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Note

Modifications to a high-speed counter-current chromatograph for improved separation capability

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Counter-current chromatography (CCC) is a liquid–liquid partition method without solid supports that permits the separation of very diverse samples[1]. The development of high-speed CCC (HSCCC) has led to a dramatic decrease in separation times by using a multilayer coil coaxially mounted on a holder rotating at high revolutional speeds[2]. HSCCC with the Ito multi-layer coil separator–extractor has found numerous applications in, for example, the fields of antibiotics[3], flavonoids[4] and alkaloids[5].

As part of our investigation into the use of centrifugal counter-current chromatography (or centrifugal partition chromatography) for the separation of natural products[6], we have introduced certain modifications to the Ito instrument in order to allow the following manipulations: (a) injection of sample without stopping the solvent flow, (b) variation of the relative proportions of the two phases in the coil and (c) reversal of phase flow.

EXPERIMENTAL

Separations were carried out at *ca.* 20°C with an Ito multi-layer coil separator–extractor (P.C., Potomac, MD, U.S.A.), equipped with a 2.6 mm I.D. coil (volume 360 ml). Ancillaries included a sample loop (with six-way valve) and a second valve to permit rapid switching of the solvent to the "head" or "tail" ends of the coil. The chromatograph was connected to two Waters Assoc. 6000A high-performance liquid chromatographic (HPLC) pumps (see Fig. 1), pump A for delivery of the upper phase and pump B for delivery of the lower phase of any biphasic solvent system.

For normal operation, the immobile coil was first filled with stationary phase. After commencing rotation, mobile phase was then introduced. When the mobile phase was the lower phase, solvent was pumped into the "head" end of the coil and when the mobile phase was the upper phase, it was pumped into the "tail" end of the coil. At the point where stationary phase no longer eluted from the coil and stable conditions were obtained, the sample was introduced via the sample loop.

By varying the speeds of the pumps, both the flow-rate of the eluate and the composition of the stationary phase in the coil could be regulated. In order to introduce a certain volume of upper (or lower) phase, the two phases were pumped

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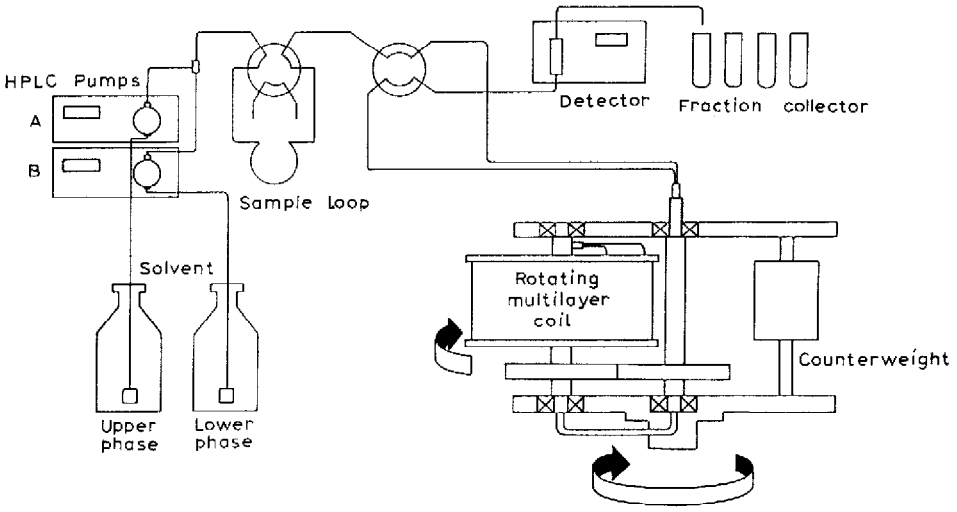

Fig. 1. Ito multi-layer coil separator–extractor and solvent pumping system.

simultaneously into the immobile coil at the required flow-rates before commencing rotation. For example, if 80% lower phase was desired in the coil, lower phase was pumped into the non-rotating coil at 8 ml/min with pump B and upper phase at 2 ml/min with pump A. When the coil had been filled, pumping was stopped and rotation was started. After allowing a few minutes for stabilization, the necessary mobile phase (upper or lower phase of the solvent system) was passed into the instrument and the sample was loaded. Solvent introduction via the "head" or "tail" ends of the coil was selected as above. At the end of a separation run, the proportions of the two phases in the coil could be checked by blowing out the solvent with nitrogen.

The instrument was connected to a Uvicord 2238 SII detector (254 nm) (LKB, Bromma, Sweden), a Model 600 chart recorder (W + W Scientific, Basle, Switzerland) and an LKB Ultrorac II fraction collector. Samples were dissolved in equal amounts of the two solvent phases before injection.

