



Advanced Chromatography

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Abstract

Since chromatography is a complex and multifaceted topic, we decided to explore more advanced topics in a detailed white paper, in which we will be building upon a previously laid foundation of basic chromatographic knowledge.

In this installment we first recap the essentials of chromatography and liquid-liquid chromatography in particular, and then move on to more advanced centrifugal partition chromatography.

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Basic Chromatography

Chromatography is the physical methodology of separating a component from a mixture. In the chromatographic process components are divided into two media: stationary phase and mobile phase. Due to the driving force (which can be pressure difference, for example) the mobile phase travels through the system and carries sample components with it after the injection of the sample.

Components which interact stronger with the stationary phase will be more retained and move through the system slower than components with weaker interactions. This results in a change in elution time, meaning a difference in the time it takes for the components to travel through the system, creating separation.

The retention profile of the column and the physicochemical properties of the mixture define the kinds of compounds that will be found in the output.

Liquid-Liquid Chromatography (LLC)

Liquid-Liquid Chromatography is a combination of liquid-liquid separation principles combined with the methodology of chromatography. In this technique, separation occurs between two immiscible liquid phases, as both the mobile and stationary phases are liquids.

In an LLC system separation takes place based on the differing partitioning behavior of the solutes between the two phases. Since LLC uses a liquid stationary phase, the technique offers many advantages over traditional liquid-solid chromatographic separation techniques.

Advantages of LLC include:

- No need for silica gel, which cuts the cost of consumables and removes a polluting and expensive material from the process
- Silica degradation is not an issue
- No need for silica regeneration cycle or replacement
- High sample loading capacity
- The possibility of lossless sample recovery
- Separation of any compound that is soluble in a liquid phase
- Quick and simple method development, with predictable outcomes
- LC analysis is usually performed at room temperature and therefore compounds that are thermally labile can be separated
- The LC detectors that are used are usually not destructive and therefore the analytes may be collected for further analysis

Liquid-Liquid Chromatography can also be used for many different target components and can be optimized for several kinds of operating modes.

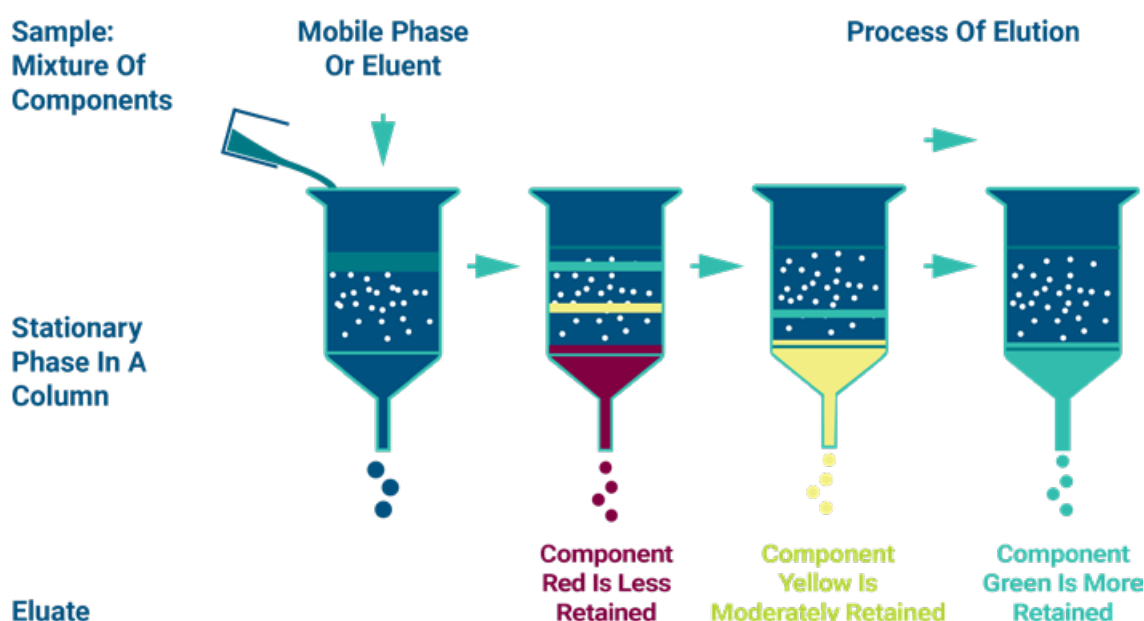


Fig. 1. Schematic representation of the chromatographic process in column chromatography.

