Journal of Chromatography A, 658 (1994) 315–341
Elsevier Science B.V., Amsterdam

CHROM. 24 978

Review

Counter-current chromatography as a preparative tool —applications and perspectives

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ABSTRACT

Counter-current chromatography is an all-liquid technique that is finding increasing use in separation problems, especially in the field of natural products. A broad selection of applications, involving both polar and non-polar compounds, is described here. Examples of the choice and utilization of aqueous and non-aqueous solvent systems are given. The emphasis is placed on new and rapid centrifugal partition techniques but droplet countercurrent chromatography is also treated, together with some applications of rotation locular counter-current chromatography.

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SSDI 0021-9673(93)E0229-N

1. INTRODUCTION

The key to any study of material from natural sources is the availability of suitable separation methods for the isolation of pure products. Over the last few decades, extremely versatile and efficient methods for the separation and purification of organic componds have appeared [1]. Although most separations are performed by chromatography on solid supports, all-liquid techniques are currently attracting considerable interest [2,3].

Counter-current chromatography (CCC) is a term introduced by Ito [4] to describe modern liquid–liquid chromatography without a sorbent, requiring two immiscible solvent phases. It is basically an outgrowth of counter-current distribution, as developed by Craig. In most reported variants of CCC, one phase remains stationary while the second phase is passed through the stationary solvent component. The principle of separation involves the partition of a solute between the two immiscible solvents, the relative proportions of solute passing into each of the two phases being determined by the respective partition coefficients.

As CCC is an all-liquid technique, it benefits from a number of advantages in comparison with the more traditional liquid–solid separation methods, such as column chromatography and HPLC: (i) no irreversible adsorption; (ii) total recovery of introduced sample; (iii) tailing minimized; (iv) minimal risk of sample denaturation; (v) solvent consumption low; and (vi) favourable economics.

2. DROPLET COUNTER-CURRENT CHROMATOGRAPHY (DCCC)

Droplet counter-current chromatography relies on the passage of droplets of a mobile phase through an immiscible stationary liquid phase for the continuous partition of a solute between the two phases [5]. A typical DCCC instrument consists of 200–600 vertical glass columns interconnected in series by capillary PTFE tubes. The columns are generally of 2 or 2.7 mm I.D.

Binary solvent systems are seldom used (except, for example, *n*-butanol–water, which has been used for the preliminary purification of a steroid glycoside sulphate from the starfish *Luidia maculata* [6]) because the formation of suitable droplets is impaired by the large difference in polarity between the two components. It is ternary (or quaternary) systems that are preferentially employed, as addition of a third (or fourth) component, miscible with the other components, diminishes the difference in polarity between the two phases. The selectivity of the system is consequently increased, allowing the separation of closely related substances.

Selection of a suitable solvent system can conveniently be performed on silica gel TLC plates by running the water-saturated organic layer of a two-phase aqueous solvent system [7]. If the R_F values of the compounds to be separated are higher than about 0.5, the less polar layer is suitable for use as the mobile phase. For higher polarity solutes ($R_F < 0.5$), the more polar layer is chosen as the mobile phase.

DCCC is ideal for the initial fractionation of

crude plant extracts because there is no possibility of irreversible adsorption on a solid support. In this context, DCCC can be chosen as the first step in a particular separation strategy. The technique can equally well be employed for the final purification of mixtures. Resolution is not high. However, in certain instances, it is possible to obtain pure substances from plant extracts by DCCC alone (one or more DCCC steps) [14,19,32]. Loadings of up to about 5 g are possible on the normal commercially available instruments.

The applications of DCCC are numerous and have been the subject of several reviews [7–10]. For this reason, only a few of the more recent examples will be given here. The explanation for the popularity of DCCC lies in its simplicity of operation; it is the most straightforward of the counter-current techniques described in this review.

A selection of solvent systems commonly used in the separation of natural products is shown in Table 1. Although certain examples involve non-aqueous solvents (for more lipophilic substances) [10,11], the major utility of DCCC is in the separation of polar compounds such as glycosides. Of special note are the separations of polyphenols, tannins, anthocyanins, cardenolides and saponins.

Some recent examples of the use of DCCC in

TABLE 1

SELECTED DCCC SOLVENT SYSTEMS FOR PREPARATIVE APPLICATION

Substance class	Solvent system
Glycosides, tannins, alkaloids, antibiotics, etc.	$CHCl_3$–MeOH–H_2O (7:13:8 and other proportions)
Saponins, iridoid glycosides, xanthone glycosides, flavonoid glycosides and anthraquinone glycosides	$CHCl_3$–MeOH–H_2O–*n*-PrOH (9:12:8:1)
Saponins	$CHCl_3$–MeOH–H_2O–*n*-PrOH (5:6:4:1)
Flavonoid glycosides	$CHCl_3$–MeOH–H_2O–*n*-BuOH (10:10:6:1)
Saponins	$CHCl_3$–MeOH–H_2O–*n*-PrOH–EtOH (9:6:8:1:8)
Alkaloids	$CHCl_3$–MeOH–H_2O–C_6H_5Me (5:7:2:5)
Alkaloids	$CHCl_3$–MeOH–acetate buffer (pH 3.6) (9:12:8)
Basic steroid saponins	$CHCl_3$–MeOH–1% aq. NH_3 (7:12:8)
Alkaloids	$CHCl_3$–MeOH–5% HCl (5:5:3)
Glycolipids	$CHCl_3$–MeOH–0.5% aq. $CaCl_2$–*n*-PrOH (50:60:40:6)
Saponins	CH_2Cl_2–MeOH–H_2O (8:13:7)
Anthocyanins, flavonoid glycosides, peptides	*n*-BuOH–HOAc–H_2O (4:1:5)
Naphthalide glycosides	*n*-BuOH–MeOH–H_2O (5:1:5)
Tannins	*n*-BuOH–*n*-PrOH–H_2O (2:1:3)
Iridoid and secoiridoid glycosides	*n*-BuOH–EtOH–H_2O (4:1:5)
Sennosides	*n*-BuOH–Me_2CO–H_2O (33:10:50)
Peptides	iso-BuOH–TFA–H_2O (120:1:160)
Gibberellins and anthraquinones	Light petroleum–EtOH–H_2O–EtOAc (5:4:1:2)
Diterpenoids and coumarins	C_6H_{14}–Et_2O–*n*-PrOH–95% EtOH–H_2O (4:8:3:5:4)
Essential oils	C_6H_{14}–EtOAc–$MeNO_2$–MeOH (8:2:2:3)
Triterpenes and steroids	C_7H_{16}–$C_2H_4Cl_2$–MeOH (47:6:72)
Triterpenes, steroids and depsides	C_7H_{16}–Me_2CO–MeOH (5:1:4)
Triterpenes and steroids	C_7H_{16}–MeCN–CH_2Cl_2 (10:7:3)

the isolation of natural products from plants are shown in Table 2; these cover a wide range of compounds with different polarities.

As pyrrolizidine alkaloids have a tendency to adsorb irreversibly on solid phases such as alumina, silica gel or chemically bonded silica gel, DCCC has often been introduced as one of the purification methods for this particular class of compound. For example, separations have been accomplished for *Arnica montana* (Asteraceae), with chloroform–methanol–water (5:6:4) (descending mode) [48], and *Crotolaria sessiliflora* (Leguminosae), with chloroform–methanol–water (5:5:3) (ascending mode) [49]. Different chloroform–methanol–water mixtures were also used for the isolation of cytotoxic

TABLE 2

SOME RECENT EXAMPLES OF DCCC SEPARATIONS OF PLANT-DERIVED COMPOUNDS

Substances separated	Plant species	Solvent system	Mode	Ref.
Phenolic glucosides	*Eurya tigang* (Theaceae)	$CHCl_3$–MeOH–H_2O (7:13:8)	Ascending	12
Phenylpropane glycosides	*Castilleja linariaefolia* (Scrophulariaceae)	$CHCl_3$–MeOH–H_2O (5:5.7:3)	Descending	13
Stilbene glycosides	*Rheum palmatum* (Polygonaceae)	$CHCl_3$–MeOH–H_2O (7:13:8)	Descending	14
Flavonol glycosides	*Strychnos variabilis* (Loganiaceae)	$CHCl_3$–MeOH–H_2O (5:6:4)	Descending	15
Quercetin 3-O-glucuronide	*Vaccinium myrtillus* (Ericaceae)	$CHCl_3$–MeOH–PrOH –5% CH_3COOH (31.2:37.5:6.2:25)	Descending	16
Flavonoid coumaroyl glycosides	*Ginkgo biloba* (Ginkgoaceae)	$CHCl_3$–MeOH–PrOH–H_2O (5:6:1:4)	Ascending	17
Polyphenols	*Cordia goetzei* (Boraginaceae)	$CHCl_3$–MeOH–H_2O (43:37:20)	Descending	18
Bianthrone C-glycoside	*Asphodelus ramosus* (Liliaceae)	$CHCl_3$–MeOH–H_2O (4:4:3)	Ascending	19
Ellagitannins	*Spondias mombin* (Anacardiaceae)	*n*-BuOH–Me_2CO–H_2O (7:2:11)	Ascending	20
Anthocyanins	*Podocarpus* sp. (Podocarpaceae)	*n*-BuOH–HOAc–H_2O (4:1:5)	Descending	21
Proanthocyanidins	*Pavetta ovariensis* (Rubiaceae)	BuOH–PrOH–H_2O (2:1:3)	Ascending	22
Hyperforin derivatives	*Hypericum revolutum* (Guttiferae)	Light petroleum–94% EtOH–EtOAc–H_2O (83:67:33:17)	Ascending	23
Phloroglucinol derivative	*Hypericum calycinum* (Guttiferae)	Light petroleum–94% EtOH–EtOAc–H_2O (83:67:33:17)	Ascending	24
Iridoid diglycosides	*Premna japonica* (Verbenaceae)	$CHCl_3$–MeOH–*n*-PrOH–H_2O (9:12:2:8)	Ascending	25
Iridoid glucoside diesters	*Premna odorata* (Verbenaceae)	$CHCl_3$–MeOH–2-PrOH–H_2O (9:12:2:8)		26
Secoiridoid glucosides	*Jasminum mesnyi* (Oleaceae)	*n*-BuOH–EtOH–H_2O (4:1:1)	Ascending	27
Ionone glycosides	*Eriobotrya japonica* (Rosaceae)	*n*-BuOH–Me_2CO–H_2O (60:18:22)	Ascending	28
β-Ionone glucosides	*Dendranthema shiwogiku* (Asteraceae)	$CHCl_3$–MeOH–*n*-PrOH–H_2O (9:12:2:8)	Ascending	29

