

Application of high-speed counter-current chromatography to the separation of coumarin and related compounds

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Abstract

Coumarin and related compounds were successfully separated by high-speed counter-current chromatography (CCC). Using the conventional technique with the cross-axis coil planet centrifuge, coumarin, esculin, 2- and 3- or 4-hydroxycinnamic acids were well resolved with a solvent system composed of ethyl acetate–10 mM potassium phosphate (1:1, v/v) at pH 6.5, while the separation between 3- and 4-hydroxycinnamic acids was difficult. The separation of 2-, 3- and 4-hydroxycinnamic acids, was achieved by pH-zone-refining CCC using a type-J multilayer coil planet centrifuge with a methyl-*tert*-butyl ether–acetonitrile–water (1:0:1, or 4:1:5, v/v) system where trifluoroacetic acid was added to the organic mobile phase as an eluter and while ammonia was added to the aqueous stationary phase as a retainer.

Keywords: Counter-current chromatography; Coumarin; Hydroxycinnamic acids; Esculin; Organic acids

1. Introduction

Counter-current chromatography (CCC) is a liquid–liquid partition technique [1] that eliminates various complications arising from the use of solid supports. Among all the existing CCC systems, high-speed CCC is the most advanced form in terms of partition efficiency and separation time [2]. During the last decade, this technique has been used for separation and purification of a wide variety of

natural products. In the mid-1980s, the cross-axis coil planet centrifuge (CPC) was developed, further extending its capability [3]. The recent model has substantially improved the retention of the stationary phase of polar solvent systems [4] including aqueous–aqueous polymer phase systems specifically designed for the separation of proteins [5,6].

The pH-zone-refining CCC technique recently developed for preparative separation has advantages over conventional high-speed CCC, including high sample loading capacity and short separation times. This method has been successfully applied to a variety of compounds [7].

In the present study, both conventional high-speed CCC and pH-zone-refining CCC techniques were

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applied to the separation of coumarin and its related compounds. Although three coumarin glycosides have been isolated by CCC from *L. dissectum* [8], no other separations of related compounds have been reported.

2. Experimental

2.1. Apparatus

The present study was conducted using two different types of the high-speed CCC centrifuge: a cross-axis coil planet centrifuge (CPC) for the conventional CCC technique and a type-J multilayer CPC for pH-zone-refining CCC.

The design of the cross-axis CPC was described in detail elsewhere [4]. In brief, the apparatus holds a pair of horizontal rotary shafts, symmetrically, one on each side, of the rotary frame at a distance of 10 cm from the centrifuge axis. A spool-shaped column holder was mounted on each rotary shaft 10 cm away from its midpoint. Each multilayer coil separation column was prepared from 2.6 mm I.D. PTFE (polytetrafluoro ethylene) tubing (Zeus Industrial Products, Raritan, NJ, USA) by winding it onto a 15.2 cm diameter holder hub forming multiple layers of left-handed coils between a pair of flanges spaced 5 cm apart. Each column consisted of 4 layers of coil with a 170-ml capacity. A pair of columns mounted on the rotary frame were connected in series to make up a total capacity of 340 ml. The speed of the apparatus was regulated at 500 rpm with a speed control unit (Bodine Electric, Chicago, IL, USA).

The high-speed CCC centrifuge is a commercial model (Ito Multilayer Coil Separator/Extractor from P.C., Potomac, MD, USA) and it was used for pH-zone-refining CCC experiments. The detailed design of the apparatus was also described elsewhere [2]. The apparatus holds a multilayer coil separation column and a counterweight symmetrically arranged at a distance of 10 cm from the central axis of the centrifuge. The separation column was fabricated by winding a single piece of 1.6 mm I.D. and ca. 160 m long Tefzel tubing (Zeus Industrial Products) directly onto the holder hub making 16 coiled layers between a pair of flanges. The total capacity of the column measures about 315 ml. Each terminus of the column

was connected to a flow tube of 0.85 mm I.D. PTFE (Zeus Industrial Products) by directly inserting it into the Tefzel tubing and tightly binding it with a piece of copper wire over the junction. A narrow-bore PTFE tube (5 m×0.3 mm I.D.) (Zeus Industrial Products) was placed at the outlet of the monitor to restrict the effluent flow. The speed of the apparatus was regulated at 800 rpm with a speed controller (Bodine Electric).

