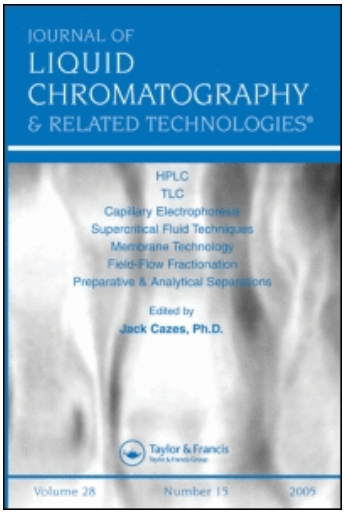

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Droplet Countercurrent Chromatography: Recent Applications in Natural Products Chemistry

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DROPLET COUNTERCURRENT CHROMATOGRAPHY: RECENT APPLICATIONS IN NATURAL PRODUCTS CHEMISTRY

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ABSTRACT

A few recent examples of our own modifications, procedures, and experiences utilizing DCCC are discussed that have enabled us to use DCCC more efficiently

INTRODUCTION

Since its development in 1970 (2), droplet countercurrent chromatography (DCCC) has built a

reputation as a very efficient method of separation. The technique has seen most use in the preparative separation of very polar compounds. Other methods of chromatography suitable for polar compounds, such as ODS-silica adsorption chromatography, tend to suffer from an inherently low sample capacity and a high cost of adsorbent. In contrast, DCCC has an inherently large sample capacity, no adsorbent, and a typically low solvent consumption. Several grams of sample can be applied in a single injection (1). Also, separation by DCCC is based upon solvent-solvent partitioning, which precludes irreversible adsorption and/or compound degradation on a solid support.

Information on the general principle of droplet counter-current chromatography, including general techniques and examples of separations, has been summarized in several excellent reviews (3,4,5). This discussion will be limited to a few recent examples of our own modifications, procedures, and experiences utilizing DCCC that have enabled us to use DCCC more efficiently.

Prepartive Use

Droplet counter-current chromatography is best known for its ability to separate large amounts of polar compounds from very crude sources, e.g., individual plant metabolites from whole plant extracts. For example, we recently examined the root bark of the West African plant Vitex madiensis for the presence of phytoecdysteroids. Following the extraction of the root bark with methanol, the resulting oil was partitioned between water and ethyl acetate to give 2.65 g of crude ethyl acetate extract. This entire sample was applied as a single injection onto a DCCC utilizing the solvent system $CHCl_3$ - CH_3OH - H_2O (13:7:4, vol/vol) in the ascending mode. This single injection gave 1.04 g pure 20-hydroxyecdysone and 0.38 g pure ajugasterone C (see Figure 1) (12).

Analytical Use

Although atypical, we have found DCCC can be useful for analytical scale separations as well. We examined a plant species related to the above mentioned V. madiensis for the presence of similar phytoecdysteroids.

