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Editorial

Separation and Purification with a Liquid Stationary Phase

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Abstract: Yoichiro Ito introduced countercurrent chromatography (CCC) in 1966, reporting the separation of blood plasma cells with a sealed helical tube. Since then, CCC has been a fertile ground for instrumental and technical innovation. The key innovation of CCC was to use centrifugal forces to retain the stationary liquid phase in the column in such a way that it is able to interact dynamically with the mobile phase without any solid support. The broad diversity of countercurrent separation terminology reflects the innovative spirit of the field, as well as the global appeal of this technique. The selection of the appropriate biphasic liquid system is the core of the CCC technique. The CCC columns must generate the centrifugal field needed to maintain the liquid stationary phase; therefore, they cannot be a simple tube with frits at both ends. Rotors, motor, gears, spools, and rotating seals are very specific things that are not needed in a classical liquid chromatography column with a solid stationary phase. The differences between the two main types of CCC columns are described. The bases of the CCC theory are also given.

Keywords: countercurrent chromatography; centrifugal partition chromatography; biphasic liquid system

1. Introduction

This text was especially prepared for the present special issue on countercurrent chromatography (CCC). Its title intentionally does not contain the words "countercurrent chromatography". Indeed, there is a problem with the naming of the technique. CCC uses a biphasic liquid system to perform chromatographic separation and/or purification of a large amount of material. The CCC column is able, without any solid support, to hold one liquid phase, the stationary phase, while the other liquid phase, the mobile phase, is flown through [1–3]. Here is the problem: there is no countercurrent phase circulation in CCC, so only one pump is used to push the mobile phase, as in any other chromatographic technique. The liquid stationary phase is held by centrifugal forces. Since it is a liquid, it may move in waves inside the CCC column. But if its volume does not change during the separation/purification process, there is no countercurrent circulation. If the liquid stationary phase volume changes during the experiment, which occurs frequently, then it is called column "bleed"; there is a small leak of liquid stationary phase due to either a too-high mobile phase flow rate, or changes in interfacial tension induced by the sample. This stationary phase "bleed" is absolutely not going in a countercurrent direction, but rather in the same direction as the mobile phase, and in reality modifying the observed chromatogram by noise in the detector and peak shifts.

The CCC technique naming problem has been discussed in international CCC symposia starting in Hangzhou (2012), and was addressed in London (2014) as follows: it is not necessary to use the countercurrent chromatography terminology and CCC acronym when performing chromatography with a biphasic liquid system and a support-free liquid stationary phase. However, considering the long use of these terms and this acronym, it is accepted that they represent all chromatographic techniques that work with two liquid phases with no solid support. In all cases, whether the CCC

terms are used or not, it is strongly recommended that the "countercurrent chromatography" keywords and/or the CCC acronym be put in the Keyword section, so that search engines can easily identify and find all relevant articles dealing with this topic [4]. Other naming, such as "centrifugal partition chromatography" for CCC using hydrostatic columns [5], or "countercurrent separations" [6], are acceptable if the appropriate CCC keywords were added in the manuscript for correct indexing [4].

To finish this introduction focusing on the confusing naming of the CCC technique, a call to common sense is made here. In the last century, Yoichiro Ito introduced a new flow-through centrifuge CCC that was ten times faster than the then-used Craig machine [7]. In 1975, more than forty years ago, it was legitimate to call it "high-speed" CCC. This CCC machine is still used today, and still needs five to twelve hours to complete the purifications, although much better and faster CCC columns have been developed. The "high speed" term, however, is obsolete today, given that the most difficult chromatographic separations are commonly measured by the second [8]. It was recommended to avoid the term "high-speed" CCC for situations where the separations last more than one hour [4]. In this special issue, you will find separations lasting more than six hours that are still dubbed "high speed". This is seen as ridiculous by our chromatographer colleagues, but it is so difficult to change long (and bad) habits that I have simply allowed the inclusion of inappropriate terms and acronyms. Please, young CCC scientists, be logical; if your CCC separation last for more than one hour, do not call it "high speed", since it is actually very low speed. Just call it CCC, period.

2. The Two Main Types of CCC Columns

Once again, there is no countercurrent fluid circulation in a CCC column. In all CCC columns, centrifugal fields are used to maintain the liquid stationary phase. The two main types of CCC columns are (i) hydrodynamic CCC columns and (ii) hydrostatic ones. Neither column looks like a tube, as in classical LC or gas chromatography (GC); centrifugal fields imply rotation, rotor, gears, pulley, connecting rotary seals, and motors with speed regulators, so the CCC "columns" are actually machines with rotating parts.

