Science Together

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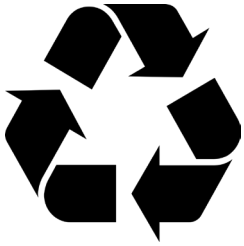
Recycle your peaks – A comparison of two recycling methods



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SUMMARY

When facing a difficult separation of two peaks, recycling chromatography can be a solution for this issue. The general principle for all approaches is to redirect the partially separated analytes through the column several times. This simulates an infinite column length. Different methods are established for this task. One example is the use of a T-fitting adapter and pumping in a closed circuit once the sample has been injected. Another technique is the alternate pumping recycling chromatography. This method uses a combination of two columns with identical dimensions and a 2-position-6-port -valve. The columns are switched in an alternating way, so that the sample enters the second column after leaving the first. Once the sample is in the second column, the valve is switched so that the first column will receive the sample again. Both methods have their advantages and disadvantages regarding to parameters such as system dead volume, detection, instrument complexity or method development time. Therefore, both techniques were evaluated using the same preparative HPLC instrument, as well as the same separation method and sample parameters. A comparison of both approaches achieves the superiority of the alternate pumping method. After five column cycles and a similar runtime this technique showed a better resolution of the target substances.

INTRODUCTION

Recycling chromatography is an interesting method for a variety of applications that have one task in common being hard to separate substances. For this application an exemplary mixture of two steviolglycosides was used, but many other examples are imaginable, for instance the separation of chiral compounds. This principle could also be used in preparative chromatography to achieve higher compound purities despite loading more sample compared to a single run. The common principle of recycling chromatography is to redirect the peaks of interest through the column multiple times. Hence, an infinite column length is simulated, leading to a better target peak resolution. Two different approaches were tested in this application. One was the classical method of peak recycling through the pump and the other was

Recycle Your Peaks – A comparison of two recycling methods

INTRODUCTION

the method of alternate pumping. In the classical method, a T-fitting adapter is mounted in front of the pump inlet and connected to a multi-position valve, that serves as a fractionation valve and is located after the detector **(Fig. 1)**. Once the sample is injected using the 2-port-6-position injection valve, the fractionation valve is switched to the recycling position. A closed circuit is established. The compounds of interest are redirected into the main flow path using the T-fitting. After passing the main pump they are separated once more on the stationary phase. This cycle is repeated until the target resolution is reached or the peak broadening prevents any further recycling. After every cycle the substances are detected with the detector and the grade of separation is visible. At the end, the target substances can be collected with the fractionation valve. With the method of alternate pumping, a redirecting of the flow through the main pump is avoided. This is achieved by using a second column of identical dimensions and another 2-position-6-port valve **(Fig. 2)**, which serves as recycling valve. The columns are connected in such a way, that when the target compounds leave the first column, theyenter the second column. Switching the recycling valve then connects the second columns exit to the first columns inlet. Similar to the recycling through pump approach, this switching cycle is repeated until the target resolution is reached or the peak broadening exceeds one column volume. The switching time of the recycling valve needs to be determined in advance. For this purpose a single run with one column is performed to determine the retention times of the target compounds. Contrary to the recycling through pump technique the detector is located outside the recycling circuit. Therefore the resolution of the target compound is detected after the separation is finished. Hence, the process cannot be monitored online unless a second detector is available. For the purpose of fractionation, a multi-position valve or a fraction collector is connected after the detector. Solvent recycling can be used with both methods. While the recycling through pump method operates in a closed circuit, the alternate pumping method requires a fractionation valve and a threshold function to recycle the solvent. To compare the performance of both recycling techniques an established preparative HPLC method (VFD0170 & VFD0171) for the separation of Rebaudioside A and Stevioside was scaled down to a semi-preparative scale.