The commercial model of type-J multilayer CPC was suitable for pH-zone-refining CCC compared with the cross-axis CPC, because the type-J CPC was equipped with the narrow tube for the separation columns and they can be rotated at high speed of 800 rpm. Consequently, the apparatus shows its ability for the high partition efficiency over a wide range of flow-rate of the mobile phase.

2.2. Materials

Hexane, ethyl acetate, methanol, acetonitrile and trifluoroacetic acid (TFA) were all glass-distilled chromatographic grade (Kanto Chemicals, Tokyo, Japan and Burdick and Jackson, Muskegon, MO, USA). Methyl-*tert*-butyl ether, 2-, 3- and 4-hydroxycinnamic acids were from E. Merck (Darmstadt, Germany). Some methyl-*tert*-butyl ether was also purchased from Burdick and Jackson. Coumarin and esculin were purchased from Wako (Osaka, Japan). Other chemicals were of reagent grade.

2.3. Measurement of partition coefficient (*K*)

The two-phase solvent systems composed of hexane, ethyl acetate, methanol and potassium phosphate (KPi) buffer were selected on the basis of partition coefficient values of coumarin, esculin and hydroxycinnamic acid isomers.

The solvent mixtures were thoroughly equilibrated in a test tube and the two phases separated. About 1.5 ml of each phase was delivered into another test tube to which about 10 µg of the sample was added. The contents were thoroughly mixed and then allowed to settle at room temperature. After two clear layers were formed (the tube was centrifuged if necessary), an aliquot (usually 1 ml) of each phase was pipetted and diluted with 0.5 ml of methanol to determine the absorbance at 276 nm using a

Shimadzu UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan). The partition coefficient, K , was expressed as the solute concentration in the upper phase divided by that in the lower phase.

2.4. Conventional CCC separation of coumarin and its related compounds

In each separation, the column of the cross-axis CPC was first entirely filled with the stationary phase (upper or lower phase) and a sample mixture containing hydroxycinnamic acid and its related compounds (each 10–20 mg in 4–6 ml solvent) was injected through a sample port. Then, the apparatus was rotated at 500 rpm while the mobile phase (lower or upper phase) was pumped into the column by an MP-311 micro pump (Lab-Quatec, Tokyo, Japan) in a proper elution mode given by a combination of the planetary motion (P_I or P_{II}), head–tail elution mode and inward–outward direction [6]. After the desired peaks were eluted, the flow was reversed, using the original stationary phase as the mobile phase to facilitate rapid elution of solutes still remaining within the column. The effluent from the outlet was continuously monitored at 276 nm with an ISCO UA-5 absorbance monitor (Instrumentation Specialties, Lincoln, NE, USA) and 3-ml fractions were collected with an LKB 2112 Redirac fraction collector (LKB Instruments, Bromma/Stockholm, Sweden). An aliquot of each fraction was diluted with methanol and the absorbance was measured at 276 nm with a Shimadzu UV-1200 spectrophotometer. The peak fractions were pooled, diluted with methanol and measured using a U-3200 Hitachi spectrophotometer (Hitachi, Tokyo, Japan) (see Fig. 1 for results).