TABLE 2 *(continued)*

Substances separated	Plant species	Solvent system	Mode	Ref.
Sesquiterpene glycosides	*Eriobotrya japonica* (Rosaceae)	$CHCl_3$–MeOH–H_2O (7:13:8)	Ascending	30
Casbane diterpenes	*Agrostistachys hookeri* (Euphorbiaceae)	Cyclohexane–Et_2O–2-PrOH –EtOH–H_2O (7:16:6:10:8)	Ascending	31
Clerodane diterpenes	*Portulaca pilosa* (Portulacaceae)	$CHCl_3$–MeOH–H_2O (13:7:4)	Ascending	32
Labdane glycosides	*Mitraria coccinea* (Gesneriaceae)	$CHCl_3$–MeOH–H_2O (7:13:8)	Ascending	33
Cucurbitacin glycoside	*Persea mexicana* (Lauraceae)	$CHCl_3$–MeOH–H_2O (13:7:4)	Ascending	34
Bufadienolides	*Urginea pancration* (Liliaceae)	$CHCl_3$–MeOH–H_2O (5:10:6)	Ascending and descending	35
Cardenolides	*Digitalis subalpina* (Scrophulariaceae)	$CHCl_3$–MeOH–H_2O (5:6:4)	Descending	36
Saponins	*Tetrapleura tetraptera* (Leguminosae)	$CHCl_3$–MeOH–H_2O (7:13:8)	Ascending	37
Saponins	*Aphloia theiformis* (Flacourtiaceae)	$CHCl_3$–MeOH–2-PrOH–H_2O (5:6:1:4)	Descending	38
Saponins	*Sargentodoxa cuneata* (Sargentodoxaceae)	$CHCl_3$–MeOH–H_2O –C_6H_6–EtOAc (45:60:40:2:3)	Ascending	39
Steroid saponins	*Balanites aegyptiaca* (Zygophyllaceae)	CH_2Cl_2–MeOH–H_2O (8:13:7)	Ascending	40
Ecdysteroids	*Leuzea carthamoides* (Asteraceae)	$CHCl_3$–C_6H_6–MeOH–H_2O (15:15:23:7)	Descending	41
Cyanogenic glycosides	*Oxyanthus pyriformis* (Rubiaceae)	$CHCl_3$–MeOH–PrOH–H_2O (5:6:1:4)	Ascending	42
Unsaturated alkamides	*Achillea ptarmia* (Asteraceae)	C_6H_{14}–$CHCl_3$–MeOH–H_2O (18:25:18:3)		43
Benzophenanthridine alkaloid	*Chelidonium majus* (Papaveraceae)	$CHCl_3$–MeOH–H_2O (5:6:4)	Descending	44
Aporphine alkaloids	*Ocotea caesia* (Lauraceae)	$CHCl_3$–MeOH–H_2O (5:5:3)	Descending	45
Chromone alkaloids	*Schumanniophyton magnificum* (Rubiaceae)	*n*-BuOH–MeOH–H_2O (5:1:5)	Descending	46
		n-BuOH–MeOH–aq. NH_3 (5:1:5)	Ascending	46
2-Pyrrolidineacetic acid	*Arnica montana* (Asteraceae)	$CHCl_3$–MeOH–H_2O (5:6:4)	Descending	47

pyrrolizidine alkaloids from *Senecio anonymus* (Asteraceae) [50]. Separation of 3 g of mixture was possible with 4 l of solvent, *i.e.*, low solvent consumption. Further, resolution of mixtures containing *cis–trans* isomeric alkaloids such as senecionine (**1**) and integerrimine (**2**) was possible. Attempts at separating these diastereoisomers by other chromatographic methods (column chromatography, reversed-phase HPLC, etc.) resulted only in slight enrichments, low recoveries and broad peaks [50].

The key step in separating the chalcone isomers okanin 3′-glucoside and okanin 4′-glucoside from the flowers of *Bidens pilosa* (Asteraceae)

was step-gradient DCCC under the following conditions: chloroform–methanol–water 13:7:4 (descending), then chloroform–methanol–2-propanol–water (26:13:1:8 and 13:6:1:4) [51].

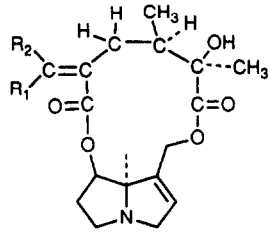
The application of DCCC to the separation of saponins has proved very successful and illustrates very well its usefulness for polar compounds. The technique is versatile and can be employed for the initial fractionation of crude extracts, for the separation of closely related saponins and/or the isolation of pure products [10]. Different solvent systems have found use for the DCCC of saponins, but chloroform–methanol–water (7:13:8) has provided the greatest number of applications [1]. Chloroform–methanol–water systems can be used either in the ascending mode for very polar saponins or in the descending mode for saponins possessing one or two sugars and few free hydroxyl groups.

Molluscicidal oleanolic acid glycosides have been isolated from a methanol extract of the fruits of the West African tree *Tetrapleura tetraptera* (Leguminosae) by a combination of open-column chromatography on silica gel and DCCC with the chloroform–methanol–water (7:13:8) solvent system in the ascending mode [37]. The saponins **3** and **4** isolated by DCCC were diglycosides containing N-acetylglucosamine. Only two separation steps were necessary for the purification, affording 50 mg of **3** and 9 mg of **4**.

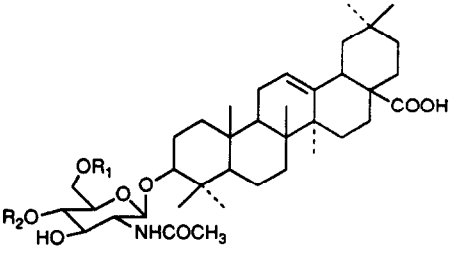
A methanol extract of the leaves of *Aphloia theiformis* (Flacourtiaceae) was found to contain tormentic acid glycosides. A total of three glycosides were isolated, including the new saponin 6β-hydroxytormentic acid ester glucoside (**5**), by a strategy involving DCCC and low-pressure liquid chromatography. Initial purification by DCCC of the methanol extract (3.5 g) was performed with the solvent system chloroform–methanol–2-propanol–water (5:6:1:4) [38].

Other tormentic acid derivatives have been isolated from *Sargentodoxa cuneata* (Sargentodoxaceae). This time two isomeric glucosides (**6** and **7**) were separated by DCCC using chloroform–methanol–water–benzene–ethyl acetate (45:60:40:2:3), with the lower phase as the stationary phase [39].

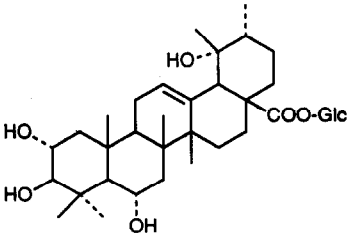
The isolation of saponins from starfish and other echinoderms has involved entensive use of a combination of DCCC and reversed-phase



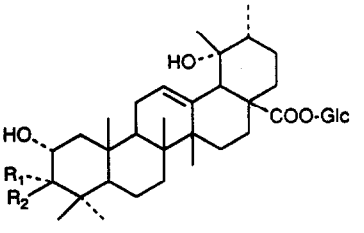
1 $R_1 = CH_3$, $R_2 = H$ (Senecionine)
2 $R_1 = H$, $R_2 = CH_3$ (Integerrimine)



3 $R_1 = H$, $R_2 = Gal$
4 $R_1 = Glc$, $R_2 = H$

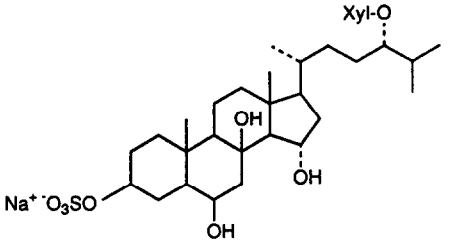


5



6 $R_1 = H$, $R_2 = OH$
7 $R_1 = OH$, $R_2 = H$

semi-preparative HPLC. The highly polar two-phase solvent system *n*-butanol–acetone–water (3:1:5) has proved of great value in these separations [52]. A recent example is the separation of steroidal glycoside sulphates (*e.g.*, **8**) and polyhydroxysteroids from the Japanese starfish *Aphelasterias japonica*. Following Sephadex LH-60 gel filtration, DCCC with the above-mentioned solvent system was performed in the ascending mode at 24 ml/h. Final purification of the required compounds was achieved by HPLC on a μBondapak C_{18} column with methanol–water (1:1) [53].



8

Another area in which DCCC has found some interest is in the field of fungal metabolites. Extracts of culture broths of *Staphylotrichum coccosporum* were first chromatographed on silica gel and then submitted to DCCC [hexane–diethyl ether–propanol–ethanol–water (4:8:3:5:8), descending mode] in order to obtain new metabolites, called spirostaphylotrichins [54].

3. ROTATION LOCULAR COUNTER-CURRENT CHROMATOGRAPHY (RLCC)

In RLCC, an assembly of sixteen glass columns (each composed of 37 compartments) is arranged around a rotational axis. Both the speed of rotation of the columns and their angle of inclination can be varied [55].

As in DCCC, one phase of a two-phase solvent system is introduced into the columns as the stationary phase and the other phase serves as the mobile phase. Unlike DCCC, the formation of droplets is not a necessary condition of RLCC. Consequently, a wider range of solvent systems is possible, *e.g.*, ethyl acetate–water systems, which are often incompatible with DCCC, are acceptable in RLCC. This fact, together with the ability to handle relatively large amounts of sample, provided the initial preference for RLCC over DCCC.

The applications of RLCC are, however, not very numerous and this particular counter-current technique has not found very widespread use. The resolution is relatively low (*ca.* 300 theoretical plates) in comparison with DCCC and problems are often encountered with the rotating seals. With the introduction of centrifugal partition chromatography, RLCC is almost certainly doomed to disappear.

However, various separations involving RLCC have been performed and even enantiomers of (±)-norephedrine have been resolved, using a chiral mobile phase [56].

In addition to triterpene saponins from *Phytolacca dodecandra* (Phytolaccaceae) [57], RLCC has been employed for the separation of steroid glycoalkaloids from ripe fruits of *Solanum incanum* (Solanaceae) [58]. RLCC with gradient elution for the large-scale separation of the crude extract preceded a final DCCC step (with its higher resolution), yielding the glycoalkaloids solamargine and solasonine. Water was used as the stationary phase for RLCC and gradient elution was carried out in the ascending mode. The first elution solvent, water-saturated ethyl acetate, to remove non-polar components, was succeeded by the upper layer of ethyl acetate–butanol–water (2:1:1), which yielded a mixture of the two glycoalkaloids. These were separated by DCCC with the solvent system chloroform–methanol–water–propanol–ammonia solution (34:65:40:5:1) in the ascending mode [58].