Part 2

Centrifugal Partition Chromatography (CPC) & How CPC Works

Centrifugal Partition Chromatography (CPC)

Centrifugal Partition Chromatography obeys classical chromatographic rules. It is a **liquid-liquid preparative chromatographic technique**, in which separation occurs between two immiscible **liquid solvent systems**. **The liquid phases act as the stationary and mobile phases and a strong centrifugal force aids the whole process, immobilizing the liquid stationary phase.**

How CPC Works

An important feature of CPC is that the solute's retention depends simply on the liquid-liquid **partition coefficient (K_d)**. The partition coefficient or distribution coefficient is the relative affinity of the solute for the stationary phase. Practically, it is the ratio of the solute concentration in the stationary phase over the solute concentration in the mobile phase.

Separation of the injected sample is based on the sample component's varying partition coefficients between the mobile and stationary phases. For a specific Compound of Interest (Col), the Partition Coefficient is equal to the ratio of its concentration in the stationary phase to its concentration in the mobile phase.

To determine the value of K_d , the shake-flask method is routinely performed during method optimization and expressed as the ratio of the concentration of the compound in the upper phase to its concentration in the lower phase by convention. By rule of thumb K_d of the Col in an optimal solvent system should be preferably in the range of 0.5-2, alternatively 0.2-5.

This means that the K_d value dictates the **amount of time each compound spends in the mobile and stationary phases**. Therefore, it also dictates the **rate at which each compound travels through the system**.

Thus, the **retention volume (V_R)** of a given compound is simply calculated by the equation:

$$V_R = V_M + K_d \times V_s$$

Here V_M and V_s are the mobile and stationary phase volumes, respectively, inside the CPC system. Moreover, the ratio of K_d values determined for a pair of components (separation factor or selectivity; e.g., for Col and its impurities) clearly predicts their separability in the CPC run.

There are several core advantages of Centrifugal Partition Chromatography over classical, solid support-based preparative liquid chromatograph.

These **advantages of CPC** include:

- simplicity of separation mechanism
- easy method development and scale-up
- high loadability and recovery
- no sample loss due to irreversible adsorption
- no need for solid stationary phase and medium solvent consumption
- cost effectiveness.

Arrangement of CPC: What

Happens Inside

In CPC, traditional chromatographic columns are replaced by cells. These **CPC cells (columns) are interconnected in series by ducts attached to a large rotor filled with the liquid stationary phase (Fig.2)**. The liquid contained in this series of cells can then be immobilized inside the rotor by a strong centrifugal force.

The other phase of the two-phase CPC system is used as the **mobile phase containing the sample to be purified**. It is introduced into the rotor under pressure and pumped through the stationary phase in the form of tiny droplets. A valve allows for a change in the direction of flow and therefore the CPC system will work in either ascending mode, where the lighter phase is the mobile phase or in descending mode when the heavier phase is used as the mobile phase.

The **success or quality** of any given separation is shown by the so-called **resolution factor (R_s)**.

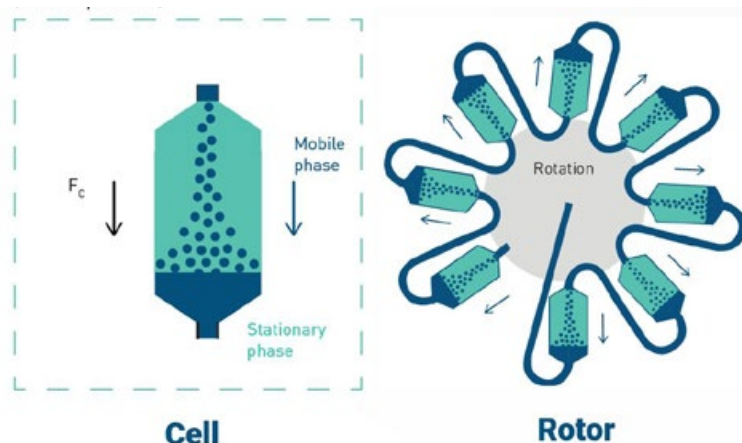


Fig. 2. Cell and rotor design, showcasing the 'descending' mode.

