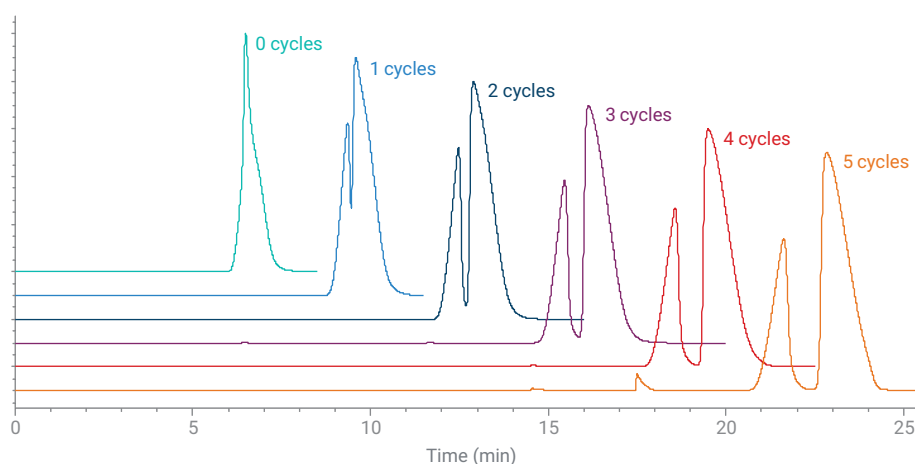


Purify With Increased Resolution by Recycling Chromatography



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Abstract

Despite continuous improvements in analytical UHPLC, some complex mixtures remain a challenge for chromatographers. If the column selectivity or bed length is not capable of separating a peak pair, recycling chromatography is a technique that can help increase the resolution. This technical overview demonstrates the application of recycling chromatography using two 50 mm-long columns, linked by a switching valve. A binary mixture of closely eluting compounds was successfully separated, and fractions were collected with a purity >99%. Despite a virtual column length of 350 mm, the system pressure was low enough to leave room for a higher flow rate to increase throughput.

Introduction

Separating binary mixtures of closely eluting compounds, such as structural or stereoisomers remains a challenge in high-performance liquid chromatography (HPLC). Although within the last five years, 36 new stationary phases for chiral separations have been commercialized¹, using a chiral separation column is not a universal solution to separation problems. The nature of the analytes, the sample solvent, or the required scale of the separation might not be compatible with the range of chiral analytical columns available today.

If the separation cannot be improved by changing the selectivity of the stationary or mobile phase, resolution can only be increased by adding more theoretical plates, which translates into using a column with smaller particles, or greater length. Extending the column length, however, is limited by the maximum pressure the instrumentation can withstand. To overcome this limitation, the concept of recycling HPLC was introduced.²

There are two ways to facilitate recycling:

1. Closed-loop recycling, where the column effluent is reverted to the column head through a valve inside the eluent pump, and
2. Twin column recycling, where the sample is separated repeatedly by two columns linked by a two-position valve.

The first method seems to have the advantage of saving solvent: during recycling, the solvent consumption is effectively zero. However, a large part of the resolution gained through recycling is lost by extracolumn dispersion: the separated compounds need to pass the entire system volume for another cycle through the column. The second method adds only minimal extracolumn volume, and is therefore the better choice for high-performance separations. Using the twin column recycling technique, the virtual column length can be extended without increasing the backpressure of the system. This will lead to a higher resolution of the analytes, at the same time leaving room for an increase of the flow rate to achieve higher throughput.

This technical overview demonstrates the separation and purification of two closely eluting compounds by means of twin column recycling chromatography. The two analytes are collected based on the UV signal. Fraction reanalysis is used to determine the purity of the collected compounds.

