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Note

Droplet counter-current chromatography with non-aqueous solvent systems

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Droplet counter-current chromatography[1] (DCCC) is a liquid–liquid separation technique which has extensively been used for the isolation of polar natural products[2,3]. As the method depends entirely upon the formation of droplets of mobile phase in small-bore columns of surrounding stationary phase, the solvent systems usually employed contain water as one of the components. These solvent systems are not suitable for the separation of 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.*[4,5] developed such a system formed from *n*-hexane–ethylacetate–nitromethane–methanol and reported some applications in the field of essential oils. Although this system provided good separations, it has the disadvantage of using nitromethane (relatively high boiling point, incompatible for UV detection, reaction with oxidizing materials, irritant).

In the present paper we report some simple non-aqueous solvent systems for DCCC formed of methanol–1,2-dichloroethane–*n*-heptane, methanol–acetone–*n*-heptane and acetonitrile–dichloromethane–*n*-heptane, and their suitability for separating weakly polar natural products such as terpenoids, steroids and depsides.

EXPERIMENTAL

The separations were achieved on a DCCC-B760 apparatus (Büchi, Flawil, Switzerland) equipped with 294 columns (2.7 mm I.D.). All the solvent systems were also tested on 2 mm I.D. and 3.4 mm I.D. columns with DCC-A and DCC-S instruments, respectively (Tokyo Rikakikai, Tokyo, Japan).

The flow-rate was 25–35 ml/h, depending on the separation mode and the solvent systems. Fractions of 9–12 ml were collected and monitored by thin-layer chromatography (TLC) on silica gel 60 F_{254} precoated aluminium sheets (E. Merck, Darmstadt, G.F.R.) with diisopropyl ether, diisopropyl ether–acetone (70:30) or toluene–ethyl acetate (6:1); detection was made with Godin reagent.

For the selection of the solvent system for DCCC, the samples were chromatographed on RP-8 F_{254} HPTLC plates (E. Merck) with the lower layer of methanol–1,2-dichloroethane–*n*-heptane (37.6:4.8:57.6), methanol–acetone–*n*-heptane (40:10:50) or acetonitrile–dichloromethane–*n*-heptane (35:15:50). Linear develop-

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ment was achieved with an HPTLC linear 28150 chamber (Camag, Muttenz, Switzerland).

RESULTS AND DISCUSSION

As basic solvents for the DCCC separations, we selected *n*-heptane–methanol and *n*-heptane–acetonitrile which form two layers. However, 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 such as dichloromethane or 1,2-dichloroethane and acetone are suitable. Thus, 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).

It is usually tedious to find the appropriate solvent system for a DCCC separation. In a previous paper[2], one of us proposed a quick method based on silica gel TLC with the water-saturated organic layer as eluent. With non-aqueous solvent systems, silica gel is not suitable as the separation is based entirely upon adsorption. However, the empirical rules described previously[2] can be used, in some cases, for the selection of a non-aqueous solvent system for DCCC when the sample is analyzed by TLC on chemically bonded phases (RP-8) with the lower layer of an *n*-heptane containing system.

The following applications were carried out.

Isolation of vulpinic acid from the petrol ether extract of the lichen Letharia vulpina (L.) Hue

A sample of 120 mg of crude extract was subjected to DCCC (294 columns, 2.7 mm I.D.) with the solvent system methanol–acetone–*n*-heptane (40:10:50) in the descending mode. The solvent front was observed after 90 ml. The main fraction (80 mg), eluted between 506 and 584 ml, yielded yellow crystals identified by UV, mass spectrometry and ^1H nuclear magnetic resonance spectroscopy as vulpinic acid[6].

Isolation of oleanolic acid and hederagenin from a hydrolyzed extract of Hedera helix L.

A sample of a crude methanolic extract of *Hedera helix L.* berries was hydrolyzed with 4 *N* hydrochloric acid. The aglycones (135 mg) were extracted with diethyl ether and subjected to DCCC (294 columns, 2.7 mm I.D.). The separation, carried out in the descending mode with methanol–1,2-dichloroethane–*n*-heptane (57.6:4.8:37.6), furnished four main fractions.

A first fraction contained polar impurities (9 mg), followed by pure hederagenin (7.8 mg), a mixture of hederagenin and oleanolic acid (1.2 mg) and pure oleanolic acid (5.7 mg). Lipophilic impurities remained in the stationary phase. As expected, by using the more polar layer as mobile phase, the more polar triterpene (hederagenin) was eluted before oleanolic acid.

Separation of a mixture of triterpenes and steroids

A mixture (80 mg) of betulinic acid, betulin, β-amyrin and cholesterol was subjected to DCCC (196 columns, 2.7 mm I.D.) with acetonitrile–dichloromethane–*n*-heptane (35:15:50), in the descending mode. The solvent front was observed after 75 ml. Betulin (7 mg) was obtained in the first fraction (100–180 ml), followed by 39 mg of pure betulinic acid (181–270 ml). Cholesterol and β-amyrin were eluted much later, namely 19 mg pure cholesterol (991–1250 ml), followed by a mixture (2 mg) of both compounds (1251–1295 ml) and finally by 9 mg of pure β-amyrin (1296–1550 ml).

When the same mixture of triterpenes and steroids was subjected to DCCC with methanol–acetone–*n*-heptane (40:10:50) in the descending mode, no separation of betulin and betulinic acid was obtained. It should be noted that this solvent is able to form hydrogen bonds with the solute, whereas the acetonitrile–dichloromethane–*n*-heptane system is aprotic.

CONCLUSIONS

The aim of the present study was to show that non-aqueous solvent systems can be used for the separation of weakly polar natural products by DCCC. Simple ternary solvent systems such as acetonitrile–dichloromethane–*n*-heptane (35:15:50) or methanol–acetone–*n*-heptane (40:10:50) 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. Thus, DCCC should find applications in the isolation of unstable natural products such as phorbol esters.

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