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Review

Applications of analytical high-speed counter-current chromatography in natural products chemistry

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ABSTRACT

Applications of analytical high-speed counter-current chromatography in natural products chemistry are reviewed and the potential of the method is discussed.

CONTENTS

1. INTRODUCTION

Counter-current chromatography (CCC) is widely used in the isolation and purification of natural products. Separations are based on liquid–liquid partitioning

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and are carried out without solid stationary phase material [1]. CCC methods have proven particularly helpful for the purification of unstable natural products, as illustrated by the isolation of hydrolyzable tannins [2], oxydizable blood pigments from a sea squirt [3], a lignan diester from *Phyllanthus acuminatus* [4], and by the purification of the prostaglandin endoperoxide PGH_2 [5]. High-speed CCC (HSCCC) [6] and centrifugal partition chromatography (CPC) [7,8] are the fastest and the most efficient CCC techniques currently available. These methods can be considered as complementary to preparative HPLC. Theory and applications of preparative CCC techniques have been extensively reviewed [9–13].

Surprisingly, little attention has been given to analytical applications of CCC. The analytical use of CCC had been suggested before [14] for natural products research and small-bore tubing had been used for the flow-through coil planet centrifuge [15], but only recently instrumentation for analytical HSCCC has become available through the work of Ito and Lee [16], and of Romanach and De Haseth [17]. The number of applications in analytical HSCCC is still small and the usefulness of the method to natural products research can not yet be fully assessed. In order to do this the following points need to be addressed: (i) is analytical CCC equivalent to HPLC?; (ii) is the technique suitable for methods development for preparative CCC?; and (iii) is analytical CCC instrumentation useful for small-scale isolation?

In this article the potential of analytical HSCCC for natural products chemistry is evaluated and applications are reviewed.

2. THEORY

Theoretical aspects of CCC have been discussed by Conway and Ito in two very comprehensive articles [18,19]. Parameters and equations presented in those articles and reproduced in this review apply to all CCC methods based on liquid–liquid partitioning. Fig. 1 shows a hypothetical separation of four compounds (**I–IV**) by analytical HSCCC assuming a coil volume of 40 ml, a flow-rate of 1 ml/min, and a stationary phase retention of 75% (S_F = 0.75, see equations 1 and 2). The separation of compounds **I–IV** is based entirely on differences in their partition coefficients K. The partition coefficient is defined as the ratio of the concentration of a solute in the stationary phase to the concentration in the mobile phase (eqn. 3). In this example (Fig.

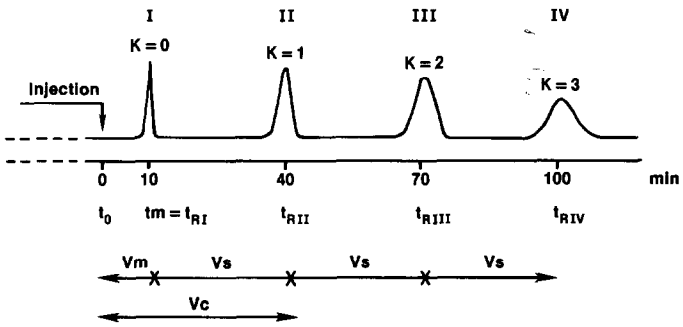


Fig. 1. Analytical HSCCC: hypothetical chromatogram and separation of compounds **I–IV** with partition coefficients of 0, 1, 2 and 3, respectively. (Modified and reproduced with permission from ref. 18).

1), compound I partitions completely into the mobile phase and is eluted with the "solvent front" at time $t_m = t_{RI}$. The corresponding elution volume V_m is equal to the amount of mobile phase in the coil. Compound II has a partition coefficient of $K = 1$ and elutes at its retention time t_{RII}. The retention time for compounds with $K = 1$ is equal to the time required for the elution of one coil volume (eqn. 4). For compounds with K values smaller or larger than 1, the retention time also depends on the stationary phase fraction S_F. Partition coefficients can be calculated from CCC chromatograms using eqn. 5.