2.5. Preparation of solvent systems and sample solutions for pH-zone-refining CCC

The solvent systems used for pH-zone-refining CCC were prepared as follows: methyl-*tert*-butyl ether–acetonitrile–distilled water at a volume ratio of either 1:0:1 or 4:1:5 were thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated. Aqueous ammonia (20 mM) was added to the lower aqueous phase which was then used as the stationary phase. The mobile

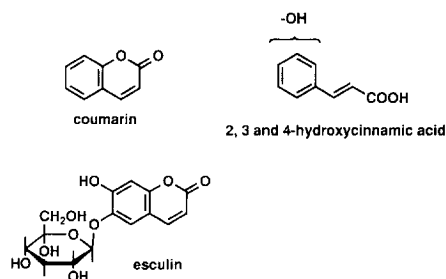


Fig. 1. Coumarin and its related compounds separated by high-speed CCC.

phase was prepared by adding TFA (10 mM) to the upper organic phase.

Sample solutions were made by dissolving 2-, 3- and 4-hydroxycinnamic acids each 100 mg in 20 ml of the solvent system.

2.6. pH-Zone-refining CCC separation of hydroxycinnamic acid isomers

A commercial model of type-J CPC was employed. The separation was initiated by filling the column entirely with the aqueous stationary phase containing 20 mM ammonia. The sample solution was injected through the sample port and the organic mobile phase was eluted through the column in the tail to head elution mode at a flow-rate of 3.0 ml/min while the apparatus was rotated at 800 rpm. The effluent from the outlet of the column was continuously monitored by both pH (pH cell, Sensitive, Stanton, CA, USA) and absorbance at 276 nm (LKB Uvicord S) and collected at 3.0 ml/min (LKB Ultrac fraction collector). After all peaks were eluted, the centrifuge was stopped and the column contents were collected to measure the volume of the stationary phase retained in the column.

2.7. Analysis

Aliquots of CCC fractions corresponding to various portions of the major peaks were analyzed by reversed-phase HPLC. The analysis was performed using a Shimadzu LC-6A system and a Whatman Partisphere C_{18} column (250×4.6 mm I.D.). A 10- μ l volume of each fraction was injected and eluted with a solvent composed of acetonitrile–methanol–water

(16:10:74, v/v) containing 20 mM TFA at a flow-rate of 1.0 ml/min. The effluent was detected by a Shimadzu SPD-6AV absorbance monitor at 276 nm.

3. Results and discussion

3.1. Selection of two-phase solvent system for the conventional CCC technique

For separation of coumarin, esculin, and 2-, 3- and 4-hydroxycinnamic acids, the partition coefficient values (K) of these compounds were determined using a series of solvent systems covering a wide range of hydrophobicities from the non-polar hexane–water system to a polar *sec.*-butanol–water system [9,10] (data not shown). Finally, we selected a solvent system composed of hexane–ethyl acetate–methanol–10 mM KPi buffer, because the solvent system provides about equal volumes of each phase so that either upper or lower phase can be used as the mobile phase without wasting the solvent.

In Fig. 2, K values for 2-, 3- and 4-hydroxycinnamic acids at pH 6.0 are plotted in a logarithmic scale against the volume ratios of the solvent systems. The K values of all isomers increase as the overall polarity of the solvent system is increased. Using a polar solvent system composed of ethyl acetate and 10 mM KPi at the volume ratio of 1:1, the influence of pH of the KPi buffer on the K values

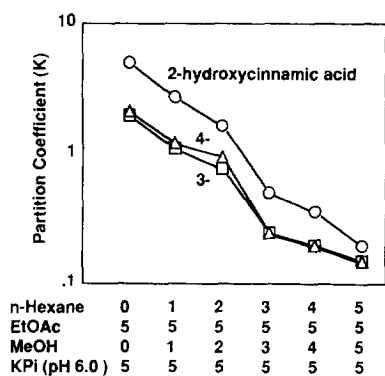


Fig. 2. Effect of volume ratio of hexane–ethyl acetate–methanol–10 mM potassium phosphate (pH 6.0) on the partition coefficients of 2- (○), 3- (□) and 4-hydroxycinnamic acid (△).

