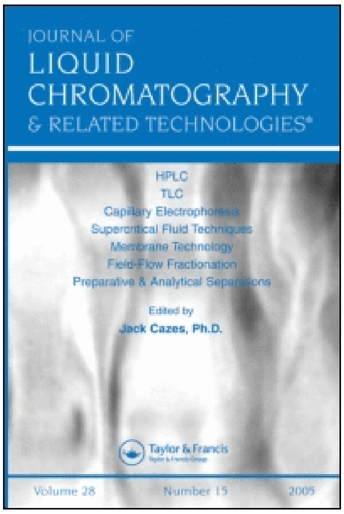

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DROPLET COUNTERCURRENT CHROMATOGRAPHY - NEW APPLICATIONS IN NATURAL PRODUCTS CHEMISTRY

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ABSTRACT

Droplet counter-current chromatography (DCCC) has found wide application for preparative separation of plant constituents and other natural products. It is particularly indicated for the isolation of polar compounds. The use of non-aqueous solvent systems allows the separation of weakly polar substances. Large-bore columns can be employed without decreasing the resolution when the flow-rate is increased. New fields of applications include essential oils, triterpenoids and steroids, plant growth regulators (gibberellins), antibiotics.

INTRODUCTION

In order to avoid complications arising from solid supports, various support-free liquid-liquid partition techniques have been recently developed (1). Among the available counter-current separation methods, droplet counter-current chromatography (DCCC) is

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becoming increasingly popular. Originally designed by Tanimura et al. (2), it has been used by Ogihara et al. (3) and Hostettmann et al. (4) for the isolation and purification of saponins and other plant glycosides. Numerous examples of separations of various classes of natural products by DCCC have been reported and recently reviewed (5, 6).

DCCC is based on the partitioning of solutes between a steady stream of droplets of mobile phase and a column of surrounding stationary phase and is carried out as follows. A pair of immiscible phases is prepared by the equilibration of a suitable mixture of solvents. Either lighter or heavier phase may be selected as mobile phase depending on the separation problem. When the lighter phase is the mobile phase (ascending mode), the instrument formed of 200-500 vertical columns interconnected in series by capillary Teflon tubes is first filled with the stationary heavier phase. The separation proceeds by delivery of the mobile phase containing the sample to the bottom of the first column. Droplets are formed which rise to the top of the column where they are delivered through the Teflon tubing to the bottom of the next column, thus generating new droplets. Under suitable conditions only the mobile phase is allowed to flow. At the end of the series of columns the mobile phase containing the components of the mixture, separated according to their partition coefficients, is collected by a fraction collector.

Although DCCC possesses limitations arising from the fact that the efficiency of the method depends entirely upon droplet formation, it has been extensively used for separation of polar compounds during natural products isolation. Since the publication of our review on DCCC in 1980 (5), more than sixty papers dealing with the application of this method have appeared. The present paper is not an exhaustive review on all these applications. We wish to report ón new interesting developements such as the use of large-bore columns and non-aqueous solvent systems which greatly increase the versatility of the technique. In addition, some selected new fields of applications in natural products chemistry will be discussed.

Use of non-aqueous solvent systems

The solvent systems generally employed in DCCC contain water as one of the components. These solvent systems allow the separation of polar compounds, but are not indicated for weakly polar or water-sensitive substances. The production of droplets possessing suitable sizes and mobilities is difficult with non-aqueous solvent systems. Recently, Becker et al. (7, 8) developed such a system formed from methanol - nitromethane - ethylacetate - n-hexane and reported successful separations in the field of essential oils. However, this system has the disadvantage of using nitromethane which is incompatible for UV detection and may react with oxidizing materials. As basic solvents for the DCCC separations, Domon et al. (9) selected methanol - n-heptane and acetonitrile - n-heptane which form two layers. But the addition of a third solvent which is miscible with both constituents is required for (i) greater selectivity by decreasing the difference in polarity between the two layers and (ii) formation of suitable droplets by decreasing the interfacial tension of both layers. Chlorinated solvents (dichloromethane or 1,2-dichloroethane) and acetone are indicated. Several ternary solvent systems could be developed. Satisfactory results were obtained with columns possessing internal diameters of 2.7 mm and 3.4 mm, respectively. The droplet formation appears to be more difficult with small-bore columns (2 mm I.D. or less).

