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THE APPLICATION OF TRUE COUNTERCURRENT CHROMATOGRAPHY IN THE ISOLATION OF BIOACTIVE NATURAL PRODUCTS

Y.W. LEE,* Q.C. FANG,[1] Y. ITO,[2] and C.E. COOK

Research Triangle Institute, Chemistry and Life Sciences, Post Office Box 12194, Research Triangle Park, North Carolina 27709

ABSTRACT.—This paper describes a highly economical and efficient chromatography system based on continuous countercurrent principles.

In the past decade, the rapid development of sophisticated spectroscopic techniques, including various 2D nmr methods, automated instrumentation, and routine availability of X-ray crystallography, has greatly simplified structural elucidation in natural products investigations. Consequently, the challenge has shifted to one's capability of isolating the bioactive components from the crude natural product extracted either from plants or animals.

The extract of crude natural products usually comprises hundreds of components with a wide range of polarities. It is essential to preserve the desired bioactivity while performing chromatographic purifications.

Countercurrent distribution has long been recognized as an effective means for purification of a wide variety of bioactive molecules (1,2). The major drawbacks have been the long separation time required and the inconvenience in operating the countercurrent distributor. Recently, countercurrent chromatography has gained increasing popularity in fractionation of natural products, and a number of highly efficient systems have been developed (3–5).

The newly developed true countercurrent chromatography (true ccc) based on continuous liquid-liquid partitioning has many advantages in dealing with crude natural products because complications arising from solid adsorbance, such as sample degradation, deactivation, and contamination, are eliminated (6,7).

Basically, the system consists of a multilayer coiled column integrated with two solvent inlets, two solvent outlets, and one sample feed line. Subjecting the system to a particular combination of centrifugal and planetary motions produces a unique hydrodynamic effect that allows two immiscible liquids to flow countercurrently through the multilayer coiled column. The sample is fed through the middle portion of the column. The extreme polar and nonpolar components are readily eluted from the opposite ends of the column followed by components with decreasing order of polarity in one phase solvent and increasing order of polarity in the other phase solvent. A component with a partition coefficient equal to 1.0 will remain inside the column. Essentially, the system permits a highly efficient method of performing conventional countercurrent distribution. The capability of this newly developed true countercurrent system has been demonstrated in the fractionation of a complex steroid mixture (5).

The capability and versatility of true ccc are further demonstrated in our isolation of bioactive lignans from an EtOH extract of *Schisandra rubriflora* Rhed. et Wils. (Schisandraceae), which has been used in Chinese herbal medicine for treatment of chronic

[1]Institute of Mareria Medica, Chinese Academy of Medical Sciences, Beijing, China.
[2]Laboratory of Technical Development, National Heart, Lung, and Blood Institute, Bethesda, Maryland 20892.

hepatitis. Lignans in the kernel were found to be responsible for lowering the SGPT levels of rats with CCl_4-induced hepatitis (8).

EXPERIMENTAL

REAGENTS AND MATERIALS.—Organic solvents used for preparation of the two phase solvent systems, including hexanes, EtOAc, MeOH, and EtOH, were glass-distilled chromatographic grade purchased from Burdick and Jackson Laboratories, Muskegon, Michigan. True ccc experiments were performed with a two-phase solvent system of hexanes-EtOAc-MeOH-H_2O (10:5:5:1). The two-phase solvent system was prepared by thoroughly equilibrating the solvent mixture in a separatory funnel at room temperature followed by filtration through a 5-μm filter and degassing.

The *Schisandra* samples were kindly provided by Professor Y.Y. Chen (Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China). The EtOH extract of the kernels of *S. rubriflora* was used for the true ccc study.

APPARATUS.—The true ccc experiments were performed with a table-top model high speed planet centrifuge equipped with a multilayer coiled column, as described in detail by Ito (4).