The partition coefficient is by far the most important parameter to control in CCC. For analytical HSCCC a partition coefficient of around 1 is desirable. Theoretically, a higher K value provides higher resolution, but practical considerations such as increased run time and peak broadening due to diffusion are against solvent systems with $K > 3$. Partition coefficients < 1 result in faster separations and are indicated if less resolution is required. Partition coefficients can be calculated from CCC chromatograms (eqn. 3) or can be determined experimentally as part of the solvent selection process. Typically, a sample is distributed between equal volumes of upper and lower phases. The concentration in each phase can be determined by measuring the corresponding UV absorption or mass distribution. For complex samples, phase distribution and HPLC quantitation of individual compounds in upper and lower phases are recommended. Similarly, bioactivity can be partitioned between the upper and the lower phases [20]. Equal distribution of bioactivity corresponds to a partition coefficient of $K = 1$, assuming there is only one active component in the sample. Retention of stationary phase, expressed as stationary phase fraction (S_F), is another important parameter and can be determined directly after the run by displacing the column contents with a flow of nitrogen. The total volume of displaced stationary and mobile phases corresponds to the coil volume (V_c) and the retention of stationary phase is calculated using eqns. 1 and 2. Instead of using nitrogen, the column contents can be displaced by pumping additional mobile phase into the coil after its rotation has been stopped. In this case, the volume of displaced stationary phase is measured and S_F can be calculated (eqn. 2), if the coil volume is known. This more practical approach offers additional rinsing of the coil and is recommended when crude extracts are separated. The importance of a high stationary phase fraction in HSCCC has been demonstrated by Conway and Ito [19], who showed that resolution increases with higher stationary phase retention.

$$V_c = V_s + V_m \tag{1}$$

$$S_F = \frac{V_s}{V_c} \tag{2}$$

$$K = \frac{C_s}{C_m} = \frac{t_R - t_m}{t_m}\left(\frac{1 - S_F}{S_F}\right) = \frac{V_R - V_m}{V_m}\left(\frac{1 - S_F}{S_F}\right) \tag{3}$$

$$t_R = \frac{V_c}{f} \text{ if } K = 1 \tag{4}$$

$$t_R = \frac{V_c}{f}[1 + S_F(K - 1)] \tag{5}$$

where

C_s = concentration of solute in stationary phase
C_m = concentration of solute in mobile phase
f = flow-rate
K = partition coefficient
S_F = stationary phase fraction
t_0 = sample injection
t_m = "solvent front"
t_R = retention time
V_c = (total) volume of coil
V_m = volume of mobile phase fraction in coil
V_R = elution (or retention) volume
V_s = volume of stationary phase fraction in coil [time in min and volumes in ml]

3. INSTRUMENTATION

Instrumentation for analytical HSCCC currently available consists of a multilayer coil plant centrifuge usually equipped with 0.85 mm I.D. PTFE tubing [16,17]. Smaller bore tubing (0.38, 0.55 mm) was used by Romanach and De Haseth [17], but yielded insufficient retention of the stationary phase. Typical operating parameters are listed in Table I. Solvent–tubing wall interactions are increased in narrow-bore tubing and thus require higher centrifugal forces and higher rotational speeds (1500–2000 rpm) in order to maintain a sufficient stationary phase retention. The revolutionary radius [10] for analytical instruments was decreased compared to preparative HSCCC in order to accomodate the higher speed. Flow-rates are typically 0.5–1.0 ml/min and are limited by back pressure and stationary phase retention, depending on the solvent system selected. Higher flow rates have been reported for chloroform–methanol–water systems [21].

Most of the early studies in analytical HSCCC were carried out with user-built instruments, the latest example being an analytical multilayer coil planet centrifuge which can be operated at 4000 rpm maximum speed [22]. Performance of this instrument, equipped with 0.55 mm I.D. tubing, was compared with results obtained

TABLE I

TYPICAL PARAMETERS IN ANALYTICAL HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

Coil	Multilayer coil planet centrifuge
Coil volume	30–40 ml
Tubing	50–70 m × 0.85 mm I.D.
Counterweights	Fixed
Flow-rate	0.5–1.0 ml/min
Back pressure	<300 p.s.i.
Retention (S_F)	50–90%
Rotation	1500–2000 rpm
Sample	μg–10 mg
Run time	20–60 min

with an analytical toroidal coil centrifuge (TCC) with 0.3 mm I.D. tubing [23]. Presently, analytical HSCCC instruments are available from two manufacturers (see footnote to Table II). Plans also exist for the introduction of an analytical instrument for centrifugal partition chromatography [24].

