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Note

Droplet counter-current chromatography for the separation of plant products

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Several chromatographic methods have been developed for the investigation of natural products. Droplet counter-current chromatography (DCC), which was initially developed by Tanimura *et al.*[1] for biochemical studies, is based on the difference of the partition coefficients of compounds in liquid–liquid phases such as counter-current distribution[2], but it has great advantages for the small-scale and semi-micro qualitative and quantitative determination of naturally occurring compounds, which is the main subject of this paper.

EXPERIMENTAL

The apparatus for DCC, illustrated in Fig. 1, consists of 500–1000 Pyrex glass tubes (40 cm long and 1.65 mm in diameter) connected to each other top-to-bottom with thin plastic tubes*** (55 cm long and 0.65 mm in diameter) that are stable against acids, alkalis and other organic solvents.

Several solvent systems that form two immiscible layers are selected for the efficient separation of compounds by DCC. Before starting the development, all of the tubes are filled with an upper layer or a lower layer of the solvent system using a micropump, to make a stationary phase. Roughly fractionated plant extracts are dissolved in a 1:1 mixture of the stationary and mobile phases and charged to a sample chamber.

In the ascending method, mobile phase is slowly pumped into the apparatus through the sample chamber to form droplets moving up from the bottom to the top of each glass tube. Partition occurs between the stationary phase and the moving droplets through the overall set of tubes, giving a well defined separation on the basis of

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*** A co-polymer of F_4-ethylene and F_6-propylene.

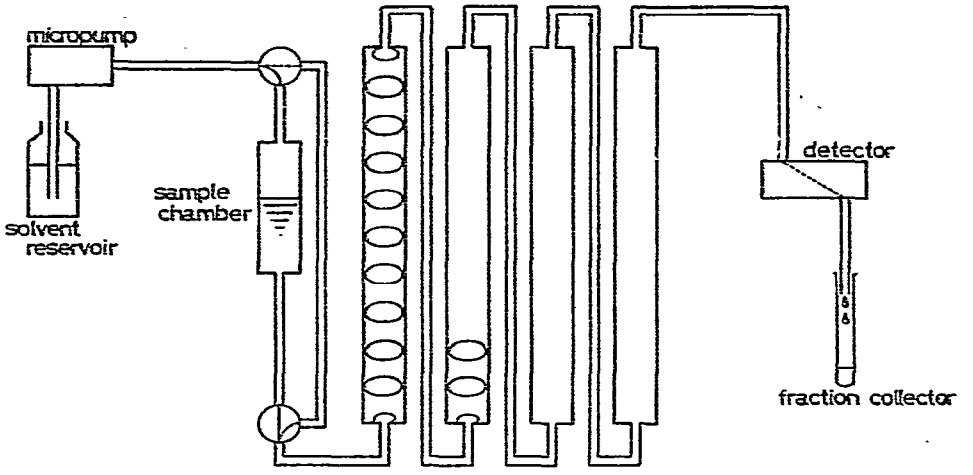

Fig. 1. Schematic model of droplet counter-current chromatography showing that droplets are ascending through the stationary phase.

the difference in the partition coefficients of the components of the sample. The end of the system is connected to a fraction collector to collect 3–5 ml fractions. The overall separation is accomplished during a period of a few days to a week, depending on the scale of experiment. Each fraction collected is evaporated to dryness and the amount present is measured gravimetrically or colorimetrically using an appropriate colour reagent. TLC is applied to each fraction in order to establish the purity and identity of the substance.

A reversed-phase system can be used for separation, in which the droplets of the lower-layer solvent move down through a stationary phase of the upper-layer solvent in the tube system. The size of the droplets in the DCC system depends on the surface tension and the difference in the specific gravities of two liquid phases, as well as on the diameter and the quality of the glass tubing. For instance, a solvent system consisting of *n*-hexane and water, which gives a high surface tension, forms a large drop of *ca.* 5 mm in diameter, resulting in plug flow of the stationary phase in the Pyrex glass tubes of the DCC system. It is desirable to use several tube systems of different diameters for different solvent systems, but it should be noted that a large diameter of the tubes decreases the efficiency of separation. The solvent systems used so far to give a suitable size of droplets for efficient separations are given in Table I.

APPLICATION OF DCC TO THE SEPARATION AND PURIFICATION OF NATURAL PRODUCTS

Ebelin lactone-producing saponins

The saponins of the seeds of *Zizyphus jujuba* Mill. and the root bark of *Hovenia dulcis* Thunb. (Rhamnaceae) are characterized by the formation of ebelin lactone[3] on acid hydrolysis, although it is obviously not the genuine sapogenin but an artifact.