2.1. Hydrodynamic CCC Columns

This CCC column design works with a special arrangement involving two axes of rotation, so that bobbins of coiled open tube can be rotated in a planetary motion: like Earth, they rotate around their own axis and simultaneously around the central axis (Figure 1A). In this arrangement, the two liquids inside the coiled tubes are subjected to a variable centrifugal force. There is (i) a decantation zone with a strong field that separates the two liquid phases, and (ii) a mixing zone with a weak and inverted field that mingles the phases (Figure 1A). The advantages of this design are (1) there is no rotary seal, (2) the mobile phase is always in contact with the stationary phase, producing a good chromatographic efficiency (thinner peaks), (3) a low driving pressure (the mobile phase pump work with less than 5 bars or 70 psi), and (4) easy cleaning of the machine with little dead or inaccessible volumes. The drawbacks of this hydrodynamic design are (1) noise and vibration can be observed, (2) some biphasic liquid systems (especially the polar ones) are not or are only very poorly retained, and (3) there is always tube wearing inside the machine requiring surveillance and periodic replacement of the leading tubes [1–3].

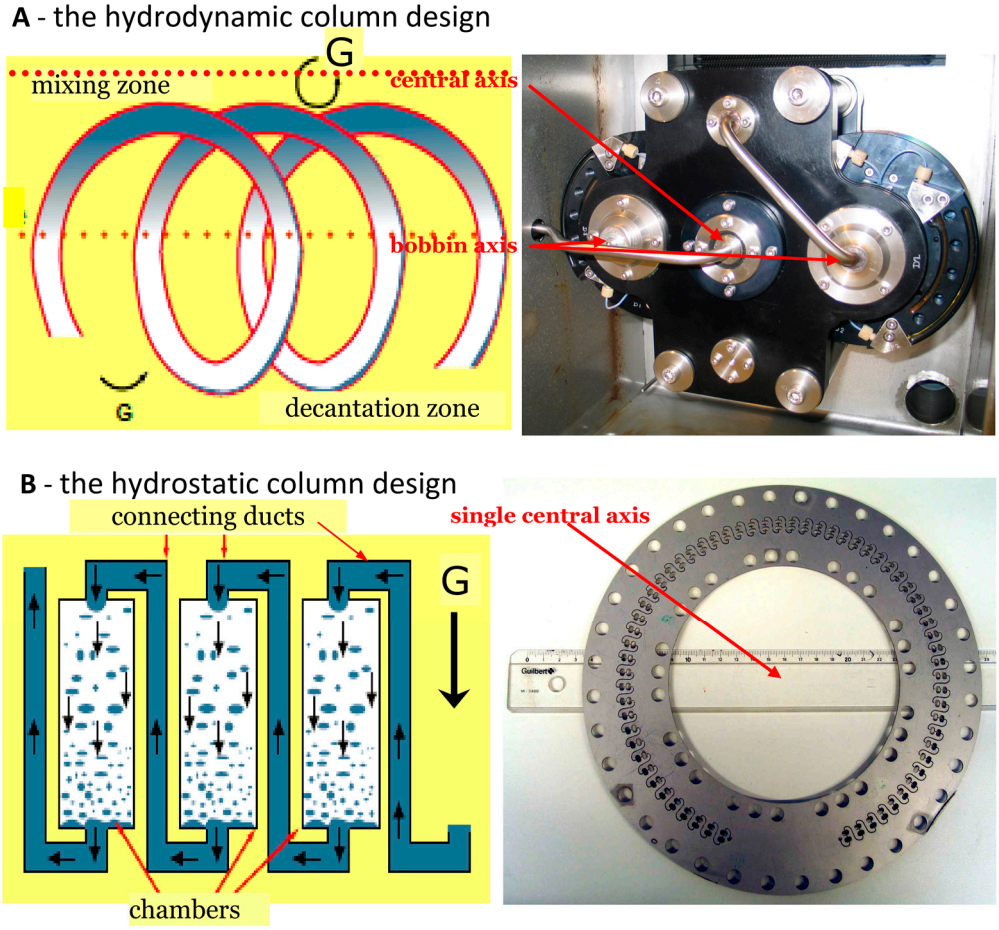

Figure 1. Countercurrent chromatography (CCC) column designs. (**A**) The hydrodynamic design combining bobbin and rotor rotation to obtain a planetary motion generating a variable centrifugal field with mixing and decantation zones. The *Spectrum* two-bobbin rotor of the Dynamic Extraction hydrodynamic column is shown on the right. (**B**) The hydrostatic design with interconnected simple rectangular chambers (left). An actual disk from a modern *Kromaton FCPC-C* hydrostatic column is shown on the right with its patented dual-cell design (64 dual cells in a disk).