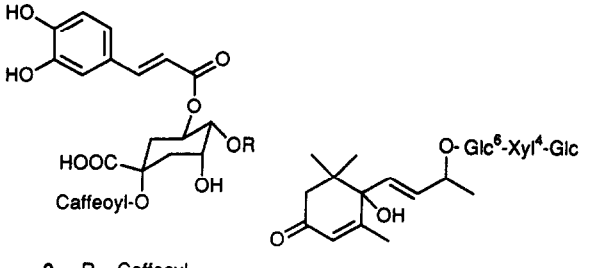
Although applications of counter-current chromatography to pyrrolizidine alkaloids have mainly involved DCCC, certain isolation strategies have employed RLCC. This is the case for the alkaloids from *Heliotropium spathulatum* (Boraginaceae), in which the RLCC solvent system was chloroform–methanol–water (5:5:3) in the descending mode [59].

RLCC was used as the first step in the isolation of ecdysteroids from the root bark of *Vitex*

strickeri (Verbenaceae). The methanol extract (1 g) was dissolved in ethyl acetate–water (1:3) and introduced into an RLCC instrument containing the aqueous layer of the solvent system ethyl acetate–*n*-propanol–water (6:1:6) as stationary phase. Before elution with mobile phase, the columns were washed with water-saturated ethyl acetate. Further purification was by means of recycling HPLC [60].

Another recent application of RLCC has been in the characterization of caffeoylquinic acids from the flowers of *Arnica montana* (Asteraceae). An ethyl acetate extract (16.6 g) of the flowers was separated into eleven fractions by RLCC with solvent system chloroform–*n*-butanol–methanol–water (10:1:10:6, descending mode). One fraction provided **9**, while a second RLCC step (ethyl acetate–*n*-propanol–water, ascending mode) was necessary to obtain **10** [61].

A considerable effort has been expended by Schreier and colleagues at the University of Würzburg to isolate and identify glycosidic precursors of compounds responsible for the aroma of different fruits [62]. As an example, a vomifoliol trisaccharide glycoside (**11**) was obtained from apple fruits (*Malus sylvestris*, Rosaceae) via an initial RLCC step applied to the methanol extract. The solvent system used was chloroform–methanol–water (9:12:8) in the descending mode and then in the ascending mode. Final clean-up was achieved by semi-preparative HPLC [51]. Two new ionone glycosides were isolated from quince fruit (*Cydonia oblonga*, Rosaceae) by a procedure which involved RLCC with chloroform–methanol–water (7:13:8) in the ascending mode [63].



9 R = Caffeoyl
10 R = H

11

4. CENTRIFUGAL PARTITION CHROMATOGRAPHY (CPC)

The two major disadvantages of DCCC which limit its potential are the long separation times (separations often require 2 days or longer) and the necessity to choose solvent systems which form droplets. RLCC is also slow and may suffer the additional complication of leaking rotating seals.

Centrifugal partition chromatography (CPC), on the other hand, relies on centrifugal force rather than on gravitation for retention of the stationary phase and solvents can be pumped at higher speeds through the instruments. This allows shorter separation times, without loss of resolution, a factor of crucial importance in the isolation of unstable or highly sensitive compounds. In CPC (or centrifugal counter-current chromatography, as it is also known) it is possible at any stage of the run to reverse the flow direction and elute with stationary phase.

4.1. Instruments

4.1.1. Rotating coil instruments

A whole family of prototype instruments have been developed which describe planetary or non-planetary rotation about a central axis [3]. These include the horizontal flow-through coil-planet centrifuge, the toroidal coil-planet centrifuge, the counter-currrent extraction coil-planet centrifuge and the high-speed counter-current chromatograph (HSCCC). Only the last type has been commercialized to any large extent. A typical HSCCC instrument consists of a PTFE tube (1.6 or 2.6 mm I.D.) wrapped as a coil around a spool. When in motion, the coil describes a planetary motion about a central axis. A seal-free system has been developed for introduction of solvent into the rotating coil in order to avoid leakages. In one form, the multi-layer coil planet centrifugal counter-current chromatograph, also known as the "multi-layer coil separator–extractor", developed by Ito and marketed by P.C. (Potomac, MD, USA), consists of a single length of 2.6 mm I.D. PTFE tubing with a capacity of about 350 ml. Rotation speeds for separation are generally around 700–800 rpm

and the single coil is balanced by a counterweight.

Another commercially available rotating coil instrument is the multi-coil countercurrent chromatograph, introduced by Pharma-Tech Research (Baltimore, MD, USA). Multi-coil chromatographs consist of two [64] or three [65] identical multi-layer coils arranged symmetrically around the rotatory frame of the centrifuge, thus eliminating the need for balancing with a counterweight. Each coiled column undergoes synchronous planetary motion in such a way that it revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity. The columns are equipped with flow tubes arranged in such a fashion that they do not twist, allowing seal-free operation of the instrument. The three-coil instrument is available in several versions, including the CCC-1000, with a capacity of 800 ml, and the CCC-3000, with a capacity of 40 ml.

A recent addition, the Kromaton, manufactured by SEAB (Villejuif, France) consists of two coils with a total capacity of 1200 ml. Rotation speeds of up to 650 rpm are possible.

Rotating coil instruments require the application of a centrifugal force to retain the stationary phase in the body of the separation unit. This results in a very effective way of using the colum space, as under these conditions almost 100% of the efficient volume is used for the mixing of the two phases. Thus the interfacial area of the phases is maximized [4].

As there is no solid support, retention of injected samples depends uniquely on the respective partition coefficients of each constituent of the mixture between the immiscible phases of the solvent system. The number of theoretical plates of the column system can be easily modified by increasing the number (or length) of the coils, by reducing the inside diameter of the coiled column or by reducing the helical diameter. Increasing the number of coils simultaneously results in an increased column capacity.

A suitable procedure for loading rotating coil instruments [66] is as follows: stationary phase is first pumped into the coil (without rotation). After the coil has been filled, it is then rotated and mobile phase is introduced. Once the necessary stationary phase has been displaced and only mobile phase exits the coil, the sample (ideally in a mixture of the two phases) is introduced via a sample loop. This "dynamic" injection procedure allows a preliminary stabilization of the mobile phase flow and avoids the noisy baseline observed when the mobile phase front appears after "stationary" injection (sample loaded prior to pumping mobile phase, while column is still immobile) [2].

A recently introduced modification of the pumping procedure now allows variation of the phase ratios in the coil, gradient operation and easy reversal of mobile and stationary phases [66]. This is achieved by using separate pumps for the mobile and stationary phases and simultaneous introduction of the two phases into the apparatus (Fig. 1).

4.1.2. Cartridge instruments

Sanki Engineering (Kyoto, Japan) manufacture counter-current chromatographs that consist of a series of cartridges located around the circumference of a centrifuge rotor, with their longitudinal axes parallel to the direction of the centrifugal force [67]. With the Model LLN, each cartridge contains the equivalent of 400 separation channels and connections between cartridges are made by narrow-bore PTFE tubes. While the rotor is spinning, stationary phase is first pumped into the cartridges, followed by introduction of mobile phase at the rotation speed required for the separation. Rotating seals at the upper and lower axes of the centrifuge allow the passage of solvent into the apparatus under pressure. When a steady flow of mobile phase alone leaves the instrument, the sample is introduced and the separation is performed. The rotational speed of the instrument can be varied but, although higher speeds generally lead to better resolution, the pumping pressure is increased. Most separations are thus achieved at speeds of *ca.* 1000 rpm. Either the heavier phase or the lighter phase of a two-phase solvent system can be employed as the mobile phase, simply by switching the direction of flow through the instrument.

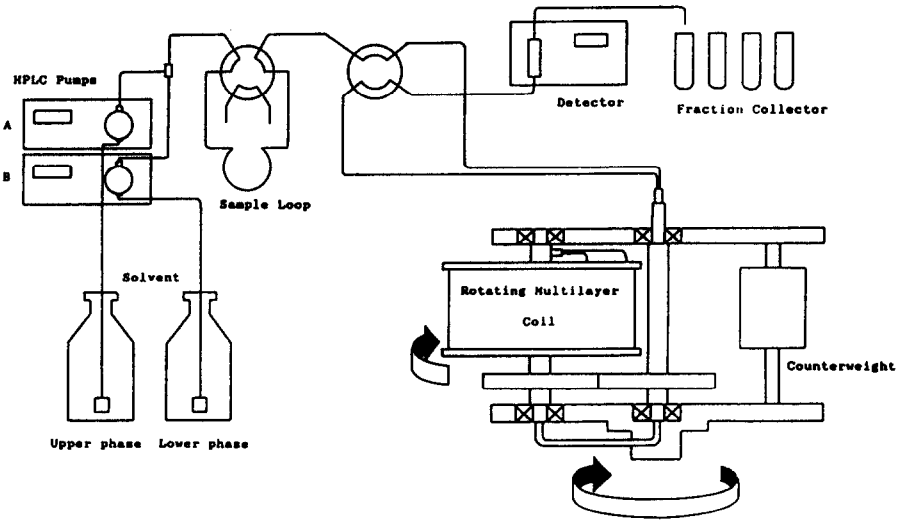


Fig. 1. Two-pump modification for the multi-layer coil separator–extractor.

4.2. Choice of solvent system

Successful counter-current chromatography depends on the correct choice of the solvent system. The most efficient solvent systems are those that yield partition coefficients of 0.2–5 [2]. Selection of the nature and composition of the solvents can be guided by several means, described below.

4.2.1. Knowledge of existing systems

A survey of the specialist literature reveals numerous examples of solvent systems used in different counter-current chromatographic separations [1–3]. Consultation of these and related references can give useful indications of systems for the separation in question. A solvent selection guide in which thirteen systems are classified by the polarity of their upper and lower phases has been described [68]. Chloroform–methanol–water or less polar *n*-hexane–ethyl acetate–methanol–water mixtures can be chosen as starting points and, by modifying the relative proportions of each individual solvent, it is possible finally to obtain the required distribution of sample between the two phases. Ternary diagrams for chloroform–methanol–water systems which, incidentally, allow the desired quantity of each phase to be prepared independently, have been published [2]. As chloroform–based systems provided large density differences and relatively high interfacial tensions between the two solvent phases, they are frequently employed in CCC. Further, their short settling times allow a reduction in the amount of displaced stationary phase and normally produce satisfactory phase retention. One drawback, however, is that chloroform–methanol–water systems can lead to overpressure problems with cartridge instruments. Another solvent combination, ethyl acetate–methanol–water, is suitable for cartridge operation but is not always compatible with the multi-layer coil planet centrifuge as the equilibrium state is difficult to reach and there is continual leakage of stationary phase.