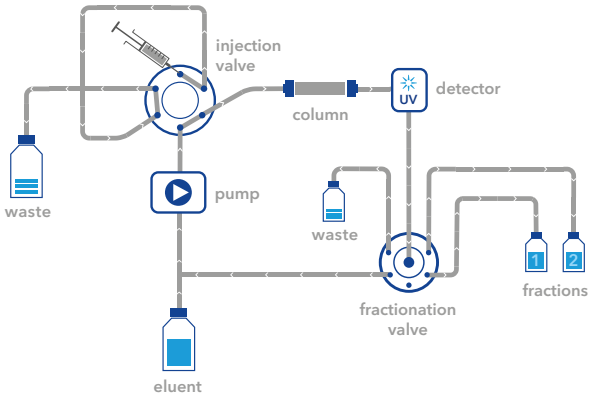


Fig. 1 Flowpath for recycling through pump approach.

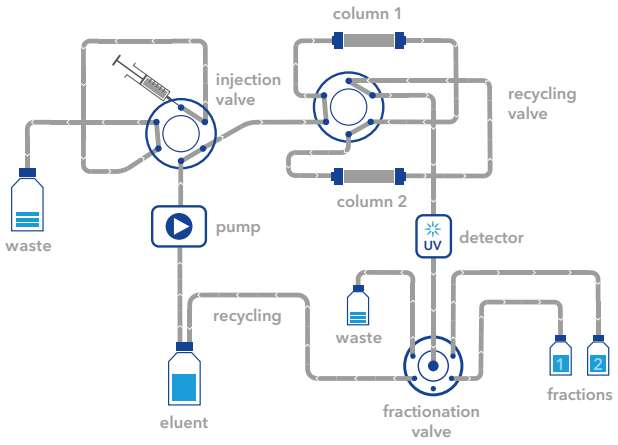


Fig. 2 Flowpath for alternate pumping approach.

RESULTS

The result of the recycling through pump method is depicted in **Fig. 3**. Since the detector is inside the closed circuit, the result of increasing cycle numbers can be acquired with one measurement. Increasing cycle numbers lead to increased peak resolution, whereas signal height decreases, and the peak width broadens. A maximum number of seven cycles was possible before peak broadening prevented another cycle. With the alternate pumping method, it can be seen, that with every subsequent cycle the target peak resolution increases as well **(Fig. 4)**. After six cycles the maximal possible resolution (1.29) is reached, since both peak widths equal one column volume. Hence the target substances can no longer be separated with the given column bed. A comparison of the resolutions is shown in **Fig. 5**, where it can be seen that the maximum resolution of 1.13 reached with the recycling through pump method was achieved in three cycles less with the alternate pumping method. With the latter method a maximum resolution of 1.29 was reached. As expected the peak areas for each cycle stay the same, whereas the peak width increases. No sample was lost during the two different peak recycling methods **(Fig. 6)**.

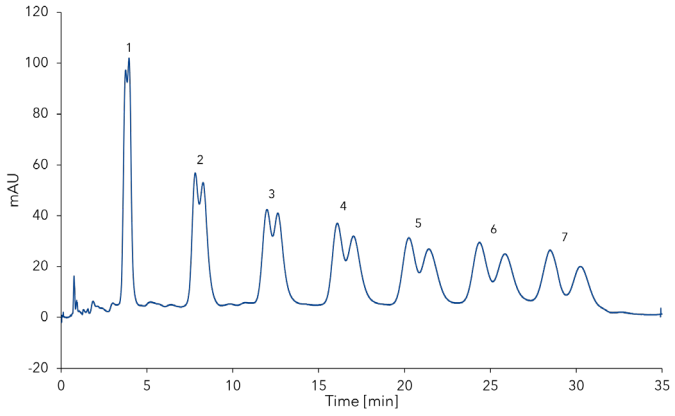


Fig. 3 Recycling through pump. One measurement with seven cycles (1 to 7); 3.5 mL/min; 100 µL injection of standard with 0.2 mg/mL Rebaudioside A and Stevioside.