4. DETECTION METHODS

4.1. UV–VIS

Carryover of droplets of non-retained stationary phase may cause high levels of detector noise if sensitive UV detectors such as photodiode array detectors are used. Most CCC chromatograms found in the literature have been redrawn from the original noisy tracings or were generated by tediously diluting and measuring the UV absorption of each of the fractions collected. An improvement has been suggested [25] by the use of a device as illustrated in Fig. 2. A solvent of intermediate polarity (methanol, isopropanol) is added to the coil effluent between the coil outlet and the detector by means of a mixing tee and a reactor coil. Droplets of non-retained stationary phase are thus "redissolved" and detector noise is significantly reduced. Applications of this method are shown in Figs. 3 and 4 by the separation of a mixture of standard compounds **1–4** and of phenolic natural products **5–8**, respectively. UV spectra of solutes were identical to those obtained with pure standards in methanol. UV spectra provide only limited structural information, but significantly enhance peak identification in analytical HSCCC.

In another approach to reducing detector noise, a piece of small-bore PTFE tubing (3 m × 0.46 mm I.D.) was inserted between the coil outlet and the UV detector [26]. This tubing was immersed in a water bath kept at 30°C. Experiments using a preparative HSCCC instrument suggested that the heating of the coil effluent significantly reduced levels of detector noise for selected solvent systems, *e.g.* chloroform–methanol–water. This method may also be applicable to analytical HSCCC.

4.2. Fourier transform infrared spectroscopy

Fourier transform infrared (FT-IR) spectroscopy has been suggested for CCC by Romanach and De Haseth [27]. Separations were carried out on a HSCCC instrument with 1.2 mm I.D. tubing (coil volume 160 ml) in chloroform–methanol–water (pH = 2) (3:1:3) and hexane–methanol–water (3:3:2) solvent systems. Flow cells with pathlengths ranging from 0.025–1.0 mm were used and solvent removal was not necessary. IR spectra were recorded on-line; however, relatively large sample sizes were required (0.2 to more than 1 mg per compound on the column). FT-IR

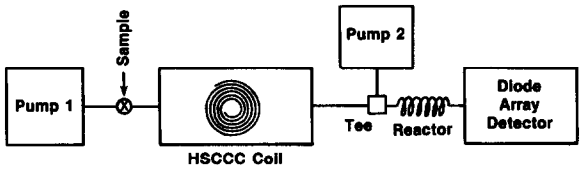


Fig. 2. Schematic diagram of analytical HSCCC with photodiode array detector and post-coil reactor.

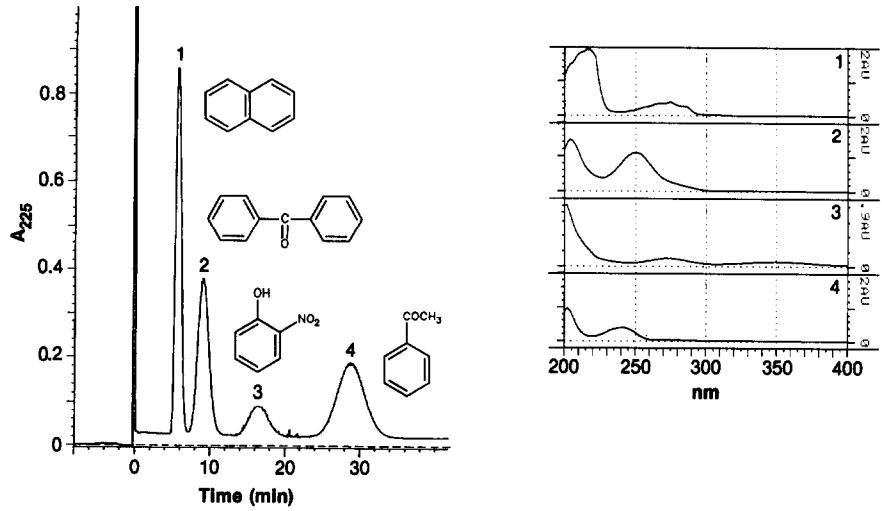


Fig. 3. Analytical HSCCC of naphthalene (**1**), benzophenone (**2**), *o*-nitrophenol (**3**), and acetophenone (**4**) (10–25 μg each); hexane–methanol–water (3:3:2), mobile phase = upper phase, 1.0 ml/min; additional flow of isopropanol at 0.5 ml/min, post-coil reactor. (Reproduced with permission from ref. 25).

