Improving the Purification and Extraction of Natural Products with Centrifugal Partition Chromatography

Natural Product Purification by Centrifugal Partition Chromatography (CPC)
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Frequently Asked Questions about Centrifugal Partition Chromatography (CPC)
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Centrifugal partition chromatography (CPC), which involves liquid–liquid partition chromatography, has a host of potential applications for the purification of large and small molecules on both pilot and bench scales. CPC technology, also known as countercurrent chromatography (CCC), provides an efficient, streamlined workflow and is an ideal technique for natural product purification because it does not denature fragile molecules. The eBook on *Improving the Purification and Extraction of Natural Products with Centrifugal Partition Chromatography* discusses a variety of considerations for natural product purification.

First, Lauren Pahnke, field applications specialist at Gilson, breaks down how CPC works, including the general principles behind the technique, how to select the best solvent system for extracting natural compounds, and the effects of flow rate and rotation speed on retention, resolution and pressure. Pahnke also discusses several possible applications for CPC systems within the natural products space such as purifying cannabinoids from cannabis oil, removing THC from cannabis oil, purifying omega-3 fatty acids from microalgae, and purifying steviosides from Stevia.

In the subsequent companion article, Pahnke answers several key questions on this topic posed by audience members during a recent *LCGC* webcast.
Introduction

Centrifugal partition chromatography (CPC), also known as counter current chromatography, systems are preparative pilot- and industrial-scale liquid–liquid partition chromatography systems for the purification of various molecules and solutions. CPC uses two non-miscible liquid phases rather than a solid stationary support to prompt the separation of analytes in a sample. Analogous to a preparative high performance liquid chromatography (HPLC) column or flash cartridge, CPC systems require a prep-scale pump for the solvents, an injector to load sample, and optional system add-ons including a detector to observe the chromatogram and the fraction collector on the backend for collection of the separated component(s).

What follows is an overview of how CPC systems work and how they provide an automated purification solution for isolating compounds from natural product crude extracts when paired with compact chromatography systems.

CPC System Overview

A basic diagram of a CPC system (Figure 1) displays the rotor in the center held on a horizontal axis to allow for rotation. At each end of this axis are two rotary seals. Connections shown on the right indicate solvent flow from the pump onto the CPC rotor (or column) and to the detector/collector.
Natural Product Purification

CPC Column

- High pressure resistance rotating chromatography column (rotor)
- Disks composed of twin cells
  - Better retention of stationary phase
  - Allows for higher flow rates for faster separations

Figure 2: CPC column

CPC General Principle

Consider each of the twin cells in the rotor disk as an individual separatory funnel. CPC employs a solvent system of two immiscible liquid phases created by mixing two or more solvents. The stationary phase is maintained by a centrifugal force and the mobile phase is pumped through the liquid stationary phase. The example diagram in Figure 3 illustrates a typical twin cell containing butanol and water. Butanol is the lighter upper mobile phase while water is the heavier lower stationary phase.

Molecules A ($K_D = 1$), B ($K_D < 1$), and C ($K_D > 1$) are added to the solvent system, mixed, and the two phases separate out to show the distribution of molecules between the two phases based on the molecules’ partition coefficients ($K_D$). The molecules with a $K_D$ close to a value of 1, are evenly distributed between the two phases which is molecule A, while the molecules with a $K_D < 1$ are mainly retained in the upper mobile phase represented by molecule B. The molecules with a $K_D > 1$ are more retained in the lower stationary phase (molecule C). Once the separation takes place in one twin
cell, the upper mobile phase is moved to the next cell in the column allowing an additional separation to take place. This process will occur hundreds of times.

Much of what occurs within the column is the result of volumes and flow rates. If molecule B has a $K_D$ of 0, molecule B will elute after one volume of mobile phase is pumped through the CPC system. Flow rate will determine when it will elute. Therefore, if molecule A is truly $K_D = 1$, it will come off following the volume of mobile phase inside the column, plus one column volume of stationary phase pumped through the system. With a $K_D$ of 2, molecule C will come off the column after a volume of mobile phase plus two column volumes. This is the scenario when run parameters have been fully optimized.