- If the R_s value is 1.5, it means that two adjacent peaks are separated with a baseline return, which is desirable.
- If the R_s value is lower than 1.5, it means that there is some peak overlap, which is not a completely pure separation.
- Resolution factors higher than 1.5 mean that there is space between the two peaks, which is the "holy grail of separation".

A major **difference between preparative HPLC and CPC** is the column - or rather the absence of a classic column in CPC. However, it is also important to mention that CPC and HPLC do not work in the same way.

The stationary phase in HPLC is an organic moiety bonded to a solid silica support and solvated mainly by the organic solvent of the aqueous organic mobile phase. Volume ratio of the stationary phase is only about 5% of the total volume of the column and it will not change significantly as the mobile phase composition varies.

In a CPC system, no silica gel, nor any organic moiety bonded to a solid support is necessary for functioning. Instead, a strong centrifugal force is introduced to solidify the immiscible organic solvent (e.g., hexane) as the stationary phase. The droplets of the water-organic mixture will then pass through the stationary phase.

The volume ratio of the stationary phase is 50-80% for most cases; the actual value depends on the instrument, the experimental conditions, and the composition of the ternary mixture used to prepare the mobile and stationary phases.

The difference in stationary phase volume between HPLC and CPC explains why the two techniques do not need the same number of plates to operate efficiently.

Ascending and Descending Modes

The column in a RotaChrom CPC machine is the rotor and the interconnected cells. This is the feature which gives way to the previously mentioned different methods of operation. The main factor which decides the mode is the density of the mobile phase.

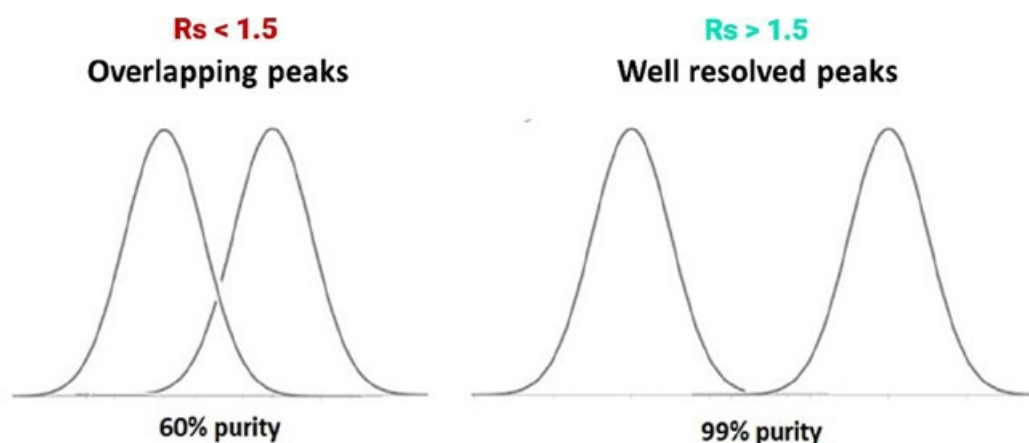


Fig. 3. R_s values visualised in two chromatograms. $R_s > 1.5$ shows baseline separation.

This is because a liquid mobile phase can be denser or less dense depending on the separation. The mobile phase's density is of course in comparison to the density of the immobilized stationary phase. This means that the mobile phase must be denser or less dense than the stationary phase.

When using the '**ascending**' mode, the mobile phase is the less-dense phase of the two phases. When this is the case, the direction of the flow becomes the opposite of the centrifugal force. When operating the machine in 'descending' mode, then the mobile phase becomes the denser phase.

Typically, operating in descending mode gives better separation

Operating a RotaChrom CPC device in both modes is possible. However, certain devices require more preparation than other ones. In the case of rCPC, one machine can be operated both ways. It doesn't need any extra parts or preparations. For the iCPC, however, the rotor must be switched depending on the mode you want to run it in. You need a different rotor for the ascending mode, and another one for the descending mode. However, the iCPC device itself otherwise can be the same.