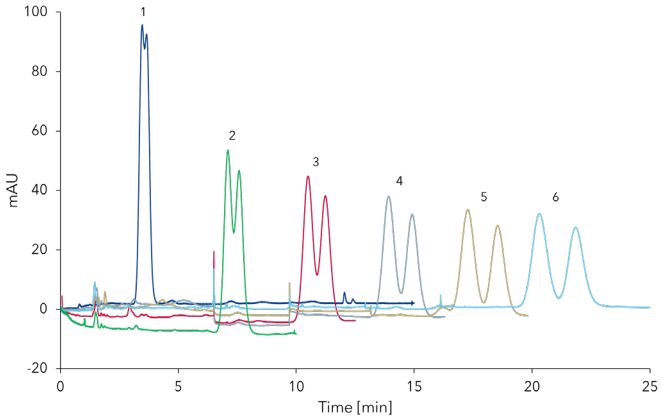


Fig. 4 Overlay of chromatograms for alternate pumping recycling chromatography. Comparison of six measurements with increasing cycle numbers (1 to 6); 3.5 mL/min; 100 µL injection of standard with 0.2 mg/mL Rebaudioside A and Stevioside.

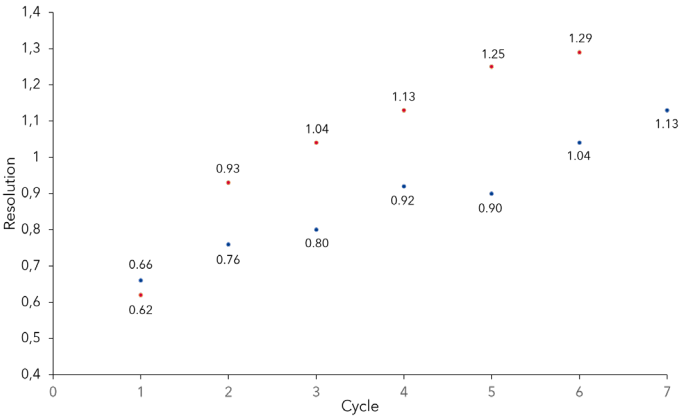


Fig. 5 Comparison of resolution for the target substances. Alternate pumping recycling (red); Recycling through pump (blue).

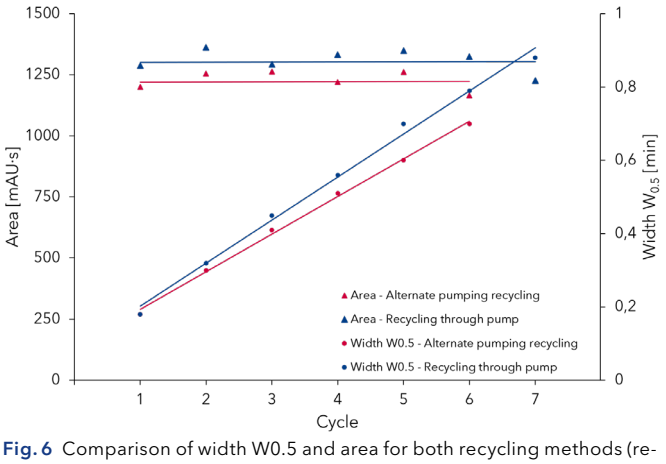


Fig. 6 Comparison of width W0.5 and area for both recycling methods (regarding Rebaudioside A peak)

Recycle Your Peaks – A comparison of two recycling methods

SAMPLE PREPARATIONS

A stock solution containing 10 mg/mL of Stevioside and 10 mg/mL of Rebaudioside A was prepared using 30:70 acetonitrile/distilled water (v/v) as solvent. The stock solution was diluted with a ratio of 1:50 with 30:70 acetonitrile/distilled water (v/v) to achieve a target concentration of 0.2 mg/mL for each compound. The sample was filtered through a 0.45 µm RC-membrane (regenerated cellulose) before injection.