2.2. Hydrodynamic CCC Columns

CCC columns that use the hydrostatic design are called centrifugal partition chromatographs (CPC). In CPCs, there are actual interconnected chambers that retain the liquid stationary phase (Figure 1B). A single axis produces a constant centrifugal field *G* that tightly holds the liquid stationary phase. The advantages of this design are (1) any biphasic liquid system (polar or less polar) is retained, (2) the mechanical arrangement is simple, allowing for fast rotation speeds, meaning that the strong centrifugal fields hence elevate the mobile phase flow rates, and (3) they are quiet and reliable machines. The drawbacks of this hydrostatic design are (1) the mobile phase is not in contact with the stationary phase in the connecting ducts (Figure 1B) where no chromatographic exchanges are possible, so the CPC's efficiency is lower, at comparable volumes, than that of a hydrodynamic CCC machine with coiled tubes, (2) a hydrostatic pressure build-up in each chamber generates significant pressure—the mobile phase pump may work with between 2 and 50 bars or 30 to 700 psi, depending on the density difference between the two phases of the biphasic liquid system used, (3) there are two rotary seals at the CPC rotor connections, and these rotating seals are subject to wearing and must be checked frequently (depending on the liquid systems used), and (4) CPC machines are more difficult to clean than hydrodynamic machines [1–3].

2.3. Which CCC Column Is Best?

People want to know in which CCC column it is best to invest. It is first important to point out that it is not the CCC column, but the selected biphasic liquid system, that allows for good and/or useful compound separation/purification. This point being clear, I would recommend having a small-volume (say 20–50 mL) modern hydrostatic column (Kromaton or Armen-Gilson) that will be able to retain any biphasic liquid system and make quick trials without wasting large volumes of solvents. When a partial separation is observed, then move the separation to a larger-volume CCC column, either hydrodynamic or hydrostatic, whose volume fits the size of the sample to be purified [9]. Small adaptations will be needed to optimize the separation.

3. What Must Be Known When Doing CCC

CCC works with a particular biphasic liquid system. The selection of a biphasic liquid system that will be able to perform the desired separation/purification is a critical aspect of the method. Indeed, it corresponds to the simultaneous choice of the stationary phase and the mobile phase. In other liquid chromatography techniques, the column choice corresponds to the selection of the appropriate stationary phase, and subsequently, a particular mobile phase is prepared. Again, in CCC, both mobile and stationary phases go together. Any change in the mobile phase composition (gradient run, for example) will change the stationary phase composition, with dramatic possible consequences.

3.1. Mobile Phase Circulation

The unique advantage of CCC is that the liquid stationary phase can be any of the two liquid phases of the selected biphasic system. However, the choice of the liquid stationary phase will dictate the circulation direction of the mobile phase. When a hydrodynamic coil full of liquid is rotated with unobstructed ends, a projection of liquid will be observed on one end, and suction on the other end. This is due to an Archimedes pumping effect [2]. The coil end where the liquid is projected is the high-pressure side, called the "head", or top; the suction side is called the "tail", or bottom. Similarly, the chambers in the CPC rotor have a top and a bottom connection to the ducts.

Now, if the selected liquid stationary phase is the denser phase, it has a tendency to sit at the bottom of the chambers; so, to have a lighter mobile phase flowing through a denser stationary phase, we must make it enter through the bottom of the chamber in an ascending direction, referred to as "tail-to-head". Conversely, if the upper or lighter phase was selected to be the stationary phase, the denser mobile phase should be introduced into the column head or top, and flown in a "head-to-tail" or descending direction [1–3].

If a mistake in flowing direction is made, the liquid stationary phase will be extruded out of the CCC column. This property was actually used to terminate CCC separations in a method called "elution-extrusion CCC", which simply consists of switching the liquid mobile phase for its liquid stationary phase counterpart without changing anything else (i.e., same rotation speed, same flowing direction) [10]. After a useful separation has been obtained in the normal way, this allows the fast recovery of all remaining solutes retained in the liquid stationary phase, leaving a CCC column full of clean stationary phase ready for the next run.

