REVIEW ARTICLE
ISSN 2278 – 2818

COUNTER CURRENT CHROMATOGRAPHY: A TECHNIQUE FOR THE MEASUREMENT OF PARTITION COEFFICIENTS

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ABSTRACT

Counter current chromatography (CCC) can be thought of as occurring in three stages: mixing, settling, and separation (although they often occur continuously). Mixing of the phases is necessary so that the interface between them has a large area, and the analyte can move between the phases according to its partition coefficient. The simplicity of counter current chromatography (CCC) is sometimes overshadowed by a complex description of its mechanical and chromatographic attributes. The universal features of chromatographic theory relevant to CCC are summarized here, using a partition coefficient elution scale to present the chromatogram in a general, readily visualized, format. The principal types of CCC apparatus are summarized, along with selected applications and an indication of the type of apparatus best suited for some specific applications.

KEYWORDS: Counter current chromatography, partition coefficient, liquid-liquid chromatography,

INTRODUCTION

Counter current chromatography was originated in the work of (Martin and Synge, 1941, Synge 1946) carried out in Britain during World War II. For their work, Martin and Synge got the Nobel Prize in Chemistry in 1952.Soon after their work, Lyman Creighton Craig and Otto Post, 1949 developed an apparatus that consisted of a series of separatory funnels (tubes). The sample was automatically transferred through the apparatus and nearly 1000 mixing and separation steps could be achieved in a day. Individual components were separated based on their partitioning behavior.

Counter Current Chromatography (CCC) is an analytical technique which is used to separate individual or group of compounds from complex mixtures. Counter Current Chromatography or partition chromatography is a liquid-liquid chromatographic technique i.e. both the stationary and mobile phase are liquids. Here separation of components of a mixture is based on their difference in affinities for mobile and stationary phases of a column. More specifically, in liquid-liquid chromatography the solid adsorbent is replaced by a stationary liquid which is only partly miscible with the flowing liquid[1].Counter Current Chromatography is very useful in the separation of natural products [2].

In CCC, the stationary phase occupies up to 90% of the total volume of the column. Due to the liquid nature of the stationary phase, Counter Current Chromatography is a liquid chromatography (LC) technique that uses special columns. The advantages of having a liquid stationary phase in chromatography are: (i) A high loading capability, (ii) A very simple solute retention mechanism (liquid-liquid partitioning), (iii) Either phase of the biphasic system can be used as a mobile phase, (iv) No irreversible solute adsorption, (v) No pH problem, (vi) Less biological solute denaturation, (vii) permanent adsorption of the analyte onto the column can be avoided, (viii)Nearly 100% recovery of the analyte can be done, (ix) Most instruments can be operated in normal or reverse-phase modes, (x) Solvent cost are cheaper than for HPLC, (xi) Cost of purchasing and disposing of solid adsorbents is completely eliminated. The high loadability is possible because the solutes reach the volume of the liquid stationary phase and not just the surface of the solid phase as in the classical Liquid Chromatography[3].

Stages of Counter Current Chromatography

Counter Current Chromatography generally occurs in three stages: mixing, settling, separation.

a) Mixing
Is done so that the interface between them has a large area, and the analyte can move between the phases according to its partition coefficient.

b) Settling
After mixing with the mobile phase, settling occurs in the stationary phase.

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c) Separation
The stationary phase retention is an important parameter. Higher quality instruments have greater stationary phase retention. The settling time is a property of the solvent system and the sample matrix, both of which greatly influence stationary phase retention[4].

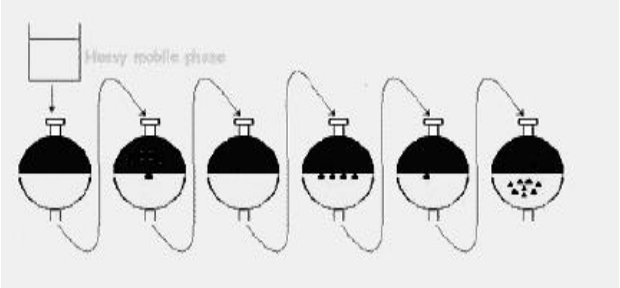

Figure 1. Various stages of Counter Current Chromatography

Theory of Counter Current Chromatography.