The saponins of this group have been studied using DCC for (a) the quantitative determination and (b) the semi-micro preparation of the pure saponins. The

TABLE I

SOLVENT SYSTEMS FOR DCC

Components	*Proportions*
n-Butanol–acetic acid–water	4:1:5
n-Butanol–pyridine–water	5:2:10
n-Butanol–pyridine–water	10:1:10
n-Butanol–0.1% acetic acid–pyridine	5:11:3
n-Butanol–*tert.*-butanol–2 *N* ammonia	3:1:4
n-Butanol–*n*-propanol–water	2:1:3
sec.-Butanol–trifluoroacetic acid–water	120:1:160
sec.-Butanol–1% dichloroacetic acid	1:1
Chloroform–acetic acid–water (or 0.1 *N* hydrochloric acid)	2:2:1
Chloroform–methanol–0.1 *N* hydrochloric acid	19:19:12
Chloroform–benzene–methanol–water	15:15:23:7
Chloroform–benzene–methanol–0.1 *N* hydrochloric acid	10:5:11:4
Chloroform–methanol–water	35:65:40
Dichloromethane–methanol–water	10:5:5
n-Hexane–ethanol–water–ethyl acetate	5:4:1:2
Ethyl acetate–*n*-propanol–water	4:2:7

semi-micro quantitative determination of the saponins of *H. dulcis* by either a gravimetric or a colorimetric method gave a good agreement. A mixture of saponins of *H. dulcis*, hovenosides (195 mg) was developed by DCC using the solvent system chloroform–methanol–water (35:65:40). After evaporation of each fraction, the residue was weighed, dissolved in 2 ml of water and then 1 ml of 5% phenol and 5 ml of concentrated sulphuric acid were added[4]. The mixture was allowed to stand for 30 min, with cooling, and then determined colorimetrically at 490 nm. The recovery of total saponins was 98% (Fig. 3a).

A comparative study of the variation in the saponin content in the root bark of *H. dulcis* using DCC showed the disappearance of hovenoside C and the appearance of G′ and H in the material collected during spring at Minakami, Gumma Pref., Japan, in contrast to that collected during autumn at Fudago, Chiba, Japan (Figs. 2 and 3).

The separation of the saponins of *Z. jujuba* seeds was attempted first with DCC using the solvent system chloroform–methanol–water (50:60:40), and jujuboside B was isolated. When *n*-propanol was added to this solvent system (chloroform–

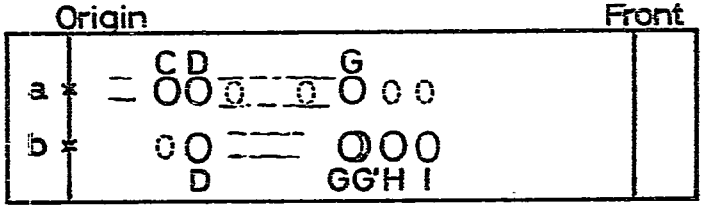

Fig. 2. TLC of the saponin fraction of *Hovenia dulcis* on a silica gel GF_{254} using the solvent system chloroform–methanol–water (65:35:10) (lower layer). Spray reagent: 10% sulphuric acid. (a) Saponins isolated from the plant collected in spring; (b) saponins isolated from the plant collected in autumn.