of 2-, 3- and 4-hydroxycinnamic acids, coumarin and esculin were examined (Fig. 3). As expected, coumarin, which contains no phenolic or carboxyl groups, exhibits a strong affinity for the upper organic phase with a high K value of over 25 over a wide pH range. Also, as expected, the K values of the three hydroxycinnamic acid isomers and esculin steadily decrease as the solvent pH is increased, falling below 0.6 at pH 7.0. The good separations should be obtained anywhere between pH 6.0 and 6.5. As shown in the diagram, 3- and 4-hydroxycinnamic acids give similar K values throughout the pH range, indicating that they are not separable under these conditions. However, separation may be possible with pH-zone-refining CCC [7] which uses two parameters of pK_a as well as hydrophobicity for peak resolution.

3.2. Conventional CCC separation

Fig. 4 shows a chromatogram obtained from a sample mixture containing coumarin, 2-, 3- and 4-hydroxycinnamic acids and esculin using a solvent system composed of ethyl acetate–10 mM KPi buffer (pH 6.0) at the volume ratio of 1:1. The separation was performed by pumping the upper organic phase at a flow-rate of 2.0 ml/min at a revolution speed of 500 rpm. The solvent front (SF) emerged at the 16th tube (3 ml/tube), while esculin with a low partition coefficient ($K < 1$), still remained in the column. After elution of 340 ml (column

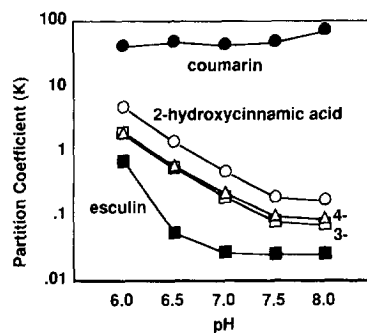


Fig. 3. Partition coefficient of coumarin (●), 2- (○), 3- (□) and 4-hydroxycinnamic acid (△) and esculin (■) in ethyl acetate–10 mM potassium phosphate (1:1, v/v) at various pH values.

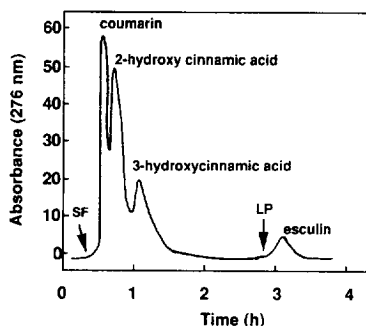


Fig. 4. Conventional high-speed CCC separation of coumarin, 2-, 3-hydroxycinnamic acids and esculin by the cross-axis CPC. Experimental conditions are as follows: columns: a pair of multilayer coils connected in series, 2.6 mm I.D. and 340-ml capacity; solvent system: ethyl acetate–10 mM potassium phosphate (pH 6.0) (1:1); mobile phase: upper organic phase; sample: a mixture of 20 mg each of coumarin, 2- and 3-hydroxycinnamic acids and esculin in 6 ml of the above solvent mixture (3 ml each phase); flow-rate: 2 ml/min; revolution: 500 rpm; SF=solvent front; LP=lower phase eluted in the reversed direction.

capacity) of the organic mobile phase, the column was eluted with the aqueous phase (initially used as the stationary phase) in the opposite direction to facilitate the elution of the retained esculin peak. The separation factor between coumarin and 2-hydroxycinnamic acids is 2.4 and that between 2- and 3-hydroxycinnamic acids is 1.2. The retention of stationary phase is estimated to 85.9% of the total column capacity at the time when the elution mode was reversed.

It is clear from the diagram (Fig. 3) that peak resolution between the above four components can be improved by operating at pH 6.5. Fig. 5 shows a similar separation performed with the solvent system at pH 6.5 where coumarin and 2-hydroxycinnamic acid peaks are completely resolved. After these two peaks were eluted, the elution mode was reversed to elute the other two peaks, esculin ($K=0.07$) and 3-hydroxycinnamic acid ($K=0.60$).