In case of CCC: For a given substance "A" the partition

Partition Coefficient K = Cs/Cm

where:
Cs: Concentration in Stationary Phase; Cm: Concentration in Mobile Phase
Good K values lies between 0.5 and 2
If K<0.5 Loss of Peak resolution [5]
If K>2 Long retention time, Peak broadening (through application of EECCC methodology) raises the K value for a good separation to much higher [6].

Equation for retention time:

$$t_R = \frac{V_C}{f}\{1 + S_F(K-1)\}$$

where:
f: flow rate; V_c: coil volume; t_R: retention time; S_f: stationary phase fraction ($Sf = V_s/V_c$)

Modes of operation

- **Head to tail:** The dense phase is pumped from beginning to end as the mobile phase.
- **Tail to head:** Here the less dense phase is used as the mobile phase.
- **Dual Mode:** The stationary and mobile phases are reversed part way through the run [7].
- **Gradient Mode:** The concentration of components in the mobile phase is varied throughout the run to achieve optimal resolution across a wide range of polarities.
- **Elution Extrusion Mode (EECCC):** The mobile phase is extruded after a certain point by switching the phase being pumped into the system.

- **pH Zone Refining:** Acidic and basic solvents are used to elute analytes based on their pKa [8].

Types of Counter Current Chromatography

1. Droplet Counter Current Chromatography (DCCC).
2. Elution Extrusion Counter Current Chromatography (EECCC).
3. Centrifugal Partition Chromatography (CPC).
4. Simulated Moving Bed Chromatography (SMBC).
5. High Speed Counter Current Chromatography (HSCCC).

1. Droplet Counter Current Chromatography [1] (DCCC)

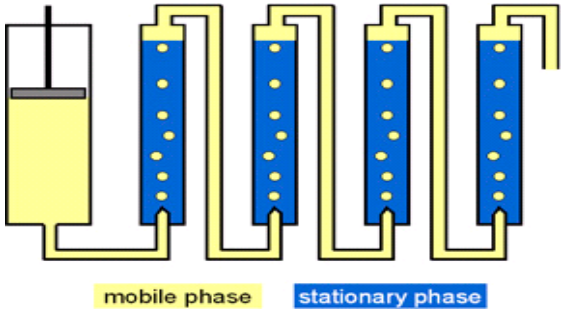

Figure 2. Diagrammatic Representation for Droplet Counter Current Chromatography

DCCC is a form of liquid-liquid chromatography where droplets of solute move through a stationary column of liquid phase by gravity i.e. it uses only gravity to move the mobile phase through the stationary phase. The mobile phase may be either dense or less dense. When dense the droplets of the mobile phase and the sample are allowed to fall through a column of the lighter stationary phase using only gravity (descending mode). If a less dense mobile phase is used it will rise through the stationary phase (ascending mode). When a droplet reaches the top of the column, it is delivered to a succeeding column of stationary phase in order to be further partitioned. More the number of columns that are used, more theoretical plates can be achieved.

ADVANTAGES

DCCC is very compacting, simplistic in design and easily operable and it can be used for the separation of milligram quantities of biochemical compounds.

DISADVANTAGES

The disadvantage of DCCC is that flow rates are low and poor mixing is achieved for most binary solvent systems, thus making this technique both time consuming and inefficient[9].

APPLICATIONS

1. Separation of glycosides ruberythric acid and lucidinprimeveroside by DCCC: The glycosides ruberythric acid and lucidinprimeveroside are commercially available as a mixture and were separated by droplet counter-current chromatography (DCCC) in ascending flow with chloroform–methanol–water (5:5:3) as eluents prior to their use as standard. Only one mixed fraction was collected. In one DCCC run 26 mg lucidinprimeveroside and 29 mg ruberythric acid were purified from 500 mg crude ruberythric acid. The separation of the glycosides was achieved far more readily with DCCC than by conventional chromatography [10].
2. Plant antiviral agents. Isolation of antiviral phenolic glucosides from Populus cultivar Beaupre by droplet counter-current chromatography
3. Separation of non-polar compounds by droplet counter-current Chromatography.
4. Isolation of virginiamycin-M1by droplet counter-current chromatography.
5. Isolation of parthenolide by droplet counter-current chromatography.
6. Macrocyclicpyrrolizidine alkaloids from Senecioanonymus. Separation of a complex alkaloid extract using droplet counter current Chromatography.
7. Application of droplet counter-current chromatography to the isolation of vitamin B12 [11].
8. Pharmacognostical studies of Tabernaemontana species.