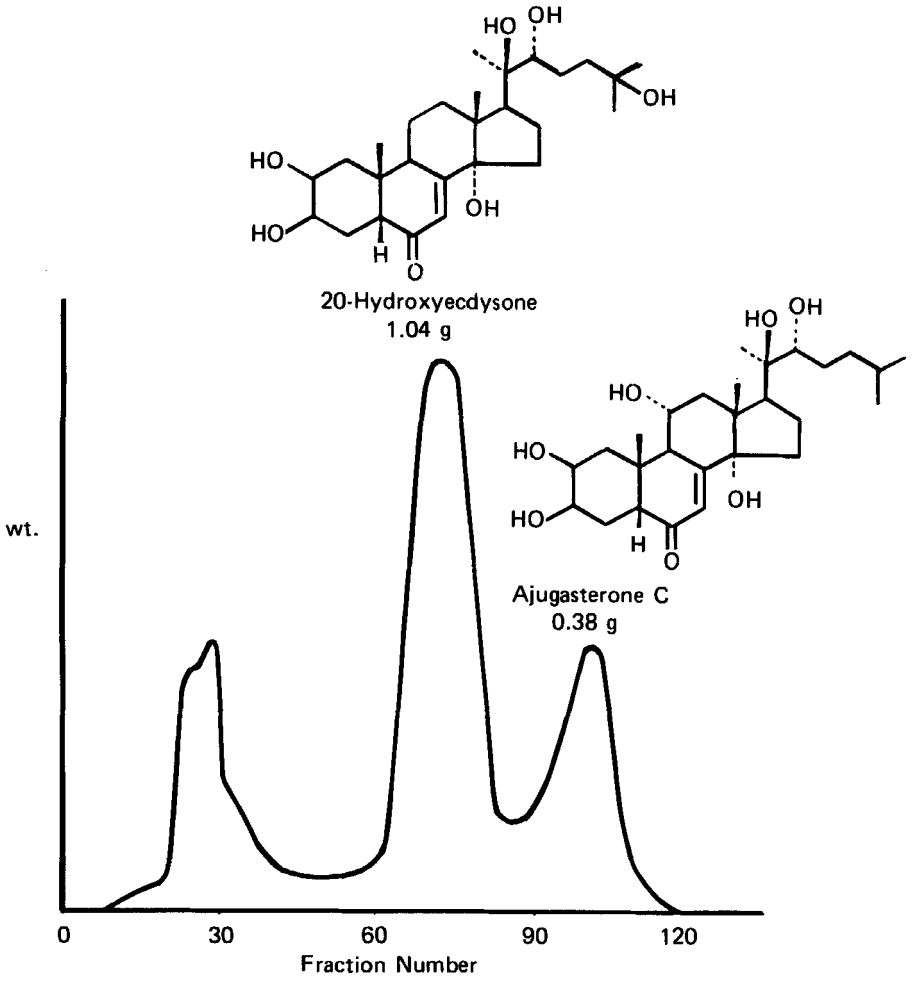

Figure 1. Constructed chromatogram of a DCCC separation of the ethyl acetate extract of V. madiensis (2.65 g) using $CHCl_3$ - CH_3OH - H_2O (13:7:4, vol/vol) by the ascending method; 1.5 ml per fraction.

A preliminary investigation of the methanol extract of the fruits showed neither 20-hydroxyecdysone nor ajugasterone C were present in amounts detectable by TLC. HPLC techniques can be used very effectively to detect the presence of small amounts of phytoecdysteroids. However, laborious steps would have been necessary to ensure all components in the injected mixture were compatible with the solvent system and column. Although the detection systems available for DCCC are generally not as sensitive as comparable devices used with HPLC systems, the high sample capacity of DCCC allows a sufficiently large enough sample to be applied so even a low percentage compound can be present in a large enough amount and, due to low solvent consumption, in a high enough concentration to enable detection. Also, column - sample incompatibility found in most types of solid support chromatographies is not problematic nor is there the chance of irreversible binding of the compounds of interest to a solid phase adsorbent. For these reasons DCCC was employed for the analytical scale detection and isolation of phytoecdysteroids in the extract of the fresh fruits of

V. thyrsiflora.

A 2.05 g portion of the crude methanol extract of the fruits of V. thyrsiflora was applied in a single injection onto a DCCC utilizing a chloroform - methanol - water (13:7:4, vol/vol) solvent system in the ascending mode. The eluent was observed with a preparative HPLC UV detector monitoring at 254 nm. The resulting chromatogram shown in Figure 2 shows near baseline separation of 12 mg of 20-hydroxyecdysone from the crude methanol extract without preliminary purification steps. This represented a yield of 0.07 % (700 ppm) of 20- hydroxyecdysone from the fresh fruits (13).

The detection limit for this separation was much lower than 12 mg and we estimate 1 mg could have been detected easily. The ratio of 1 mg to the 2 g of injected sample is comparable to the 10 ng HPLC detection limit of 20-hydroxyecdysone in an analytical scale injection of 0.02 mg (14). However, such a direct injection of the crude methanol extract on an HPLC column would most probably result in permanent damage to the column due to the very wide range of compounds present.