RESULTS AND DISCUSSION

Variation of phase ratios

By altering the proportion of stationary phase in the coil (by changing the relative flow-rates of the two HPLC pumps; see Experimental), the retention times of peaks in the chromatogram could be changed at will. This phenomenon is illustrated by a separation of anthranoid pigments from a root bark light petroleum (b.p. 60–80°C) extract of *Psorospermum febrifugum* (Guttiferae)[6] (Fig. 2). With the upper phase of the non-aqueous solvent system hexane–acetonitrile-methanol (40:25:10) as the mobile phase, the elution order was as shown on the left-hand side of Fig. 2. Use of the lower phase as the mobile phase gave the reverse elution profile. In Fig. 2a–c, increasing amounts of lower phase in the coil increased retention times, whereas in

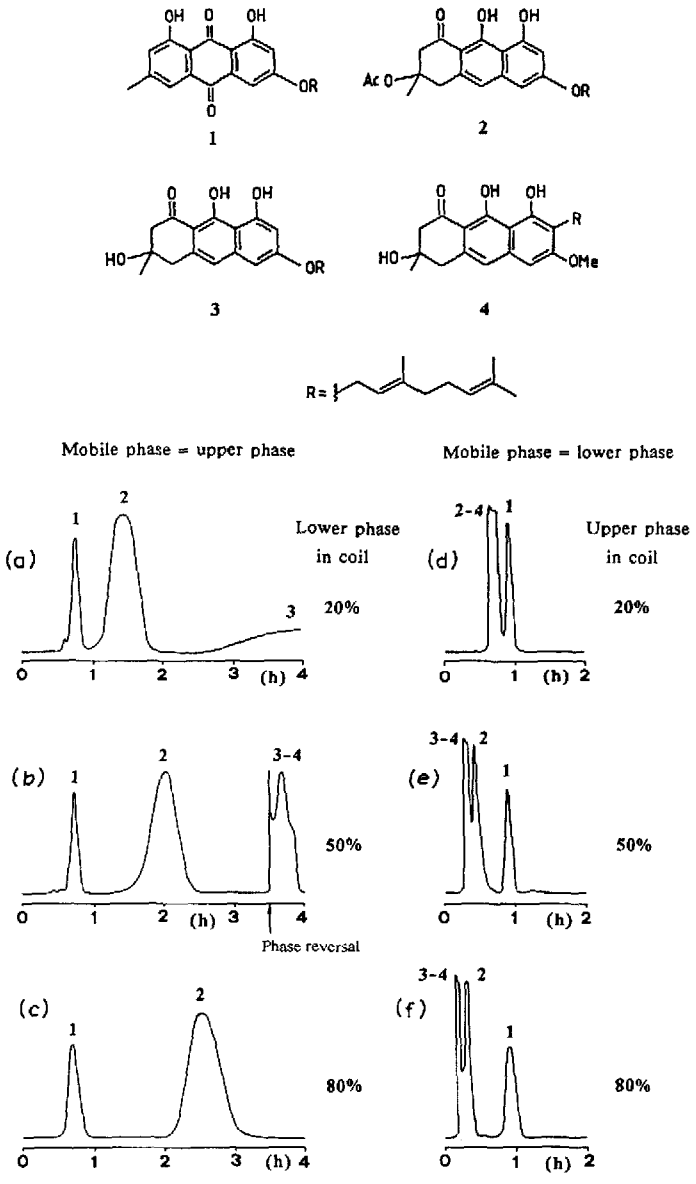

Fig. 2. Separation of *P. febrifugum* root bark pigments on an Ito CCC instrument with different compositions of the stationary phase. Solvent system, hexane–acetonitrile–methanol (40:25:10); sample 100 mg of light petroleum extract; flow-rate, 4 ml/min; rotational speed, 700 rpm; detection, 254 nm. Ac = Acetyl; Me = methyl.

Fig. 2d–f, increasing amounts of upper phase in the coil decreased retention times. Interestingly, the elution time of **1** was hardly affected by changing the stationary phase composition in either of the two modes.

Phase reversal

Fig. 3a shows the separation of the flavonoids hesperetin (**5**), kaempferol (**6**) and quercetin (**7**) with the lower phase of the solvent system chloroform–methanol–water (33:40:27) as the mobile phase. By using the upper phase as the mobile phase (Fig. 3b), a considerably longer separation time resulted.

Starting the separation of flavonoids **5**–**7** with the upper phase as mobile phase and then changing to the lower phase after elution of quercetin (**7**) gave a dramatic increase in separation speed (Fig. 3c). This phase reversal during the separation (or "reversed-phase" operation) was achieved by activating a four-way valve between the solvent delivery system and the coil (Fig. 1), without stopping the instrument. The only difference between Fig. 3b and c (apart from the separation time) is the change in order of elution of **5** and **6**. In this example, the coil was filled at the beginning of the run with 50% of each component of the two-phase solvent system by pumping upper phase with pump A (5 ml/min) and lower phase with pump B (5 ml/min) simultaneously, before sample injection. Other initial phase ratios for "reversed-phase" operation were feasible and could be achieved by adjusting the relative flow-rates of pumps A and B.

