along the inner portion of each segment. Since the column is rotating with respect to the centrifugal force field, the above finding indicates that the two solvent phases present at any portion in the coiled column is subjected to a repetitive mixing and settling process at a high frequency synchronous with the revolution, *i.e.*, thirty times a second in the present experiment. Consequently, the solutes are subjected to an efficient partition process even under a high flow-rate of the mobile phase. This powerful partitioning capability of the high-speed CCC system was clearly demonstrated in the present experiment by the fact that a five-fold increase of the flow-rate from 60 to 300 ml/h resulted in the reduced separation time from 90 to 15 min without significant loss in resolution between major peaks as shown in Figs. 3 and 5. Loss of resolution among the minor peaks may be partly caused by reduced retention volume of the stationary phase from 86 to 68% at the maximum flow-rate of 300 ml/h.

Rapid and efficient separations attained in the present experiments may be largely attributed to the favorable physical properties of the chloroform solvent system which provides large density difference and relatively high interfacial tension between the two solvent phases. Because of their short settling time, chloroform–methanol–water solvent systems can produce satisfactory phase retention even under a unit gravitational field as well documented in droplet CCC[9]. In order to obtain comparable results with other types of two-phase solvent systems such as hexane–ethyl acetate–methanol–water at various volume ratios, it may be necessary to apply much higher revolutional speed to accelerate phase settling in the rotating column. This goal will be achieved by further refining the present coil planet centrifuge system.

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