Table 1. Non-aqueous solvent systems for DCCC

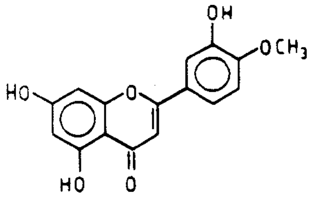
Solvent	Separated compounds	Reference
acetonitrile : dichloromethane : n-heptane (7 : 3 : 10 v/v)	triterpenoids steroids	(9)
methanol : acetone : n-heptane (4 : 1 : 5 v/v)	depsides triterpenoids steroids	(9)
methanol : 1,2-dichloroethane : n-heptane (12 : 1 : 8 v/v)	triterpenoids	(9)
methanol : nitromethane : ethyl acetate : n-hexane (3 : 2 : 2 : 9 v/v)	essential oil of chamomile	(8)
methanol : nitromethane : ethyl acetate : n-hexane (3 : 2 : 2 : 8 v/v)	valepotriates essential oil of *Echinacea angustifolia*	(7)

Some typical non-aqueous DCCC solvent systems are given in Table 1. Simple ternary solvent systems form droplets with suitable sizes and mobilities. However, it should be noted, that, in general, lipophilic compounds are more easily separated by classical chromatographic methods. The interest of DCCC with non-aqueous solvents lies in the separation of weakly polar substances which are unstable in the presence of water or decompose during chromatography on silica gel.

Use of large-bore columns and influence of the flow-rate on the separation.

Most of the DCCC-separations reported until now were achieved on small-diameter glass columns (2 mm I.D.) which limited the sample loading capacity. By using small-bore columns, the choice of solvent systems is restricted as the formed droplet must have a smaller size than the internal diameter of the column. Recently, columns with 2.7 mm, 3.0 mm and 3.4 mm I.D. have become commercially available. These columns allow the use of numerous solvent systems which could not be employed previously.

We have demonstrated that an increase of the internal diameter of the columns does not necessarily result in a decrease of the resolution. The following experiments were carried out. A mixture formed of three coumarins (herniarin, scopoletin, ombelliferon) and a flavanone (hesperetin) was submitted to DCCC. Separation was achieved on 2.7 mm I.D. columns (294) at different flow rates varying from



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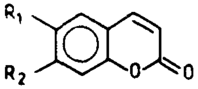


1: $R_1 = OCH_3$ $R_2 = H$

3: $R_1 = OH$ $R_2 = OCH_3$

4: $R_1 = OH$ $R_2 = H$

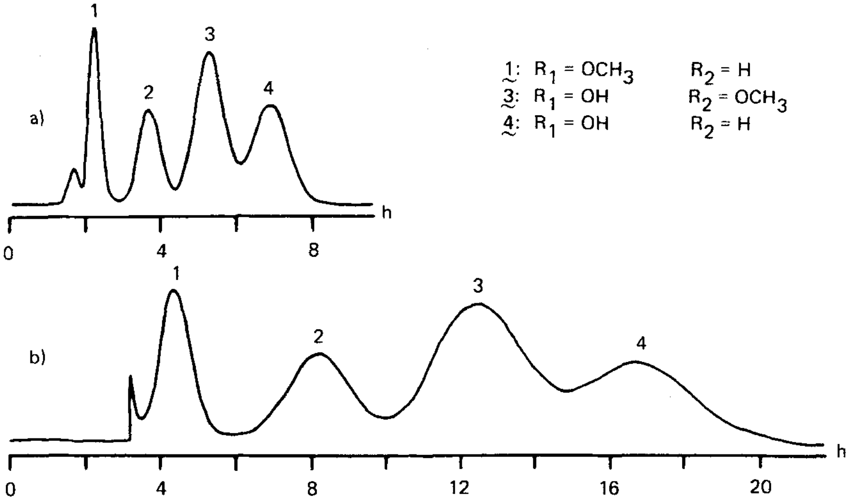
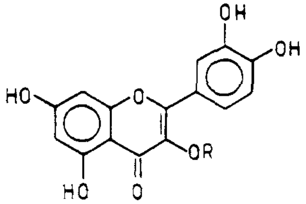