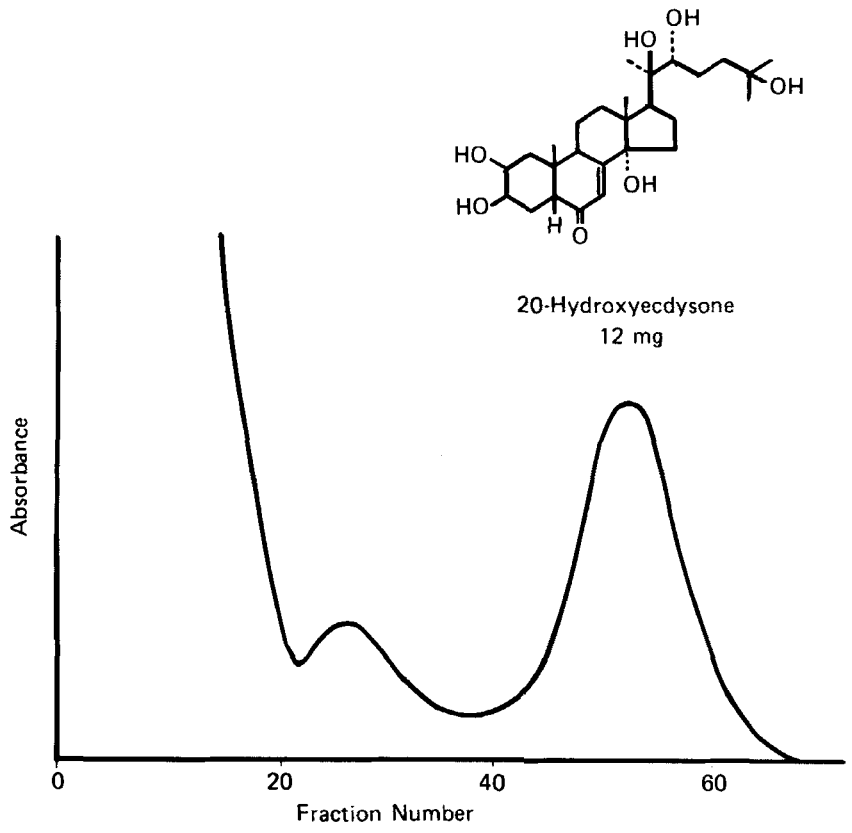

Figure 2. DCCC separation of the methanol extract of V. thyrsiflora (2.05 g) using $CHCl_3$ - CH_3OH - H_2O (13:7:4, vol/vol) by the ascending method; 2.1 ml per fraction, detection at 254 nm.

We also applied analytical scale DCCC to an isolation of ecdysone and 20-hydroxyecdysone from the pupae of the silkworm Bombyx mori (15). The increased complexity of animal metabolites tend to make animals

more difficult to work with than plant extracts. An injection of the ethyl acetate soluble portion (0.65 g) of a methanol extract of 405 (654 g) male pupae and monitored at 254 nm is shown in Figure 3 (chloroform - methanol - water; 13:7:4, vol/vol, ascending mode). This resulted in the isolation of 1.4 mg of ecdysone and 0.8 mg of 20-hydroxyecdysone. The utility of this separation is most clearly seen when it is compared to the original solid support adsorption chromatography isolation of ecdysone and 20-hydroxyecdysone from an extract of 500,000 g of B. mori pupae (25 mg and 0.33 mg, respectively) (16).

Increasing Injection Rate

Typical DCCC separations take from 1 to 3 days. The system operates at low flow rates and pressures and does not require attention during the elution period. However, the time required for separation can become a limitation if other laboratory operations can not be performed during the elution period. We recently reported an inexpensive modification to the commercial instrument that allows a substantial decrease in the time required between sample injections (17).