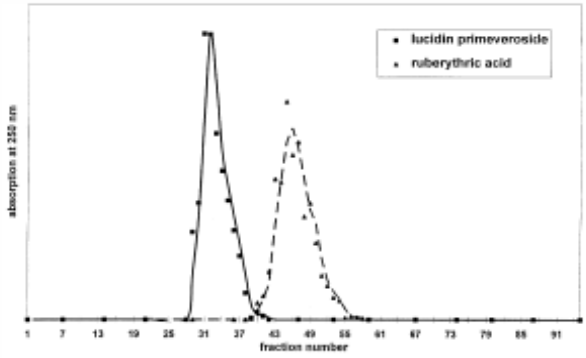

Figure 3. Reconstructed chromatogram of DCCC separation.

2. Elution Extrusion Counter Current Chromatography (EECCC).

In EECCC the mobile phase is extruded after a certain point by switching the phase being pumped into the system. A CCC column is used in which a sample mixture of seven compounds is injected. Separation starts almost immediately after injection.

The affinity of each compound for stationary and mobile phase is different. The dark blue compound as shown in the figures has high affinity for mobile phase hence is eluted out faster as it is travelling at same speed as that of the mobile phase. This is a normal elution process. Slower moving compounds have high affinity towards the stationary phase therefore take longer time to elute out.

In order to speed up the run and to eliminate excessive peak broadening we now start the extrusion step. To begin this process we simply switch the phases we are pumping into the system. The stationary phase now becomes the mobile phase. Now the compounds travel at a faster rate through the column and are extruded faster than if we have simply continued with the classical elution process. In fig 9 extrusion step is started earlier even before the first compound has been eluted. Once the compound are fully separated inside the column there is no reason to continue elution, we simply push them out.

Figures Showing Full Elution Extrusion Counter Current Chromatography [12]

Figure 4. Representation of a CCC column which has been equilibrated in its ready for sample to be injected

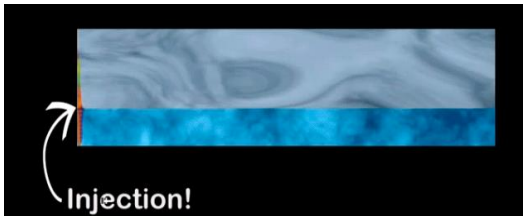

Figure 5. Injection of a sample mixture of seven compounds

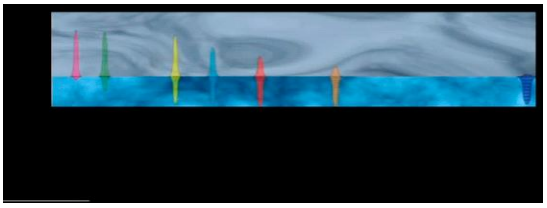

Figure 6. Separation starts almost immediately after injection

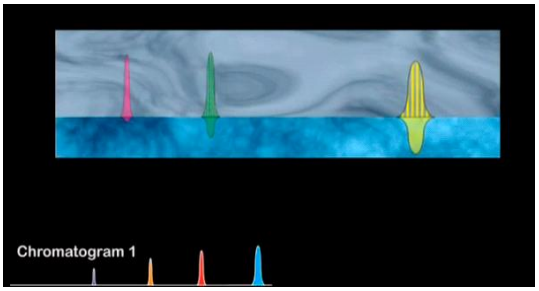

Figure 7. Each sample on elution from the system gives a chromatogram peak

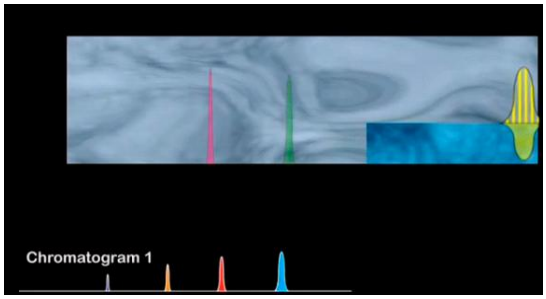

Figure 8. Extrusion step (the phase being pumped into the system is switched)