**Solvent System Determination**

Choosing the solvent system in CPC is basically the same as selecting the column and eluent in HPLC. Depending on the end goal, it should be decided whether to operate the CPC in ascending or descending mode. Ascending is akin to normal phase, whereas descending is like reverse phase. This flexibility provides more options regarding solvent system selection.

When selecting a solvent system to use, the solvent system polarity must match the polarity of the molecules to be separated. Next, find the solvent system in which the partition coefficient for the compound of interest is 0.5–3. Note that if $K_D$ is too large, a long run time and a broad peak will result. A long run time increases solvent consumption and reduces productivity. If the $K_D$ is too small, coelution with other compounds is likely. The third objective when determining the solvent system is recognizing that the $K_D$ of the target compound must be different from other molecules’ $K_D$, thus avoiding coelution. Compounds having the same partition coefficient will pass through the column in a similar manner, resulting in no separation.

Once potential solvent systems are identified, the “shake flask” method can be performed to see if they are suitable for CPC analysis. For the “shake flask” method, first create the solvent system by mixing together the appropriate proportions of each solvent. Next, transfer a volume of both the upper and lower phases to a different vessel. Then, add the crude extract to the vessel and mix. Once cantation is complete, there will be partitioning of the molecules within the crude amongst the two phases. Finally, analyze the upper phase and the lower phase (typically with thin-layer chromatography [TLC] or HPLC equipment).

The partition coefficient for each molecule can then be determined within the crude for this particular solvent system tested by a direct comparison of the concentration of each spot on TLC in the upper phase and lower phase. This is followed by comparing the percent area of each peak in HPLC in the upper or lower phase.

**Steps of a CPC Purification Run**

Once a solvent system is determined, a CPC run consists of six main steps, which can be scaled up to a larger production-scale unit:

- Choose descending or ascending mode and program the mode into the method
so an automatic valve can switch to accommodate the setting.

- Load the CPC system with the stationary phase.
- Equilibrate the column with the mobile phase to determine the operating pressure of the current system as well as the retention of the stationary phase with current run parameters.
- Perform a sample injection.
- Assuming that a good operating pressure and good retention of stationary phase during the equilibration step is obtained, elute using the same conditions as equilibration. During elution, primary fraction collection will occur.
- Extrusion is performed and 100% stationary phase will be pumped back onto the column. This extrusion step accomplishes replenishing fresh stationary phase on the column while also allowing for fraction collection of molecules heavily retained in the stationary phase.

Once the methodology is optimized, CPC runs can be performed in an elution extrusion mode. Following the initial loading of stationary phase and equilibration at the beginning of the day, load the sample, elute, and extrude, and then repeat. This can be accomplished because fresh stationary phase is loaded on that CPC column during the extrusion step.

Effects of Flow Rate and Rotation Speed on Retention, Resolution and Pressure

Figure 4 illustrates that retention of the stationary phase will decrease with increased flow rate; however, retention will increase with an increased rotation speed. Once the solvent system is determined, the right balance between flow rate and rotation speed to ensure appropriate retention of that stationary
phase on the column must be found.

Resolution increases with rotation speed, while resolution decreases with increased flow rate (see Figure 5). Operating at too high a flow rate will cause loss of stationary phase, thus poor retention. An ideal separation cannot be achieved if attempting to operate the CPC with poor retention of the stationary phase.

As flow rate and rotation speed increase, pressure rises. This is one reason why the equilibration step is performed when developing methods. Changing the solvent system, flow rate, or rotation speed of the method, results in a change in the operating pressure of the system. Knowing the pressure at equilibrium is important when working toward optimizing the sample load capacity of that column.

“An ideal separation cannot be achieved if attempting to operate the CPC with poor retention of the stationary phase.”

