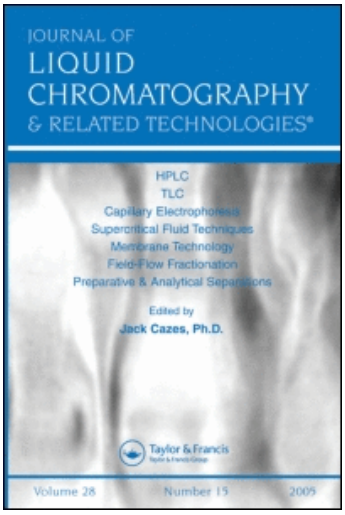

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COUNTERCURRENT CHROMATOGRAPHY IN THE PREPARATIVE SEPARATION OF PLANT-DERIVED NATURAL PRODUCTS

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EXTRACTIONS AND PURIFICATIONS

COUNTERCURRENT CHROMATOGRAPHY IN THE PREPARATIVE SEPARATION OF PLANT-DERIVED NATURAL PRODUCTS

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ABSTRACT

The versatility of high-speed countercurrent chromatography (HSCCC) makes it an ideal method for the separation of plant-derived natural products. The technique can handle complex polar or apolar crude plant extracts equally well as purified fractions. Sample quantities can vary from several milligrams to tens of grams on the same instrument. Difficult isolation problems, with very similar compounds, can be attempted. Different applications that explore these possibilities are described here.

INTRODUCTION

Plants can contain hundreds, or even thousands, of constituents, varying from high molecular weight compounds, from carbohydrates or proteins to low molecular weight compounds, such as monoterpenes or simple phenolic secondary metabolites. Their separation poses a multitude of problems, especially,

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when a single compound is targeted for its potential therapeutic or other functions.

Different methods are available for the separation of natural products and the most commonly used involve chromatography on solid phases (Table 1).[1] While certain of these techniques, such as semi-preparative HPLC, provide high resolution, the all-liquid methods share a number of their own advantages. The most important of these is the elimination of complications associated with a solid support.[2] Furthermore, apart from the outlay on instruments, the only cost involved is the purchase of solvents. Several reviews have highlighted examples of separations of natural products using different countercurrent chromatographic methods.[3-6] The fastest and most widely-used variant is high-speed countercurrent chromatography (HSCCC), both in the form of rotating coils or rotating disks and cartridges.

It is the intention of this article, to illustrate some of the wide-ranging possibilities when using centrifugal countercurrent chromatography in the fractionation of samples derived from plants and their extracts. The versatility of the method is important, especially, when one considers the enormous range of solvent systems which are applicable.[1]

EXPERIMENTAL

Preparative-scale separations were performed at room temperature with a CCC-1000 instrument (Pharma-Tech Research Corp., Baltimore, MD, USA) equipped with three coils of total capacity 650 mL and running at 1000 rpm. The instrument was equipped with two model 300LC pumps (Scientific Systems, Inc., State College, PA, USA) for pumping upper and lower phases. The flow-rate of mobile phase was 3 mL/min. Fractions were monitored at 254 nm by a variable wavelength UV detector (Knauer, Berlin, Germany), connected to a model 600 integrator (W+W Scientific, Basle, Switzerland), and a Pharmacia 2070 Ultrorac II fraction collector (Uppsala, Sweden).

Table 1. Preparative Methods for the Chromatography of Natural Products

Solid-Liquid Chromatography	Liquid-Liquid Chromatography
Planar chromatography (TLC, Chromatotron, OPLC)	Countercurrent distribution
Open-column chromatography	Droplet countercurrent chromatography
Pressure liquid chromatography (LPLC, MPLC, HPLC, SFC)	Centrifugal countercurrent chromatography

The instrument was filled by pumping equal amounts of upper and lower phases, simultaneously, into the stationary coils, giving a 1:1 proportion of the two phases in the apparatus.

RESULTS AND DISCUSSION

Fractionation of Crude Plant Extracts

CCC is very effective for initial fractionation of crude plant extracts. It can be used for all ranges of polarities, but has special advantages for the handling of polar extracts, which are often difficult to process by more classical techniques. Saponin-containing extracts can be efficiently fractionated by HSCCC, as exemplified by *Phytolacca icosandra* (Phytolaccaceae). Various plant species of the genus *Phytolacca*, and most notably *P. dodecandra*, have been intensively studied for the molluscicidal effects of saponins from their fruits.[7] The snail-killing activity of the saponins is of importance for the control of the parasitic disease schistosomiasis, which involves a snail intermediate host in the life cycle of the parasites. A methanol extract of the fruits of *P. icosandra* from Indonesia, was first partitioned between n-butanol and water, to concentrate the saponins in the organic phase. The butanol fraction (3 g) was subjected to HSCCC, giving the fractions shown in Fig. 1. TLC monitoring of fractions was necessary because