A similar mixture containing 4-hydroxycinnamic acid instead of 3-hydroxy isomer was separated by CCC under the same conditions (Fig. 6). These four components were completely resolved in 4.5 h. However, the separation of 3- and 4-hydroxycinnamic acids isomers is difficult using the conventional CCC mode with the solvent system because

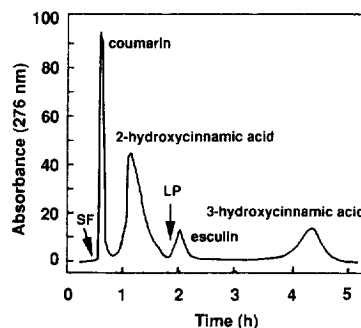


Fig. 5. Conventional high-speed CCC separation of a mixture of coumarin, 2- and 3-hydroxycinnamic acids and esculin. Experimental conditions as in Fig. 4 except that the pH of the solvent system was raised to pH 6.5.

the partition coefficients of these isomers are very close.

3.3. pH-zone-refining CCC of 2-, 3- and 4-hydroxycinnamic acids

Fig. 7 shows a chromatogram of pH-zone-refining CCC of 2-, 3- and 4-hydroxycinnamic acids. The separation was performed with a solvent system composed of methyl-*tert*-butyl ether–water in which 20 mM ammonia (retainer base) was added to the aqueous stationary phase and 10 mM TFA (eluter acid) to the organic mobile phase. Three hydroxycinnamic acid isomers (each 100 mg) were eluted as

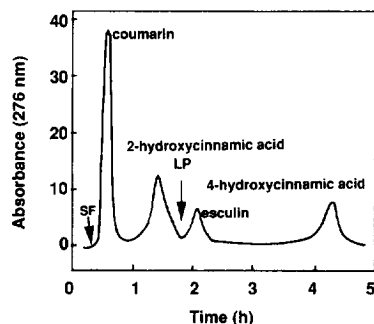


Fig. 6. Conventional high-speed CCC separation of a mixture of coumarin, 2- and 4-hydroxycinnamic acid and esculin. Experimental conditions as in Fig. 5 except for substituting 4-hydroxycinnamic acid for 3-cinnamic acid in the same solution.

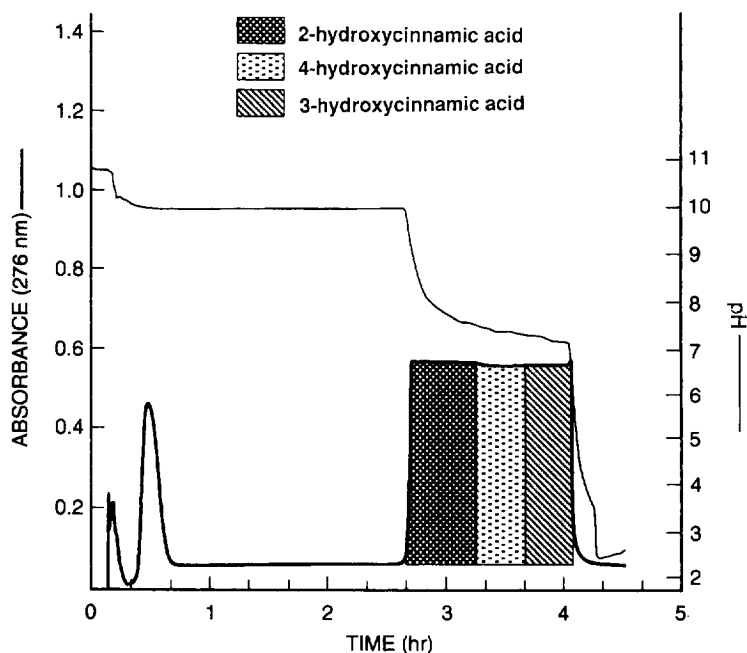


Fig. 7. Separation of 2-, 3- and 4-hydroxycinnamic acids by pH-zone-refining CCC. Experimental conditions: apparatus: multilayer coil high-speed CPC with a semipreparative Tefzel column of 1.6 mm I.D. and 315-ml capacity; solvent system: methyl-*tert.*-butyl ether–water (1:1), 20 mM NH₃ in aqueous stationary phase and 10 mM TFA in organic mobile phase; sample: three isomers, each 100 mg dissolved 20 ml solvent system (10 ml each phase); flow-rate: 3.0 ml/min in the tail-to-head elution mode; revolution: 800 rpm; stationary phase retention: 92%.