Figure 1 : Separation of hernianin 1, hesperetin 2, scopoletin 3 and ombelliferon 4 using chloroform : methanol : water (13 : 7 : 8 v/v) in the descending mode on 2.7 mm I.D. columns; eluate monitored at 254 nm.

a) at 50 ml/h b) at 18 ml/h.

15 to 80 ml/h with chloroform : methanol : water (13 : 7 : 8 v/v) in the descending mode. Figure 1 shows the results obtained at 50 ml/h and 18 ml/h.

An improvement of the separation efficiency with increasing flow rates was observed until 60 ml/h. The number of theoretical



1: R = α-L-rhamnosyl(1 6)β-D-glucosyl
2: R = β-D-galactosyl
3: R = α-L-rhamnosyl

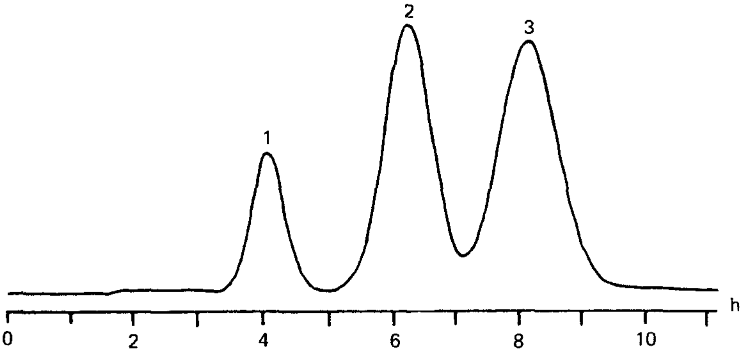


Figure 2 : Separation of rutin 1, hyperoside 2 and quercitrin 3, using chloroform : n-butanol : methanol : water (10 : 1 : 10 : 6 v/v) in the ascending mode on 2.7 mm I.D. columns at a flow rate of 48 ml/h; eluate monitored at 278 nm.

plates was improved from 120 at 18 ml/h to 240 at 60 ml/h. For higher flows no significant changes could be noted, but some stationary phase was eluted. Similar results were obtained for the separation of hesperetin and kaempferol using chloroform : methanol : water (33 : 40 : 27 v/v) in the descending mode and for the resolution of a flavonoid glycoside mixture (rutin, hyperoside, quercitrin) with chloroform : n-butanol : methanol : water (10 : 1 : 10 : 6 v/v) in the ascending mode. Figure 2 shows the glycoside separation carried out at 48 ml/h. A base-line separation is completed within 9 h.

These results can be explained by a decrease of the longitudinal diffusion and a better mass transfer between the droplets and the surrounding stationary phase when the flow rate is increased. Thus 60 ml/h seems to be an optimum flow for working with 2.7 mm I.D. columns; in such conditions the time required for a separation can be reduced to a few hours.

It should be noted that all above mentioned experiments were achieved with chlorinated solvents which have high densities and low viscosities. High flow-rate can not be obtained with very viscous solvent systems.

We have not yet studied the sample loading capacity of large-bore columns. But it appears that more than 6 g of sample can be handled in one separation process when 3.4 mm I.D. columns are employed. This has been exemplified by the isolation of pure flavonoid glycosides from a methanolic extract (6.4 g) of *Lomatogonium carinthiacum* (Gentianaceae) with chloroform : methanol : n-propanol : water (5 : 6 : 1 : 4 v/v) in the descending mode, using 288 columns (length 40 cm) (10).