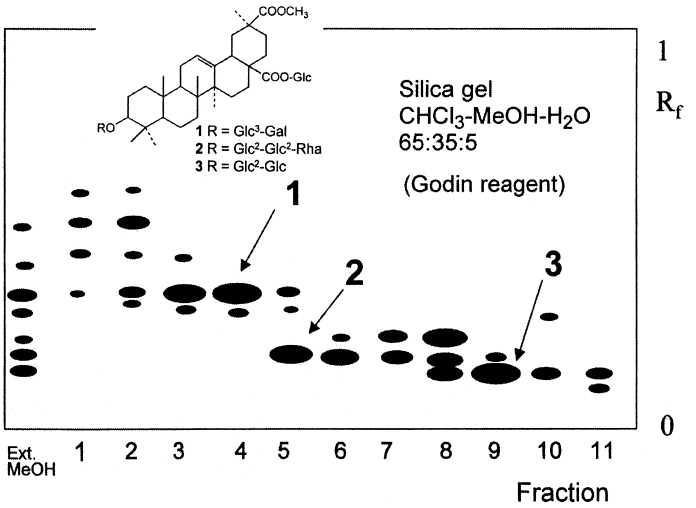
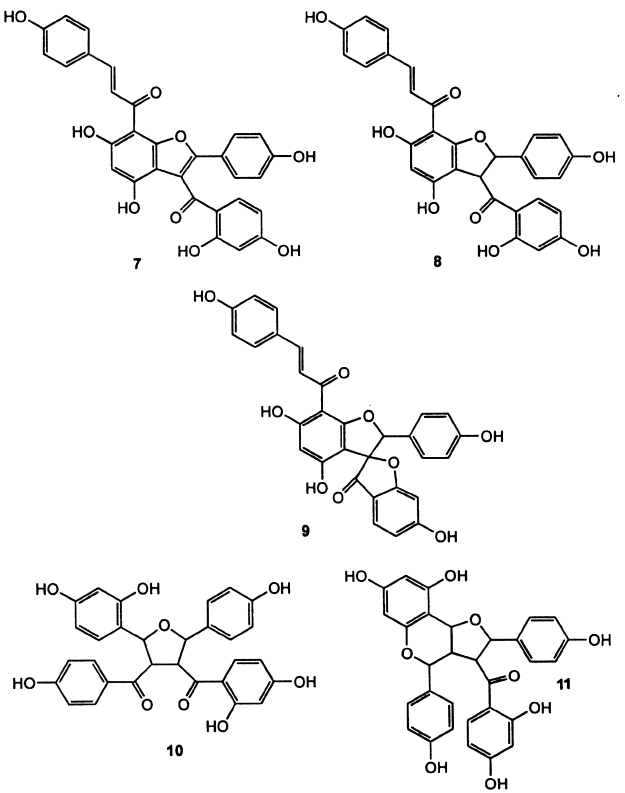


Figure 1. TLC monitoring of fractions from the HSCCC separation of a methanol extract of *Phytolacca dodecandra* (Phytolaccaceae) fruits. HSCCC conditions : solvent chloroform-methanol-isopropanol-water 5 :6 :1 :4 (lower phase as mobile phase); sample weight 3 g.

saponins were not detected at 254 nm. Saponins, **1** (236 mg), **2** (23 mg) and **3** (100 mg), were obtained after a final chromatographic step – over Sephadex LH-20 for **1** and over silica gel for **2** and **3**.



A big problem when dealing with crude extracts of plants is the fractionation of large amounts of sample without suffering too much material loss. Solid supports, and most of all, silica gel, are notorious for irreversibly absorbing large proportions of sample. When the absorbed material is the required bioactive component of a sample, the complications are evident. Countercurrent chromatography provides a means of avoiding this problem. While HSCCC instruments have been used, generally for separations with quantities of sample less than 5 g, it is surprising just how much can be loaded onto one of these chromatographs. For instance, large quantities of naphthalene glycosides could be isolated from a methanol extract of *Euclea mayottensis* (Ebenaceae) root bark. This small tree finds many uses in traditional medicine in the Comoros Islands. The glycosides are relatively labile, furnishing naphthoquinones, which have a

variety of biological activities, including fungicidal activities. These glycosides, which are rather like pro-drugs, were very difficult to isolate by conventional methods and broke down to give naphthoquinones. By HSCCC, 26 g of crude methanol extract (dissolved in 30 mL of upper and 30 mL of lower phase, and introduced into a 100 mL sample loop) could be chromatographed in one run (Fig. 2). The three major constituents (**4** – **6**) of the extract were thus separated. Their final purification was achieved by gel filtration on Sephadex LH-20, with different methanol-water mixtures. In this way, sample loss was cut to a minimum.