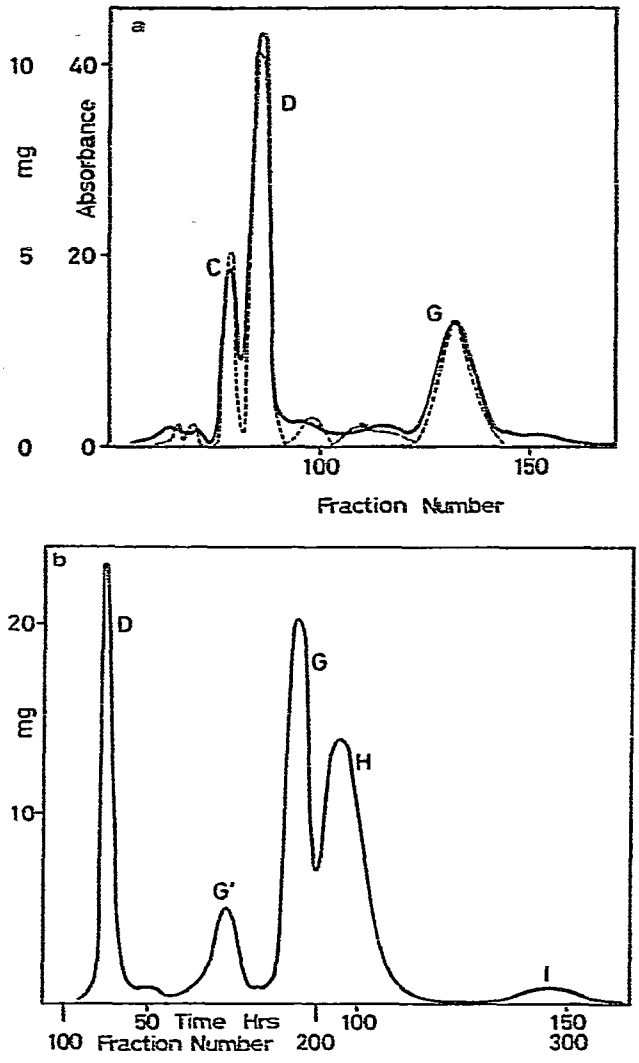

Fig. 3. DCC elution diagrams of saponins of *Hovenia dulcis* (hovenosides). (a) Obtained in spring at Minakami using 509 tubes (40 cm × 1.65 mm I.D.), solvent system chloroform–methanol–water (35:65:40), lower layer for stationary phase and upper layer for mobile phase in the ascending method. A saponin mixture (195 mg) was charged for separation to collect fractions of 4 g each. Solid line, measured colorimetrically at 490 nm using phenol–sulphuric acid as the reagent; broken line, measured gravimetrically. (b) Obtained in autumn at Fudago using 509 tubes (40 cm × 1.65 mm I.D.), solvent system chloroform–methanol–water (35:65:40), lower layer for stationary phase and upper layer for mobile phase in the ascending method. A saponin mixture (1 g) was fractionated into 2-ml fractions for gravimetric determination.

methanol–*n*-propanol–water, 45:60:5:40), jujuboside A was also obtained in a pure crystalline state (Fig. 4).

Sennosides A, B and C of Senna and Rhubarb

A mixture of sennosides A, B and C[5] (3 mg each) was submitted to DCC using

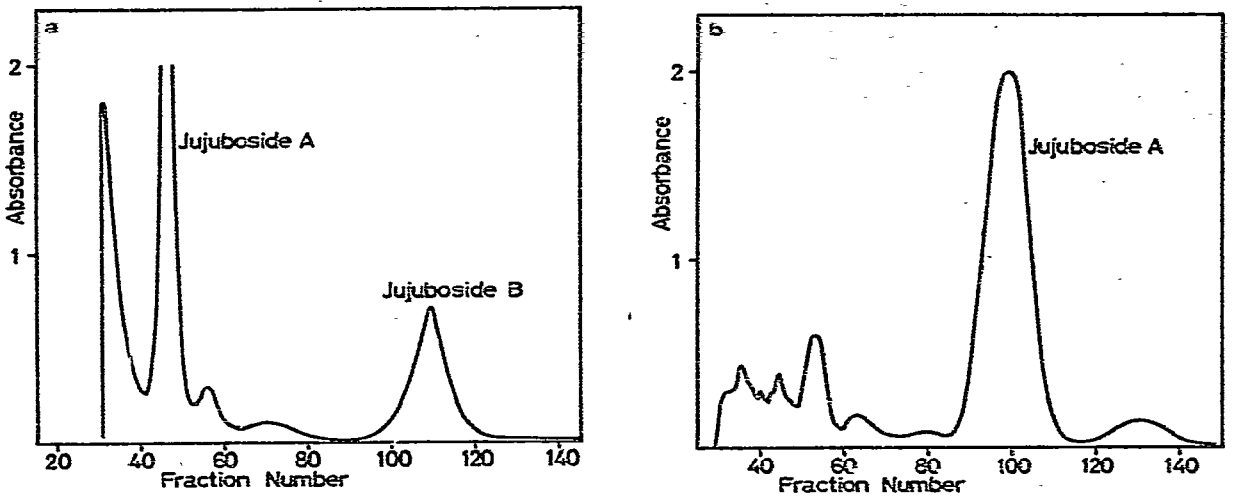

Fig. 4. (a) Separation of saponins of the seeds of *Zizyphus jujuba* (jujubosides). A saponin mixture (25 mg) was developed using 509 tubes and the solvent system chloroform–methanol–water (50:60:40) in the ascending method. The mobile phase eluted was fractionated into 4-ml fractions for colorimetric determination using phenol–sulphuric acid as the reagent. (b) Purification of jujuboside A. Fractions 30–70 (12 mg) containing mainly jujuboside A, which were separated by the previous DCC (see Fig. 4a), were developed again through 509 tubes (40 cm × 1.65 mm I.D.) by the ascending method using the solvent system chloroform–methanol–*n*-propanol–water (45:60:5:40) into 4-ml fractions for colorimetric determination using phenol–sulphuric acid as the reagent.

