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REVIEW PAPER

The use of centrifugal partition chromatography in the separation of natural products

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Centrifugal partition chromatography (CPC) belongs to the family called countercurrent chromatography (CCC) which involves liquid-liquid partition, and does not employ a solid support matrix. Although it has been applied to separate and purify a broad range of synthetic and naturally occurring chemical species, CPC offers distinct advantages for the separation of bioactive components from crude extracts of plants, marine organisms and microbial fermentation media. This paper will give some examples of the different applications of CPC.

Key Words: counter current chromatography, centrifugal partition chromatography, natural products

NATURAL PRODUCTS ARE A RICH SOURCE OF CHEMICAL DIVERSITY with a great potential for the discovery of bioactive compounds such as insecticides, pesticides, and drugs. Screening of natural products which may lead to these compounds presents special problems not found with pure compounds. The enormous chemical complexity of extracts contributes non-specific responses and synergistic phenomena resulting in so called "false positives". For this reason, a bioassay-directed fractionation of an active extract does not always lead to the isolation of active components. The loss of activity due to the separation of synergistically acting components of low individual potency cannot be easily distinguished from the loss of activity due to chemical changes induced by a particular isolation technique. Thus, it is highly desirable to use the least destructive isolation method. CPC fulfills this condition.

Principles

Centrifugal partition chromatography is a liquid/liquid chromatographic method employing two immiscible phases in which the liquid stationary phase is retained by a centrifugal field rather than by a solid support. Separation of a mixture is achieved by introducing the sample into the stationary phase and eluting with the mobile phase. Since there is no solid support, the retention of components depends only on their partition coefficients. Those components which have partition coefficients in favor of the mobile phase will be eluted first. The technique was developed in order to obtain separations analogous to stepwise liquid/liquid extraction but in a continuous mode (1).

Other countercurrent chromatographic methods such as droplet countercurrent chromatography (DCCC) or rotation locular countercurrent chromatography (RLCC), which rely on gravitational field rather than a centrifugal field for retention of the stationary phase, are considerably more time-consuming than CPC techniques. Furthermore, in DCCC, solvent systems are limited to two-phase combinations which form droplets when the mobile phase is passed through the stationary phase.

The following are some desirable characteristics of CPC (2,3): 1) does not require solid supports, which in many cases are very costly, since it relies on easily available solvents; 2) accommodates a wide range of solvent systems in both normal and reversed phase modes; 3) the absence of solid support avoids problems due to irreversible adsorption or decomposition on the solid/liquid interface often encountered with conventional packed columns. Thus, this leads to total recovery of samples; 4) high flow rates may be used, without appreciable loss of resolution, leading to short separation times; 5) the volume ratio of the stationary phase to the total column volume is greater in CPC than in conventional liquid chromatography. A comparison between HPLC and CPC was given by Focault and Nakanishi (4) and is shown in Figure 1. Thus for CPC, larger quantities of sample can be used; 6) no pH constraint; 7) both normal-phase and reversed-phase elution may be conducted with the same solvent pair; and 8) compatibility with all molecular sizes, from small ions to macromolecules.

Instruments

There are two alternative designs of the apparatus presently available (3,5-7):

a) *The Sanki centrifugal partition chromatographic system.* This is a system of 12 cartridges arranged in series around the circumference of a centrifuge (Fig. 2). Each cartridge contains 400 separation channels, the top of each channel is connected to the bottom of the next one. The cartridges are connected by narrow bore Teflon tubes. It is not necessary to use the maximum number of cartridges and they can removed easily until the desired number remains. By this means, the speed of separation can be increased when high separation efficiencies are unnecessary. The apparatus is enclosed in a constant temperature box and experiments can be performed with heating or cooling.

b) *PC INC. Multilayer Coil CCC,* called the high speed countercurrent chromatograph which was developed by Ito, Y., NIH, Bethesda, Maryland, USA (6). This is a sepa-