Natural Product Applications
The are several possible applications for CPC systems within the natural products space, including purifying cannabinoids from cannabis oil, removing THC from cannabis oil, purifying omega-3 fatty acids from microalgae, and purifying steviosides from stevia.

An example comparing CPC and HPLC techniques for the purification of Caulerpenyne from seaweed (Caulerpa...
taxifolia) is shown in Figure 6. The HPLC chromatogram of the crude shows many impurities and additional purification by CPC analysis using Gilson’s CPC 250 and PLC 2250 Purification Systems results in the purified product. There is an increase in the isolated mass and yield, a reduction in solvent consumption, and a reduction in run time when the CPC is used as the purification method.*

Another example is a microalgae crude extract. During CPC purification, the fractions are collected and those fractions are dried down to obtain purified products. Several groups of fractions obtained by CPC are pooled, and spotted by TLC, resulting in a yield of 23% with 99% pure EPA fractions, and a 10% yield with 98% pure myristic ethyl ether fractions.

**Conclusion**

Centrifugal partition chromatography is a cost-effective solution for natural product purification. Having no column to replace or silica to recycle, there is low solvent consumption when compared with traditional chromatography techniques, and no extensive pre-treatment of the sample is requested. The crude material is diluted, filtered, and loaded directly onto the CPC system with no initial clean-up methods being necessary. CPC systems allow for high injection capacities ranging from milligrams to multi-kilogram scale. The technique provides the capability to produce enriched extracts and pure molecules.

*This work were done in collaboration with University of Nice and is published as “Caulerpenyne from Caulerpa taxifolia: A comparative study between CPC and classical chromatographic techniques,” Phytochemistry Letters, Vol.20, 2017, p.406-409.
During a recent LCGC webcast, Lauren Pahnke, field applications specialist at Gilson, discussed key aspects of centrifugal partition chromatography (CPC) that analysts should consider when determining the best parameters for natural product purification goals. A summary of that presentation appears on page 4 of this eBook.

In this article, Pahnke answers several key questions posed by audience members during the LCGC webcast.

What kind of solvents will I use with the CPC and in what quantities?

**Pahnke:** The average range we use includes heptane, ethyl acetate, methanol, and water. Once you start doing your research on solvent systems, it will branch out from there. As far as the kind of solvents, it’s really up to you and your research.

In terms of the general quantities, our typical guideline is 1.5 times the column volume. The model number of a Gilson CPC system indicates the milliliters of column volume. For instance, CPC 1000 PRO means 1,000 mL or 1 L of column volume. So, 1.5 x 1 L means you will use about 1.5 L of mobile phase and 1.5 L of stationary phase per run.

This amount can decrease once you fully optimize your method and determine routes to decrease runtime and so forth.

**Can sites expect to be up and running within a couple of days following the installation of a CPC system?**

**Pahnke:** If the initial shake flask testing is completed before the CPC system is on site, you’ll be a step ahead; however, I wouldn’t expect to be running at full production capacity within a couple of days. It will take at least a couple of weeks to optimize your solvent system and load (e.g., what concentration and volume of that sample can be loaded per run), and then optimize your workflow to reduce solvent consumption. From there,
“For some applications, we have methods to start you off with and we’ll provide tools/guidance for successfully optimizing methods to meet your purification goals.”

you’ll have to do back-end analysis of those fractions to get the ideal purity you’re looking for.

How does flow rate affect separation efficiency?

Pahnke: If you have an increased flow rate, you can expect the retention of that stationary phase to decrease, meaning that some stationary phase will be lost over the course of the run, which will then negatively affect the separation. It will decrease the resolution and cause a loss in separation capabilities.

Natural product crude samples often have components that cause foaming. How do you deal with this in CPC?

Pahnke: I’d like to highlight how samples are loaded into the column. Crude cannot be loaded directly onto the column because it is often viscous. So, we dilute that down, whether it’s in the mobile phase, the stationary phase, or a 50/50 mixture of both. If we see additional foaming from there, we’d have to proceed on an application by application basis.