A large quantity of crude methanol extract of *Brackenridgea zanguebarica* (Ochnaceae) inner stem bark could also be efficiently fractionated by HSCCC. This shrub, which is found in Southern and Central Africa, has a variety of traditional uses. The stem bark methanol extract was shown to be active against the plant pathogenic fungus *Cladosporium cucumerinum*, and a bioactivity-guided fractionation procedure was undertaken. The first step was a large-scale separation by HSCCC. A charge of 20 g was introduced into the instrument and the resulting TLC analysis of the grouped fractions is shown in Fig. 3. A good separation of the five main compounds was achieved, including resolution of the closely running components, **7** and **8**. The benzofuran, **7,** was obtained in the pure state after simple crystallization, while the other compounds required an

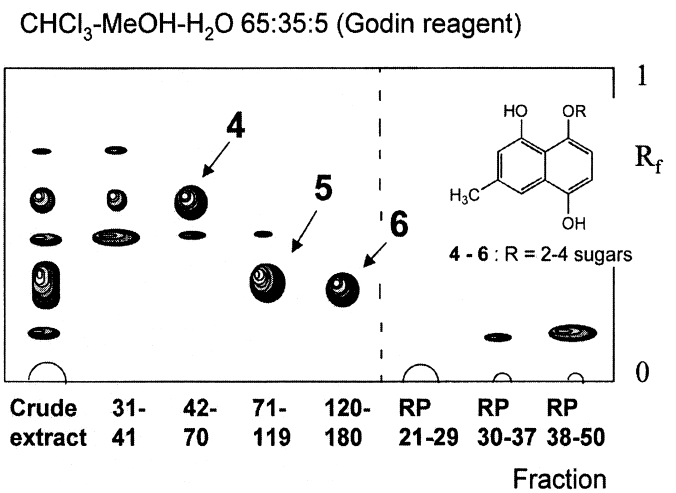


Figure 2. TLC monitoring of fractions from the HSCCC separation of *Euclea mayottensis* (Ebenaceae) root bark methanol extract. HSCCC conditions: solvent chloroform-methanol-n-butanol-water 7 :6 :3 :4 (lower phase as mobile phase; after fraction 180, mobile phase changed to upper phase); sample weight 26 g.

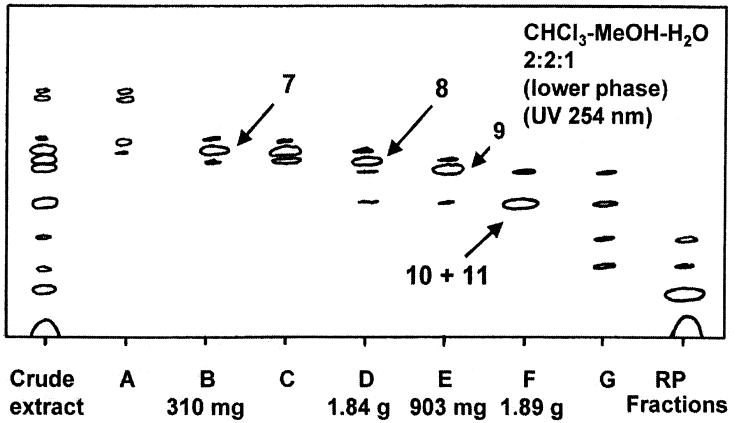


Figure 3. TLC monitoring of fractions from the HSCCC separation of *Brackenridgea zanguebarica* (Ochnaceae) stem bark methanol extract. HSCCC conditions: solvent cyclohexane-ethyl acetate-methanol-water 7 :8 :6 :6 (upper phase as mobile phase; remaining material flushed out by phase reversal, giving reversed-phase (RP) fractions); sample weight 20 g.

additional chromatographic step for final purification. Thus, the four other polyphenols, **8** – **11,** were all isolated after low-pressure liquid chromatography on RP-18, with methanol-water as solvent. The spiro-derivative, **9,** can be considered as deriving from intramolecular cyclization of the dihydrobenzofuran derivative, **8**. All five compounds were effective against *Cladosporium cucumerinum*, and while they also had antibacterial activity against *Bacillus subtilis*, the spiro-compound, **9,** only showed borderline activity.[8]

Separation of Closely Related Substances

Although, resolution by HSCCC is not high when compared with methods like semi-preparative HPLC, the countercurrent technique can give very good selectivities.