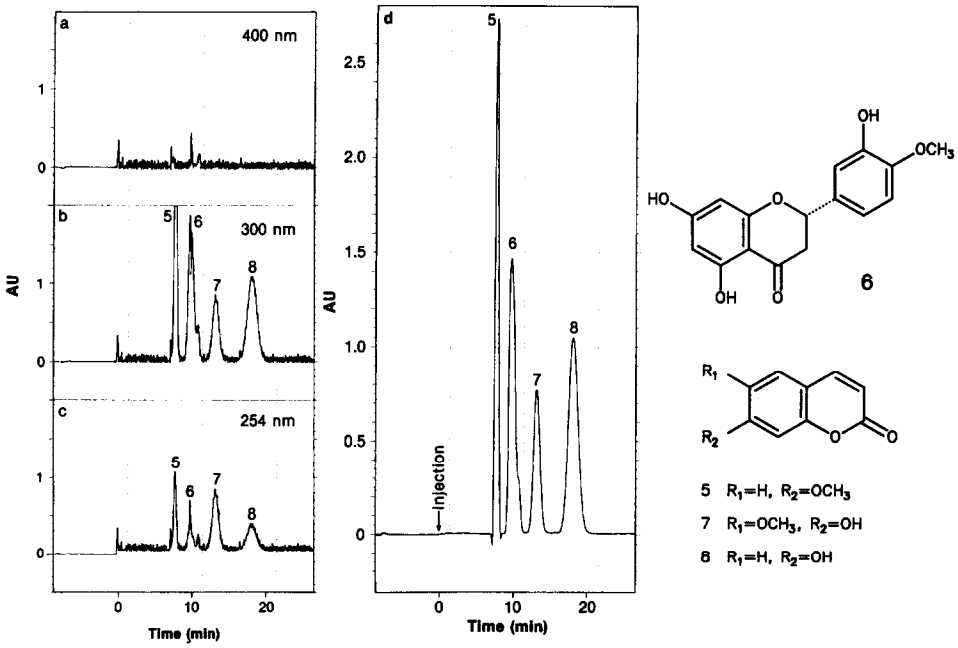


Fig. 4. Analytical HSCCC of herniarin (**5**), hesperetin (**6**), scopoletin (**7**), and umbelliferone (**8**), 50 μg each; chloroform–methanol–water (13:7:8), lower phase at 0.8 ml/min; post-coil reactor with additional flow of methanol (0.8 ml/min); original chromatograms (a–c) recorded with a photodiode array detector; chromatogram (d) obtained by subtracting (a) from (c) and smoothing. (Reproduced with permission from ref. 25).

spectroscopy represents an excellent method for the identification of solutes. On the other hand the large sample sizes make an application of CCC–FT-IR in natural products chemistry (*e.g.* separation of crude extracts/fractions) rather unlikely.

4.3. Mass spectrometry

The combination of analytical HSCCC with thermospray fast atom bombardment (FAB) mass spectrometry (MS) has been described by Lee and co-workers [28–30]. An experimental instrument for analytical HSCCC (planet centrifuge with 0.85 mm I.D. tubing) was interfaced with a thermospray quadrupole mass spectrometer. The high back pressure generated from the thermospray vaporizer was overcome by an additional high-performance liquid chromatography (HPLC)-type pump placed in-line between the CCC instrument and the mass spectrometer. Ion chromatograms and mass spectra allowed the specific detection of compounds in complex samples such as crude extracts (see applications).