Do you provide onsite demonstrations?

Pahnke: We do not provide onsite demonstrations right now, but we do have a short-term lease option available for our small-scale CPC units (e.g., the CPC 250 paired with PLC 2050) in the United States. This is a good option for laboratories that aren’t quite sure if CPC is the right technology for them.

Do you have ready-to-go methods that you offer for the separation and collection of cannabinoids in large-scale production?

Pahnke: Yes, we have some rough methods, but they are not fully optimized because each crude and the purification goals are different. For some applications we have methods to start you off with and we’ll provide tools/guidance for successfully optimizing methods to meet your purification goals. This is part of the application training provided during install of the instrumentation.
Can you load a sample solubilized in a solvent system different from the ones used for separation?

Pahnke: You can certainly try that when you have the CPC at your site. The one thing to be aware of is that changing the solvent you’re using to prepare your sample could have a negative effect on the column stability. It could cause a significant loss in pressure after the sample is loaded onto the column, meaning stationary phase is being lost.

Our sample doesn’t need the pre-treatment. How can you inject sample in CPC?

Pahnke: When we say you don’t need to pre-treat the sample, we’re saying you don’t need to perform an initial clean up by, for example, solid phase extraction or some other manner. When you get the crude, minor pre-treatment of your sample involves dilution in either the mobile phase, stationary phase, the 50/50 mixture, or any other solvent followed by filtration to remove particulates or dissolved solids. Sample preparation is something you’ll need to pay attention to when optimizing run parameters to ensure each variable modified doesn’t negatively impact your separation.

Do you have any experience with new aqueous phase systems in CPC?

Pahnke: Yes, we have done some isolation of natural products with this at Gilson. If you’d like more specifics about what molecules Gilson personnel have worked with and the results, please reach out to us and we would be happy to take a deeper look into your application with you.

Since the fractions are selected in a mobile phase solvent, how would you recommend removing them?

Pahnke: Many customers remove the solvent using a rotovap, and others use spray evaporation. Since the primary fractions are collected in a mobile phase solvent, you need to properly choose the solvent system implemented by CPC as well as the mode in which the CPC operates—ASC (normal phase) or DSC (reverse phase)—as these factors determine which solvents you’ll be tasked with removing from the final purified product(s).
What grade solvent would we use?

**Pahnke:** We recommend ACS reagent grade or higher, though a higher grade will typically provide better results. We also recommend that you keep the grade consistent from run to run for consistency.

What are typical power requirements for the CPC 1000 PRO?

**Pahnke:** Typical power requirements for the CPC 1000 PRO in the United States are just three 110V outlets. You can visit our website (www.gilson.com) or reach out to any of our Gilson representatives to receive user’s guides for the CPC and PLC, which provide more specifications.

How pure should the cannabis oil be to maximize removal of THC?

**Pahnke:** I have worked with many kinds of hemp oil that are about 2–3% THC and about 60–80% CBD with the goal of removing THC. We have customers operating the systems for more than 20 hours a day for at least five days a week and they are able to successfully remove the THC below that 0.3% threshold.

As for a cannabis sample that may contain more cannabinoids and other impurities, we’d have to address it on a case-by-case basis. Typically, the less impurities or dissolved solids present in the starting material, the better the instrumentation will perform, which will result in a better separation of your material. Reach out to us directly for more information.

Do you offer method development?

**Pahnke:** Yes, we have two options. We have a Gilson applications lab in France, where they can receive samples, and perform CPC development. To complete this, Gilson will need certain information pertaining to the crude material safety information, end goal, HPLC methodology, etc. Please contact Gilson for more information on this process. We currently do not have something like that set up in the United States, but we would be able to help you along in your optimization process for an application and will provide the tools you’ll need to complete this that on your own.
COST-EFFECTIVE PURIFICATION ALTERNATIVE

Advantages of CPC Equipment:

- Environmentally friendly, silica-free technique
- One-step purification process
- High recovery and purity