Experimental

Instrumentation

The HPLC system used in this experiment consisted of the following modules:

- Agilent 1290 Infinity II Preparative Binary Pump (G7161B) with 50 mL pump heads (option #202)
- Agilent 1290 Infinity II Preparative Open-Bed Sampler/Collector (G7158B)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A) with 0.3 mm preparative flow cell (option #024)
- Agilent 1290 Infinity Valve Drive (G1170A) with preparative 2-position/10-port valve (G4730A)
- Agilent 1290 Infinity II Preparative Column Compartment (G7163B)

The columns were connected with the valve using capillary kits designed for column selection valves (0.5 × 650 mm, part number 5067-6868). These capillary kits are also available with other inside diameters (id) to suit higher or lower flow rates and match the id of the system capillary kit.

Columns

Two Agilent InfinityLab Pursuit XRs C18 columns, 21.2 × 50 mm, 5 μm (part number INF6000050X212)

Software

Agilent OpenLab CDS ChemStation edition for LC and LC/MS Systems, Rev. C.01.10 [272] or later versions

Solvents

LC-grade methanol was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system, equipped with a 0.22 μm membrane point-of-use cartridge (Millipak).

Method settings

Table 1. Chromatographic conditions.

Parameter	Value
Flow Rate	25 mL/min
Eluents	A) 0.1% Formic acid in water B) 0.1% Formic acid in methanol
Solvent Composition	Isocratic 45% B
Injection Volume	250 µL
Sampler Method Preset	Preset 1: Polar sample matrix
Temperature	Ambient
UV Detection	260 nm Peak width >0.1 min (2 s response time, 5 Hz data rate)
Fraction Collection	Peak-based UV threshold: 5 mAU UV upslope: 1 mAU/s UV downslope: 1 mAU/s

Results and discussion

A sample containing two closely eluting compounds was separated isocratically using two columns that were connected through a valve to enable recycling. The system was configured so that - without recycling - the sample was separated by both columns (see Figure 1). Upon switching the valve, the effluent from the second column was diverted to the head of the first column to undergo another separation cycle. The timing when to switch the valve was determined experimentally: just before the first peak is visible in the detector, it can be assumed that the sample has eluted from the first column and is retained on the second column.

