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Improved recycling chromatography – how to make faster and automatic separations of peak pairs

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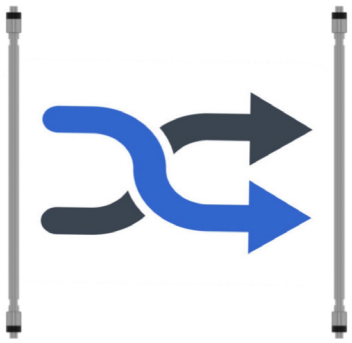

SUMMARY

Recycling chromatography is an interesting technique to separate two close eluting peaks by increasing the column bed length. It can be applied as an alternative strategy when other optimization approaches as decreasing the flow rate or changing the solvent gradient do not result in separation of the target peaks. Two recycling systems have been described in literature: close loop recycling and alternative pumping recycling. The first is a classic and simple method which uses one column, the second is an improvement of the first one by using two identical columns and a 2-position valve. Here, we describe an optimization of the latter method by the addition of a second UV detector resulting in faster method development. The method was further improved allowing automated recycling which works also with changes in sample concentration under the PurityChrom® 6 software. These changes significantly improved the recycling chromatography technique.

INTRODUCTION

Recycling chromatography could be the solution for one of the most common challenges in HPLC: separation of two substances characterized by close retention times. To lengthen the column bed is a strategy to consider, if the goal is to obtain the best resolution for two compounds, expetially when optimizing other influential parameters, such as flow rate and/or solvent gradient, did not result in their separation. However, the use of a long column has some restrictions, like costs, dimensions, and back pressure which could bring the system to its limits. Recycling chromatography could overcome the co-elution issue by using one or two identical short columns and a 2-position valve. The principle of peak recycling process is to re-inject the eluted target peak back on a column forming a circuit which can be repeated several times simulating one long column. Two recycling systems have already been studied: close loop recycling [1] and alternative pumping recycling [2] 1. Close loop recycling is considered the classic peak recycling method. It uses one column, and the eluted target peak is re-injected into the same column through the pump by switching of the 2-position valve.

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The disadvantage of this set-up is the peak broadening by the flow through the pump which fast prevents any further recycling. Alternative pumping recycling is an improvement of the close loop recycling, as it has a better peak resolution. It is composed of two identical columns and a 6-port 2-position valve. By switching the valve, which is placed between the two columns, the eluted target peak from one column is re-injected into the second column without passing through the pump. To compare the performace of these two known systems, the resolution of the two peaks were compared after each cycle. The alternative pumping recycling achieved a better resolution after less cycles[2]. Nevertheless, the method development takes certain time as only one detector is included in the system. Here, an upgrade of the alternative pumping recycling system by implementing a second UV detector between the two columns, to monitor the separation online, is presented. In addition, the method was automazised using an automatic valve switch by the PurityChrom® 6 software. A standard solution of Stevioside and Rebaudioside A was used for testing the new recycling configuration.

SAMPLE PREPARATION

A standard 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 standard solution was filtered through a 0.45 µm RC-membrane (regenerated cellulose) before injection. This solution was diluted with 30:70 acetonitrile/distilled water (v/v) to achieve the desired concentration.

RESULTS

The addition of a second UV detector **(Fig. 2 UV 1)** permits to observe the separation chromatogram during the recycling process. Monitoring the process in real time is an advantage compared with the standard alternative pumping system, where several measurements are needed to determine the cycle number for the best peak separation. The incorporation of the additional UV detector was possible due to the exchange of the 6-port-2-position valve by a 8-port-2-position valve to connect the two columns and UV 1.