5. SOLVENT SYSTEMS

Most of the solvent systems used so far in analytical HSCCC are hexane–water-based with a variable third, and occasionally fourth component (ethyl acetate, propanol, methanol; see Table II). Mixtures of chloroform–methanol–water represent the most frequently used solvent systems in CCC (see ref. 31 for a ranking of these solvents by polarity) and can also be used in analytical HSCCC. The solvent system chloroform–methanol–water (13:7:8) was used for the separation of phenolic compounds by analytical HSCCC and good retention (>80%) of the stationary (upper) phase was obtained [25]. Ito [32] studied the retention of stationary phase in analytical HSCCC (0.85 mm I.D. coil) as a function of the holder diameter, rotational speed, and flow-rate. Sufficient retention of the stationary phase was observed for hexane–water, hexane–ethyl acetate–methanol–water (1:1:1:1) and chloroform–water mixtures when flow-rates of less than 1 ml/min were used at speeds over 1500 rpm (holder diameter of 5 cm). Butanol–water systems were found to yield rather poor retention (< 50 %) even at flow-rates of less than 0.4 ml/min. Most recently, gradient elution has been suggested for CCC [33,34]. Gradient elution compensates for peak broadening of late-eluting compounds and shortens the run time in separations of samples which cover a wide range of polarity. So far, no experimental data are available for gradient elution in analytical HSCCC.

6. APPLICATIONS

Applications of analytical HSCCC in natural products chemistry are summarized in Table II. All separations were carried out on instruments using 0.85 mm I.D. tubing. Applications cover a wide range of classes of compounds, mostly from higher plants.

6.1. Alkaloids

An artificial mixture of the *Vinca* alkaloids vincamine (**9**) and vincine (**10**) was separated by analytical HSCCC in hexane–ethanol–water (6:5:5) and the separation was compared with results from analytical reversed-phase HPLC [28]. Baseline

TABLE 2

ANALYTICAL CCC OF NATURAL PRODUCTS

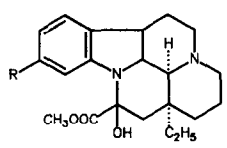
Compound	Source	Conditions[a]	Ref.
Alkaloids	*Stephania tetrandra* (Menispermaceae)	Hexane–ethyl acetate methanol–water (3:7:5:5), lower and upper, A	15
	Vinca minor (Apocynaceae)	Hexane-ethanol-water (6:5:5), lower, B	28
	Indole plant hormones	Hexane–ethyl acetate–methanol–water (3:7:5:5), lower, B	36
Anthraquinones	*Rheum palmatum* (Polygonaceae)	Hexane–ethanol–methanol–water (9:1:5:5), lower and upper, A	37
Coumarins	–	Chloroform–methanol–water (13:7:8), lower, C	25
Flavonoids	*Hippophae rhamnoides* (Elaeagnaceae)	Chloroform–methanol–water (4:3:2), lower, A	21
Lignans	*Podophyllum peltatum* (Podophyllaceae)	Hexane–ethyl acetate–methanol–water (1:1:1:1), lower, C	38
	Schisandra rubriflora (Schisandraceae)	Hexane–ethanol–water (6:5:5), lower, B	29,30,43
Macrolides	*Bugula neritina* (Bugulidae)	Hexane–isopropanol–methanol–water (4:1:1.6:0.4), lower, C	42

[a] Solvent system, mobile phase and instrumentation. A = CCC-2000 analytical high-speed counter-current chromatograph, *ca.* 70 m × 0.85 mm I.D. (*ca.* 40 ml coil volume); Pharma-tech, Baltimore, MD, U.S.A. B = Experimental instrument, 0.85 mm I.D. tubing, see corresponding references for further description. C = "Micro" high-speed counter-current chromatograph for analytical HSCCC, *ca.* 50 m × 0.85 mm I.D. (*ca.* 30 ml coil volume) operated at 1800 rpm; P.C. Inc., Potomac, MD, U.S.A.

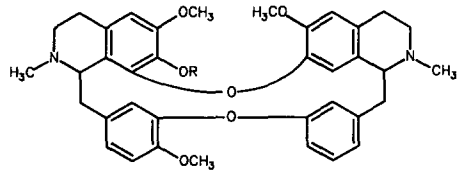
resolution was obtained with both methods and an isomer of vincine was separated by analytical HSCCC, but not by HPLC. The sample was also analyzed by CCC–MS. In another example [35], an artificial mixture of the bisbenzylisoquinoline alkaloids tetrandrine (**11**), fangchinoline (**12**), and the protoberberine type alkaloid cyclanoline (**13**) was separated in two *n*-hexane–ethyl acetate–methanol–water solvent systems. Alkaloids **11–13** were originally isolated from *Stephania tetrandra* S. Moore, a Chinese medicinal plant with antiinflammatory properties. Lee *et al.* [36] studied the separation of indole acetic acid and of three homologues. These plant growth hormones were resolved to baseline in hexane–ethyl acetate–methanol–water (3:7:5:5) with the lower phase as mobile phase.