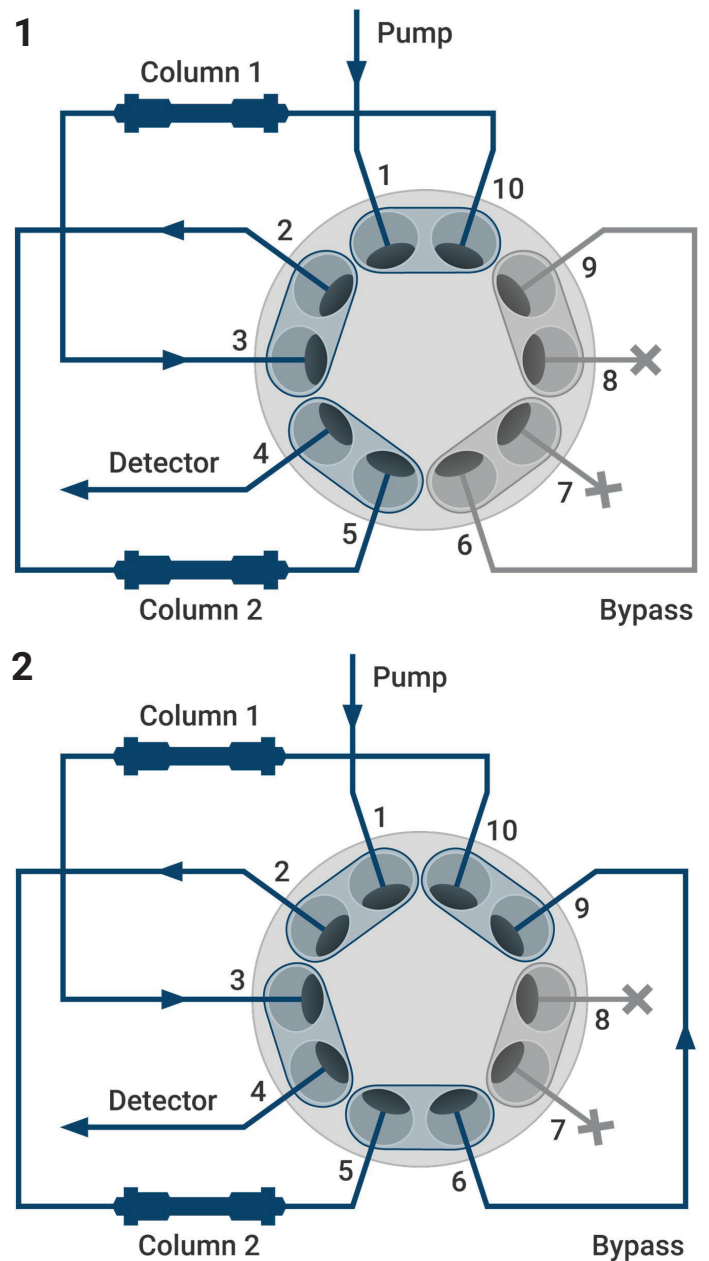


Figure 1. Plumbing scheme of a 2-position/10-port preparative valve to enable recycling preparative chromatography. The bypass was facilitated using a 0.5 × 105 mm capillary (part number 5065-9927). Ports 7 and 8 were closed by blank nuts (part number 01080-83202).

A second injection with one recycling step (3 columns) was necessary to determine the timing when to switch the valve back to position 1. The time difference between the first and second switch was then added to the timetable. By switching alternately between positions 1 and 2, each switch will add another virtual column. Note that this regular pattern of switching only applies in case a pair of identical columns is used. Performance differences between the two columns may cause different dwell times of the sample on each column. This in turn might require different valve switching intervals that compensate for the different dwell times.

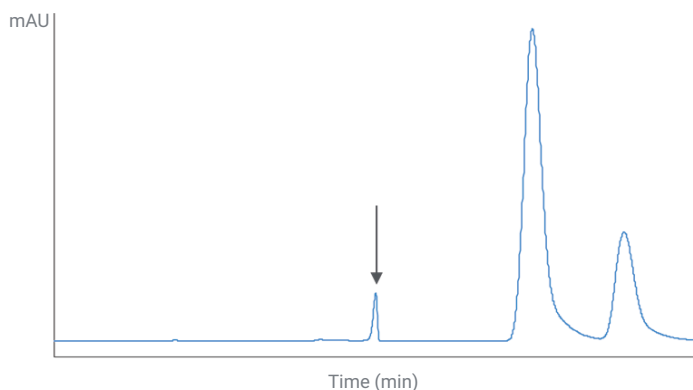


Figure 2. Too many switching cycles will cause a part of the peak to be cut off (arrow).

Recycling can only be repeated up to a certain point. With each cycle added, the peaks will disperse, and the time window necessary to elute both compounds will increase. When the elution time window is larger than the time both compounds are retained on a single column, switching the valve to the other column will cut off the part of a peak that exceeds the separation space of one column (see Figure 2).

Figure 3 shows a chromatogram overlay of the separation with increasing recycling steps. Starting with co-eluting peaks in the twin column setup without recycling, the increase in resolution is visible with each cycle. The final method was programmed with four recycling steps, because a significant portion of one compound was already cut off after five cycles.