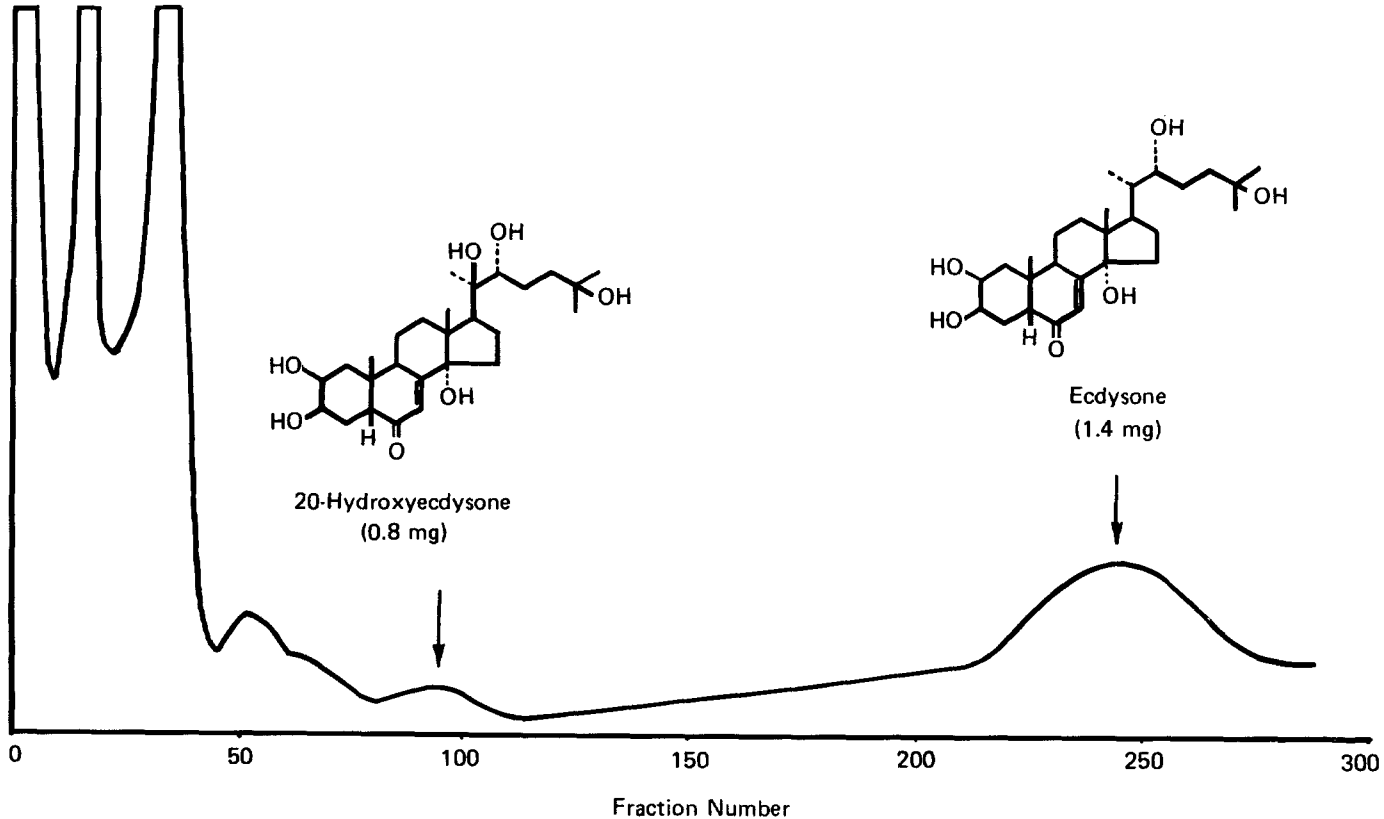

Figure 3. DCCC separation of the ethyl acetate soluble portion of a methanol extract (.065 g) of 654 g of B. mori male pupae using $CHCl_3$ - CH_3OH - H_2O (13:7:4, vol/vol) by the ascending method; 1.0 ml per fraction, detection at 254 nm.