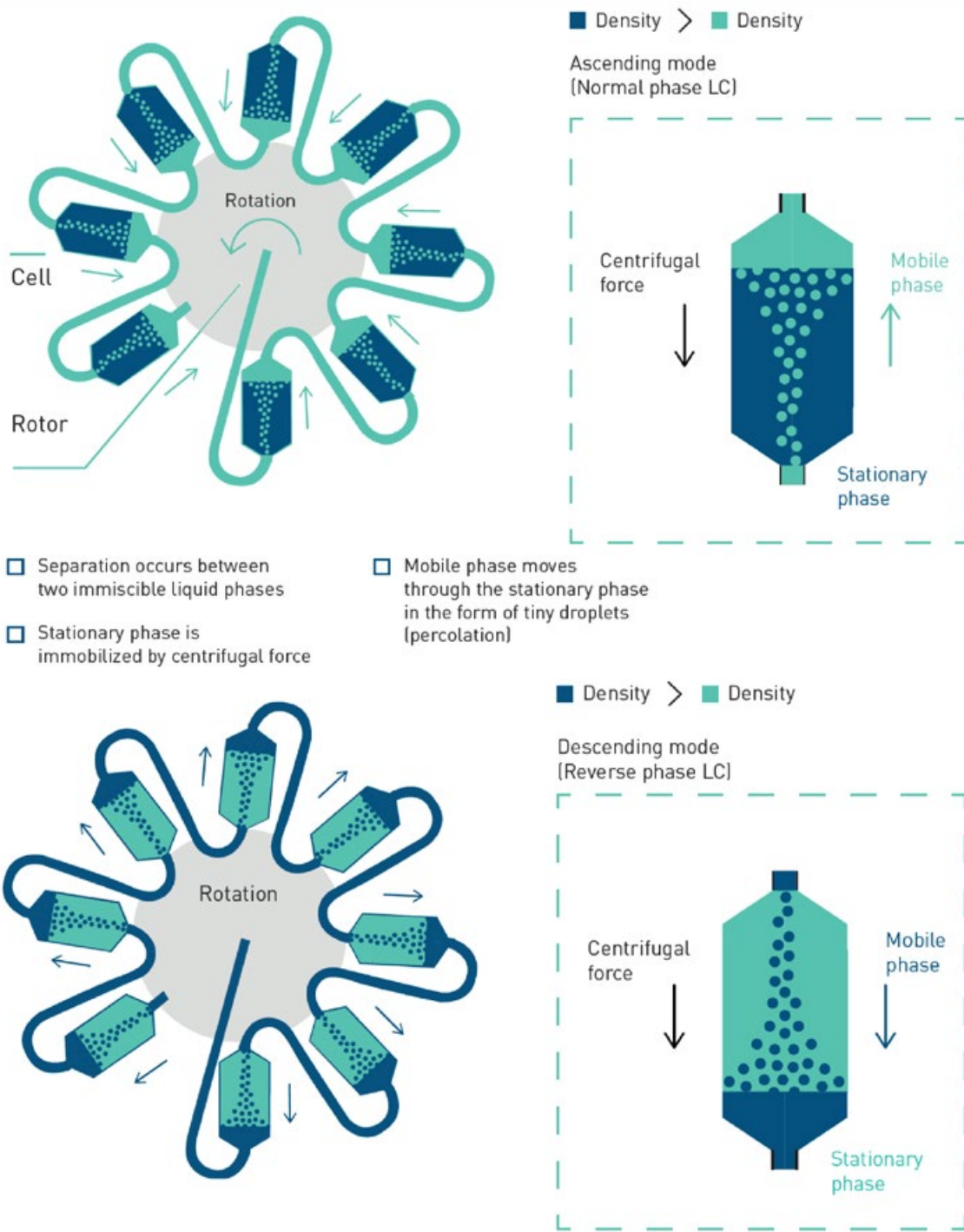


Fig. 4. Ascending (upper) and descending (lower) modes visualised.