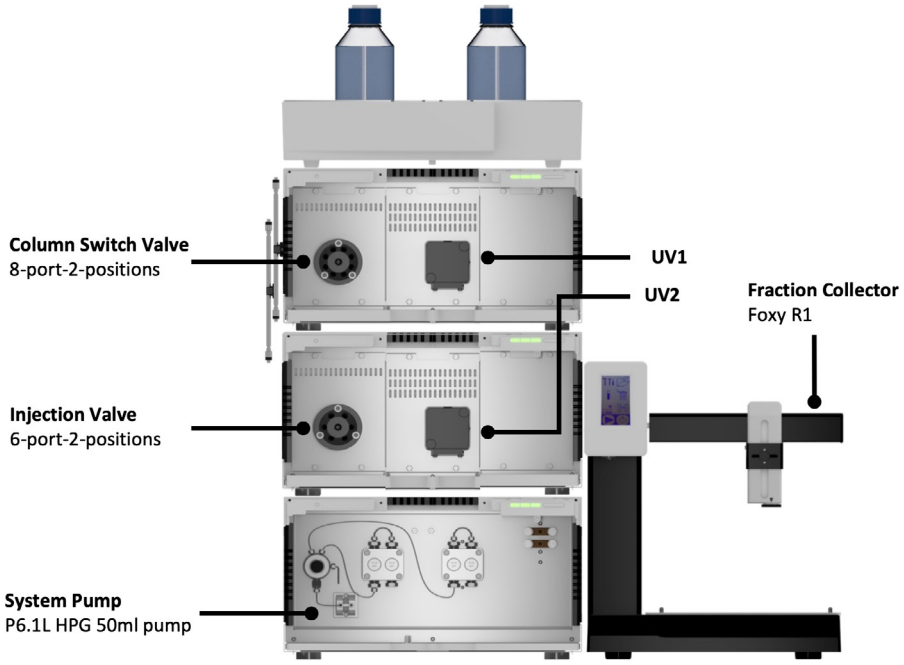

Fig. 1 New set-up for alternative pumping recycling system

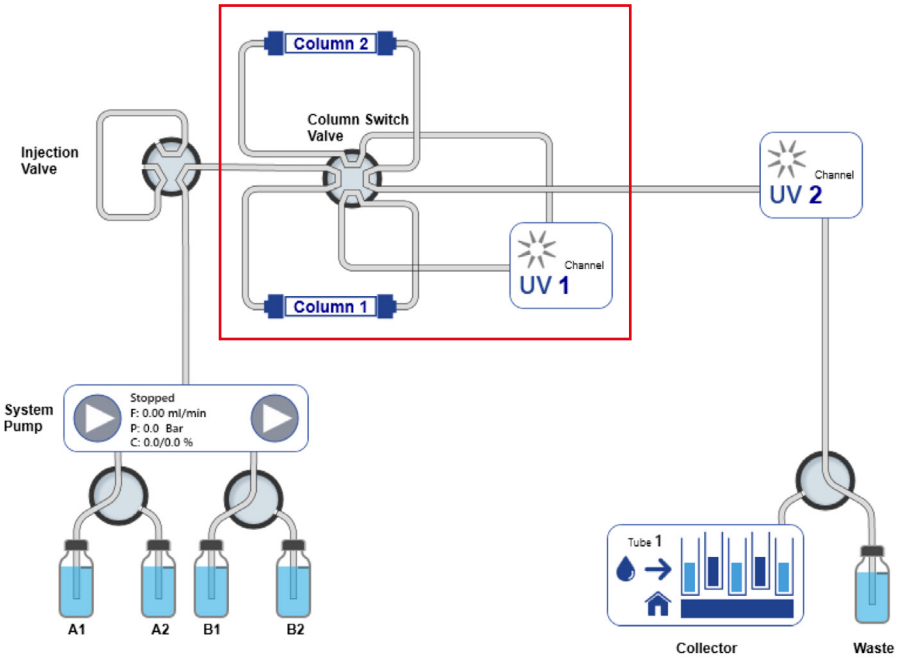

Fig. 2 PurityChrom® 6 visualization of new set-up for alternative pumping recycling system. Red rectangle – recycling circuit.