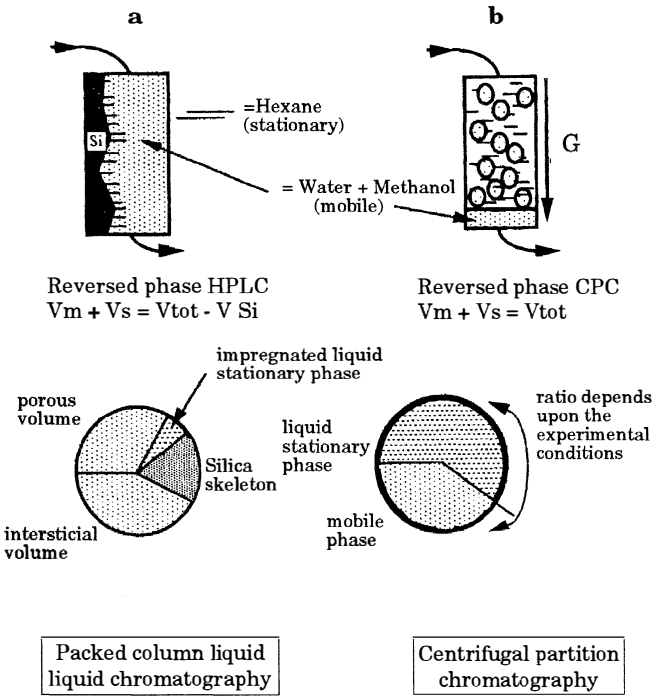


Figure 1. Comparison between conventional liquid/liquid reversed phase chromatography and reversed phase centrifugal partition chromatography. **a.** Surface of silica is bonded with hexyl silane, and saturated with methanol, which constitutes the impregnated liquid stationary phase of hexane/methanol (only = 5% of the total volume) in equilibrium with the water/methanol mobile phase (which occupies the interstitial and most of the porous volume, i.e., 75% of the total volume). The bulk volume of silica, which does not participate in the partition process, occupies 20% of the volume. **b.** Silica is eliminated and a strong gravity field allows the heavy droplets of water/methanol to flow through the hexane (saturated mainly with methanol) light stationary phase. The total volume is used for the partition process, and the ratio stat./mobile, depending upon the experiment is approximately 50/50.

ration coil made of Teflon and incorporates an anti-twisting flow assembly which avoids the use of a rotating seal.

Solvent Systems

Various approaches in the choice of solvent systems are the following: 1) Consideration of known systems (1,8). Alternatively, a classical chloroform: methanol: water or a (less polar) n-hexane: ethyl acetate: methanol: water system can be chosen as the starting point; 2) the method of screening by tlc (silica) for solvent systems used in DCC which was devised by Hostettmann, can be used in CPC (9). Rf values should lie between 0.2 and 0.4. for the Sanki instrument and a little lower for the P.C. Inc. CCC; 3) analytical HPLC method to determine the partition coefficients of the components of the mixture (9); and 4) partition ratio of biological activity. A solvent system which gives a fairly good distribution of activity between the two phases can be considered a candidate for the separation (10).

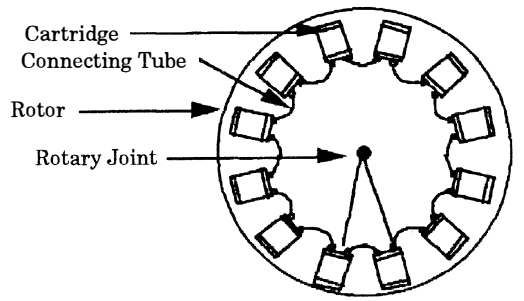


Figure 2. Disposition of cartridges in the Sanki instrument.

Figure 3 shows the mode of operation. In ascending mode, the upper lighter phase is used as mobile phase; in descending mode, the heavier lower phase is the mobile phase.

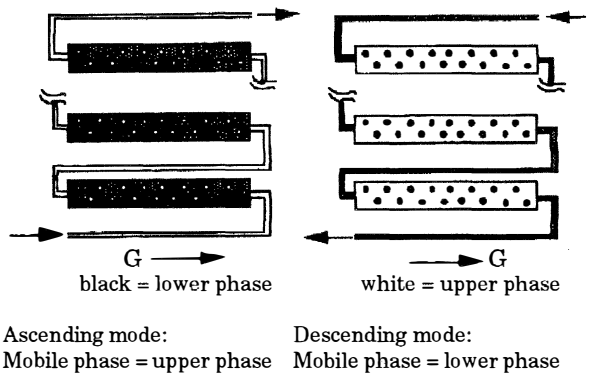


Figure 3. Operation modes in CPC.

Preparative Applications

The list of examples of natural product separations by CPC is becoming quite lengthy. A selection of these for the two CPC instruments is shown in Tables 1 and 2 (3).