RotaChrom's CPC design comes with several **advantages**, including:

- Exceptional Purity Over 95% purity of Col can be achieved.
- Scalability and Capacity: Our individual cell design allows easy scalability without impacting performance.
- Cost Effective No more silica gel or resin needed. Recover and reuse 95% of the solvent.
- Customizable and tunable chemistry of the stationary and mobile phases to fix low resolving power due to limited selectivity
- Versatile operation modes with increased efficiency to fix low resolving power (purity and/or yield) due to limited efficiency
- Mild solvent system, no degradation-triggering adsorption phenomena in the system
- Recyclable solvent systems (evaporation, membrane separations), miniature-scale solvent handling in the Pilot CPC Pro platform
- No solid waste, only solvents, fresh stationary phase for every run
- Option for continuous batch operation (24/7 on pCPC)

CPC Cell Design

To examine CPC's cell design, we will explore how RotaChrom Technologies uses its CPC cell and what advantages it has over traditional chromatographic solutions.

RotaChrom's CPC design takes advantage of the space provided by cylindrical chambers. Large bore cylinders are easy to manufacture and provide linear scalability thanks to their unique, patented dimensions and architecture. The individual extractions cells are scaled in a way that the performance is not impacted, allowing our extraction cells to be scaled from 10 to 100 millimeters without impacting performance and providing flow increases from milliliters per minute to hundreds of milliliters per minute.

Other advantages of our cell design include:

- Direct and easy scalability between the product portfolio members.
- Accessible cell and rotor environment for easy maintenance and cleaning.

- Tailor-made cleaning procedure (CIP) for convenience.
 - Reduced eluent back-mixing in the cells.
- We have also improved designs beyond the extraction cells. Our injection plugs provide enhanced, stable droplet dispersion of the mobile phase to the stationary phase. And, our collection plug increases solvent retention, increasing loading capacity for the same column size by 20 percent.

RotaChrom designed its top-performing CPC cells with computational fluid dynamics (CFD) software. After thousands of simulations, we found drawbacks of conventional cell designs, highlighting the unparalleled load capacity and scalability of iCPC.



Discoveries include:

- The circular motion of liquid inside planar cells induces back mixing.
- Shorter cells with less surface area provide less space for separation to occur.
- Fluid dynamics change when scaling up production with conventional systems.
- Poor fluid dynamics lead to poor stationary phase retention and theoretical plate numbers.
- Our cell design remedies the issues found in planar cells. There is more space for separation since the surface area inside the cell has increased. Relying on cylindrical columns rather than planar cell shapes also reduces back mixing. The improved fluid dynamics enabled by the unique cell design allows for scaling production while maintaining efficiency. Additionally, the optimized fluid dynamics contribute to the greater stationary phase retention and plate numbers, providing high loadability and productivity for industrial scale processes.

Typical uses of chromatography can be separation, isolation and remediation. All 3 are complex purification goals. In general, a solution can be optimized to perform just one of them. And if you want your machine to do more than one consecutively or even parallel, it takes painstaking method development and hardware modifications.

Isolation

Isolation is performed when you focus on a specific Compound of Interest (Col) from a complex matrix. This is a very challenging goal, which requires resolving power and capability to fractionate the Col selectively. Targeting a single Col within a complex matrix, which contain compounds with a wide variety of physico-chemical properties requires meticulous research and method optimization.

Separation

During separation, we anticipate that the input matrix is simplified to a certain extent, and that it also contains at least 2 Cols that are both in focus. However, they might often denote a chromatographic challenge during separation. Some of the best examples for such challenges are the separation tasks of isomers.

With isomers, the individual Cols demonstrate little to no differences that could help tackle the separation challenge.

Remediation

Remediation simply means the removal of components that are often unwanted. This is mainly because of the negative influence on the Col's efficacy. In other cases, the original matrix in its original form might be harmful to the environment or to the user. Remediation can also be the decontamination process of a valuable matrix. You can remediate virtually any kind of impurity. It could be a health threat, a carcinogen, an environmental pollutant or a legally challenging compound. While remediation takes place within the iCPC part of the platform, the solvent handling system must be up for the challenge as well to ensure that the eliminated constituents are not reintroduced into the loop.