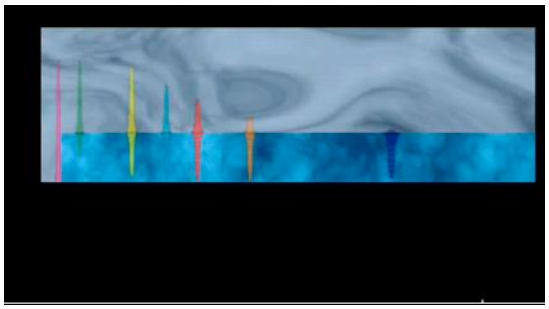

Figure 9. Extrusion step started earlier (even before the first compound has eluted).

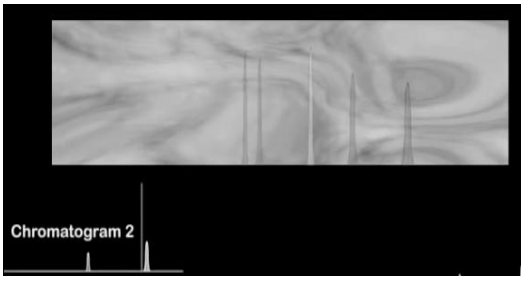

Figure 10. Final Chromatogram of Elution Extrusion Chromatography Technique

ADVANTAGE

EECCC is time saving, less solvent is used and it eliminates problem to the peak broadening.

3. Centrifugal Partition Chromatography (CPC)

Centrifugal partition chromatography (CPC) utilizes centrifugal force to enhance phase separation, and thus provides a new dimension in the area of separation science [13]. CPC is based on liquid–liquid partitioning technique and is an excellent alternative to evade the problems associated with solid phase adsorbents, and helps in preserving the chemical integrity of mixtures subjected to fractionation and isolation [14].The CPC is comprised with a unique rotor (column) which rotates on its planetary axis, offers a wide rotation speed range (500 to 2000 rpm). The rotor is filled with the stationary phase and the mobile phase is pumped through it i.e. one liquid phase is made mobile while the other one is made stationary inside the column by a constant centrifugal force. Centrifugal Partition Chromatography can be operated in both ascending and descending mode and the mode of operation depends upon the force generated by the rotor. CPC is gaining importance as a preparative separation method [15].

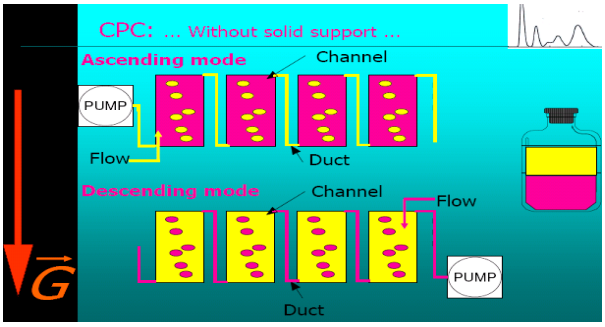

Figure 11. Centrifugal Partition Chromatography

4. Simulated Moving Bed Counter Current Chromatography

Simulated moving bed counter current chromatography is an important technology applied to difficult separation problems in petrochemical, fine chemical and pharmaceutical industries. The process employs a series connection of several columns. These are switched periodically against the fluid flow (Fig 12), which 'simulates' a counter-current of the two phases. Thus allowing continuous separation and often superior performance when compared to classical single-column chromatography [16].