The multilayer coiled column was prepared from 2.6 mm i.d. PTFE tube (Zeus Industrial Products, Raritan, NJ) by winding it coaxially onto the holder with a total volume capacity of 400 ml. In order to facilitate the countercurrent process, the head terminal was located at the outer layer and the tail terminal at the inner layer of the coiled column. Each terminal is equipped with a 3-way adaptor that connects inlet and outlet flow tubes to the coiled column, whereas at the middle portion of the column a sample feed tube opens through another 3-way adaptor. At each terminal the feed line is passed through the adaptor to extend into the separation column for about 50 cm or one complete helical turn. This prevents the introduced phases from flowing back toward the immediate outlet opening at each terminal. The liquid collection lines were made of 0.85 mm i.d. PTFE tubes, and the other three feed lines were 0.55 mm i.d. tubes. As mentioned earlier (7), these 5 flow tubes from the separation column are free from twisting.

The rotational speed of the apparatus is continuously adjustable up to 1000 rpm, but the rate was limited to 700 rpm throughout the present study. Two Model 6000A hplc pumps (Waters Assoc., Milford, MA) were used to pump the liquid phases. The flow rate through the liquid collection line (upper phase) was regulated by the use of a needle valve.

METHOD.—In a typical operation mode, the multi-layer coiled column is first filled with two solvents simultaneously from opposite ends of the coiled column, while the two collection lines are closed and the sample feed line connected at the middle of the coiled column is left open to let the air out. After the coiled column is filled up, the lighter phase solvent is connected to the solvent inlet line at the tail end and the lower phase solvent is connected to the solvent inlet line at the head end. The sample feed line is then closed; the two solvent outlet lines are left opened. The system is revolved at 700 rpm while the two solvents are pumped into the system at same flow rates until the eluents from both ends of the column are clear and free from the other phase. The revolution then is stopped and sample solution is introduced through the sample feed line. Afterward, the revolution is resumed at 700 rpm and the effluents from the head and tail ends are simultaneously fractionated into a series of test tubes with a Buchler LC 200 fraction collector. Each fraction is monitored with tlc and with an analytical hplc system [Zorbax-ODS 4.6 mm × 25 cm; MeOH-H_2O (85:15)].

RESULTS AND DISCUSSION

One distinct feature of the true ccc system is that the two fresh mobile phases are continuously countercrossing each other; thus a multicomponent mixture can be fractionated simultaneously at both ends of the eluents according to the partition coefficients of individual components. A component with a partition coefficient of 1.0 will remain inside the column.

The resolution and versatility of true ccc have been successfully demonstrated in the preparative separation of plant indoles and steroid mixtures (7).

Figure 1 shows an analytical hplc trace of the crude extract from *S. rubriflora*. Because the major bioactive lignan schisanhenol (peak 6) is closely eluted with its acetate (peak 5), it has been a major problem to isolate these bioactive lignans on a preparative scale. When the crude *Schisandra* sample was fed through the middle portion of the column, the extreme polar and nonpolar components were readily eluted from the opposite end of the column followed by components with increasing order of polarity in the

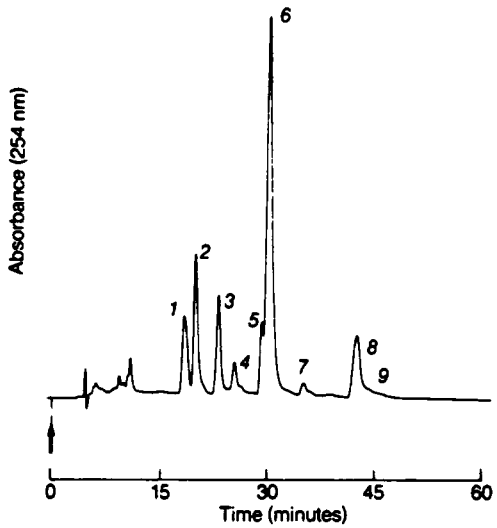

FIGURE 1. Hplc chromatogram of crude EtOH extract of kernels of *Schisandra rubiflora*. Column conditions: Zorbax ODS, 250 × 4.6 mm i.d.; mobile phase, MeOH-H_2O (75:25).

upper mobile phase and decreasing order of polarity in the lower mobile phase. As shown in Figure 2, the fractions eluted from upper phase and from lower phase were monitored by tlc. The upper phase, being less polar than the lower phase, provides a sequence of elution similar to normal phase chromatography (Figure 2A). At the other end, the lower (polar) phase provides a sequence of elution resembling reversed-phase chromatography (Figure 2B).