RotaChrom's pCPC device is suited for isolation, separation and remediation tasks and can be optimized to perform all of them.

Part 4

Applications

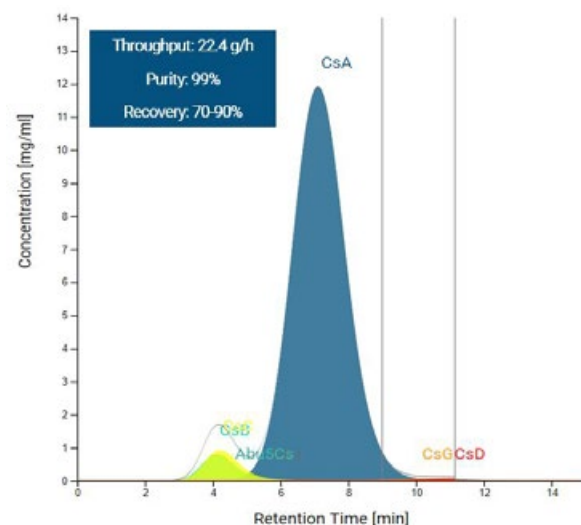
Isolation of an Antibiotic Oligopeptide (Cyclosporine A)

Active Pharmaceutical Ingredient (API):
Cyclosporine A (CsA)

Goal: Purification

Cyclosporine A is a cyclic oligopeptide, with a lipid side-chain. It is produced by fermentation. The result of fermentation is usually a mixture of products with closely related structural properties. These are usually purified by classical purification methods: a combination of crystallization and preparative HPLC.

To avoid costly two-step purification methods, RotaChrom developed a CPC solution which can produce pure Cyclosporin in 15 minutes on an industrial- scale.



Sample	crude cyclosporine A
Apparatus	rCPC
Solvent system	Alkane-based solvent system
Flow rate	200 mL/min
Rotation	1000 rpm
Mode	descending

Polishing an Anticancer API (Anastrozole)

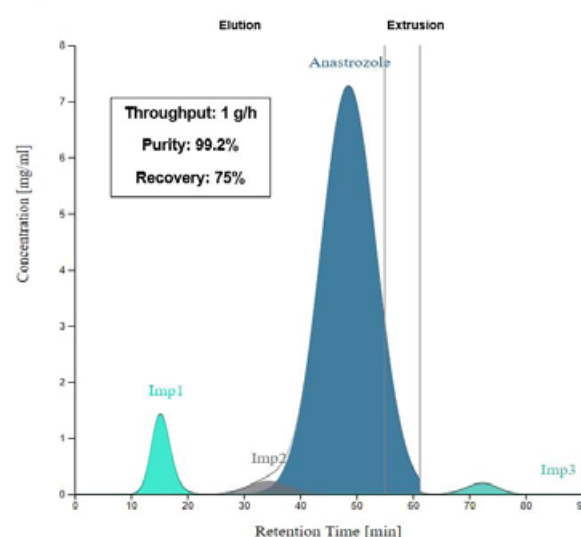
API: Anastrozole

Goal: Polishing the API

Anastrozole is a pharmaceutically active agent, acting as a selective nonsteroidal aromatase inhibitor.

Guidelines for API manufacturers mention that impurities must be kept below set limits. The study's goal was to replace a purification step which needed column chromatography.

At the end of the study, RotaChrom's method could produce Anastrozole with higher than 99% purity, all in one step. After CPC purification, >99.9% purity can be achieved in a single crystallization step.



