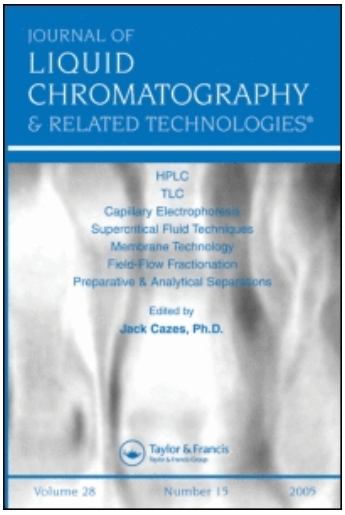

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COMPARISON OF SOME CENTRIFUGAL PARTITION CHROMATOGRAPHY SYSTEMS FOR A GENERAL SEPARATION OF PLANT EXTRACTS

K. Ingkaninan[a]; A. C. J. Hermans-Lokkerbol[a]; R. Verpoorte[a]
[a] Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands

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K. Ingkaninan,* A. C. J. Hermans-Lokkerbol, R. Verpoorte

Division of Pharmacognosy
Leiden/Amsterdam Center for Drug Research
Gorlaeus Laboratories
Leiden University
P. O. Box 9502
2300 RA, Leiden, The Netherlands

ABSTRACT

In order to find a Centrifugal Partition Chromatography (CPC) solvent system that can be used as a general prefractionation step for crude plant extracts, twelve two-phase systems were compared. The butanol: ethyl acetate: water 3:2:5 (v/v/v) system gave good separation for polar compounds, and the heptane: ethyl acetate: methanol: water 6:1:6:1 (v/v/v/v) system was found to be effective for the separation of nonpolar compounds and somewhat less for polar compounds.

INTRODUCTION

Presently, the interest from pharmaceutical industries to screen natural product extracts for new biologically active compounds is increasing due to the availability of high-throughput screening methods. The access to a large number of chemical structures is required and biodiversity is a rich source for

such screens. However, the efficiency of finding novel leads in the receptor binding assays or the enzyme assays used in the screenings suffers from the occurrence of well-known active compounds or compounds that cause a false-positive reaction in the assays. For example, phenolic compounds show non-specific binding activity to proteins, while gamma aminobutyric acid (GABA), glutamic acid, tryptamine, and adenosine cause positive responses in some assays.[1] Regarding this problem and the fact that the number of samples to be tested is not a limiting step in the high-throughput screening method, a general and reproducible prefractionation prior to biologically activity screening might be an interesting approach.

By means of a reproducible prefractionation, the false-positive fractions or interfering fractions can be identified in an early stage. Moreover, the chance of finding leads among minor compounds is increased. Centrifugal partition chromatography (CPC) might be suited for such a prefractionation.

Centrifugal partition chromatography is a countercurrent liquid-liquid partitioning chromatography method in which the stationary phase is immobilized by centrifugal force, while the mobile phase is pumped through at high flow rates. Any two immiscible solvents may be used to perform a two-phase system. Sample components are partitioned between the mobile and stationary phases, and are separated on the basis of differences in their partition coefficients. The method was first described by Murayama[2] and the theoretical aspects were discussed by Berthod and co-workers.[3-8]

CPC offers particular advantages for the isolation of natural products. Since it does not involve solid adsorbents such as silica gel, the denaturation of sensitive compounds is minimized and there is no irreversible retention. Compared to conventional solid supported column chromatography, CPC has a higher capacity because of the large volume of stationary phase involved in the separation process. It also has an economic advantage, since smaller amounts of solvents are used at comparable mass throughputs and no packing materials are required.

As both mobile and stationary phases are liquid in CPC, the so-called dual-mode operation can be used. The elution mode can be changed by turning the stationary phase into the mobile phase during the operation. In this way both polar and nonpolar compounds are eluted in a short single run. CPC can be used on analytical scale as well as on preparative scale.

