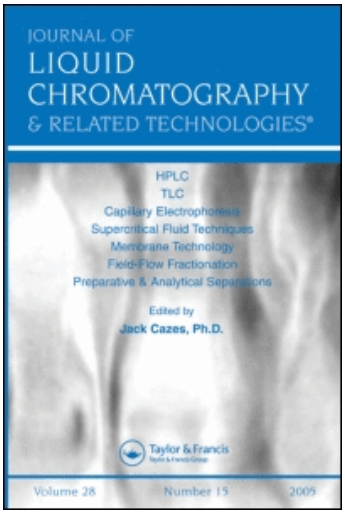

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SEPARATION OF FLAVONOIDS IN CRUDE EXTRACT FROM SEA BUCKTHORN BY COUNTERCURRENT CHROMATOGRAPHY WITH TWO TYPES OF COIL PLANET CENTRIFUGE

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ABSTRACT

Flavonoid constituents in a crude ethanol extract from dried fruits of sea buckthorn (Hippophae rhamnoides) were subjected to countercurrent chromatography with a two-phase solvent system composed of chloroform, methanol and water at a 4:3:2 volume ratio. Separations were performed with two different types of the coil planet centrifuge: One is called the multilayer coil planet centrifuge and the other, the horizontal flow-through coil planet centrifuge. Both instruments permit continuous elution through the rotating column without the use of rotary seals. Although the horizontal flow-through coil planet centrifuge produced efficient peak resolution for five flavonoid components, the multilayer coil planet centrifuge yielded much superior results in terms of partition efficiency, separation time and sample loading capacity.

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INTRODUCTION

Countercurrent chromatography (CCC) is an efficient technique of partition chromatography that totally eliminates the use of solid supports. Since 1970, Ito and his coworkers have developed a series of flow-through coil planet centrifuge schemes for performing CCC (1-8). These instruments are especially suitable for separating natural products in both analytical and preparative purposes. In the early 1980's the authors began to use a horizontal flow-through coil planet centrifuge in China for separating medicinal herbs, such as the mixture of quercetin and rutin, squalidine and platyphylline, etc. (9).

This paper introduces the results of separations of a crude flavonoid sample extracted from dried fruits of the sea buckthorn (Hippophae rhamnoides) by our horizontal flow-through coil planet centrifuge and a commercial model of the multilayer coil planet centrifuge. Five flavonoid constituents were successfully separated by coil planet centrifuge techniques. Performance of the above two instruments are compared in terms of partition efficiency, separation time, sample loading capacity, etc.

EXPERIMENTAL

Apparatus

The present studies were carried out with two different types of the coil planet centrifuge: one is called the horizontal flow-through coil planet centrifuge and the other, Ito multilayer coil separator and extractor (P. C. Inc., Potomac, MD). Both

instruments provide identical planetary motion of the column holder, i.e., rotation about its own axis and revolution around the central axis of the centrifuge at the same angular velocity in the same direction. This particular mode of synchronous planetary motion of the holder prevents twisting of the flow tubes and therefore permits continuous elution of the mobile phase through the rotating column without the use of a conventional rotary seal device which would become a potential source of various complications such as leakage, contamination, etc.

The horizontal flow-through coil planet centrifuge was manufactured by New Technology Application Research Institute of Beijing, China and the detailed design of the apparatus has been described elsewhere (9). The rotary frame of the apparatus hold a pair of column holders symmetrically at a distance of 14 cm from the central axis of the centrifuge. A short coiled column or column unit was prepared by winding a piece of PTFE (polytetrafluoroethylene) tubing, 2.5 mm I.D. and 0.5 mm wall thickness, onto a stainless steel pipe of 1.25 cm O.D. and 50 cm in length, making about 117 helical turns. Eight column units were connected in series and mounted symmetrically around the column holder at a distance of 3.2 cm from the holder axis with a β value of 0.23. The β value is given by a ratio of the radius of rotation (r) to the radius of revolution (R) or $\beta = r/R$ which is an important parameter to determine hydrodynamic distribution of the two solvent phases in the rotating coiled column. The total capacity of the coiled column measured approximately 200 ml.

In the second apparatus called the Ito multilayer coil separator and extractor (a commercial model of the multilayer coil planet centrifuge), the column holder is positioned at a distance of 10 cm from the central axis of the centrifuge. The separation column was prepared by winding a long piece of PTFE tubing, 1.6 mm I.D. and 0.3 mm wall thickness, directly onto the holder hub of 10 cm diameter making multiple coiled layers. The β value ranges from 0.5 at the internal terminal to 0.8 at the external terminal. The total capacity of the multilayer coil measures about 280 ml.

Preparation of Two-Phase Solvent System and Sample Solution

A two-phase solvent system composed of chloroform/methanol/water at a 4:3:2 volume ratio was exclusively used in the present experiment. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and separated before use.