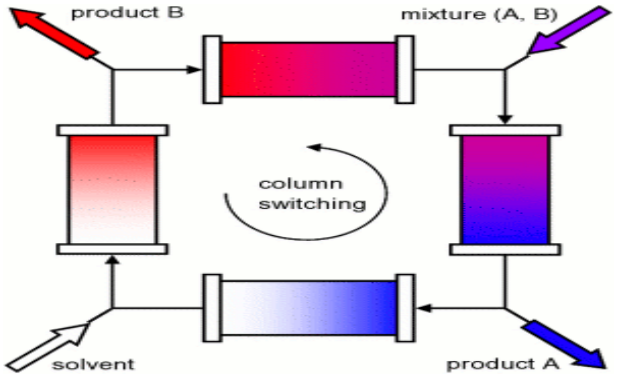

Figure 12. Figure showing general principle of SMBCCC

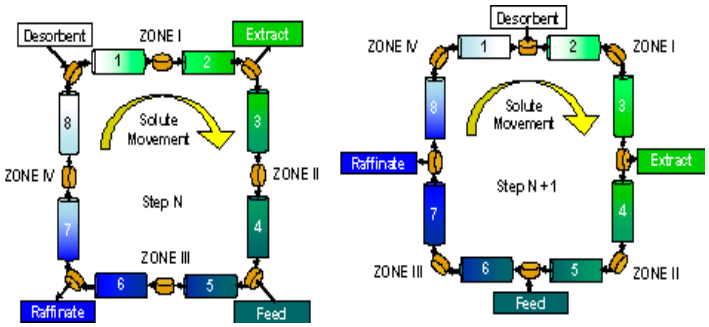

Figure 13. Figure showing working of Simulated Moving Bed Counter Current Chromatography

5. High-Speed Counter Current Chromatography (HSCCC)

The high-speed counter current chromatography consists of a helical coil of inert tubing which rotates on its planetary axis and simultaneously rotates eccentrically (Not symmetrically with respect to the center) about another solar axis. These axes can be made to coincide. The effect is to create zones of mixing and zones of settling which progress along the helical coil at altering speed. This produces a highly favorable environment for chromatography [17].

Comparison of Centrifugal Partition Chromatography with HSCCC

The rotor in case of Centrifugal Partition Chromatography rotates on its central axis while in case of HSCCC column rotates on its planetary axis and simultaneously rotates eccentrically about another solar axis. CPC offers a broader rotation speed range from 500 to 2000 rpm than HSCCC thus allowing a better decantation and retention for unstable biphasic system.

High Performance Counter Current Chromatography (HPCCC)

High Performance Counter Current Chromatography (HPCCC) is an orthogonal and complementary liquid chromatographic technique. In comparison to solid phase techniques, like reverse or normal phase liquid chromatography e.g. (prep-HPLC) or SFC, the HPCCC stationary phase is a liquid rather than a solid (as it is a type of CCC). High resolution separations are possible due to differences in the liquid-liquid partitioning behavior of sample solutes i.e. difference in partition coefficient. Preparative scale (mg to kg) chromatographers who use conventional chromatography techniques should consider using this technique as this technique can provide productivity improvements in many applications.

Comparison of High Performance Counter Current Chromatography (HPCCC) with HPLC [18]

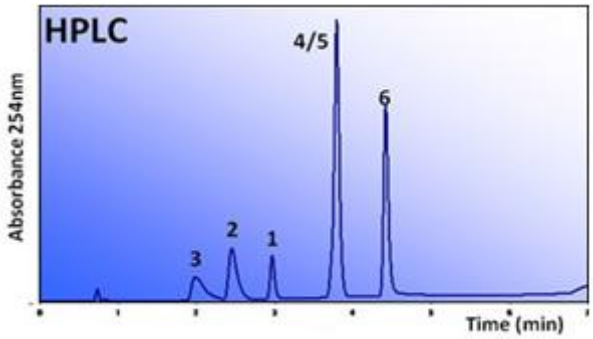

Figure 14. Shows the RP-HPLC chromatogram of a test mixture in which components 4 and 5 co- elute

The test mixture comprises:
1. Dipyridamole
2. 4-Bromobenzamide
3. Methyl 4-amino-3-methylbenzoate
4. Warfarin
5. Methyl 2-acetamido-5-bromobenzoate
6. Biphenyl

Figure 14. RP-HPLC of Test Mixture
HPLC separation conditions:
Gemini NX, 3µm, C18, 110 A (50 x 4.6mm). A= water+0.1% TFA, B= methanol +0.1% TFA
Gradient: 0 - 3.5min 40-100%B; 1ml/min; UV 254nm.