4.2.2. Thin-layer chromatography

As in DCCC, the selection of appropriate biphasic solvent systems can be made by TLC. When using the organic layer of a two-phase aqueous system as TLC solvent, the R_F values should lie between 0.2 and 0.5. However, this method gives only an approximate indication of the utility of a particular solvent because TLC involves both partition and adsorption mechanisms, whereas counter-current chromatography is based on purely liquid–liquid partition phenomena.

For the separation of tannins, there have been reports of the use of cellulose TLC plates for the selection of solvent systems [69].

4.2.3. High-performance liquid chromatography

A knowledge of the partition coefficients of the solutes in question is of great benefit for the choice of suitable solvent systems. Measurement is possible by UV spectrophotometry, as used for the classification of certain solvent systems [70]. An analytical HPLC method has been described for the determination of partition coefficients of the components of a mixture [71]. The solutes are partitioned between two immiscible liquid phases and their respective concentrations are established by reversed-phase HPLC. The partition coefficient of each component is calculated from the detector response following injection of a solution of the compounds before and after extraction with an immiscible solvent. This procedure is of value for natural product mixtures, in which the identities of the individual components may not be known.

4.2.4. Partition ratio of biological activity

This method, limited to bioactive compounds, is based on the distribution of the biological activity of the mixture to be separated. The sample is first shaken with the two-phase solvent system under test and then the upper and lower phases are screened for the activity in question. A solvent system which gives a fairly good distribution of activity between the two phases can be considered a candidate for the separation. One disadvantage is the length of time required for collection of the results from the biological test. The method has found certain applications, mainly in the isolation of antibiotics [72].

4.2.5. Analytical counter-current chromatography

A valid method for the choice of solvent systems for preparative-scale separation is the use of analytical CCC. For example, rapid information on the suitability of a solvent system can be obtained on a Pharma-Tech Model CCC-3000 analytical instrument (coil capacity *ca.* 40 ml).

Care should be taken, however, as differences between the instruments (coil I.D., etc.) can lead to variations. Although carry-over of a viscous stationary phase may not affect the quality of a preparative separation, leakage of only a very minor amount of this phase in the small coil of an analytical apparatus results in a dramatic decrease in the retention potential of the stationary phase.

4.3. Preparative applications

CPC is now widely accepted as a routine preparative technique in both industrial and university laboratories. In the field of natural products, both crude extracts and semi-pure fractions can be successfully chromatographed. Aqueous and non-aqueous solvent systems are used and the separation of compounds with a wide range of polarity is possible. CPC is particularly useful, however, for polar substances. The resolution is not always very high but samples ranging in size from microgram to gram amounts can be separated on the range of instruments available. Although certain separations have been performed on prototypes or laboratory-made installations, most of the applications reported have used one of the following three systems: the multilayer coil separator–extractor (P.C.), the CCC-1000 (Pharma-Tech) or the Sanki cartridge instruments.

An extensive range of applications of the various CPC instruments is now available (see Tables 3–6) and several reviews containing a selection of these applications have appeared [1–3,73–75].

4.3.1. Flavonoids

Liquid–liquid chromatography is ideal for the separation of flavonoid aglycones and glycosides because problems of irreversible adsorption and tailing (found with the usual sorbents, such as silica gel and polyamide) are absent. Quantitative recovery of samples is possible.

The first step of the separation of flavonol glycosides from *Epilobium parviflorum* (Onagraceae) involved the introduction of a methanol extract (2 g) on to an Ito multilayer coil separator–extractor eluted with the lower phase of the solvent system chloroform–methanol–

TABLE 3

SEPARATIONS OF PLANT-DERIVED NATURAL PRODUCTS WITH ROTATING COIL INSTRUMENTS

Sample	Solvent system	Ref.
Flavonoids	$CHCl_3$–MeOH–H_2O (33:40:27)	66
	$CHCl_3$–MeOH–H_2O (4:3:2)	76, 77
Flavonoid glycosides	$CHCl_3$–MeOH–H_2O (7:13:8)	78
	EtOAc–H_2O → EtOAc–iso-BuOH–H_2O	79
Lignans	*n*-C_6H_{14}–CH_3CN–EtOAc–H_2O (8:7:5:1)	80
Lignan glycosides	$CHCl_3$–MeOH–H_2O (7:13:8)	81
	$CHCl_3$–MeOH–H_2O (5:5:3)	82
	n-C_6H_{14}–CH_2Cl_2–MeOH–H_2O (2:4:5:2)	82
Phenolic glycosides	C_6H_{12}–Me_2CO–EtOH–H_2O (7:6:1:3)	83
	$CHCl_3$–MeOH–H_2O (7:13:8)	81
	n-C_6H_{14}–EtOAc–MeOH–H_2O (3:7:5:5)	84
Tannins	*n*-BuOH–0.1 *M* NaCl (1:1)	85
Coumarins	*n*-C_6H_{14}–EtOAc–MeOH–H_2O (3:7:5:5)	84
Coumarin glycosides	$CHCl_3$–MeOH–H_2O (13:23:16)	86
Anthranoids	*n*-C_6H_{14}–CH_3CN–MeOH (8:5:2)	66
Iridoid glycosides	$CHCl_3$–MeOH–2-PrOH–H_2O (5:6:1:4)	87
	$CHCl_3$–MeOH–H_2O (9:12:8)	88
Sesquiterpenes	iso-C_8H_{18}–EtOAc–MeOH–H_2O (7:3:6:4)	89
Clerodane diterpenes	*n*-C_6H_{14}–EtOH–H_2O (2:1:1)	90
Taxane diterpenes	Light petroleum–EtOAc–MeOH–H_2O (50:70:80:65)	91
Quassinoids	$CHCl_3$–MeOH–H_2O (5:6:4)	83
Triterpenes	*n*-C_6H_{14}–EtOAc–MeOH–CN_3CN (5:5:4:2)	92
	n-C_6H_{14}–94% EtOH–H_2O (6:5:2)	77
Saponins	$CHCl_3$–MeOH–iso-BuOH–H_2O (7:6:3:4)	93
	$CHCl_3$–MeOH–H_2O (7:13:8)	94
	$CHCl_3$–MeOH–2-PrOH–H_2O (5:6:1:4)	95
Cardiac glycosides	$CHCl_3$–MeOH–HOAc–H_2O (5:3:1:3)	96
Alkaloids	*n*-C_6H_{14}–EtOH–H_2O (6:5:5)	97
	$CHCl_3$–0.07 *M* sodium phosphate (1:1)	98
	$CHCl_3$–MeOH–H_2O (10:10:1)	99
	$CHCl_3$–MeOH–H_2O (5:4:3)	100
	$CHCl_3$–MeOH–0.5% aq. HBr (5:5:3)	101
	$CHCl_3$–MeOH–0.5% aq. HCl (5:5:3)	102
	n-BuOH–Me_2CO–H_2O (8:1:10)	103
	n-BuOH–0.1 *M* NaCl (1:1)	104
Carotenoids	CCl_4–MeOH–H_2O (5:4:1)	105,106
Gingerols	*n*-C_6H_{14}–EtOAc–MeOH–H_2O (3:2:3:2)	107
Cyclohexadienone derivatives	*n*-C_6H_{14}–94% EtOH–EtOAc–H_2O (83:67:33:17)	108
Humulone derivatives	*n*-C_6H_{14}–CH_3CN–*tert.*-BuOMe (10:10:1)	109
Phthalides	*n*-C_6H_{14}–EtOAc–MeOH–H_2O (70:30:14:10)	110
	n-C_6H_{14}–CH_3CN–*tert.*-BuOMe (10:10:1)	110
Polyacetylene alcohols	*n*-C_6H_{14}–CH_3CN–*tert.*-BuOMe (10:10:1)	109,111
Glucose nitropropanoyl esters	C_6H_{12}–EtOAc–H_2O (3:3:4)	112
	C_6H_{12}–EtOAc–H_2O–CH_3CN (5:1:4:1)	112

TABLE 4

SEPARATIONS OF PLANT-DERIVED NATURAL PRODUCTS WITH CARTRIDGE INSTRUMENTS

Sample	Solvent system	Ref.
Flavonoids	$CHCl_3$–MeOH–H_2O (33:40:27)	113
Flavonoid glycosides	EtOAc–94% EtOH–H_2O (2:1:2)	113, 114
	EtOAc–*n*-BuOH–H_2O (2:1:2)	114
Xanthone glycosides	$CHCl_3$–MeOH–H_2O (4:4:3)	88
Phenolic glycosides	*n*-C_6H_{14}–EtOAc–MeOH–H_2O (3:7:5:5)	113
Polyphenols	C_6H_{12}–EtOAc–MeOH–H_2O (7:8:6:6)	115
Chalcones	$CHCl_3$–MeOH–H_2O (7:13:8)	116, 117
Tannins	*n*-BuOH–*n*-PrOH–H_2O (4:1:5)	116
	n-BuOH–*n*-PrOH–H_2O (2:1:3)	69, 118
	n-BuOH–HOAc–H_2O (4:1:5)	119
Coumarins	*n*-C_6H_{14}–EtOAc–MeOH–H_2O (3:7:5:5)	113
Anthranoids	*n*-C_6H_{14}–CH_3CN–MeOH (8:5:2)	113
Naphthoquinones	*n*-C_6H_{14}–CH_3CN–MeOH (8:5:2)	113
Iridoid glycosides	$CHCl_3$–MeOH–H_2O (9:12:8)	115
Norditerpenes	$CHCl_3$–MeOH–H_2O (5:6:4)	120
Saponins	$CHCl_3$–MeOH–H_2O (7:13:8)	113
Retinals	C_6H_6–*n*-C_5H_{12}–CH_3CN–MeOH (500:200:200:11)	121
Cyclohexadienone derivatives	*n*-C_6H_{14}–94% EtOH–EtOAc–H_2O (83:67:33:17)	108

water (7:13:8). Three major bands were obtained: one containing lipophilic compounds, one containing quercitrin and one containing myricitrin, isomyricitrin and gallic acid. After Sephadex LH-20 gel filtration, all four phenolics could be obtained in a pure state [78]. For CPC, the coil of the instrument was filled with 80% upper phase and 20% lower phase by the two-pump modification [66].