Selected applications in natural products chemistry

Gibberellins

These diterpenes of the kaurane group are very widely used plant growth-promoting substances and are manufactured on a large scale by fermentation. Bearder and MacMillan (11) have shown that the separation of these important natural products is simpler and more economic by DCCC than by classical chromatographic techniques. Excellent separations of various gibberellins could be achieved with dichloromethane : methanol : water (5 : 6 : 4 : v/v) or with chloroform : methanol : water (7 : 15 : 8 v/v) in the descending mode. Mixtures of gibberellin GA_3 and gibberellin GA_{13} were resolved with the solvent system chloroform : acetic acid : water (2 : 2 : 1 v/v) used in the descending mode.

Antibiotics

DCCC is of particular interest for the preparative separation of antibiotics and their metabolites. A complex mixture of various tetracyclines has been successfully analysed with chloroform : methanol : n-propanol : aq. HCl 0.01 N (9 : 12 : 1 : 8 v/v) in the ascending mode (300 columns; I.D. : 2.o mm; length 40 cm). The crude macrolide antibiotic niphithricin could be separated into niphitricin A and niphithricin B by Keller-Schierlein and coworkers (12). A sample of 628 mg of crude material afforded 37 mg of pure niphithricin B and 144 mg of pure niphithricin A.

These compounds were active against Gram-positive bacteria and fungi. The solvent system benzene : chloroform : methanol : water (15 : 15 : 23 : 7 v/v) was employed in the descending mode for the separation of the closely related antibiotics narbomycin, picromycin, methymycin and neomethymycin (13). The formula of the separated antibiotics are represented in Figure 3. A further antibiotic separation was reported recently, namely the resolution of ni-

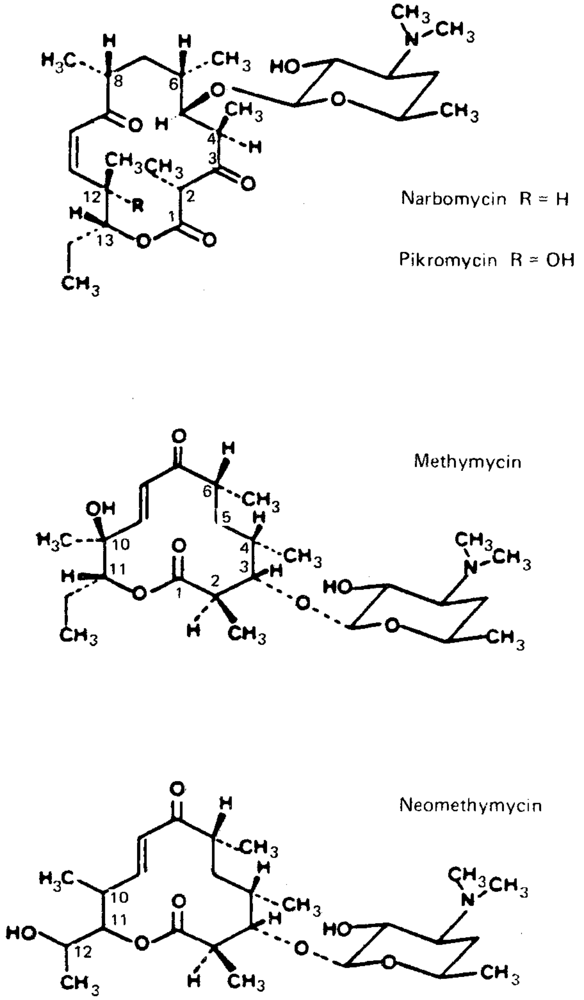


Figure 3 : Structures of macrolide antibiotics separated by DCCC with benzene : chloroform : methanol : water (15 : 15 : 23 : 7 v/v) in the descending mode (13).

phymycin I in niphymycin Iα and niphymycin Iβ with chloroform : methanol : water (35 : 65 : 40 v/v) used in the ascending mode (14).

CONCLUSION

Although DCCC possesses limitations due to the fact that its efficiency depends entirely upon droplet formation, it is increasingly applied in the field of natural products. Recent developements such as the use of large bore columns, the use of non-aqueous solvent systems and the reduction of the separation time by working at an optimum flow-rate will contribute to a wide extension of this simple counter-current method.

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