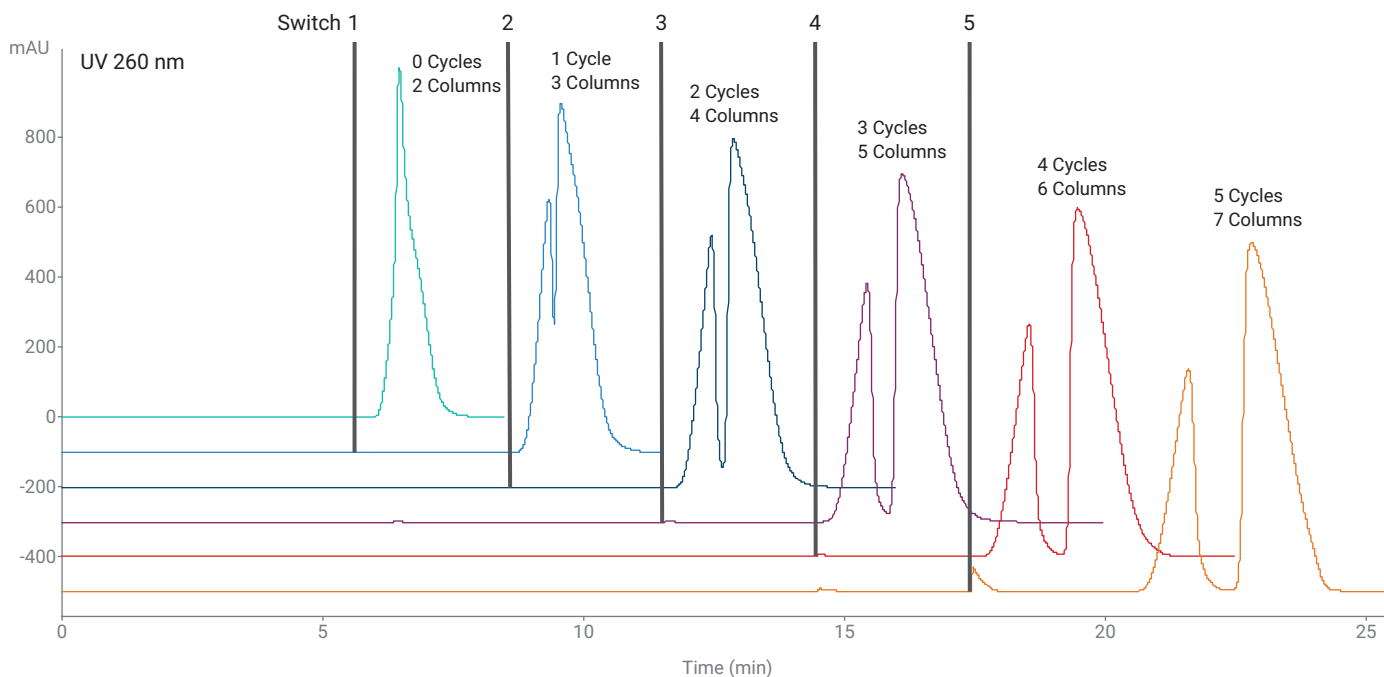


Figure 3. Chromatogram overlay of the separation with increasing number of recycling switches.

A chromatogram, along with the valve switching timetable of the final separation, is depicted in Figure 4. By recycling the sample four times (equaling six columns), the resolution of the two compounds had increased to about 1.1, which was sufficient to collect the peaks in two separate fractions. To achieve this separation on a standard system without recycling capabilities would require a column length of 300 mm. Extrapolating the observed system pressure during the experiments, this column would need to be operated at a pressure above 400 bar. These numbers outline the benefits of a system capable of column recycling: two short columns keep the operating pressure low, while enabling high resolution and increased plate numbers by virtually extending the column length.

The increased resolution between the compounds enabled fraction collection with a low threshold, which in turn maximized recovery. Both fractions were injected into an analytical LC system to assess their purity. With these collection settings, both fractions reached a purity of >99%, see Figure 5.