Sample	crude Anastrozole
Apparatus	labCPC
Solvent system	Ethereal solvent system
Flow rate	14 mL/min
Rotation	2000 rpm
Mode	descending

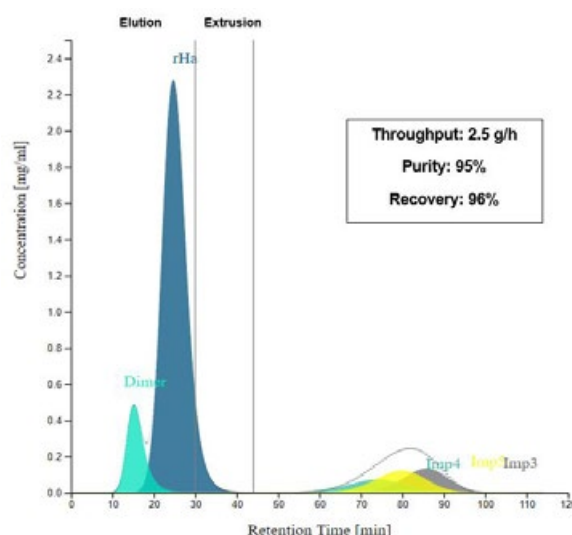
Isolation of Plasma Proteins

Col: Recombinant Human Albumin (rHA)

Goal: Isolation of the Col

To this day, the Cohn method is the most widely used core fractionation technique for fractionation. However, it comes with high initial investment and maintenance cost.

At the end of the study, the resulting rCPC method could isolate rHA with higher than 95% purity and high recovery. Also, this low-temperature process with sequential precipitations induces substantial loss of proteins.



Apparatus	rCPC
Solvent system	Aqueous two-phase system
Flow rate	40 mL/min
Rotation	1000 rpm
Mode	descending

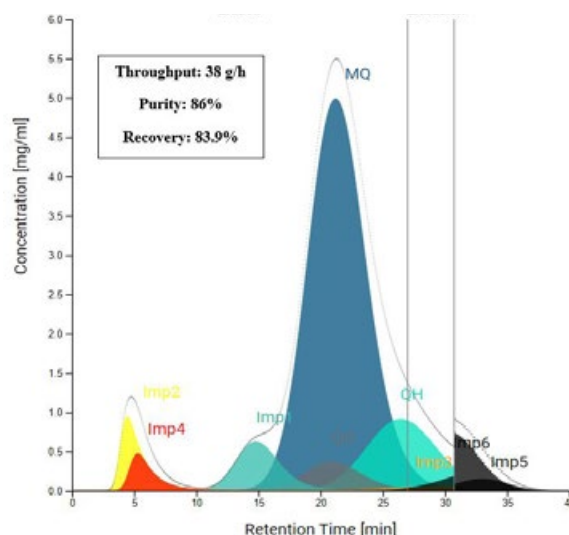
Purification of Quercetin Glycosides

Col: Quercetin Glycosides (isolated from grape tannin)

Goal: Feasibility of the isolation of the Col

The primary objective was to achieve a purity value greater than 75% by weight for quercetin-3- β -D-glucoside (QG) quercetin-3-O- β -D-glucuronide (MQ) and a quercetin hexoside (QH) using food-grade solvents. After extraction of grape tannin the developed CPC method was capable of isolating the three target compounds together with purity greater than the expected.

It had a high throughput using only food grade chemicals. The end product could be re-crystallized from water which could further increase purity.



Sample	crude grape tannin
Apparatus	rCPC
Solvent system	Alcohol-based solvent system
Flow rate	150 mL/min
Revolution speed	1000 rpm
Mode	descending

References

2022

Gergo Ignacz, Robert Orkenyi, Arpad Konczol and Gyorgy Szekely, 2022, '**Contemporary Chemical Approaches for Green and Sustainable Drugs – 1st Edition: Sustainable separations in pharmaceutical manufacturing**', Book Chapter <https://doi.org/10.1016/B978-0-12-822248-5.00008-5>

Arpad Konczol, Gergo Dargo, 2022, '**Sustainable Features of Centrifugal Partition Chromatography**', Sustainable Separation Engineering: Materials, Techniques and Process Development, Volume 1, Book Chapter <https://doi.org/10.1002/9781119740117.ch7>

2020

Laszlo Lorantfy, Dora Rutterschmid, Robert Orkenyi, David Bakonyi, Jozsef Farago, Gergo Dargo, and Arpad Konczol, 2020, '**Continuous Industrial-Scale Centrifugal Partition Chromatography with Automatic Solvent System Handling: Concept and Instrumentation**', Organic Process Research & Development, article <https://dx.doi.org/10.1021/acs.oprd.0c00338>



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