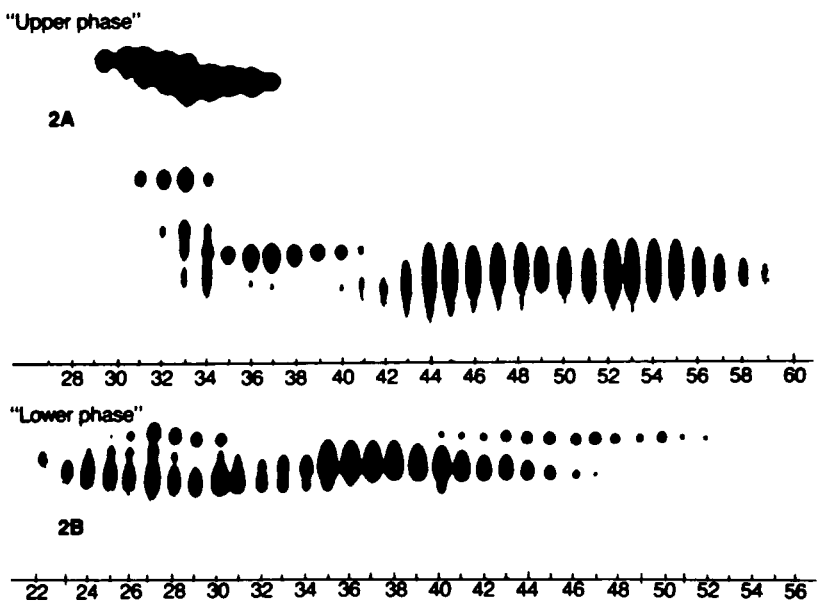

FIGURE 2. Tlc chromatogram of fractions eluted from upper phase (2A) and lower phase (2B) of a true ccc separation of 125 mg the crude EtOH extract of kernels of *Schisandra rubiflora*. True ccc solvent system: hexanes-EtOAc-MeOH-H_2O (10:5:5:1), flow rate 2 ml/min; tlc solvent system: CH_2Cl_2-Me_2CO (95:5).