The aim of this study is to develop a simple and fast reproducible method as a general prefractionation step which is able to separate a broad range of compounds with quite different polarities. Ethanol extracts from *Tabernaemontana pandacaqui* Poir. (Apocynaceae) cell suspension cultures were used as a model. A series of CPC two-phase systems was selected based

on the difference in polarity of the two phases and the overall polarity. The separations of the extract by these CPC systems were evaluated by means of TLC analyses. The two best systems were then evaluated with ethanol extracts from two plants and a plant cell culture.

EXPERIMENTAL

Extraction

Cell suspension cultures

The cell suspension cultures, *Tabernaemontana pandacaqui* Poir. (cell line 6OriB13)[9] and *Catharanthus roseus* L. (cell line A12A2),[10] were grown in 2 liter flasks. They were harvested and stored at -20°C. After thawing they were washed with pH 7 phosphate buffer. The cells were then extracted with ethanol (5 mL per gram fresh weight) using a Turrax at high speed for 3 min. The ethanol extract was evaporated till dryness under reduced pressure.

Plant materials

Dried leaves of *Tabernaemontana pachysiphon* Stapf (Apocynaceae) and roots of *Aconitum xhenryi* E.Pritz 'Spark' (Ranunculaceae) were macerated with 5 mL ethanol per gram for 1 week. Then they were filtered and the filtrates were evaporated till dryness under reduced pressure.

CPC Apparatus

A modular Sanki (Kyoto, Japan) centrifugal partition chromatography (type LLN) was used. It consisted of a power supply (Model SPL), a triple-head constant-flow pump (Model LBP-V) and a centrifuge (Model NMF). The centrifuge could contain up to 12 cartridges with a total volume of 250 mL. A Panasonic Pen-recorder (Model VP 67222A) was connected to a UVIS 200 detector (Linear Instruments, Reno, NV, USA). Fractions were collected by means of a LKB 2211 Superrac fraction collector.

CPC Separation

In all experiments, six cartridges (total internal volume 125 mL) were used. The pressure was limited to 60 bar. The flow rate was set to 2 mL/min. The fraction size was 8 mL. In each run, 100 mg of sample dissolved in 2 mL of each of the two phases was injected.

Table 1

The Composition of the Stationary Phase and the Mobile Phases of Solvent System 9, Hexane:1-Butanol:Water

		%Hexane	% 1-Butanol	%Water	Volume of Mobile Phase (mL)
Stationary phase		1	7	92	
Mobile phase	1	100	0	0	80
	2	88	12	To be saturated	40
	3	78	20	2	40
	4	67	30	3	40
	5	45	50	5	40
	6	34	60	6	40
	7	5	80	15	40
	8	0	82	18	40

Table 2

The Composition of the Stationary Phase and the Mobile Phases of Solvent System 10, Ethyl Acetate:1-Butanol:Water

		%Ethyl Acetate	% 1-Butanol	%Water	Volume of Mobile Phase (mL)
Stationary phase		5	3	92	
Mobile phase	1	96	0	4	64
	2	74	18	8	64
	3	51	36	13	64
	4	32	52	16	64
	5	14	68	18	64
	6	0	82	18	80

For each isocratic system, dual mode operation was used. Ascending mode elution was performed to collect 160 mL of eluate including the void volume. Then the mode of elution was changed to descending mode to collect another 160 mL.

The stepwise gradients of systems **9** and **10** were based on the ternary diagrams.[11] The different compositions of mobile phase were made according to Table 1 and 2 and introduced to the column one by one. Mode reversion was performed as the final step to collect 120 mL of water rich phase as a mobile phase.

The gradient systems **11** and **12** were made by the linearly gradual replacement of the initial mobile phase by the final mobile phase. The initial mobile phase of system **11** was heptane:1-butanol:water 85:14:1 (v/v/v) and the final mobile phase was heptane:1-butanol:water 41:54:5 (v/v/v). The stationary phase of this system was constant at heptane:1-butanol:water 91:8:1 (v/v/v). After 200 mL of the gradient elution of heptane rich mobile phase, the mode of elution was reversed to collect 120 mL of 1-butanol rich phase.

The initial mobile phase of system **12** was methanol:water 30:70 (v/v) saturated with heptane and the final mobile phase was heptane:methanol:water 3:80:17 (v/v/v). The stationary phase of this system was heptane saturated with methanol and water. After 160 mL of the gradient elution of methanol rich mobile phase, the mode of elution was reversed to collect 120 mL of heptane rich phase.