6.2. Anthraquinones

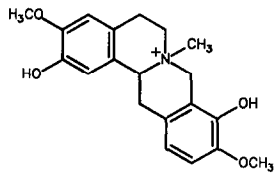
Five anthraquinones from *Rheum palmatum* L. were separated by analytical HSCCC using *n*-hexane–ethyl acetate–methanol–water (9:1:5:5) as the solvent system [37]. The sample mixture (1 mg of extract containing about 0.5% free hydroxy-anthraquinones) was analyzed in a 0.85 mm I.D./40 ml volume coil. The elution was first carried out with the lower phase as the mobile phase for 20 min at 1 ml/min, then the mode was reversed. Elution was then carried out with the upper phase for an



9: R = H
10: R = OCH_3



11: R = CH_3
12: R = H



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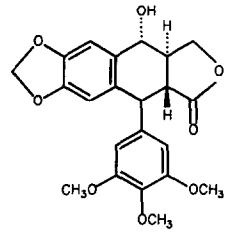

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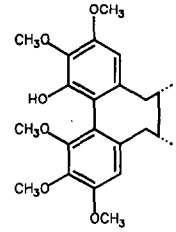

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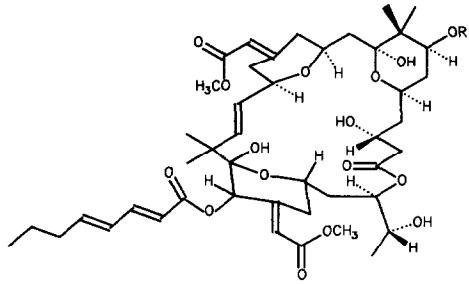

16: R = $COCH_3$
17: R = H

additional 50 min, thus eluting less polar anthraquinones which otherwise would have remained in the stationary phase. This example shows the advantage of reversed elution in the case of complex samples having a wide polarity range. Aloe-emodin, chrysophanol, emodin, physcion, and rhein were identified by MS but no information about peak purity was provided.

6.3. Lignans

Podophyllotoxin (**14**) is a major cytotoxic lignan of the mayapple, *Podophyllum peltatum* L. The dichloromethane extract of the rhizomes was separated (3 mg sample) by analytical HSCCC in hexane–ethyl acetate–methanol–water (1:1:1:1) [38]. Elution was carried out with the lower phase at 0.5 ml/min and fractions (1 min each) were collected. The thin-layer chromatographic (TLC) analysis of selected fractions is shown in Fig. 5.

Fraction 100 was a 1:1 mixture of podophyllotoxin (identified by HPLC, ^{1}H NMR, FAB-MS) with an unidentified lignan. Another compound (fraction 70) of

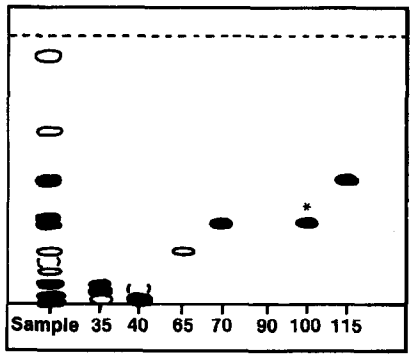

Fig. 5. TLC analysis of selected fractions from analytical HSCCC of *Podophyllum peltatum* extract (3 mg sample). Hexane–ethyl acetate–methanol–water (1:1:1:1), lower phase at 0.5 ml/min; fractions: 1 min; retention 50%. TLC: silica gel; dichloromethane–methanol–water (1:1:1, lower phase), UV 254 (empty circles) and anisaldehyde spray reagent (full circles); * marks a fraction containing podophyllotoxin.

almost the same R_F value as podophyllotoxin was easily separated by analytical HSCCC. Retention of the stationary phase was 50%.

The root extract of *Schisandra rubriflora* Rhed et Wils. was separated in hexanes–ethanol–water (6:5:5) with the lower phase being the mobile phase (0.8 ml/min). A thermospray mass spectrometer was used as a detector and schishanenol (**15**), along with five structurally related lignans were detected by means of selective ion chromatograms and mass spectra [29]. Schishanenol and its acetate derivative were separated by HSCCC, but not by reversed-phase HPLC with methanol–water. The lignans of *S. rubriflora*, a traditional Chinese medicinal plant for the treatment of hepatitis, were also isolated by true CCC, a promising new type of CCC [39].