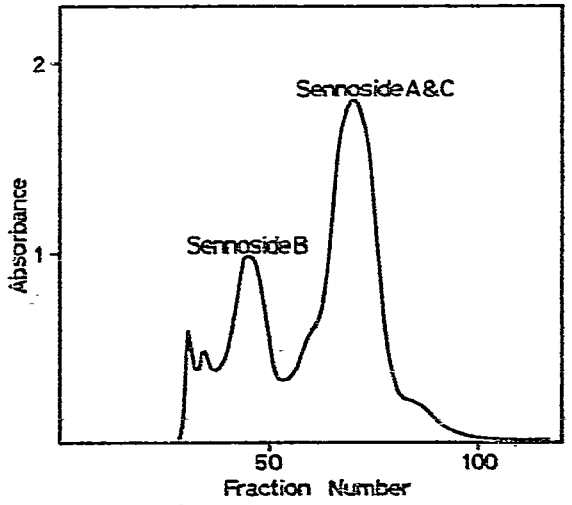

Fig. 5. Separation of sennosides. A mixture of sennoside A, B and C (3 mg each) was separated by ascending DCC with 509 tubes (40 cm × 1.65 mm I.D.) using the solvent system chloroform–methanol–*n*-propanol–water (45:60:10:40) into 4-ml fractions for densitometric determination at 260 nm in 50% ethanol solution.

the solvent system chloroform–methanol–*n*-propanol–water (45:60:10:40). The mobile phase was collected in the fraction tubes in volumes of 4 ml each, the solvent was evaporated to dryness and the residue was dissolved in 5 ml of 50% ethanol, followed by photometric measurement at 260 nm. In this instance the geometric isomers, sennoside A and B, were separated well, whereas sennoside A and C had identical retention times (Fig. 5).

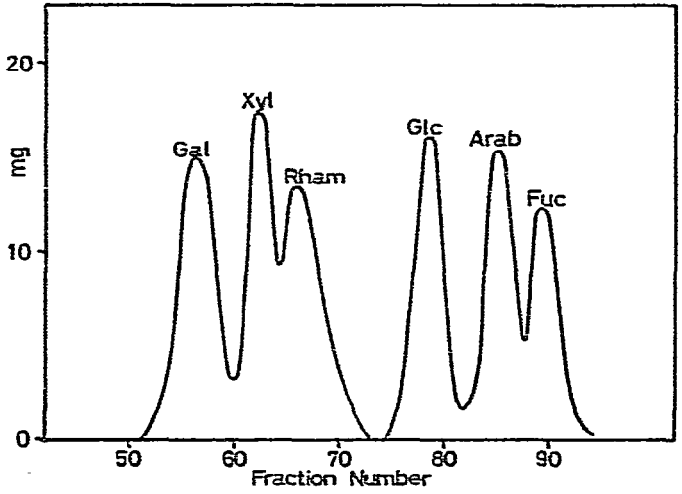

Fig. 6. Separation of monosaccharides. In the first stage a mixture of galactose, xylose and rhamnose (100 mg each) was developed by ascending DCC with 600 tubes (40 cm × 1.65 mm I.D.) using the solvent system chloroform–methanol–water (35:65:40), and in the second stage, starting 24 h after the charge of the first sample, a mixture of arabinose, glucose and fucose (100 mg each) was added and developed in the same way, collecting 2-ml fractions for gravimetric determination.

Monosaccharides

The separation and identification of monosaccharides are often required for the investigation of saponins and other glycosides.

A mixture of galactose, xylose and rhamnose (100 mg each) was subjected to DCC and, after running for 24 h, a mixture of glucose, arabinose and fucose (100 mg each) was administered. The use of the solvent system chloroform–methanol–water (35:65:40), with 1200 theoretical plates, gave a satisfactory separation of the components (Fig. 6).

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REFERENCES

1 T. Tanimura, J. J. Pisano, Y. Ito and R. L. Bowman, *Science*, 169 (1971) 54.
2 C. C. Craig, *J. Biol. Chem.*, 155 (1944) 519.
3 K. Kawai, T. Akiyama, Y. Ogihara and S. Shibata, *Phytochemistry*, 13 (1974) 2829.
4 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, 28 (1956) 350.
5 A. Stoll and B. Becker, *Helv. Chim. Acta*, 32 (1949) 1897.