An example is provided by the separation of two secoiridoid isomers, vogeloside (**12**) and isovogeloside (**13**), from the South American herb *Halenia campanulata* (Gentianaceae). After defatting with dichloromethane and extraction with methanol, the methanol extract was fractionated by open-column chromatography over silica gel. One fraction contained a mixture of the isomers, **12** and **13**, which were very difficult to separate by the usual chromatographic methods. However, by HSCCC with a chloroform-methanol-water solvent system,

baseline separation of the two isomers was achieved (Fig. 4). From a total sample load of 240 mg, 32 mg of pure **12** and 24 mg of pure **13** were obtained.

Repeated Injection of Samples

When the separation between peaks is small (selectivity low) in HSCCC, one solution is repetitive injection, when several small quantities of the sample are injected one after the other. This strategy was used successfully for the isolation of vincamine and vincine from *Vinca minor* (Apocynaceae). Twenty successive injections, each of 1.7 mg, gave a final yield of 16.5 mg vincamine and 14 mg vincine. [9]

This method is also useful for the separation of closely related compounds, which have similar retention times. In the case of *Gentiana campestris* (Gentianaceae), collected in Switzerland, the methanol extract of the whole plant, after chromatography and gel filtration, gave a mixture of two xanthones (**14** and **15**). Repeated injection (three times) of 50 mg quantities of mixture enabled the isolation of 63 mg of **14** and 51 mg of **15** (Fig. 5).

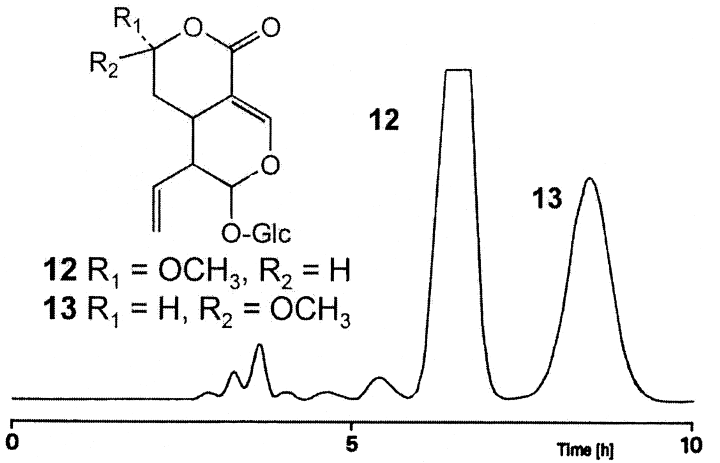


Figure 4. Isolation of vogeloside (**12**) and isovogeloside (**13**) from *Halenia campanulata* (Gentianaceae). HSCCC conditions: solvent chloroform-methanol-water 9 :12 :8 (lower phase as mobile phase); sample weight 240 mg.

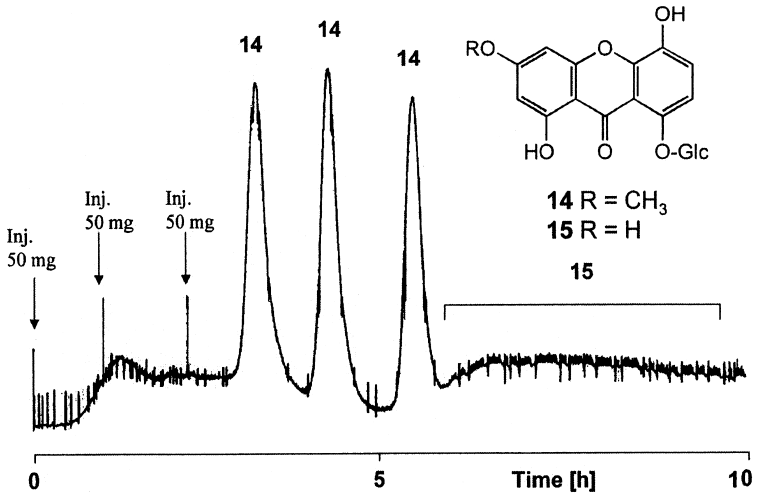


Figure 5. Separation of xanthones **14** and **15** by repetitive injections of 50 mg batches of sample. HSCCC conditions: solvent chloroform-methanol-ethanol-water 5 :3 :3 :4 (lower phase as mobile phase).