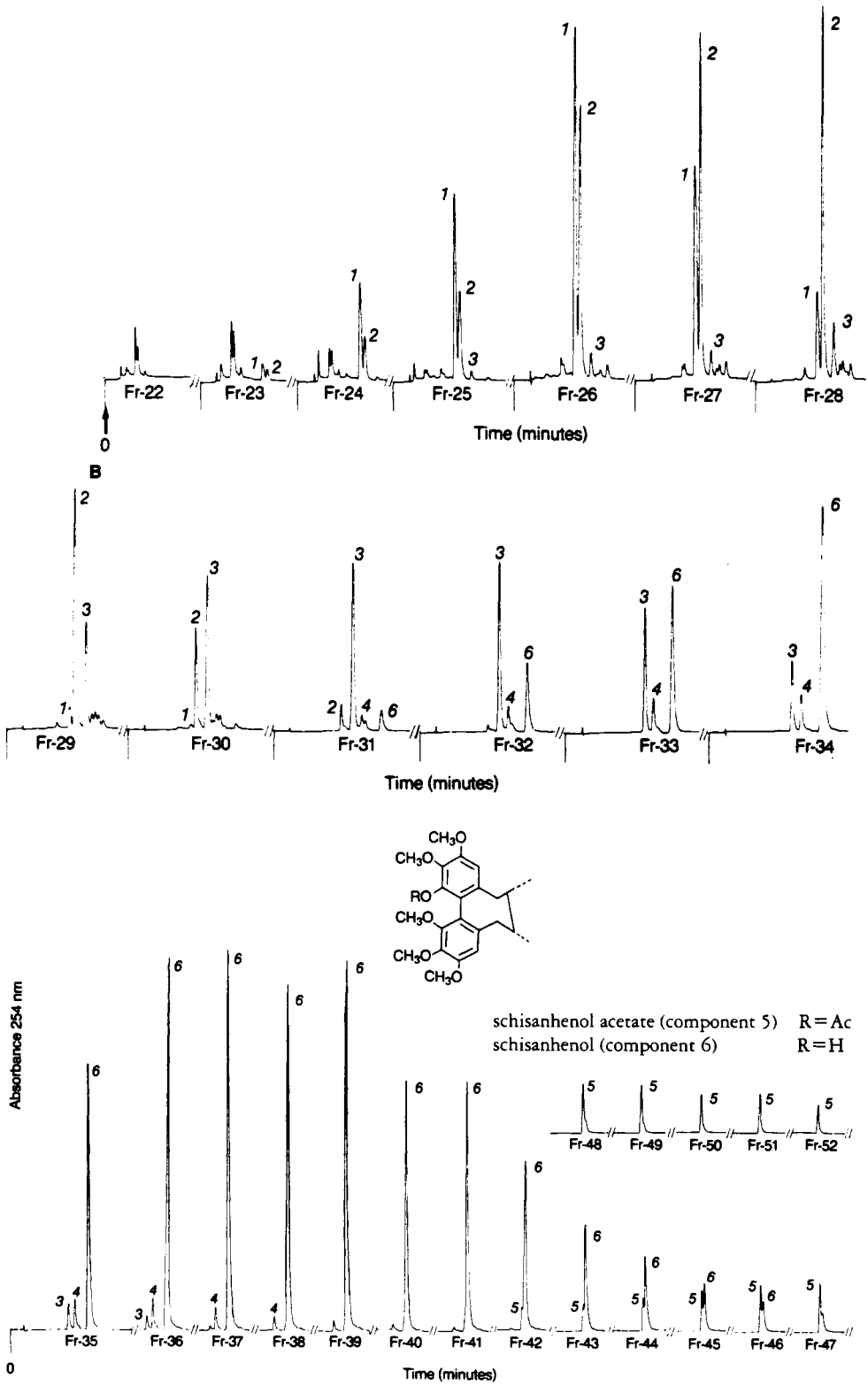

FIGURE 3. Hplc traces of individual fractions eluted from lower phase of a true ccc of the crude EtOH extract of *Schisandra rubiflora*. Column conditions the same as for Figure 1.

Because our desired bioactive lignans, schisanhenol and schisanhenol acetate, were eluted from the lower phase, a reversed-phase analytical hplc system was employed in addition to tlc to monitor the purity of each individual fraction.

It is important to note that the sequence of elution from countercurrent chromatography is according to the partition coefficient of each component in the two immiscible solvents employed. Therefore, it is not unusual to observe that certain fractions eluted from ccc show a quite different pattern of polarities when examined with a tlc which is based entirely on an adsorption-deadsorption process on Si gel. For example, the component eluted from upper phase F-43 to F-59 in Figure 2A must have a high partition coefficient in the upper phase; nevertheless, on the tlc plate it showed low R_f values. Furthermore, the lower phase fractions from F-22 to F-34 in Figure 2B showed similar R_f values and no separations; however, these fractions are quite different from each other when examined by hplc analyses.

As shown in Figure 3, the rise and fall of each component peak in the hplc trace reveals not only the sequence of elution but also the resolution of true ccc. For example, the lower phase fractions 22 and 23 contain mainly the extremely polar impurities. Components 1 and 2 eluted from F-23 and reached a maximum in fraction 26. Gradually, the component 1 decreased and components 2 and 3 increased. Fraction 28 showed a majority of component 2, and fraction 31 showed a majority of component 3. The desired components, schisanhenol (component 6) and its acetate (component 5), were also successfully resolved, as shown from lower phase fractions 35 to 52 in which essentially pure schisanhenol (32 mg) was obtained from F-36 to F-40 and pure acetate (4 mg) was isolated from F-50 to F-57.

The sequence of elution on reversed-phase hplc (component 5 followed by 6) implies the phenolic compound 6 is less polar than its acetate 5. This may be due to formation of an intramolecular hydrogen bond in compound 6. However, in the true ccc, the phenol 6 appears to be more polar than acetate 5, as compound 6 elutes first in the polar phase. Thus, there is a difference in the interaction of these compounds with the solid phase vs. their interaction with the liquid-liquid partition of true ccc.

In conclusion, true ccc offers an excellent method for preparative isolation of bioactive components from natural products, as evidenced in the isolation of pure bioactive lignans, schisanhenol and its acetate, from a crude natural product extract. Its capability of dealing with very crude samples with a wide range of polarity, its high resolution and sample loading capacity, and its mild operating conditions for fractionation of bioactive components are the unique features of this new countercurrent technique.

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