A comparative separation of the flavanone hesperetin (1) and the flavonols kaempferol (2) and quercetin (3) by RLCC, DCCC and two CPC methods is shown in Figure 4 (11-12). While DCCC and RLCC required more than 30 h for complete separation in the descending mode, the two CPC methods took only about 3 h. Doing the same CPC separations but in ascending mode as shown in Figure 5 (a,c), a longer separation time was required. However, both are amenable to phase reversal leading to shorter separation time as shown in Figure 5 (b,d) (11-12).

Tannins, as a class, lends itself particularly well to CPC techniques since column chromatography of these and related polyphenols on solid supports often leads to adsorption and hydrolysis problems (13,14). Okuda carried out much work on tannin separation on a Sanki instrument using the solvent system n-BuOH : n-PrOH : water (4:1:5).

CPC (Sanki) alone was employed in the isolation of 4 new saponins, Alysicarpins A-D, from the extract of an African plant, *Alysicarpus* sp. (15). CPC is particularly well suited for the separation of alkaloids. Quaternary indole alkaloids, for example, are difficult to purify due to their polarity and their interaction with solid support matrices (16).

Table 1. Selected applications of the Sanki cartridge instrument in the separation of natural products. (3)

Substances separated	Solvent System
Flavonoids	$CHCl_3$-MeOH-H_2O (33:40:27)
Flavonoid glycosides	EtOAc-94% EtOH- H_2O (2:1:2)
	EtOAc-n-BuOH- H_2O (2:1:2)
Tannins	n-BuOH-n-PrOH-H_2O (4:1:5)
	n-BuOH-n-PrOH-H_2O (2:1:3)
	n-BuOH-HOAc-H_2O (4:1:5)
Chalcones	$CHCl_3$-MeOH-H_2O (7:13:8)
Coumarins	n-C_6H_{14}-EtOAc-MeOH-H_2O (18:42:30:30)
Phenolic acids	n-C_6H_{14}-EtOAc-MeOH-H_2O (18:42:30:30)
Anthranoids	n-C_6H_{14}-CH_3CN-MeOH (40:25:10)
Naphthoquinones	n-C_6H_{14}-CH_3CN-MeOH (40:25:10)
Cyclohexadienone der.	n-C_6H_{14}- EtOAc-94% EtOH-H_2O (83:33:67:17)
Retinals	Cyclohexane-n-C_5H_{12}-CH_3CN-MeOH (500:200:500:11)
Norditerpenes	$CHCl_3$-MeOH-H_2O (5:6:4)
Saponins	$CHCl_3$-MeOH-H_2O (7:13:8)
Tunichromes	i-AmOH-n-BuOH-n-PrOH-H_2O-HCOOH-(t-Bu)2S (32:48:40:120:1:4)

Table 2. Selected applications of the multilayer coil separator- extractor in the isolation of natural products. (3)

Substances separated	Solvent System
Flavonoids	$CHCl_3$-MeOH-H_2O (4:3:2)
	$CHCl_3$-MeOH-H_2O (33:40:27)
Flavonoid glycosides	EtOAc-H_2O— EtOAc-i-BuOH-H_2O
Lignan glycosides	n-C_6H_{14}-CH_2Cl_2-MeOH-H_2O (1:5:4:3)
Tannins	n-BuOH-0.1M NaCl (1:1)
Coumarins	n-C_6H_{14}-EtOAc-MeOH-H_2O (18:42:30:30)
Coumarin glycosides	$CHCl_3$-MeOH-H_2O (13:23:16)
Phenolic acids	n-C_6H_{14}-EtOAc-MeOH-H_2O (18:42:30:30)
Alkaloids	n-BuOH-Me_2CO-H_2O (8:1:10)
	n-BuOH-0.1M NaCl (1:1)
	CCl_4-MeOH-H_2O (20:20:2)
	$CHCl_3$-0.07M sodium phosphate (1:1)
Carotenoids	CCl_4-MeOH-H_2O (5:4:1)
Sesquiterpenes	i-C_8H_{18}-EtOAc-MeOH-H_2O(7:3:6:4)
Cardiac glycosides	$CHCl_3$-MeOH-HOAc-H_2O (5:3:1:3)
Anthranoids	n-C_6H_{14}-CH_3CN-MeOH (40:25:10)
Antibiotics	$CHCl_3$-MeOH-H_2O (7:13:8, 1:1:1)
	CCl_4-$CHCl_3$-MeOH-H_2O (5:5:6:4)
	CCl_4-$CHCl_3$-MeOH-H_2O (4:1:4:1)
	Et_2O-n-C_6H_{14}-MeOH-H_2O(5:1:4:5)
Marine natural products	$CHCl_3$-MeOH-H_2O (5:10:6)
	CH_2Cl_2-MeOH-H_2O (5:5:3)
	$ClCH_2CH_2Cl$-$CHCl_3$-MeOH-H_2O (2:3:10:6)
	n-C_7H_{16}-CH_2Cl_2-CH_3CN (10:3:7)