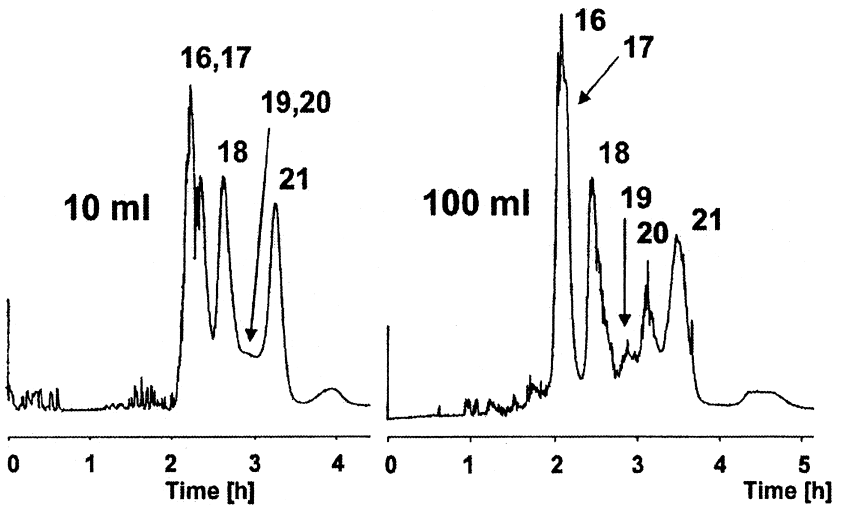
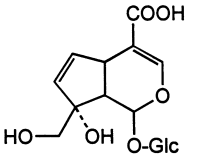


Figure 6. Effect of changing sample volume on separation in HSCCC. Injection of 500 mg of *Pyrola elliptica* (Rosaceae) methanol extract via a 10 mL or a 100 mL injection loop. HSCCC conditions: solvent hexane-ethyl acetate-methanol-water 4 :12 :4 :5 (upper phase as mobile phase).

Changing Sample Volume

There are several ways of extending the flexibility of CCC separations. These include operation in both normal and « reversed-phase » modes, employing solvent gradients and changing the proportion of mobile and stationary phases in the coils.[10]

Another possibility is to change the injection volume of the sample. This is illustrated by the separation of constituents of the Canadian plant *Pyrola elliptica* (Rosaceae) (Fig. 6). A portion of the methanol extract (500 mg) of the whole plant was injected into the chromatograph and the principal constituents were collected. When a 10 mL sample loop was used and the extract was dissolved in 2 mL of lower phase and 2 mL of upper phase, there was incomplete separation of taxifolin 3-*O*-arabinoside (**19**) and taxifolin 3-*O*-xyloside (**20**). Monotropein (**16**) and isohomoarbutin (**17**) eluted together, while the flavonol glycosides quercetin 3-*O*-galactoside (**18**) and quercetin 3-*O*-arabinoside (**21**) were well separated.



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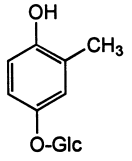

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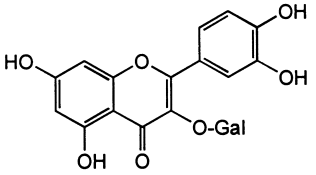

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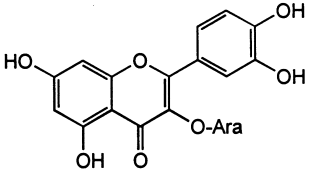

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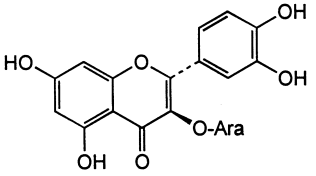

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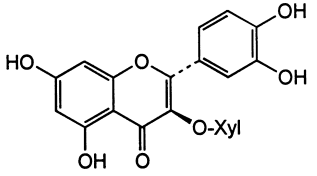

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Surprisingly, when the same amount of sample in the same volume of upper and lower phases was injected through a 100 mL sample coil, the resolution improved and the two taxifolin glycosides (**19** and **20**) were separated. This unusual effect, which is contrary to the situation found in chromatography on solid supports (a larger sample volume leads to lower resolution of the individual components), is of potential importance, especially if the sample is sparingly soluble in the solvent system and has to be diluted. This also implies that injection of crude extracts in large volumes of eluent is not necessarily disadvantageous.

CCC, since its inception in the 1960s, has proved its value in many fields. In particular, high-speed CCC has many applications in the separation of natural products. It can be employed as an initial fractionation step or as an alternative to other chromatographic methods when these fail to produce the necessary results.

HSCCC has come of age and should provide an indispensable additional tool in the laboratory of any separations scientist. It will, of course, never provide a universal solution to separation problems but maximum use can be made of the various aspects outlined above.

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