6.4. Macrolides

Bryostatin 1 (**16**) is an antitumor macrolide isolated from the marine Bryozoan *Bugula neritina* [40] and currently in preclinical development. The detection of bryostatin 1 by reversed-phase HPLC in crude extracts or fractions of *B. neritina* requires several sample clean-up steps [41]. The extract (10 mg sample) was separated by analytical HSCCC using hexane–isopropanol–20% aqueous methanol (4:1:2) with the lower phase as mobile phase at 0.6 ml/min. Fractions eluting between 30–34 min contained bryostatin 1, but not bryostatin 2 (**17**) and were pure enough for direct HPLC analysis.

6.5. Phenolics

A mixture of three plant coumarins and one flavanone (see Figs. 2 and 4) was used to evaluate protodiode array detection for analytical HSCCC [25]. See section on Detection methods for further discussion.

Flavonoids from *Hippophae rhamnoides* L. fruits were studied by Zhang *et al.* [21] using a 0.85 mm I.D., 40 ml coil. The separation of the crude ethanolic extract (3 mg sample) by analytical HSCCC was accomplished with chloroform–methanol–water (4:3:2) within 100 min. Elution with the lower phase at 1 ml/min at 1800 rpm

yielded excellent retention of the stationary phase (86%). Isorhamnetin was detected as the major flavonoid present in the extract. The separation was repeated at different flow-rates and the analysis time was shortened to 15 min. A flow-rate of 5 ml/min provided sufficient resolution and acceptable stationary phase retention (68%). The authors also separated a sample (100 mg) on a preparative HSCCC instrument and obtained the same elution profile with similar resolution.

7. DISCUSSION

Typical instrumentation for analytical HSCCC consists of a multilayer coil planet centrifuge with 0.85 mm I.D. tubing and a total coil volume of 30–40 ml. High rotational speeds (1500–2000 rpm) are required to retain a high stationary phase fraction in the coil. Future instrumentation is expected to have smaller and shorter tubing and to be operated at even higher speeds [22]. Several detection systems have been used in conjunction with HSCCC, including UV [25,26], FT-IR [27] and thermospray MS [28–30]. Among those, CCC–MS is a very promising technique, although not readily available. Analytical HSCCC separations are based on differences in partition coefficients (K) between individual compounds and separations are therefore predictable. Partition coefficients represent the most important parameter to be controlled by the chromatographer. It is recommended to determine K values as part of the solvent selection process prior to carrying out separations. K values can also be calculated from chromatograms, using eqn. 5.

Applications of analytical HSCCC reviewed in this article emphasize a number of successful solutions to difficult natural products separation problems. Various biphasic solvent systems provide high resolution and relatively short separation time. An almost unlimited (but not yet fully explored) choice of solvent systems allows excellent control over selectivity. Theoretical plate counts are less than 2000 and suggest that analytical HSCCC at its present state can not compete with modern HPLC. Yet there are examples in which analytical HSCCC has given results which could not have been achieved on normal- or reversed-phase solid supports.

Analytical HSCCC represents a fast and reliable way of methods development for preparative CCC separations. This was demonstrated by Zhang *et al.* [21] by analytical and preparative HSCCC of flavonoids from *Hippophae rhamnoides*. Small-scale isolations of natural products in μg to mg quantities are yet another interesting application for analytical HSCCC instrumentation. Complex samples, *e.g.* crude extracts are conveniently fractionated with complete sample recovery. Enriched fractions or semipure compounds may be obtained in a single step, as illustrated by the separation of a crude *Podophyllum peltatum* extract [38] and by the enrichment of bryostatin 1 from *Bugula neritina* [42]. To our knowledge, analytical HSCCC has not yet been used for quantitative work with natural products.

Analytical HSCCC is ideal for methods development in preparative HSCCC (possibly also for other types of CCC) and represents a convenient method for micro- or small-scale purifications. This new analytical method is complementary to HPLC and most valuable in cases where resolution can only be obtained by changing the selectivity of the chromatographic system. A large number of applications of analytical HSCCC can be expected for natural products chemistry and for related areas where difficult separation problems are common.

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