TLC Analysis

All fractions from CPC were spotted on 20x20 cm silica gel plates F254 No. 5554 (Merck, Darmstadt, Germany) and developed in saturated normal chambers (saturation time 1 hour). Two TLC solvent systems, i.e. chloroform: methanol 95:5 (v/v) and dichloromethane: ethyl acetate: water 4:4:0.5 (v/v/v) were used.

The visual detection was done under UV 254 nm and UV 366 nm. Then the TLC plate was sprayed by modified anisaldehyde-sulphuric acid spray reagent.[12] After spraying, the plates were heated with a hot air blower for 2 minutes. Color changes during the heating were noted.

Evaluation of Separation

The separation efficiency of each CPC system (see Figure 1) was evaluated as follows:

> The CPC collected eluates (after the void volume was discarded) were combined into 5-10 fractions according to TLC patterns. A total of about 20 compounds were observed on the TLC plates.

no.	two-phases system	elution mode	separation efficiency: nonpolar fractions	separation efficiency: polar fractions
1	EtOAc:water	isocratic	-	-
2	methyl *tert* butyl ether:water	isocratic	-	-
3	$CHCl_3$:MeOH:water 1:1:1	isocratic	+	+
4	BuOH:EtOAc:water 3:2:5	isocratic	-	+++
5	BuOH:PrOH:water 4:1:5	isocratic	+	+
6	EtOH:EtOAc:water 3:7:10	isocratic	-	++
7	Hep:EtOAc:MeOH: Water 6:1:6:1	isocratic	++	+
8	Hep: MeOH	isocratic	++	-
9	Hex:BuOH:water	stepwise gradient	-	++
10	EtOAc:BuOH:water	stepwise gradient	-	++
11	Hep:BuOH:water	gradient	-	++
12	Hep:MeOH:water	gradient	++	-

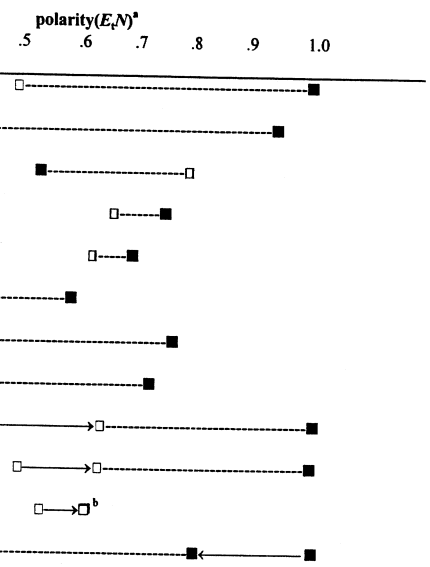

Figure 1. Comparison of the two-phase systems used to separate the alcohol extract from cell suspension cultures of *T. pandacaqui*. [a]The E_tN data are from S.J. Gluck *et al.*[13] (system **2**), measured in our laboratory (systems **9-12**), and from A.P. Foucault[14, 16](other systems). The E_tN value of system **4** is the estimation from the E_tN value of BuOH:EtOAc:water 4:1:4. [b]The E_tN value of the upper phase increases from 0.53 to 0.6 and the E_tN value of the lower phase is 0.6. □ upper phase, ■ lower phase. Abbreviations: BuOH, 1-butanol; $CHCl_3$, chloroform; EtOAc, ethyl acetate; EtOH, ethanol; Hep, heptane; Hex, hexane; MeOH, methanol; PrOH, 1-propanol.

The separation score of each fraction was evaluated as follows:

Separation Score	No. of Obs. Compounds	Vol. of Fraction (mL)
3	1-2	up to 80
2	3-5	8-48
1	3-5	56-80
	or >5	8-24
0	3-5	>80
	or >5	32-80

The fractions were categorized into two groups; nonpolar fractions which eluted by a less polar organic phase and polar fractions which eluted by a more polar aqueous phase.