3.2. Fundamental Retention Equation

The theory of CCC is very simple, since solute partitioning between the two liquid phases is a unique retention mechanism. No pore volume retention, no silanol interaction, and no reduced phase overloading are observed in CCC, in contrast to classical preparative LC. The CCC retention equation relates the solute retention volume, V_r, to the solute partition coefficient, K, and the CCC "column" mobile, V_M, and stationary, V_S, phase volumes [1–3]:

$$V_r = V_M + KV_S \quad (1)$$

There is nothing other than mobile and stationary liquid phase inside the CCC "column", so the column volume, V_C, is:

$$V_C = V_M + V_S \quad (2)$$

The unique property of CCC compared to all other chromatographic techniques is that the stationary phase volume is variable. It depends on numerous experimental parameters, including (i) the biphasic liquid system selected, (ii) the centrifugal field (rotor rotation speed), (iii) the mobile phase flow rate, and (iv) the CCC column used (hydrostatic or hydrodynamic). A special parameter, the stationary phase retention ratio, S_f, was introduced to make comparisons between CCC columns. *Sf* is defined as the ratio of the experimental volume of stationary phase retained in a CCC column over the column volume V_C:

$$S_f = V_S/V_C \quad (3)$$

If Equation (1) is rearranged using Equation (2), it becomes:

$$V_r = V_C + (K - 1)V_S \quad (4)$$

Equation (5) is an interesting form of the CCC retention Equation (1), showing that a compound with a *K* value of 1 is eluted at the column volume with $V_r = V_C$ for any CCC column and S_f condition [1]. Solutes with partition coefficient *K* higher than 1 have retention volumes that increase when more stationary phase is retained by the CCC column (higher S_f). The S_f contribution is negative for solutes with partition coefficient *K* lower than 1.

3.3. Peak Positions

The retention equations (either Equation (1) or Equation (5)) show that the mobile phase volume needed to elute a particular sample component depends on the CCC column condition (V_S volume) and *K*, the solute partition coefficient. The partition coefficient, *K*, is also called distribution constant and, in CCC, it is conventionally expressed as [1–3]:

$$K = \frac{[solute\ in\ e\ stationary\ phase]}{[solute\ in\ the\ mobile\ phase]} \quad (5)$$

where these coefficients are more often expressed as a concentration ratio of the solute in the organic phase over that in the aqueous phase, e.g., $K_{o/w}$ octanol/water partition coefficient [11]. It is important to realize that the position of any component of the sample can be predicted if its partition coefficient and the S_f ratio of the column are accurately known. The chromatogram cannot be entirely predicted, since the peak shapes depend on the CCC column efficiency. However, the peak position obtained in a separation by a given biphasic liquid system can be exactly predicted when this liquid system is used with the same sample on another (i.e., a larger) CCC column of whatever type.

It is important to realize that the solute partition coefficients are extremely dependent on the experimental conditions. In a recent study, we showed that the tabulated heptane/water benzoic acid partition coefficient was 0.2 at 20 °C. In CCC experiments, the observed benzoic acid *K* values could be as low as 0 with high pH aqueous mobile phases, because benzoic acid was carried as benzoate anion, rather than being retained by the heptane stationary phase. The benzoic acid experimental CCC *K* value could also be as high as 25 with an acidic mobile phase and benzoic acid dimerization in the heptane phase [12].

4. Conclusions

Countercurrent chromatography is a liquid chromatographic method that uses a liquid stationary phase and an immiscible liquid mobile phase to perform large-scale (preparative) separation or purification of high-value compounds. There is no countercurrent circulation of fluids in CCC, the name being somewhat of a misnomer. The term centrifugal partition chromatography would be much

better as a generic term for CCC. For historical reasons, CPC is reserved for hydrostatic CCC columns, which doesn't make things easy to understand for newcomers to the technique. The modern trend is to develop reliable CCC "columns" of different volumes (between 20 mL for "analytical" columns and mg-scale purification, up to 5 L for larger columns and kg-scale purification) either based on hydrostatic or hydrodynamic designs. Small CCC "columns" allow the fast determination of the right biphasic liquid system to be adapted to the purification. Scale-up to a larger-volume CCC instrument is relatively straightforward, being mostly linear. It is pointed out that the CCC practice implies the knowledge of biphasic liquid system physico-chemical behavior and solute partitioning, which is poorly taught today.

Conflicts of Interest: The authors declare no conflict of interest.

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