The time required between injections can be divided into periods of washing, loading, and elution. Washing the solvent path is necessary to collect former sample that has remained in the stationary phase. Loading of a new stationary phase is necessary to prevent contamination from previous samples and to ensure a proper composition of the stationary phase. The maximum elution flowrate is limited by droplet formation and is characteristic of a particular solvent system. Little can be done to hasten the elution time short of introducing a larger gravitational type force (18). However, the maximum flowrate for the washing and loading of the commercial instrument was seen to be an unnecessary and time-limiting factor that could easily be resolved. In the current commercial DCCC design (Eyela DCC-300, Tokyo Rikakikai Co., Ltd.) the maximum flow rate for washing and loading exists because of the pressure limitation of the system's coupling system (300-450 psi) and the large back pressures of solvents at high flowrates. The solvent back pressure is a result of the very long path length of teflon capillary tubing (0.5 mm I.D., 160 m) and vertical glass columns (2.0 mm

Table 1. Maximum washing and loading flowrates of the commercial DCCC instrument equipped with 2 mm I.D. vertical columns as determined by the system's pressure limitations.

Solvent	Viscosity, cP	Maximum flowrate, ml/min	
		normally	with valves
Hexanes	0.31	2.4	13.4
Methanol	0.55	2.4	13.4
Chloroform	0.57	2.4	13.4
Water	1.0	1.4	12.0
n-Butanol	2.98	0.4	6.9

I.D., 120 m). The maximum washing and loading flow rates in the commercial instrument vary from 0.5 to 2.5 ml/min and depends on the viscosity of the stationary phase component solvents (see Table 1). A system equipped with 300 2.0 mm I.D. vertical glass columns (400 ml total volume) would take from 3.5 to 16 h to wash or load, depending upon the stationary phase composition, just to displace the previous solvent. More time would be necessary to account for incomplete washing and to counter any mixing at the interface of the new and old solvent systems when loading the new stationary phase.

One solution to this flow limitation was to reduce the amount of back pressure by shortening the solvent path during washing and loading. This was accomplished through the use of five chemically inert valves. The valves allow the operator to alternate between a normal solvent path of 300 head to tail vertical columns attached in a series and a modified solvent path where the columns were divided into 4 groups, with each group consisting of 75 head to tail vertical columns (see Figure 4). The groups of columns are attached in parallel so that the length of the solvent path was 1/4 the normal distance. The associated drop in back pressure allows many times the normal maximum flowrate (see Table 1).

For example, the washing of columns with methanol and the loading of a water based stationary phase could now be performed in 1 h rather than in 7.5 h. A methanol washing and the loading of an n-butanol based stationary phase could now be completed in 1.5 h rather than the normal 19.5 h. Previously, the usual practice was to first wash the system and then load the stationary phase overnight, since it was rare that both operations could