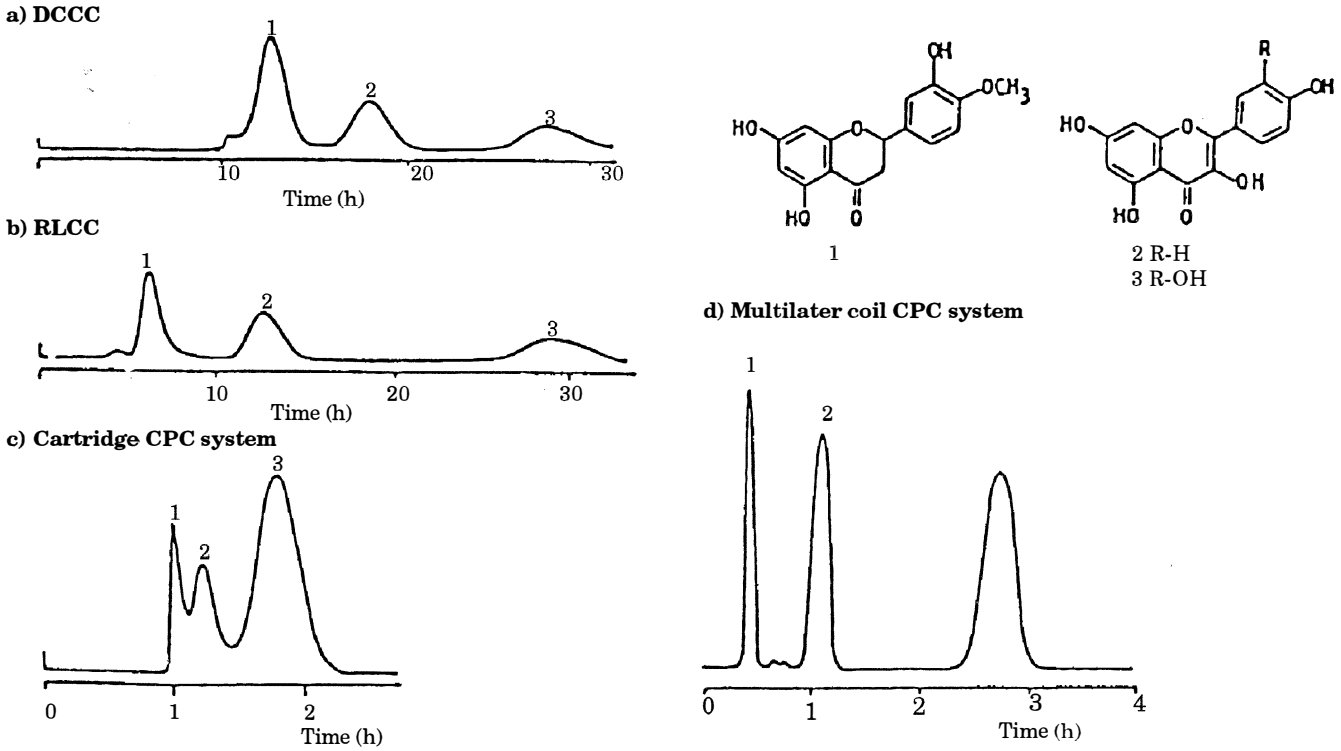


Figure 4. Separation of hesperetin (1), kaempferol (2) and quercetin (3). Solvent system: chloroform + methanol + water (33:40:27). Mobile phase: lower phase. Detection: 254 nm. (a) Flow rate: 48 ml/h; (b) flow rate: 18 ml/h; (c) flow rate: 2 ml/min. Rotational speed: 600 rpm. 6 cartridges; (d) flow rate: 3 ml/min. Rotational speed: 700 rpm.