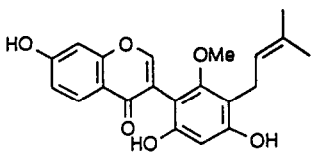
Extracts of *Gingko biloba* (Gingkoaceae) leaves have important therapeutic implications in certain geriatric problems, including treatment of circulatory disorders of the brain. Flavonoids from the leaves have been isolated by a combination of CPC and semi-preparative HPLC. The liquid–liquid step involved gradient elution, starting with water as stationary phase and eluting with ethyl acetate. Increasing amounts of isobutanol were then added to the ethyl acetate, reaching ethyl acetate–isobutanol (6:4) at the end of the elution. Seven flavonol glycosides were obtained from 500 mg of leaf extract [79].

Other applications of CPC (Table 4) include the isolation of flavonoid glycosides from *Tephrosia vogelii* (Leguminosae) [113] and from *Crossopteryx febrifuga* (Rubiaceae) [114].

Among several new phenolic compounds isolated from licorice originating from northwest China is an isoflavone, glicoricone (**12**), with a phloroglucinol-type substitution pattern in the B-ring. For its separation, 20 g of the ethyl acetate extract were chromatographed on a Sanki L-90 instrument equipped with twelve 1000-ml cartridges (capacity 850 ml), using the solvent system chloroform–methanol–water (7:13:8). Final purification was by chromatography on MCI gel CHP-20P and preparative TLC [155].

4.3.2. Xanthones

The xanthones are a class of polyphenolics with potential therapeutic use in the inhibition of monoaminooxidases. A three-step isolation procedure involving a cartridge CPC instrument gave three new xanthone glycosides (**13–15**) from *Halenia campanulata*, a South American species of the Gentianaceae. Initial gel filtration was followed by centrifugal partition chromatography with the solvent system chloroform–methanol–water (4:4:3), using the lower phase as mobile phase, at 3.2 ml/min and 300 rpm. As xanthone glycosides **14** and **15** eluted together, and **13** was still contaminated with a small



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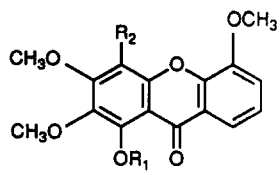


13 R_1 = Glc^6-Glc, R_2 = H
14 R_1 = Glc^6-Xyl, R_2 = H
15 R_1 = Glc^6-Xyl, R_2 = OCH_3

amount of impuritiy, final purification by semi-preparative HPLC was necessary [88].

4.3.3. Tannins

This is another substance class which poses considerable problems for separation by classical chromatography on solid supports [69,116]. Preparative HPLC is also accompanied by considerable sample loss and deterioration or contamination of the column [117]. Centrifugal partition chromatography, on the other hand, has proved to be an ideal technique for the resoltuion of these particular difficulties.

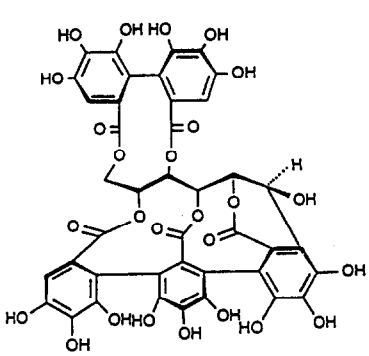
Among the examples of successful separations performed on a Sanki cartridge instrument are the isolation of water-soluble tannin geraniinic acid A from *Geranium thunbergii* (Geraniaceae) [119] and the isolation of the ellagitannin liquidambin from *Liquidambar formosana* (Hamamelidaceae) [156]. In the latter instance, up to 3 g of sample were loaded on to the chromatograph and eluted with *n*-butanol–*n*-propanol–water (4:1:5).

A remarkable separation is that of castalgin (**16**) from vescalagin (**17**), as they are diastereoisomers differing only in the configuration of the hydroxyl group of the central carbohydrate moiety. They were extracted from *Lythrum anceps* (Lythraceae) leaves and chromatographed on a Sanki cartridge instrument with the solvent system *n*-butanol–*n*-propanol–water (4:1:5), using the upper phase as the mobile phase [69].

A readily hydrolysable trimeric tannin, nobotanin J, and a tetrameric tannin, nobotanin K, from *Heterocentron roseum* (Melastomataceae) were obtained in the pure state by chromatography with the solvent system *n*-butanol–*n*-propanol–water (4:1:5). When purification of nobotanin J was attempted by gel filtration, extensive hydrolysis occurred [16,17].

4.3.4. Lignans

An immunomodulatory lignan, eleutheroside E (**18**), has been isolated from the roots of Siberian ginseng (*Eleutherococcus senticosus*, Araliaceae) by a strategy involving CPC. An initial open-column chromatographic step on silica gel of the methanol extract gave 1.3 g of a fraction containing the desired lignan glycoside.



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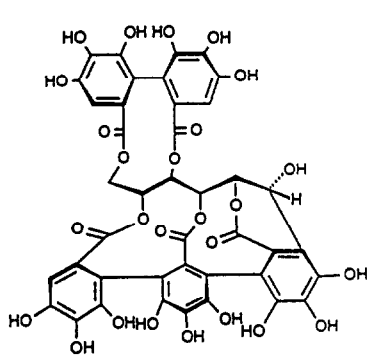

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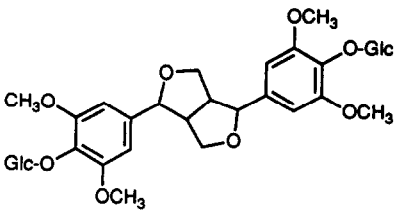

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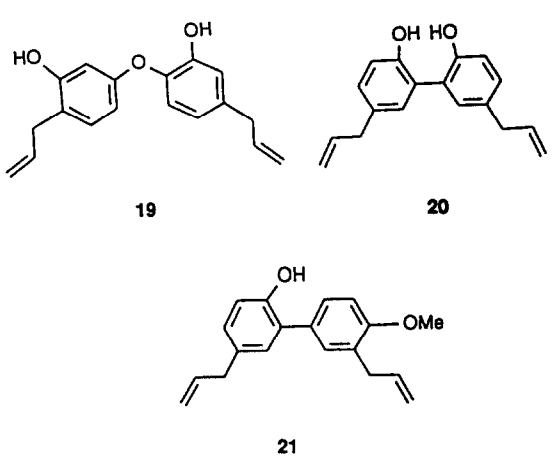
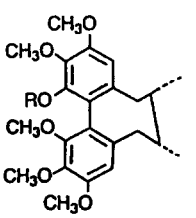
A portion (730 mg) of this fraction was chromatographed on a multi-layer coil separator–extractor filled with 50% of each phase of a chloroform–methanol–water solvent system to give 35 mg of pure eleutheroside E (**18**) [115].

Initial purification of insecticidal neolignans from *Magnolia virginiana* (Magnoliaceae) by centrifugal chromatography was claimed to be better, less expensive and more efficient than traditional open-column or more recent flash chromatographic methods [80]. A hexane extract of the leaves was chromatographed using the lower layer of the solvent system hexane–acetonitrile–ethyl acetate–water (8:7:5:1) as mobile phase. This very interesting solvent system contains only a small proportion of water, probably to provide compatibility with the very lipophilic extract. Subsequent purification of the fractions provided a biphenyl ether (**19**) and two biphenyls (**20** and **21**), which were not only insecticidal to *Aedes aegypti*, the vector of yellow fever, but also fungicidal, bactericidal and toxic to brine shrimp [80].

Dual counter-current chromatography is very useful for the separation of crude extracts and fractions which are composed of many constituents with a wide range of polarities. Sample is fed into the middle portion of a multi-layer coil and the polar and non-polar components are eluted from appropriate ends of the column, *i.e.*, this is true counter-current chromatography. By this means, normal- and reversed-phase elutions can be performed simultaneously. Separation of schisanhenol (**22**) (32 mg) and schisanhenol acetate (**23**) (4 mg) from a crude ethanol extract (125 mg) of *Schisandra rubriflora* (Schisandraceae) kernels was possible by dual CCC on a 400-ml, 2.6 mm I.D. PTFE coil with the solvent system hexane–ethyl acetate–methanol–water (10:5:5:1) at a flow-rate of 2 ml/min [157].



19 20 21



22 R = H
23 R = Ac

4.3.5. Phenolic acids

The small-scale separation of cinnamic (**24**), ferulic (**25**) and caffeic acid (**26**) was possible with a Sanki LLN cartridge instrument (six catridges, rotational speed 1000 rpm). The solvent system *n*-hexane–ethyl acetate–methanol–water (3:7:5:5; mobile phase = upper phase) was used at a flow-rate of 3.2 ml/min. Cinnamic acid ($N = 300$) eluted first and the separation, in order of increasing polarity, was complete within 2.5 h [113]. The same solvent system on a multi-layer coil separator–extractor (700 rpm, 3 ml/min) gave a much slower separation but with improved peak resolution ($N = 700$ for cinnamic acid). In view of the excellent peak resolution, scale-up was attempted and a sample size of 1 g could be injected, still with baseline separation of the three components (Fig. 2).

4.3.6. Anthracene derivatives

Lipophilic extracts of the African medicinal plant *Psorospermum febrifugum* (Guttiferae) ex-

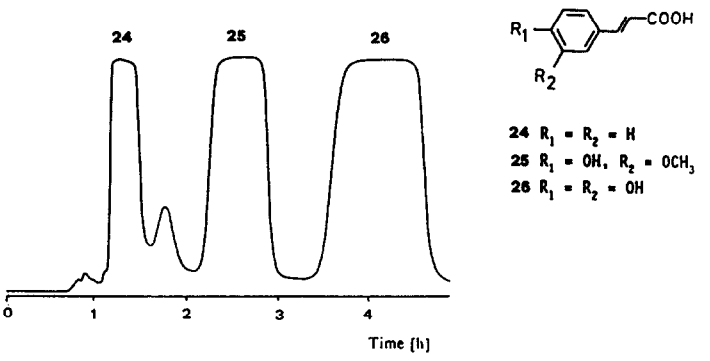


Fig. 2. Large-scale separation of cinnamic (**24**), ferulic (**25**) and caffeic acid (**26**) on the multi-layer coil separator–extractor. Sample, 1 g; solvent system, *n*-hexane–ethyl acetate–methanol–water (3:7:5:5); mobile phase, upper phase; flow-rate, 3 ml/min; rotational speed, 700 rpm, detection, 254 nm.

hibit strong growth inhibition of cancer cells and have antimalarial activity. Separation of the active anthranoid pigments by flash chromatography and low-pressure liquid chromatography resulted in considerable material losses, owing to irreversible adsorption on the sorbents. However, in a single CPC step (Sanki cartridge system), three pure compounds (**27–29**) and a mixture of a fourth anthranoid pigment (**30**) with an unidentified constituent were obtained without loss of product (Fig. 3). A non-aqueous solvent system was employed for the separation [113]. Increasing the number of cartridges permitted a better resolution of the peaks but required a longer separation time. With twelve cartridges, the separation could be scaled up to a 500-mg sample size [158].