Crude flavonoid sample used for CCC separation was yellow powder prepared from dried fruits of sea buckthorn (H. rhamnoides) by ethanol extraction. The sample is insoluble in water. The sample solution was prepared by dissolving the above sample in the solvent mixture at a concentration of 2.2 g%.

Experimental Procedures

Separation of crude flavonoid sample was performed by the two types of CCC instruments using the lower nonaqueous phase as the mobile phase. Separation with the multilayer coil planet centrifuge was performed as follows: The coiled column was first entirely filled with the stationary upper phase followed by

injection of sample solution through the sample port. Then, the apparatus was rotated at the optimum revolutional speed of 800 rpm while the lower mobile phase was pumped into the head end of the column at 200 ml/h flow rate. Effluent from the tail of the coiled column was continuously monitored with a UV monitor at 278 nm and fractionated into test tubes with a fraction collector. Aliquot of each fraction was diluted with methanol and the absorbance was determined at 260 nm with a spectrophotometer. Separation with the horizontal flow-through coil planet centrifuge was similarly performed except that the column was first eluted with the mobile phase for about 5 min before injection of the sample solution. Detailed experimental conditions applied to both separations are listed in Table 1.

TABLE 1

Comparison of experimental conditions applied to separations on two types of coil planet centrifuge (CPC)

Apparatus	Sample (mg)	Column Capacity (ml)	Revolution (rpm)	Flow Rate (ml/hr)	S.P* Retention (%)	Time (hr)
Multilayer CPC	100	280	800	200	65.7	2.5
Horizontal Flow-through CPC	20	200	300	60	35.0	4.5

*S.P. - Stationary Phase

Partition Efficiency Determination

Partition efficiency of the multilayer coil was calculated from the chromatogram according to the conventional gas chromatographic formula.

$$N = (4R/w)^2 \quad (1)$$

where N is partition efficiency expressed in terms of theoretical plates (TP); R, retention time of the peak maximum; and w, the peak width expressed in the same unit as R.

Partition Coefficient Determination

Once the solute peaks are well resolved, the partition coefficient of each component can be estimated from the chromatogram by measuring the retention time of the peak maximum and using the following equation

$$K(Cm/Cs) = (C - R_f)/(R - R_f) \quad (2)$$

where K is the partition coefficient obtained by solute concentration in the mobile phase divided by that in the stationary phase; C, the total column capacity; R_f, the retention volume of the solvent front; and R, the retention volume of the solute peak. The partition coefficient of each component is also experimentally determined by redistributing the peak fraction and measuring the absorbance in the upper and the lower phases. The above methods were used to identify the quercetin and isorhamnetin from the fractions obtained from the multilayer coil separation.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of the crude flavonoid sample obtained with the horizontal flow-through coil planet centrifuge. Five flavonoid peaks were resolved and eluted out in 4.5 hours. The results obtained with the present CCC method are substantially better than those from the conventional column chromatography in terms of both peak resolution and separation time.

Fig. 2 shows a similar chromatogram obtained with the multilayer coil planet centrifuge. Compared with the results obtained with the horizontal flow-through coil planet centrifuge, separation are much improved. All components were completely resolved from each other as symmetrical peaks and eluted out in 2.5 hours. Partition efficiencies computed from equation (1) range from 800 TP (2nd peak) to 530 TP (5th peak). By calculating the partition coefficients of each peak according to equation (2) and comparing the obtained values with those of the pure compounds, quercetin and isorhamnetin peaks were identified as labelled in the chromatogram.

The above results clearly indicate superior performance of the multilayer coil planet centrifuge. In spite of five-fold sample loading, the method yielded much higher peak resolution and the separation was completed in a considerably shorter period of time.

Partition efficiency in CCC highly depends upon the applied centrifugal force field and the column geometry on the holder.

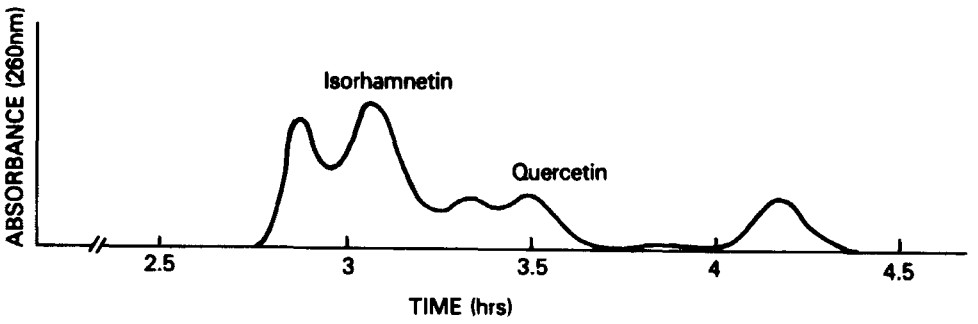

Figure 1: Countercurrent chromatogram of flavonoids in crude extract from dried fruits of sea buckthorn by a horizontal flow-through coil planet centrifuge. Conditions for CCC: Sample size, 20 mg; solvent system, chloroform/methanol/water (4:3:2); mobile phase, lower phase; flow rate, 60 ml/hr; revolution, 300 rpm; fraction volume, 3 ml.