continuous rectangular peaks. HPLC analysis of fractions corresponding to each plateau revealed that 2-hydroxy isomer eluted first followed by the 4- and 3-hydroxy isomers. Fractions 84–98 (2-hydroxy isomer), fractions 102–108 (4-hydroxy isomer), and fractions 112–122 (3-hydroxy isomer) were over 98% pure (HPLC) while three fractions of 6 ml each contained mixture.

In pH-zone-refining CCC, increasing the polarity of the solvent system may improve the resolution of the polar analytes [11]. In order to improve the yield of 4-hydroxy isomer fractions, methyl-*tert.*-butyl ether–acetonitrile–water (4:1:5, v/v) was used for the separation of these isomers under otherwise identical conditions (Fig. 8). HPLC analysis of the fractions corresponding to the second peak revealed that the yield of 4-hydroxy isomer was much improved, measuring over 98% pure from an increased number of fractions 106 through 116, while the width of the mixing zone remained the same.

The cross-axis CPC has also been used for pH-zone-refining CCC under similar experimental conditions. However, the resolution of the three cinnamic acid isomers was substantially lower than those obtained with the type-J high-speed CPC described above.

4. Conclusion

The results show that with this particular solvent system the conventional CCC technique is useful for the separation and purification of coumarin, esculin and 2- and 3- or 4- hydroxycinnamic acids, but separation of the 3- and 4-isomers is difficult which is due to their partition coefficients being too close. However, the three hydroxycinnamic acid isomers are well resolved by pH-zone-refining CCC, since the analytes are separated according to their difference in pK_a and hydrophobicity.

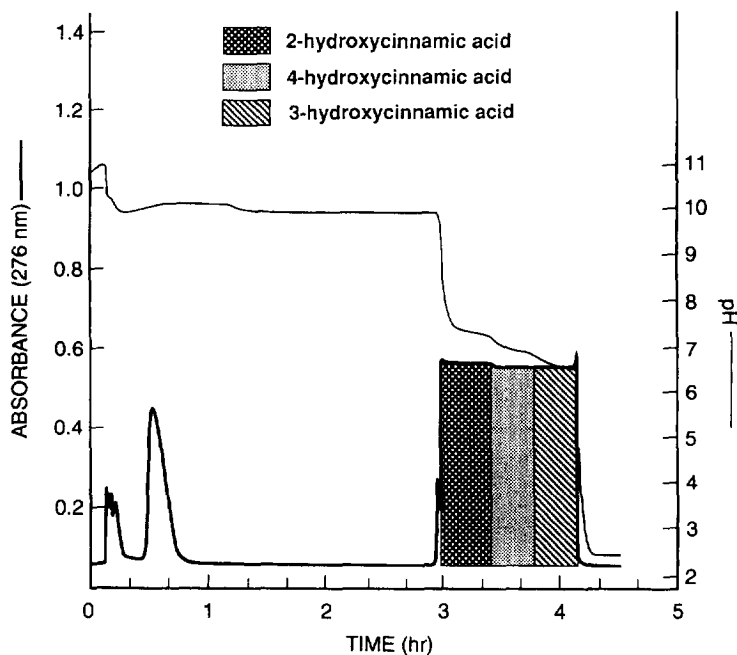


Fig. 8. Separation of three isomers by pH-zone-refining CCC. Experimental conditions: solvent system: methyl-*tert*-butyl ether–acetonitrile–water (4:1:5). For other conditions, see Fig. 7.

Acknowledgments

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