"Gradient" elution

By pumping simultaneously the lower phase with one HPLC pump and the upper phase of a two-phase system with the other HPLC pump, it was possible to change the proportions of phases in the coil during a separation run. In the example shown (Fig. 4), the coil of the chromatograph was first filled with equivalent amounts

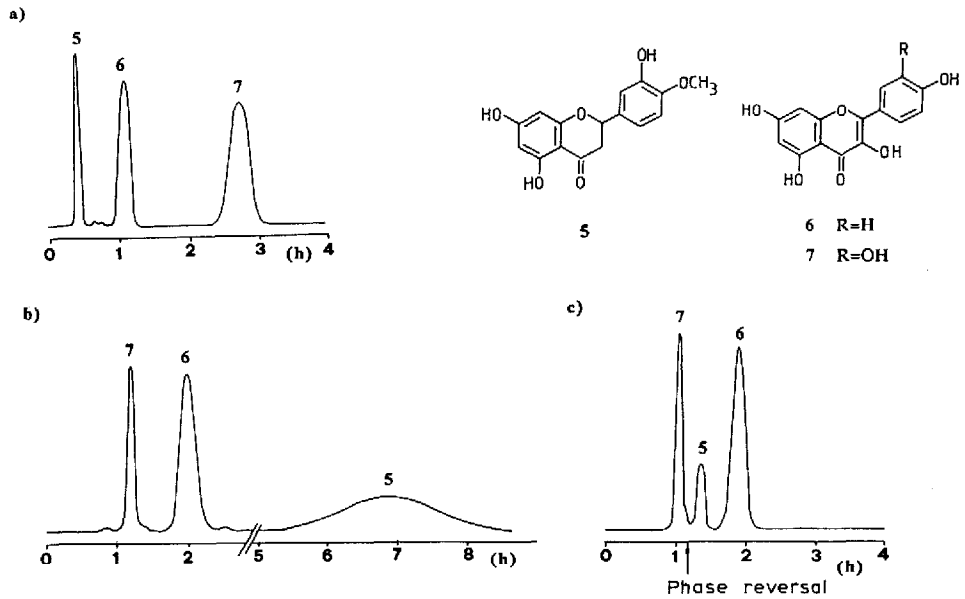

Fig. 3. CCC separation of hesperetin (**5**), kaempferol (**6**) and quercetin (**7**) with the Ito instrument. Solvent system, chloroform–methanol–water (33:40:27); detection, 254 nm; rotational speed, 700 rpm; flow-rate, 3 ml/min; sample, 15 mg. (a) Mobile phase, lower phase; (b) mobile phase, upper phase; (c) mobile phase, upper phase to 70 min, then lower phase.

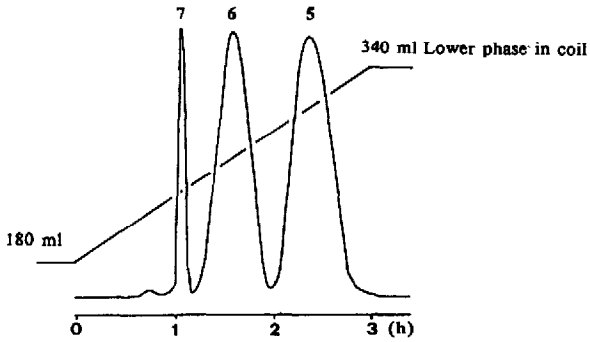

Fig. 4. CCC separation of flavonoids 5–7 with the Ito instrument using "gradient" elution. Mobile phase: upper phase (4 ml/min) and lower phase (1 ml/min). Conditions as in Fig. 3.

of upper and lower phases of the solvent system chloroform–methanol–water (33:40:27) (see Experimental). A mixture of flavonoids **5**–**7** was then injected. By pumping upper phase through the apparatus at 4 ml/min and lower phase at 1 ml/min, the content of lower phase in the coil increased from 180 to 340 ml over 3 h. The separation of the three flavonoids was thus achieved in a time 5 h shorter than that shown in Fig. 3b.

CONCLUSION

The above examples show some of the separation possibilities available to the multi-layer coil separator-extractor with separate pumping of the upper and lower phases of a biphasic solvent system. Choice of solvents can be guided by silica gel thin-layer chromatography (with the water-saturated non-aqueous phase as solvent)[6] and, when the biphasic system has been decided upon, the rates of elution of injected compounds can be varied by changing the ratio of stationary to mobile phase. Once the sample has been injected, the required mobile phase can be pumped through the machine with no subsequent loss of stationary phase from the coil. Accurate control of the flow-rate and of the composition of stationary phase in the separation coil is permitted, allowing "gradient" elution and phase reversal. In addition, phase reversal, previously described for analytical HSCCC[7,8], has now been applied to the preparative Ito instrument. With the aid of these developments, it is possible to expand the area of operation of the Ito multi-layer coil separator–extractor and introduce a greater degree of flexibility when establishing experimental parameters.

ACKNOWLEDGEMENT

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