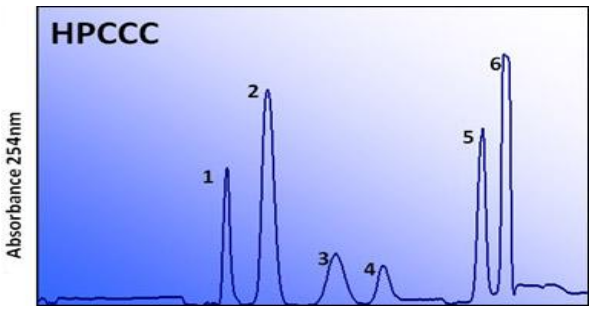

Figure 15. Shows the HPCCC chromatogram of the same mixture where the elution order has changed and components 4 and 5 are well resolved.

Figure 15. HPCCC of Test Mixture
HPCCC separation conditions:
Hexane/Ethyl acetate/Methanol/Water:: 1:1: 1:1, HEMW at SS 17 + 0.1%TFA2ml/min classical elution for 12min. Extrusion for 8 minutes at 4ml/min.

The differences are a consequence of the different selectivities of the two techniques: HPLC selectivity depends primarily on a partition mechanism between the mobile phase and the chemically bonded stationary phase, which in this case is C18, but is also influenced by secondary mode interactions with the support matrix (silica) such as ionic interactions of basic compounds with acidic silanols and gel permeation (molecular sieving).

Applications of HCCC/CPC Technologies

HSCCC/CPC Technologies have Applications in the Following Industries:
1. Nutraceuticals
2. Fine Chemicals
3. Pharmaceutical
4. Bio-Medical
5. Biotechnology
6. Fats and Oils
7. Fermentation

Compounds That Can Be Isolated in High Purity by HSCCC/CPC Technologies:
1. Saponins
2. Alkaloids
3. Chlorophylls
4. Tannins
5. Carotenoids
6. Phospholipids
7. Fat soluble vitamins
8. Mono/Oligo-saccharides
9. Anthocyanins
10. Lignans
11. Phenolic compounds
12. Synthetic Compounds
13. Other active compounds present in MAP's

The Advantages of HSCCC/CPC Technologies over HPLC

Figure 16. HPLC

1. Expensive columns
2. Irreversible adsorption
3. Poor loadability
4. Loss of biological activity
5. Ratio is low

HSCCC/CPC

Figure 17. HSCCC/CPC

1. No column
2. High recovery
3. High throughput
4. Retention of fragile Compounds (molecular integrity)
5. Volume ratio of stationary/mobile very high (better resolution)

CCC as a liquid–liquid reactor for catalytic reactions

A CCC machine, which is a chromatographic column with a liquid stationary phase, can be used as a liquid–liquid reactor for chemical reaction involving a liquid catalyst. The reduction of benzaldehyde into benzyl alcohol by sodium formatewascarried out at room temperature in an aqueous phase when a ruthenium–triphenylphosphine–trisulfonate complex liquid catalyst was used.

The CCC machine works as a plug-flow reactor. There are two significant advantages to use the CCC chemical reactor. First, the chemical process is continuous. The products may interact with the stationary phase and be retained. This could displace the chemical equilibrium toward the desired product formation.

Second, the CCC reactor can be used to evaluate rapidly the properties and capabilities of a new liquid catalyst. This capability should allow a rapid optimization of the use of a new liquid catalyst in a given chemical reaction.

The CCC chromatographs are made with Teflon tubes that are oxygen permeable. It is also difficult to raise the temperature. Specially adapted CCC machines with stainless steel tubing should be designed for chemical reactions.

CONCLUSION

1. Solid supports are eliminated in case of counter current chromatography.
2. Permanent adsorption of analyte onto the column is avoided and thus 100% recovery of analyte can be achieved in case of CCC.
3. More than 99 % purity of compounds from complex samples can be achieved.
4. Counter Current Chromatography is useful in the separation of natural products.
5. Solvent costs in case of CCC are also generally cheaper than for HPLC.
6. CCC offers cost savings in equipment cost by using lower flow pumps, and also savings in solvent and process cost.
7. Scale up is linear and predictable in case of CCC.
8. Quick (high throughput in preparative separation)
9. Inexpensive (only solvent costs, which is 5 times less than other LC techniques)
10. Gentle and versatile, for separation of varied compounds, with less chance of decomposition

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