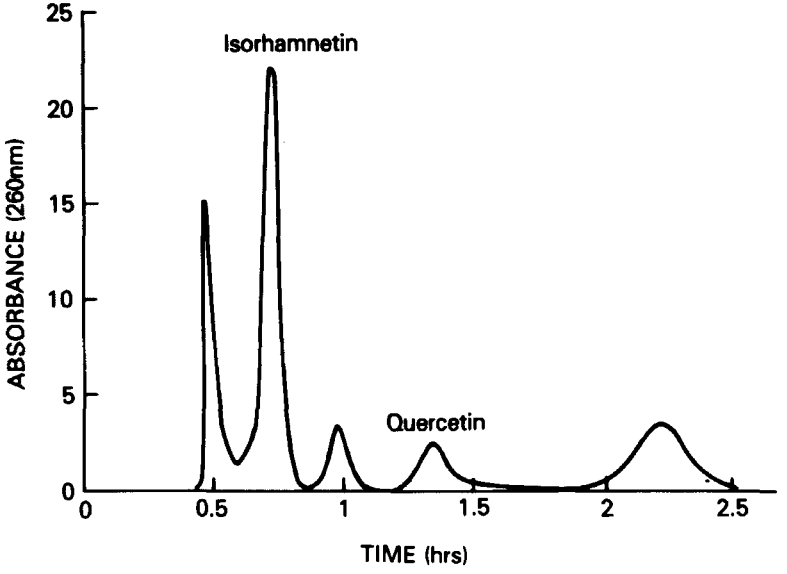

Figure 2: Countercurrent chromatogram of flavonoids in crude extract from dried fruits of sea buckthorn by a multilayer coil planet centrifuge. Conditions for CCC: sample size, 100 mg; solvent system, chloroform/methanol/water (4:3:2); mobile phase, lower phase; flow rate, 200 ml/hr; revolution, 800 rpm; fraction volume, 6ml.

The above two types of the coil planet centrifuge produce an identical mode of planetary motion which yields a highly complex heterogeneous distribution of centrifugal force vectors. Except for the vicinity of the holder axis, each vector radiates outwardly from the holder while periodically changing its magnitude and direction during each revolutional cycle (10). This centrifugal force field produces different hydrodynamic distribution of two solvent phases according to the location and orientation of the coiled column on the holder.

In the horizontal flow-through coil planet centrifuge, each coiled column is mounted on the periphery of the holder. Under this eccentric coil orientation, the radially directed centrifugal force field distributes two solvent phases in the coil in such a way that the lighter phase is held in the inner loop and the heavier phase in the outer loop of each helical turn. Consequently, elution with either phase through the coil retains the other phase in each helical turn while the fluctuating centrifugal force field steadily agitates the two solvent phases to promote the partition process. While the system provides an efficient solute partitioning, the volume of the stationary phase retained in the column is always less than 50% of the total capacity of the column which further decreases with higher flow rate of the mobile phase. This limits the application of a high flow rate of the mobile phase in the horizontal flow-through coil planet centrifuge.

In the multilayer coil planet centrifuge, the coiled column is coaxially mounted around the holder which creates completely

different hydrodynamic effects. Under this coil orientation, the outwardly directed centrifugal force field separates the two solvent phases in the coil in such a way that the lighter phase is layered over the heavier phase along the length of the coil while the undulating centrifugal force field moves one of the phases (head phase) toward the head to establish a unilateral hydrodynamic distribution of the two solvent phases in the coil. This unilateral phase distribution can be effectively utilized in performing CCC in the two different ways: The coil is first filled with the tail phase followed by elution with the head phase from the tail toward the head of the coil. Alternatively, the coil is first filled with the head phase followed by elution with the tail phase from the head toward the tail of the coil. In either elution mode the system permits retention of a large volume of the stationary phase in the coil against a high flow rate of the mobile phase. Furthermore, stroboscopic observation of the rotating column revealed a vigorous local agitation of the two solvent phases in each helical turn at the vicinity of the centrifuge axis. Each mixing zone travels through the coil toward the head at a rate of one turn per one revolution. Thus the two solvent phases at any portion of the coil are subjected to a typical partition process of repetitive mixing and settling at an extremely high frequency of over 13 times per second while the mobile phase is constantly passing through the coil (8). Consequently, the system enables highly efficient chromatographic separation of solutes under a high flow rate of the mobile phase.

The large difference in performance between the two CCC centrifuges, as demonstrated in the present studies, may well be explained on the basis of the above hydrodynamic phenomena.

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