4.3.7. Monoterpene glycosides

A one-step CPC procedure has also been employed for the isolation of the secoiridoid glycoside gentiopicrin (**31**) from the roots of gentian (*Gentiana lutea*, Gentianaceae). The roots were extracted with methanol and, after evaporation of solvent, the residue was taken up in water and partitioned with light petroleum, ethyl acetate and *n*-butanol. HSCCC of the butanol extract (500 mg) gave the pure bitter

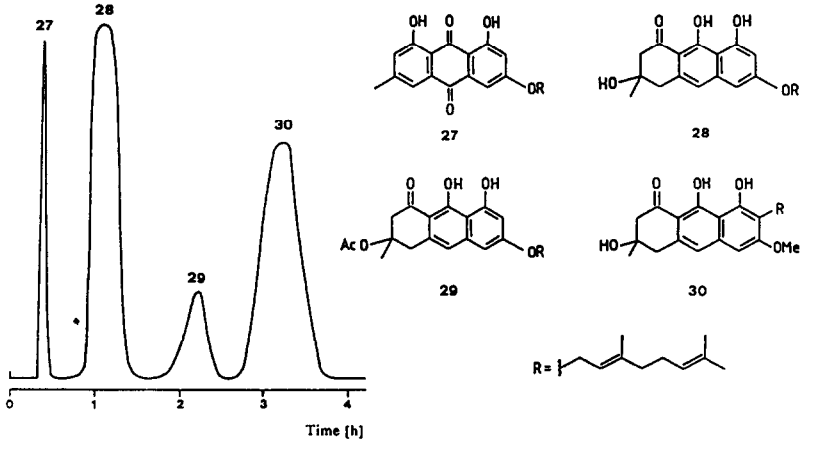
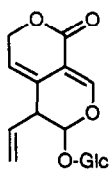


Fig. 3. CPC separation of a light petroleum extract of *Psorospermum febrifugum* (Guttiferae) root bark with a Sanki LLN cartridge instrument. Solvent system, *n*-hexane–acetonitrile–methanol (40:25:10); mobile phase, upper phase; flow-rate, 5.5 ml/min; rotational speed, 1500 rpm; detection, 254 nm.



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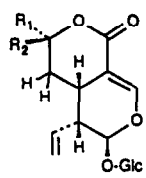


32 $R_1 = OCH_3$, $R_2 = H$
33 $R_1 = H$ $R_2 = OCH_3$

principle gentiopicrin (113 mg) after an elution time of *ca.* 6 h, using 20% of lower phase and 80% of the upper phase of the solvent system chloroform–methanol–water (9:12:8) as the composition of the coil [115].

Two secoiridoid glycosides (**32** and **33**) were obtained form *Halenia campanulata* (Gentianaceae) after Sephadex LH-20 gel filtration and CPC on a Pharma-Tech CCC-1000 instrument (700 rpm, 3 ml/min) with the solvent system chloroform–methanol–water (9:12:8; lower phase as mobile phase). The two isomers differ only in the configuration at C-7 but were well separated by liquid–liquid chromatography [88].

4.3.8. Diterpenes

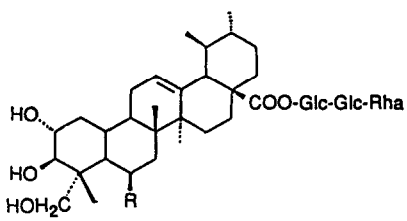
The diterpene taxol exhibits high activity against ovarian cancer in phase II clinical trials but the isolation and purification of the drug is complex. For this reason, attempts have been made to find a rapid isolation procedure using CPC, with the aim of scaling up the extraction of taxol from plant material. First experiments involved a preliminary partition and column chromatographic step, to give an enriched taxane fraction. A portion of this (300 mg) was chromatographed on a multi-layer coil separator–extractor with the solvent system light petroleum (b.p. 40–65°C)–ethyl acetate–methanol–water (50:70:80:65; upper phase as mobile phase). One fraction contained pure taxol and another fraction contained a mixture of taxol and cephalomannine, which could be separated by HPLC. At the end of the process, 43 mg of taxol were obtained [91].

4.3.9. Saponins

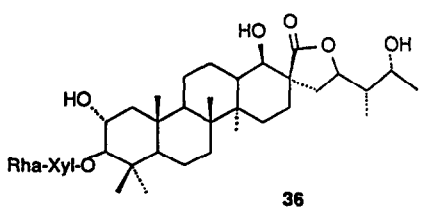
As with DCCC, centrifugal partition chromatography is ideal for the separation of saponins. In one example, 2 g (4 × 0.5 g) of an 80% methanol extract of leaves of *Abrus fruticulosus* (Leguminosae) were chromatographed on a P.C. instrument using chloroform–methanol–water (7:13:8; lower phase as mobile phase) at a flow-rate of 1.5 ml/min. Final purification by flash chromatography or over-pressured liquid chromatography gave four sweet-tasting triterpene glycosides [94].

Two saponins, asiaticoside (**34**) and madecassoside (**35**), which differ only in the presence of one hydroxyl group, were separated from an extract of the medicinal plant *Centella asiatica* (Umbelliferae) by HSCCC. The two-phase solvent system used was chloroform–methanol–isobutanol–water (7:6:3:4), with the lower phase as mobile phase, at a flow-rate of 4 ml/min. A 400-mg sample of the evaporated mother liquors of *C. asiatica* was injected. Detection of the non-UV-active saponins was achieved by direct coupling to TLC [93].

CPC was employed as a separation step in the isolation of saponins from the African plant *Sesamum alatum* (Pedaliaceae). After chromatography of a methanol extract of the aerial parts on a silica gel column, a fraction (1.25 g) was injected on to a multi-layer coil separator–extractor [chloroform–methanol–2-propanol–water (5:6:1:4), with the organic phase as the mobile phase] before final purification by low-pressure LC on an RP-8 column. A novel 18,19-secoursane disaccharide (**36**) was obtained [95].



34 R = H (Asiaticoside)
35 R = OH (Madecassoside)



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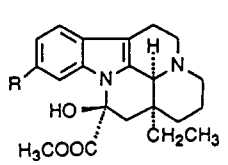
4.3.10. Alkaloids

Certain alkaloids (*e.g.*, the quaternary indole alkaloids) are difficult to purify because of their polarity and their interaction with solid chromatographic supports. However, CPC provides an alternative method for their purification, as has been shown by work on the isolation of curare alkaloids [103].

An anti-HIV and cytotoxic alkaloid has been isolated from the leaves of *Buchenavia capitata* (Combretaceae). The final separation step involved CPC on a Sanki NMF cartridge instrument (twelve cartridges, 300-ml capacity, 400 rpm) with the solvent system chloroform–methanol–0.5% HCl (5:5:3) [102].

Sleepygrass (*Stipa robusta*, Graminae) infected with an *Acremonium* endophyte produces ergot alkaloids. An intermediate step in their isolation and characterisation was liquid–liquid chromatography on a Pharma-Tech CCC-2000 triple-coil instrument (column capacity 200 ml). The solvent system chloroform–methanol–water (5:4:3) was used for the separation of alkaloid mixtures [100].

Repetitive sample injections are possible for the separation of close-running compounds on rotating coil instruments. This has been shown by the separation of vincamine (**37**) and vincine (**38**), alkaloids of *Vinca minor* (Apocynaceae). After twenty successive injections (at 42-min intervals), each of 1.7 mg of sample mixture, 16.5 mg of **37** and 14 mg of **38** were obtained on a 230-ml instrument. The solvent system used was *n*-hexane–ethanol–water (6:5:5; lower phase as mobile phase). Interestingly, the resolution of the HSCCC system was not changed when it was shut down overnight and re-started the next day with the same stationary phase in the column [97].



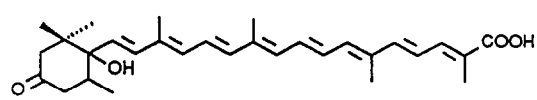
37 R = H (Vincamine)
38 R = OCH_3 (Vincine)

4.3.11. Carotenoids

The pigments cochloxanthin (**39**) and dihydrocochloxanthin (**40**) from *Cochlospermum tinctorium* (Cochlospermaceae) could not be isolated by either HPLC or preparative TLC. However, with a multi-layer coil separator–extractor they were obtained in one step from a methanol extract of the roots. The solvent system was carbon tetrachloride–methanol–water (5:4:1; upper phase as mobile phase), the flow-rate 4 ml/min and the rotational speed 800 rpm. The extract (800 mg) was first dissolved in a 1:1 mixture (10 ml) of the two solvent phases before injection. Separation was achieved within 2 h [106].

4.3.12. Polyacetylenic alcohols

For the separation of apolar compounds, as has been reported for anthranoids and naphthoquinones [158], it is sometimes advantageous to use non-aqueous solvent systems, particularly when solubility is a problem. This approach has also been adopted for the isolation of polyacetylenic alcohols from parsley root (*Petroselinum crispum*, Umbelliferae). When 350 mg of diethyl ether extract were subjected to HSCCC, the antifungal compounds falcarinol (**41**, 17 mg) and falcarindiol (**42**, 4 mg) were



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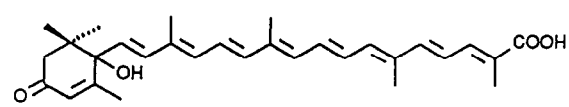

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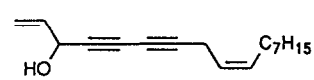

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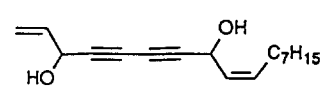

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obtained. As mobile phase, the acetonitrile phase of hexane–acetonitrile–*tert.*-butyl methyl ether (10:10:1) was employed. This one-step procedure minimized the problems that occur during the usual multi-step separation of these oxygen-, light- and heat-sensitive compounds [111]. Two C_{20} compounds, crispane and crispanone, were isolated directly from a diethyl ether extract of parsley seeds by HSCCC with the same solvent system [159].