CONCLUSION

Both methods show that difficult separations are possible using recycling chromatography. The recycling through pump approach has the advantage that the instrument configuration is rather straightforward. Method development is easy since the process can be monitored online with the detector. Because of the closed circuit the solvent consumption is low. The main disadvantage of the recycling through pump approach is the large dead volume. Any tubing before and after the column, the pump head inside, or the mixing chamber increases the dead volume. This causes the substances to partially remix after leaving the stationary phase. In consequence a fast peak broadening is observed. Another disadvantage is that the pump and eluent delivery system is contaminated with the sample. Depending on the intended application, this can be a major drawback. Compared to the classic alrecycling through pump approach, the alternate pumping technique eliminates some of the drawbacks mentioned before. First and foremost, the sample never comes into direct contact with the eluent delivery system, thus avoiding its contamination. Furthermore, the dead volume is significantly smaller since only the tubing between the column inlets and outlets and the dead volume of the recycling valve are accounted for. Hence, peak broadening happens not as fast with increasing cycle numbers as with the cycling through pump method. Thus, the resolution of the target peaks increases also faster. In the given example, the same resolution can be achieved three cycles earlier. This results in a shorter overall runtime. One of the disadvantages of the alternate pumping method is, that a second column and valve are needed, leading to higher instrument complexity. The process cannot be easily monitored online, since the detector is usually outside of the recycling circuit. Nevertheless, the alternate pumping technique shows a better resolution of the target substances and is therefore more suitable for separating the sample.

MATERIALS AND METHODS

Tab. 1 Instrument setup

Column temperature	ambient
Injection volume	100 µL
Injection mode	Full loop
Detection	UV 210 nm
Data rate	2 Hz

Tab. 2 Pump parameter

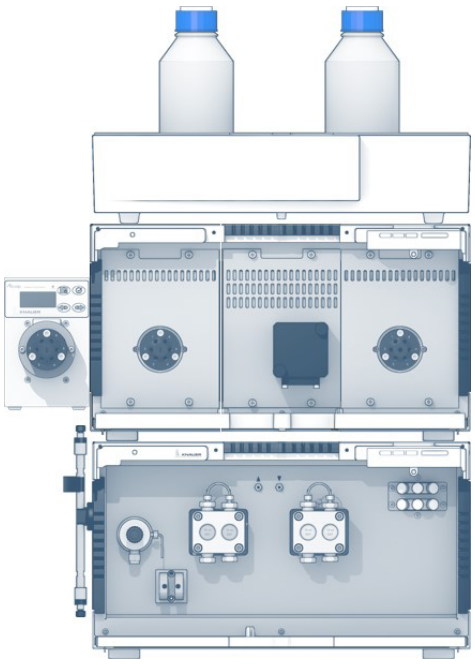
Eluent (A)	30:70 Acetonitrile/water (v/v)
Flow rate	3.5 mL/min

Tab. 3 Optimal system configuration (Alternate pumping)

Instrument	Description	Article No.
Pump	AZURA P6.1L HPG 50 mL pump head sst	APH38FA
Assistant	AZURA Assistant ASM 2.2L Left module: Valve drive VU 4.1 Middle module: UV detector Right module: Valve drive VU 4.1	AY00593
Valve	2-position valve, 6 Port	AVD26AE
Valve	Multiposition valve, 6 Port	AVS26AE
Valve Drive	AZURA Valve Unifier VU 4.1	AWA01
Valve	2-position valve, 6 Port	AVD26AE
Flow Cell	Semi-preparative 3 mm UV Flow Cell 3 mm path length, 1/16", 2 µl volume, 300 bar, stainless steel	A4042
Columns	Vertex 3 Eurospher 100-10 C18, Column 125 x 8 mm Vertex 3 Eurospher 100-10 C18, Column 125 x 8 mm	12GE181ESN
Software	PurityChrom 5.09.069	A2650

Tab. 4 Optimal system configuration (Recycling through pump)

Instrument	Description	Article No.
Pump	AZURA P6.1L HPG 50 mL pump head sst	APH38FA
Assistant	AZURA Assistant ASM 2.2L Left module: Valve drive VU 4.1 Middle module: UV detector UVD 2.1S Right module: Valve drive VU 4.1	AY00593
Valve	2-position valve, 6 Port	AVD26AE
Valve	Multiposition valve, 6 Port	AVS26AE
Flow Cell	Semi-preparative 3 mm UV Flow Cell 3 mm path length, 1/16", 2 µl volume, 300 bar, stainless steel	A4042
Column	Vertex 3 Eurospher 100-10 C18, Column 125 x 8 mm	12GE181ESN
Software	PurityChrom 5.09.069	A2650



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VFD0171 – Evaluating preparative online SPE for the purification of stevia leave extracts