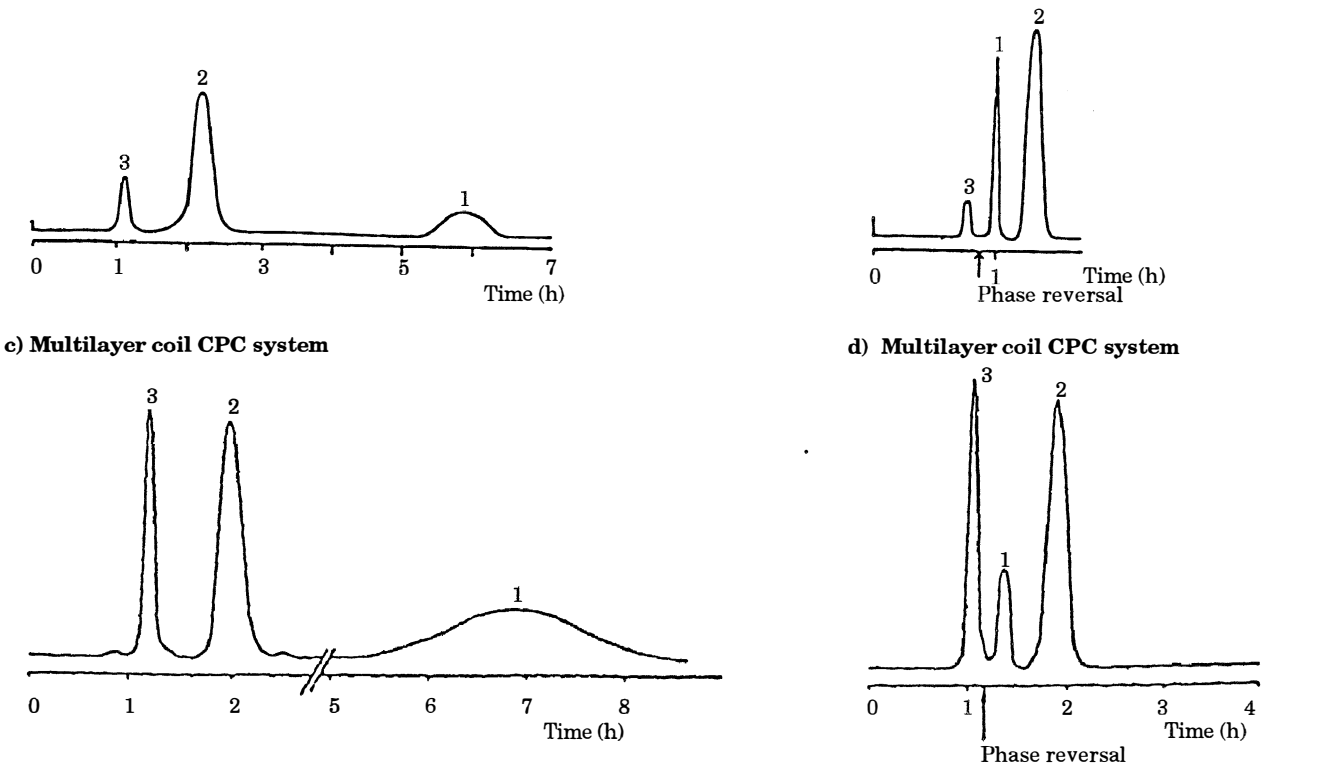
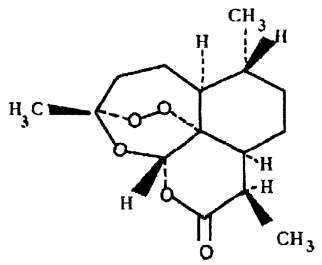
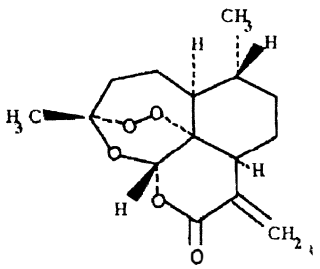


Figure 5. Separation of hesperetin (1), kaempferol (2) and quercetin (3) with phase reversal. Solvent system: chloroform + methanol + water (33:40:27). Detection: 254 nm. (a) Mobile phase: upper phase. Flow rate: 1 ml/min. Rotational speed: 600 rpm. 6 cartridges; (b) Mobile phase: upper phase to 55 min, then lower phase. Flow rate: 1.6 ml/min. Rotational speed: 600 rpm. 6 cartridges; (c) Mobile phase: upper phase. Flow rate: 3 ml/min. Rotational speed: 700 rpm; (d) Mobile phase: upper phase to 70 min, then lower phase. Flow rate: 3 ml/min. Rotational speed: 700 rpm.