4.3.13. Antibiotics

CPC has been associated with the field of antibiotics since its inception. Liquid–liquid partition techniques are particularly suitable for the separation of antibiotics (Table 5) because

TABLE 5

SEPARATIONS OF ANTIBIOTICS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

Substances separated	Instrument	Solvent system	Ref.
Polyene antibiotics	Synchronous coil planet centrifuge	$CHCl_3$–MeOH–H_2O (4:4:3)	122
Glycoside antibiotics	Shimadzu CCC	*n*-BuOH–Et_2O–H_2O (10:4:2)	123
Trichoverrins	CCC-1000	*n*-C_6H_{14}–$CHCl_3$–MeOH–H_2O (1:1:1:1)	124
	HSCCC	*n*-C_6H_{14}–$CHCl_3$–MeOH–H_2O (1:1:1:1)	124
Benarthin	Sanki NMF	*n*-BuOH–HOAc–H_2O (15:1:15)	125
Pristinamycins	SFCC CPHV 2000	$CHCl_3$–EtOAc–MeOH–H_2O–HCOOH (12:8:15:10:2)	126
Siderochelin A	HSCCC	$CHCl_3$–MeOH–H_2O (7:13:8)	127
Efrotomycin	HSCCC	CCl_4–$CHCl_3$–MeOH–H_2O (5:5:6:4)	127
Pentalenolactone	HSCCC	$CHCl_3$–MeOH–H_2O (1:1:1)	127
BU 2313B	HSCCC	*n*-C_6H_{14}–CH_2Cl_2–MeOH–H_2O (5:1:1:1)	127
Tirandamycin	HSCCC	*n*-C_6H_{14}–EtOAc–MeOH–H_2O (70:30:15:6)	127
Dunaimycins (Macrolide antibiotics)	HSCCC	*n*-C_6H_{14}–EtOAc–MeOH–H_2O (70:30:15:6)	128
		n-C_6H_{14}–EtOAc–MeOH–H_2O (8:2:10:5)	128
		n-C_6H_{14}–EtOAc–MeOH–H_2O (8:2:5:5)	128
Actinomycins	HSCCC	*n*-C_6H_{14}–Et_2O–MeOH–H_2O (1:5:4:5)	129
Valinomycin	HSCCC	*n*-C_6H_{14}–MeOH–H_2O (10:9:1)	130
Pipericidin A1	HSCCC	*n*-C_6H_{14}–Et_2O–MeOH–H_2O (4:1:4:1)	130
Concanamycin	HSCCC	*n*-C_6H_{14}–EtOAc–MeOH–H_2O (1:1:1:1)	130
Tomaymycin	HSCCC	*n*-C_6H_{14}–EtOAc–MeOH–H_2O (1:3:1:3)	130
Acetoxycycloheximide E73	HSCCC	$CHCl_3$–C_6H_5Me–MeOH–H_2O (5:4:5:4)	130
Tiacumicin	HSCCC	CCl_4–$CHCl_3$–MeOH–H_2O (7:3:7:3)	131
Benzanthrins	HSCCC	CCl_4–$CHCl_3$–MeOH–H_2O (4:1:4:1)	132
Coloradocin	HSCCC	$CHCl_3$–MeOH–H_2O (1:1:1)	133

these bioactive metabolites are often produced in very small amounts and have to be removed from other secondary metabolites and unmetabolized media ingredients. Antibiotics are normally biosynthesized as mixtures of closely related congeners and many are labile molecules, thus requiring mild separation techniques with a high resolution capacity.

Siderochelin A, efrotomycin, pentalenolactone, Bu2313B and tirandamycin A and B have been isolated from crude fermentation extracts of *Bacteroides fragilis* and *Staphylococcus aureus* with the aid of a 325-ml multi-layer coil. Sample loads of up to 670 mg were injected [127].

Recently, dunaimycins, spiroketal 24-membered macrolides, were isolated from *Streptomyces diastatochromogenes* by a strategy involving HSCCC with different compositions of the solvent system *n*-hexane–ethyl acetate–methanol–water [128].

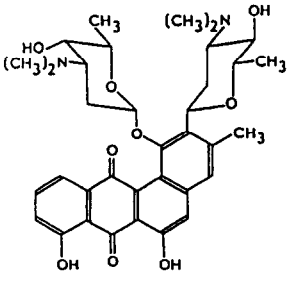
Actinomycins have been separated on multi-layer coil instruments. Pure actinomycins C_1 (2.3 mg), C_2 (18.9 mg) and C_3 (22.3 mg) were obtained from an actinomycin C complex. CCC is thus capable of separating very closely related products: an ethyl group in C_2 replaces a methyl substituent in C_1, while a second ethyl group in C_3 replaces a methyl group in C_2 [129].

Two isomeric benz[*a*]anthraquinones, benzanthrin A (**43**) and B (**44**), have been isolated from *Nocardia lurida* by procedure which involved a final HSCCC step, using the solvent system carbon tetrachloride–chloroform–methanol–water (4:1:4:1) [132].

The sporaviridins, water-soluble basic glycoside antibiotics, have complex structures, are unstable under basic conditions and exist as mixtures of closely related compounds. A sample of six sporaviridins was resolved on a Shimadzu prototype counter-current chromatographic instrument (total capacity 325 ml, 800 rpm). Selection of the solvent system was based on partition coefficient data from chloroform–methanol–water, chloroform–ethanol–methanol–water and *n*-butanol–diethyl ether–water mixtures. After HPLC analysis, the final system adopted was *n*-butanol–diethyl ether–water (10:4:12). Sample was introduced by so-called "sandwich" injection (sample was injected after filling with stationary phase and before mobile phase elution was begun). The six components were separated within 3.5 h, employing a total elution volume of 500 ml [123].

4.3.14. *Trichothecene mycotoxins*

Two species of the genus *Baccharis* (Asteraceae) contain highly cytotoxic trichothecenes similar to those found in the common soil fungi *Myrothecium* [124]. They are only present in small amounts and their isolation presents a number of problems, which are often not resolved by TLC and HPLC [160]. The application of CPC has provided the possibility of overcoming some of these difficulties. Using a Pharma-Tech CCC-1000 instrument with interchangeable columns, it was possible first to determine the separation conditions on 55-ml analytical coils. By changing to 350-ml semi-preparative coils, the transposition of the conditions enabled difficult separations to be performed. Thus, trichoverrins of the series A, B and C, very closely-related 2′,3′-*trans*-trichoverrins and isomeric 2′,3′-*cis*-trichoverrins could all be separated from one another. Aliquots (400 mg) of crude tri-



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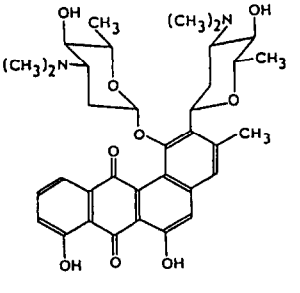

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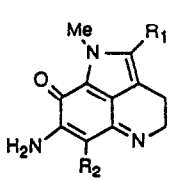
choverrin mixture were injected into the CCC-1000 instrument and chromatographed with the solvent system hexane–chloroform–methanol–water (1:3:3:2; lower phase as mobile phase). A second separation using gradient elution [dichloromethane–carbon tetrachloride–methanol–water (2:3:3:2→5:2:3:2; lower phase as mobile phase)] completed the procedure [160]. Jarvis stated [160] that the relative retention times in CPC were not easy to predict by TLC or HPLC. On silica gel, the relative order of retention times was roridin A > roridin D > roridin E > verrucarin A. On reversed-phase column the order was roridin E > roridin D > roridin A > verrucarin A. In contrast, the order for CPC with the solvent system carbon tetrachloride–methanol–water (5:3:2) was roridin A > roridin D > verrucarin A > roridin E. On the one hand, this shows that analytical CPC is the best method of selecting a suitable solvent system, and on the other, this is a good example of obtaining a different selectivity for a separation which does not necessarily work by established liquid–solid chromatography.

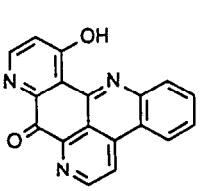
4.3.15. Marine natural products

The mild and rapid conditions achieved with CPC are ideal for separations of delicate marine natural products (Table 6). Attempts at purification of antitumour ecteinascidins from the tunicate *Ecteinascidia turbinata*, for example, by normal- or reversed-phase chromatography usually led to extensive loss of activity. CPC, however, proved to be a very effective means of separating these light- and acid-sensitive alkaloids [161].

A series of pyrroloquinoline alkaloids isobatzelline A (**45**), B (**46**) and C (**47**), have been isolated from a *Batzella* sponge. They exhibited



45 R_1 = SCH_3, R_2 = Cl
46 R_1 = SCH_3, R_2 = H
47 R_1 = H, R_2 = Cl



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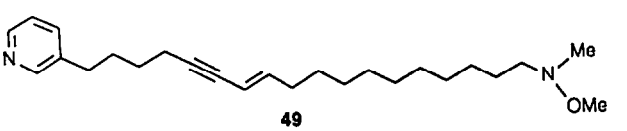
in vitro cytotoxicity against the P-388 leukaemia cell line and antifungal activity against *Candida albicans*. Centrifugal countercurrent chromatography was used in the purification, after extraction and solvent partitioning. The HSCCC involved elution with the upper phase of the solvent system heptane–chloroform–methanol–water (2:7:6:3) [139].

A polycyclic aromatic alkaloid, meridine (**48**), has been isolated from the ascidian *Amphicarpa meridiana*, collected off the coast of South Australia. The alkaloid was obtained directly from a methanol–chloroform extract after chromatography on a multi-layer coil separator–extractor with chloroform–methanol–5% HCl (5:5:3; lower phase as mobile phase) [138].

The non-aqueous solvent system heptane (or hexane)–dichloromethane–acetonitrile (10:3:7) has been used for a variety of marine natural products (Table 6), including a cytotoxic sesquiterpene–methylene quinone from a deep-water sponge [162]. A long-chain methoxylamine pyridine, xestamine A (**49**), has been isolated from the sponge *Xestospongia wiedenmayeri* via CPC with a heptane-containing solvent mixture [137].



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4.3.16. Other applications

Counter-current chromatographic techniques have been employed to solve a variety of separation problems in the areas of dyes, long-chain fatty acids, inorganics, drug metabolites, cell components, etc. [3].

Separations of amino acids, peptides and proteins have been well documented [2]. Synthetic peptides can be purified by CPC, often using *n*-butanol–acetic acid–water systems [163]. CPC is also useful for the purification of organic chemicals after synthesis.