-nonpolar fractions: fractions obtained from the ascending mode elution using the organic phase as the mobile phase except which in system **3**. In system **3**, which consisted of chloroform, the nonpolar fractions were obtained from the descending mode as the nonpolar phase was the heavy phase.

-polar fractions: fractions from the descending mode using the aqueous phase as the mobile phase of every system except system **3**. In system **3**, polar fraction obtained from the ascending mode.

The separation efficiencies for both polar and nonpolar fraction were calculated separately from the mean of the separation score, which was the sum of separation scores divided by the number of fractions:

Mean of the Separation Scores	Separation Efficiency
0-0.4	-
0.5-1.4	+
1.5-2.4	++
2.5-3	+++

Measurement of Polarity

In each of the two phases of a solvent system, a small amount (*ca.* 0.02 mg ml^{-1}) of Reichardt's dye (Aldrich, Steinheim, Germany) was dissolved. The absorption maximum between 400 and 800 nm (λ_{max}) was determined by a Varian Cary 1Bio UV-visible spectrophotometer.

Polarity, expressed as E_tN was calculated according to the method used by Gluck and Wingeier.[13]

$$E_tN = (28590\lambda_{max}^{-1} - 30.7)\,(32.4)^{-1}$$

Nonpolar liquids have an E_tN close to zero and E_tN of polar liquids is close to 1.

RESULTS AND DISCUSSION

Eight isocratic and four gradient CPC systems were chosen based on the polarity of the two phases. They were used in the separation of the ethanol extract from *T. pandacaqui* cells. The results are compared in Figure 1 together with the polarities of the two-phase systems reported in E_tN values.[13,14,16] The separation efficiency of each solvent system was evaluated by means of TLC analyses. It is difficult to quantitatively evaluate the efficiency of a chromatographic separation for crude plant extracts. Therefore, an evaluation criterion was developed as described in the experimental part. This evaluation is based on the number of compounds per fraction, i.e. the more fractions with a low number of compounds, the higher the efficiency of the separation is.

In every experiment, the dual mode operation was used in order to minimize the run time and maximize the partition efficiency. According to Berthod and Armstrong,[3] the retention volume (v_r) of any compound can be calculated by the equation:

$$v_r = v_m + Kv_s \qquad (1)$$

in which K is the partition coefficient of the compound between the mobile and the stationary phase, v_m is the mobile phase volume, and v_s is the stationary phase volume. To minimize the run time, the mode of elution should be reversed when compounds with K=1 are eluted. At that point, the elution volume is equal to the total volume, v_t ($v_t = v_m + v_s$). After mode reversion, all compounds that are left in the CPC have a partition coefficient ≤ 1, and they are then eluted within another elution volume equal to v_t. Therefore in each isocratic system (i.e. systems **1**-**8**), we changed the elution mode at 160 mL of the retention volume (including the void volume). In the case that the void volume is more than 35 mL, at that turning point, K is still less than 1. So in every experiment, we collected the eluate after mode reversion more than the total volume (v_t) to make sure that no compounds were left in the column. In this way, we could finish a run with the complete elution of all compounds in 160 minutes. The mode reversion technique was also used as a final step in all gradient systems (i.e. systems **9**-**12**).

The solvent systems were selected based on their polarity range. The polarity of each phase and the polarity of a solute determine the partition coefficient of that solute.[13] For a high separation efficiency, a broad range of partition coefficients is required. Crude plant extracts contain various compounds over a wide range of polarity, from nonpolar compounds such as lipids and steroids to very polar compounds such as glycosides and sugars. This implies that the ideal CPC system for our application should be able to cover all polarities.

The investigation was started with solvent systems **1** and **2** which have a great difference in polarity between two phases (Figure 1). However, neither system could achieve a good separation of the tested extract. The high polarity of the aqueous phases (1 and 0.94 respectively) of these systems might be the reason for the poor separation. Similar to other liquid chromatography methods, polar compounds were rapidly eluted by too polar eluents. The separation efficiency was improved when we used less polar solvent systems such as the systems **3**-**7**, which have *EtN* values of the aqueous phase between 0.58-0.8. Another factor that affected the separation was the solvent selectivity. Although the polarities of aqueous phases were similar, for example system **4** and system **7**, the elution sequence and the separation efficiency were different.