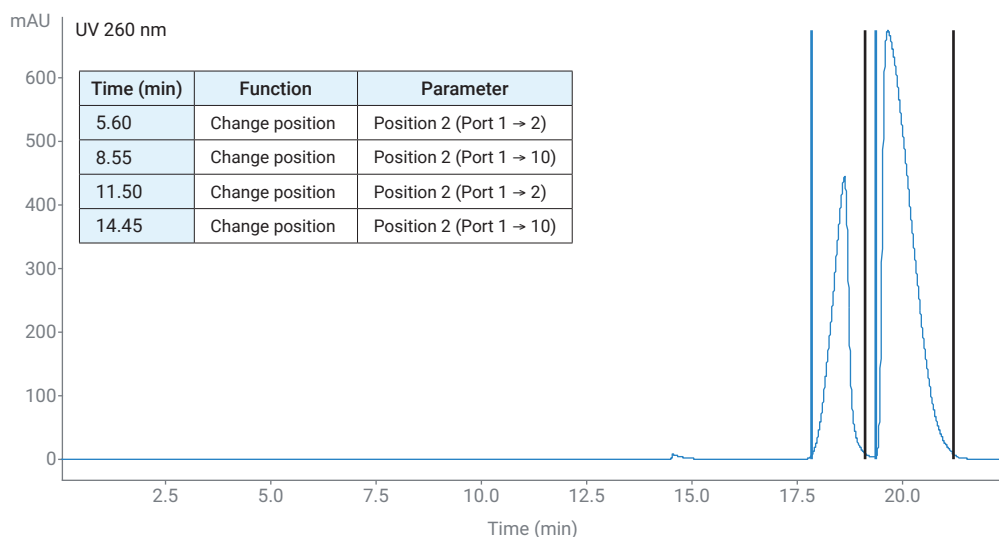


Figure 4. UV chromatogram of the final separation method, with four recycling switches as depicted in the timetable. Fraction collection events are represented by horizontal bars.

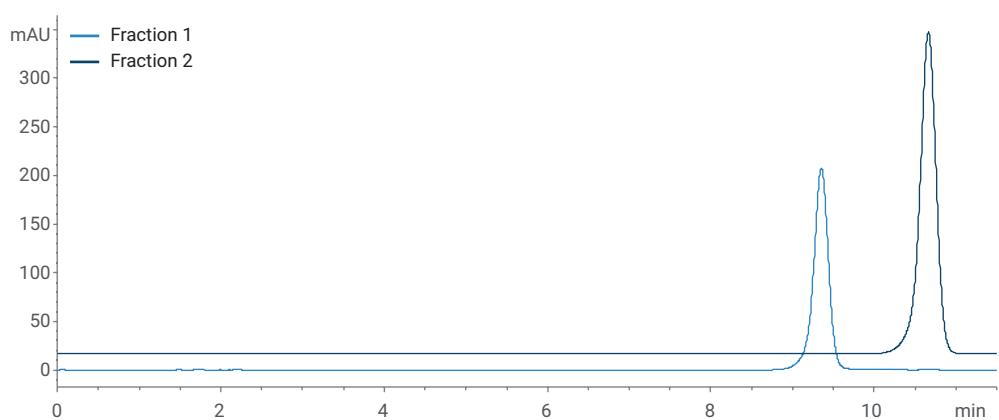


Figure 5. UV chromatogram (260 nm) of the fraction re-analysis.

Conclusion

This technical overview demonstrates the implementation and benefits of recycling preparative chromatography. Two columns were linked with a valve, connecting the outlet of the second column to either the downstream flow path or back to the first column. Using multiple valve switches, a sample was successfully recycled through six virtual column lengths. This technique enabled a significant increase in resolution between two closely eluting compounds, facilitating fraction collection with purities > 99%. The increase in virtual column length did not come at the expense of higher system pressure, which leaves room for increasing the flow rate and throughput of the separation method.

References

1. Bell, D. S. New Liquid Chromatography (LC) Columns and Accessories for 2021. *LCGC Europe* **2021**, 34(5), 197–207.
2. Martin, M. *et al.* Theoretical and Experimental Study of Recycling in High-Performance Liquid Chromatography. *J. Chrom. A* **1976**, 125(1), 17–41.

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