4.4. Comparison of techniques

A comparative separation of the flavanone hesperetin (**50**) and the flavonols kaempferol

TABLE 6

SEPARATIONS OF MARINE NATURAL PRODUCTS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

Organism	Class of compound	Solvent system	Ref.
Sponge *Tedania ignis*	Indoles, carbazole, β-carboline, phenolics	$CHCl_3$–MeOH–H_2O (25:34:20)	134
Sponge *Tedania ignis*	Diketopiperazine	$CHCl_3$–MeOH–H_2O (25:34:20)	135
Sponge *Calyx podatypa*	*N*-Methylpyridinium salts	*n*-C_7H_{16}–CH_3CN–CH_2Cl_2 (10:7:3)	136
Sponge *Xestospongia wiedenmayeri*	Methoxylaminopyridines	*n*-C_7H_{16}–CH_3CN–CH_2Cl_2 (50:30:15)	137
Ascidian *Amphicarpa meridiana*	Alkaloids	$CHCl_3$–MeOH–5% aq. NH_3 (5:5:3)	138
Sponge *Balzella* sp.	Pyrroloquinoline alkaloids	*n*-C_7H_{16}–$CHCl_3$–MeOH–H_2O (2:7:6:3)	139, 140
		n-C_7H_{16}–EtOAc–MeOH–H_2O (4:7:4:3)	139, 140
		$CHCl_3$–iso-Pr_2NH–MeOH–H_2O (7:1:6:4)	140
Sponges *Axinella* sp. and *Hymeniacidon* sp.	Pyrrololactams	*n*-BuOH–(0.01 *M* K_3PO_4–0.01 *M* K_2HPO_4) (1:1)	141
Bryozoan *Bugula neritina*	Bryostatin (macrocyclic lactone)	*n*-C_6H_{14}–EtOAc–MeOH–H_2O (14:6:10:7)	142
Sponges *Dercitus* sp. and *Stellatta* sp.	Acridine alkaloids	CH_2Cl_2–MeOH–H_2O (5:5:3)	143
Sponge *Spongosorites ruetzleri*	Imidazolediylbisindoles	*n*-C_7H_{16}–EtOAc–MeOH–H_2O (4:7:4:3)	144
		n-C_7H_{16}–EtOAc–MeOH–H_2O (5:7:4:3)	144
Sponge *Discodermia polydiscus*	(Aminoimidazolinyl)indole	$CHCl_3$–MeOH–H_2O (5:10:6)	145
Sponge *Theonella* sp.	Cyclic peptide	*n*-C_6H_{14}–EtOAc–MeOH–H_2O (3:7:5:5)	146
Sponge *Theonella* sp.	Arginine derivative	$ClCH_2CH_2Cl$–$CHCl_3$–MeOH–H_2O (2:3:10:6)	147
Sponge *Plakortis lita*	Cyclic peroxides	*n*-C_7H_{16}–CH_2Cl_2–CH_3CN (5:1:4)	148
Sponge *Dercitus* sp.	Indolinium chloride	$CHCl_3$–MeOH–H_2O (5:10:6)	149
Sponge of Spongiidae family	Sesquiterpene	*n*-C_7H_{16}–CH_3CN–CH_2Cl_2 (10:7:3)	150
Tunicate *Ascidia nigra*	Tunichromes	iso-AmOH–*n*-BuOH–*n*-PrOH–H_2O–HCOOH–*tert.*-butyl sulphide (32:48:40:120:1:4)	151
Tunicate *Clavelina picta*	Quinolizidines	*n*-C_6H_{14}–CH_3CN–CH_2Cl_2 (10:7:3)	152
Tunicate *Clavelina picta*	Indolizidines	*n*-C_6H_{14}–CH_3CN–CH_2Cl_2 (10:7:3)	153
Marine bacterium *Alteromonas*	Pseudomonic acid	$CHCl_3$–MeOH–H_2O (25:34:20)	154

(**51**) and quercetin (**52**) by DCCC, RLCC and cartridge and multi-layer coil CPC methods is shown in Fig. 4 [66,113]. Elution was in order of increasing polarity when the lower phase of the solvent system was used as the mobile phase. Whereas DCCC and RLCC required more than 30 h for complete separation, the two centrifugal methods took only *ca.* 3 h. Solvent consumption for RLCC was *ca.* 1500 ml and for DCCC and the CPC separations *ca.* 550 ml. With the six-cartridge system baseline separation was not achieved, but with twelve cartridges the resolution of the mixture improved considerably but the separation time was 3.5 h. Scaling up (as long as there are no solubility problems) would be possible for DCCC, RLCC and for the multi-

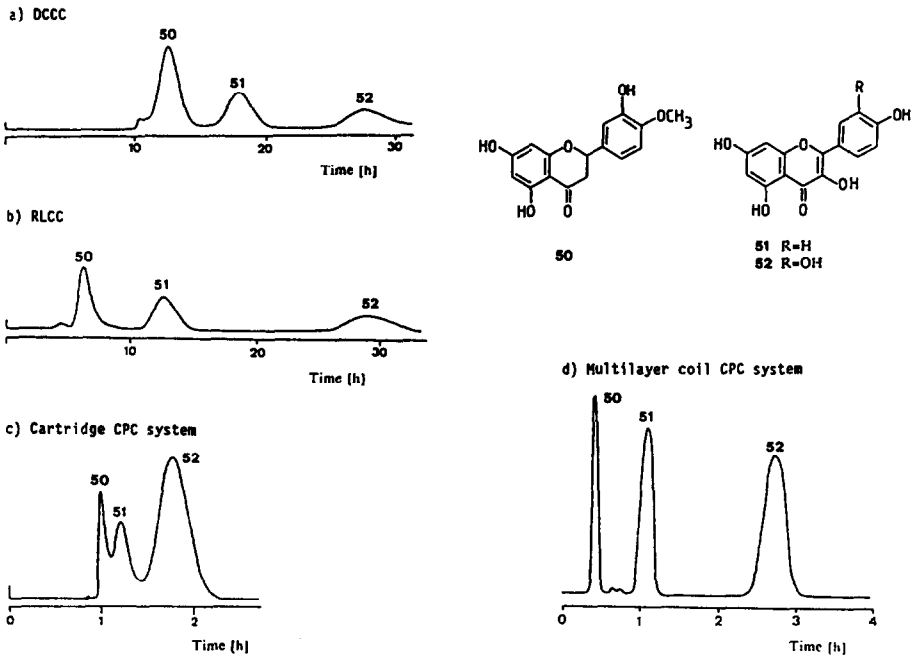

Fig. 4. Separation of hesperetin (**50**), kaempferol (**51**) and quercetin (**52**) by different counter-current chromatographic methods. Solvent system, chloroform–methanol–water (33:40:27); mobile phase, lower phase; detection, 254 nm. Flow-rate: (a) 48 ml/h; (b) 18 ml/h; (c) 2 ml/min; (d) 3 ml/min. Rotational speed: (c) 600 rpm, six cartridges; (d) 700 rpm.

layer coil separator–extractor. In the case of phenolic acids (see Fig. 2), scale-up was achieved for the rotating coil method because of the improved analytical separation when compared with the cartridge system.

Another difficulty with cartridge instruments is the rotating seal, a weak point in the design of the apparatus, as leakages are not an infrequent phenomenon. On the contrary, the rotating coil instruments have, in general, been designed with seal-free connections to the coil assembly.

The back-pressures in the multi-layer coil instruments are generally considerably lower than in the cartridge machines. Consequently, difficulties of overpressuring can be encountered when using viscous solvent mixtures (butanol, etc.) in the cartridge set-up. On the other hand, there are certain combinations of solvents that are compatible with cartridge instruments and not with coil instruments (*e.g.*, some ethyl acetate–methanol–water mixtures).

A comparison of fundamental chromatographic parameters for cartridge and multi-layer coil instruments has been reported [164].

5. PERSPECTIVES

Counter-current chromatography is now a well established preparative separation technique, as is evident from the numerous applications surveyed here. There is still a place for DCCC and no doubt some further examples of RLCC will appear, but the real future belongs to the new generation of centrifugal instruments. Not only are they considerably faster than DCCC or RLCC, but also the choice of suitable solvent systems is larger.

In some instances (*e.g.*, the separation of anthranoids from *Psorospermum febrifugum*, Guttiferae [113], and the isolation of clerodane diterpenes from *Portulaca pilosa*, Portulacaceae [22]), it is possible to purify compounds in one step from a crude extract. Although it is claimed that CPC has a high partition efficiency, the resolution is not always high and there is certainly no comparison with HPLC techniques from this viewpoint. However, the method possesses considerable advantages over HPLC in that the instruments have a high loading capacity without

the need for sample clean-up procedures. Crude extracts and semi-pure mixtures can equally well be chromatographed, without any material loss arising from irreversible adsorption on solid matrices. Samples with a wide range of polarities can be injected, including both highly polar and apolar compounds. A high degree of versatility is also possible with the solvents used; aqueous and non-aqueous systems are equally applicable. Non-aqueous solvent systems are of particular interest for mixtures of apolar substances.

The excellent reproducibility of CPC is well illustrated by the isolation of alkaloids from *Vinca minor* (Apocynaceae) [97].

At present the number of manufacturers of centrifugal countercurrent chromatographs is very limited (P.C. and Pharma-Tech in the USA, SEAB in France and Sanki Engineering in Japan). Further, the design and construction of certain instruments leave much to be desired and leakages and breakdowns are frequent. If widespread acceptance and use of the technique are to come about, modifications and improvements of existing models need to be undertaken. One disadvantage is the need to operate existing CPC instruments manually. There is little process monitoring and automatic control, which is an unattractive aspect for many potential users [165]. That being said, CPC is still in its infancy and, based on the practical experience already available, there is enormous potential for the future of this type of chromatography. While great efforts are being put into the development and applications of analytical-scale CPC instruments, there is a need for chromatographs that can accommodate higher loads (on the 100 g to the kg scale). Some advances have been made in this direction: samples of 1–5 g can be loaded on to Pharma-Tech instruments [158] without any particular problems and 20 g of an ethyl acetate extract of licorice has been introduced into a Sanki L-90 instrument (equipped with twelve 1000E cartridges) in order to obtain biologically active phenolic compounds [155]. However, the reports on this type of application are limited and more work needs to be done in order to develop true preparative instruments. This will require a good deal of thought and a better understanding of the mechanisms of separation that actually occur in a separation coil or cartridge.

6. ACKNOWLEDGEMENT

Thanks are due to the Swiss National Science Foundation for financial support.

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