Sequiterpenes

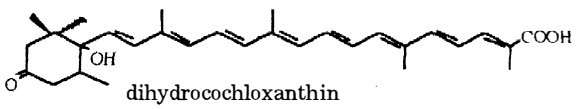


Artemisinin

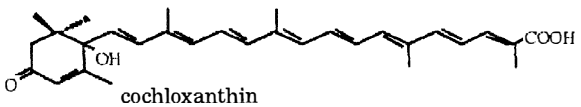


Artemisitene

Carotenoids

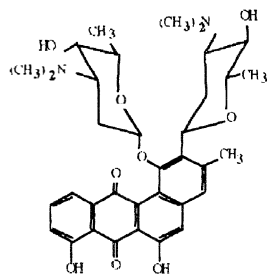


dihydrocochloxanthin

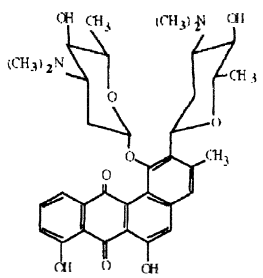


cochloxanthin

Antibiotics

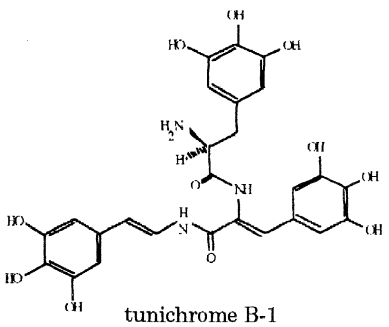


benzanthrin A



benzanthrin B

Marine Natural Products



tunichrome B-1

Figure 6. Some compounds isolated by CPC.

Figure 6 shows some compounds that have been isolated by CPC. A promising antimalarial agent, the sesquiterpene lactone endoperoxide artemisinin was separated from a contaminant, artemisitene by CPC. Neither silica gel chromatography nor crystallization was successful (17).

The isomeric carotenoids, dihydrocochloxanthin and cochloxanthin were separated within 2 h with a multilayer CCC from a methanol extract of the roots of *Cochlospermum tinctorium* (Cochlospermaceae) (18-19). Both HPLC and preparative TLC proved unsuitable for the isolation.

CPC is particularly useful for the isolation of antibiotics from microbial fermentation media (10). Antibiotics are very often produced in small quantities and have to be separated from other secondary metabolites and unmetabolized media ingredients. Furthermore, they are usually biosynthesized as mixtures of closely related congeners and many labile products. Therefore, mild techniques with high resolution are needed for isolation. CPC was used in the final purification step for benzanthrins A and B from *Nocardia lurida* (20).

The tunichromes are yellow blood pigments involved in the irreversible accumulation of vanadium in the sea squirt, *Ascidia nigra*. They are extremely labile and decomposed during HPLC. Using CPC, the isolation of tunichrome B-1 was achieved (21). A number of other marine natural products have also been isolated by CPC.

CPC is applicable to the entire range of polarity of natural products. Both aqueous and non-aqueous solvent systems have been employed (22-24). Biopolymers may be readily fractionated by CPC with aqueous two-phase solvent systems consisting of mixed aqueous solutions of water-soluble polymers such as dextran and polyethylene glycol (25).

CPC can handle milligram as well as multigram quantities and is also of practical use for the extraction of compounds from a large volume of solvent. In this case, the solvent is used as the mobile phase and a stationary phase which has a large affinity for the compound to be extracted is chosen. The extraction of metabolites from urine is an example of this application (26).

Conclusions

CPC has become an indispensable tool for the separation and purification of natural products. The wide range of applications, varying from apolar to very polar substances and from milligram to gram quantities, proves the versatility of CPC. The speed of the method, high recovery and high purity of isolated compounds make CPC an ideal separation tool in natural products research.

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