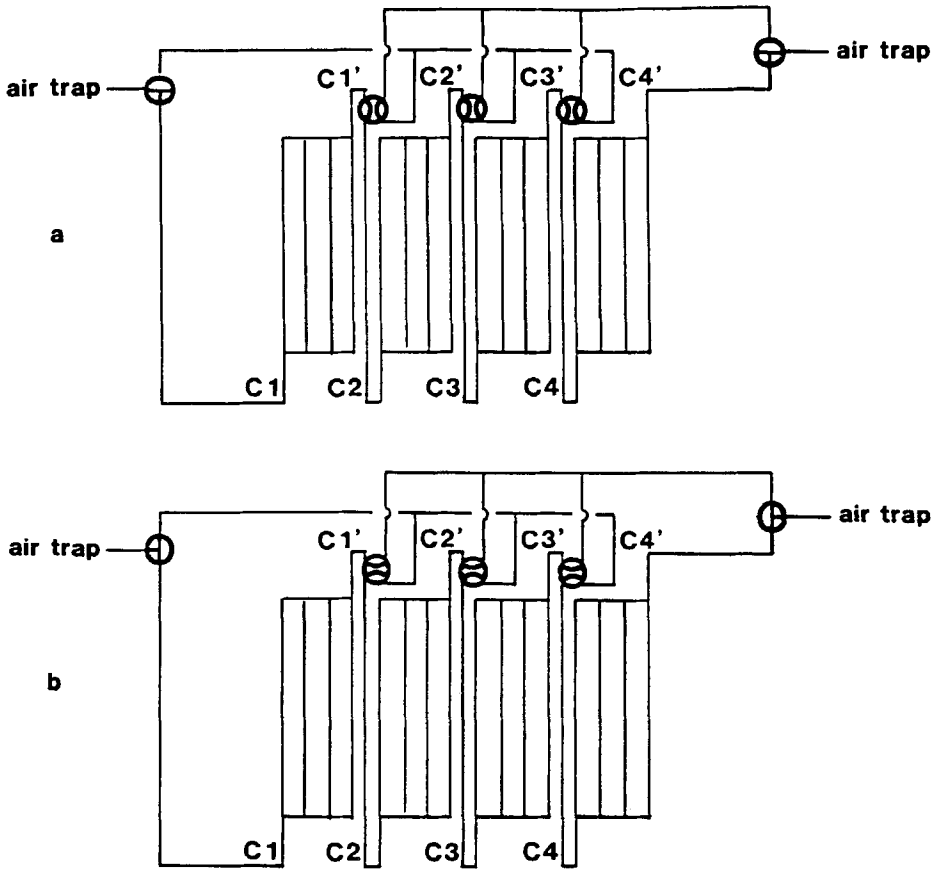

Figure 4. The DCCC solvent path after the installation of the valves: a) valves in the separation mode, with a normal solvent path of 300 head to tail columns; b) valves in the washing and loading mode, with 4 groups of columns having a solvent path length of 75 head to tail columns.

be completed within one work day. Hence, the greatly reduced back pressure allowed a 30 % decrease in the overall time required between injections for a typical 3 day separation.

Problems With Droplet Formation

We have noticed that it is common to observe a decrease in the number of droplets that would form in a given vertical glass column after repeated injections of very large amounts of crude plant extracts. We believe this is due to the surface of the glass becoming wettable by the droplets of mobile phase. When this occurs the droplets that would normally remain suspended in the stationary phase glide along the surface of the vertical columns. Although these droplets go to the end of the column, coalesce, and enter the capillary tubing as they would normally, droplets traveling by this method have a greatly reduced surface area that is available for partitioning. We have tried washing the affected columns by pumping water, methanol, ethyl acetate, and hexanes. Neither these solvents nor a 0.1 N HCl acid solution had any effect. The disassembling of all 300 columns and their sonication in an ammonia based

detergent solution also had no effect on their ability to form suspended droplets. The problem was finally eliminated by following a normal methanol wash with a solution of NaOH. The pumping of one liter of a 1.0 N NaOH washing solution eliminated all wetting of the glass by the mobile solvent, and normal droplet formation was immediately observed in all columns. We now routinely wash our DCCC instruments with a 1.0 N NaOH solution containing 0.5 % sodium hypochlorite and have found this mixture to be quite capable of ensuring proper droplet formation.

CONCLUSION

We have described the use of DCCC for the preparative and analytical scale isolation of polar compounds from very crude sources. Its main utility can be seen by its wide range of solute compatibility (water soluble to hexane soluble compounds), and in its large sample capacity. It is especially useful in dealing with very crude sources since it is not possible to irreversibly bind a portion of a sample onto the instrument. Although the method is slow when compared to

more traditional methods of chromatography, it is usually not necessary to carefully monitor the status of the instrument since it operates at a very low pressures and flow rates. Also, the time necessary between injections on commercial instruments can be greatly decreased at a modest price with the described modification. Very little maintenance is generally required, and the system is easily washed free of any remaining sample or contaminant. Periodic maintenance requires little more than cleaning the columns by pumping through a basic solution to ensure droplet formation.

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