For the separation of polar compounds, solvent system **4**, butanol: ethyl acetate: water 3:2:5 (v/v/v), was the most effective one. When the mode of elution was reversed, using the water rich phase as a mobile phase, glucose, sucrose, phenyl alanine, and tryptophan were separated. They were identified by means of ^{1}H-NMR and comparison with standard compounds. With the ethyl acetate rich phase, most of the nonpolar compounds came out in the same fraction.

For nonpolar compounds, best separations were obtained by using solvent systems **7** and **8**, which were the most nonpolar solvent systems examined. Both systems have *EtN* values of the nonpolar phase of 0.23. However, solvent system **7**, heptane: ethyl acetate: methanol: water 6:1:6:1(v/v/v/v), gave a higher separation efficiency for the polar compounds than solvent system **8** (Figure 1).

A possible method to fractionate compounds of widely differing polarities and partition coefficients is gradient elution.[15] Four gradient systems, **9**-**12** were tested. According to ternary diagrams,[11] these systems provide an opportunity to vary the composition of the mobile phase while the stationary phase remains constant. The systems **9**, **10** and **11** are favorable for a normal phase gradient run since a water rich phase is used as the stationary phase. After the gradient, we reversed the mode of elution to retrieve polar compounds that were retained in the column.

Table 3

Separation Efficiency for Both Polar and Nonpolar Fractions of Solvent System 4 (Butanol: Ethyl Acetate: Water 3:2:5) and Solvent System 7 (Heptane: Ethyl Acetate: Methanol: Water 6:1:6:1)

Samples	System 4		System 7	
	Nonpolar	Polar	Nonpolar	Polar
Cell biomass of *Tabernaemontana pandacaqui* Poir. (Apocynaceae)	-	+++	++	+
Cell biomass of *Catharanthus roseus* (L.) G. Don (Apocynaceae)	+	++	++	++
Dry leaves of *Tabernaemontana pachysiphon* Stapf (Apocynaceae)	-	++	++	+
Roots of *Aconitum xhenryi* E.Pritz "Spark" (Ranunculaceae)	+	++	+	+

As indicated in Figure 1, all nonpolar compounds eluted at the same time directly after the injection peak, due to a too high polarity of the organic mobile phases, even though the polar compounds were separated reasonably well. The ternary diagrams of the gradient solvents, however, do not show any possibility for further decreasing the polarity of the mobile phases.

Gradient system **12** (heptane: methanol: water) which is favorable for a reversed phase run was tested. From the ternary diagram,[11] it is clear that the polarity of the aqueous mobile phase can be increased gradually while the composition of the organic stationary phase (heptane saturated with methanol and water) remains constant. Since the organic phase of system **8**, heptane:methanol, gave a good separation of nonpolar compounds, we expected that the separation of polar compounds could be improved by this gradient system. As the result shows in Figure 1, we could not achieve a better separation. The aqueous phase was still too polar to give a good separation.

After the trial of these eight isocratic and four gradient systems, we found that the best system for the separation of polar compounds was solvent system **4**. And, solvent system **7** gave the best separation for nonpolar compounds and some separation for polar compounds. These two systems were tested again with 3 other extracts. The samples were separated effectively as shown in Table 3.

CONCLUSIONS

Testing a series of CPC systems for their ability to separate compounds with a wide range of polarity, two CPC systems came out best. Polar compounds are effectively separated with the butanol: ethyl acetate: water 3:2:5 (v/v/v) two-phase system, but the separation efficiency for the nonpolar compounds is poor. The heptane: ethyl acetate: methanol: water 6:1:6:1 (v/v/v/v) system, is effective for the separation of nonpolar compounds and somewhat less for polar compounds. Both systems performed better than the gradient systems tested. It is clear that not only polarity but also solvent selectivity affects the separation efficiency of CPC. Further studies will be on the application of these systems including the determination of the retention of various known-bioassay-interfering compounds to further validate the systems as a possible prefractionation step in biological activity screening.

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