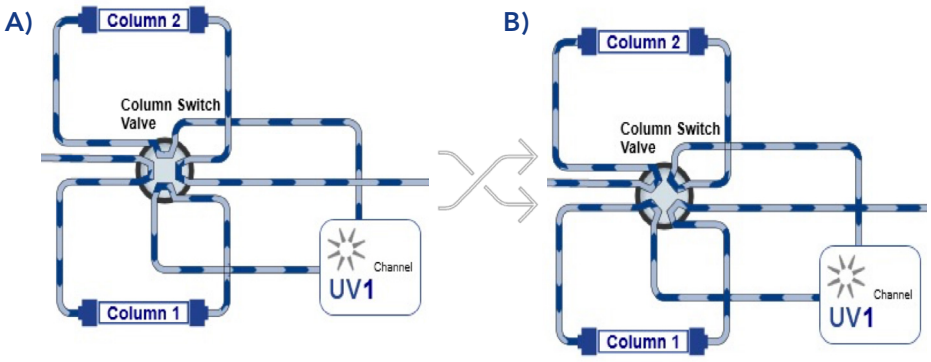

Fig. 3 Two flow paths. **A:** Flow from column 1 to UV 1 to column 2. **B:** Flow from column 2 to UV 1 to column 1.

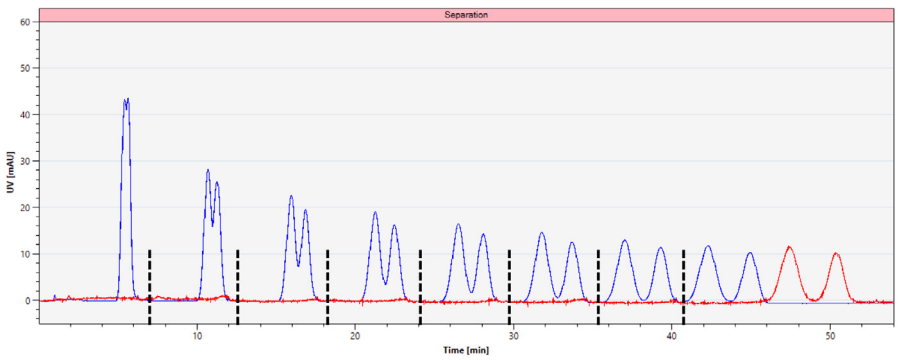

Fig. 4 Method development chromatogram from alternative pumping recycling system with two UV detectors. Black dash line – manual column switching; blue – UV 1, red - UV 2; 3.5 ml/min; 100 µl injection of standard solution 0.2 mg/ml Stevioside and Rebaudioside A.

By switching the valve, the eluted peaks follow two flow paths: from column 1, to UV 1 to column 2, and from column 2, to UV 1 to column 1 **(Fig. 3)**. After each switch the peaks can leave the column to go to the second UV detector (UV 2) and then the target peak can be collected by fractionation.

The chromatogram shown in **Fig. 4** is the result after one measurement, where the 8-port-2-position-valve was switched manually several times until satisfactory resolution of the two peaks was reached. The manual switching was carried out during the measurement in one run. The valve position was manually switched few seconds after the second peak reached the baseline. This short delay is required to ensure that the peaks leaving the UV 1 detector are injected into the next column.

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By using the function "Sampling Window" of the PurityChrom® 6 software peaks are recognized automatically which enables automated switching of the 8-port-2-position-valve during the recycling method. The recognition of the peaks by the software is possible by using the integration parameters (filter length, slope sensitivity and maximum baseline level) which are determined in the method development chromatogram **(Fig. 5, red rectangle)**. With these three values, the end of the second peak is detected and consequently the valve is switched generating the recycling circuit **(Fig. 5)**.

Fig. 6 shows the resulting chromatogram from automatic recycling method. The UV 1 channel (blue) is the reference for peak recognition in the defined Sampling Windows (green rectangles) defining the switch time of the valve. The UV 2 channel (red) is the reference for a threshold that initiates the fractionation of the target peaks after the recycling process.

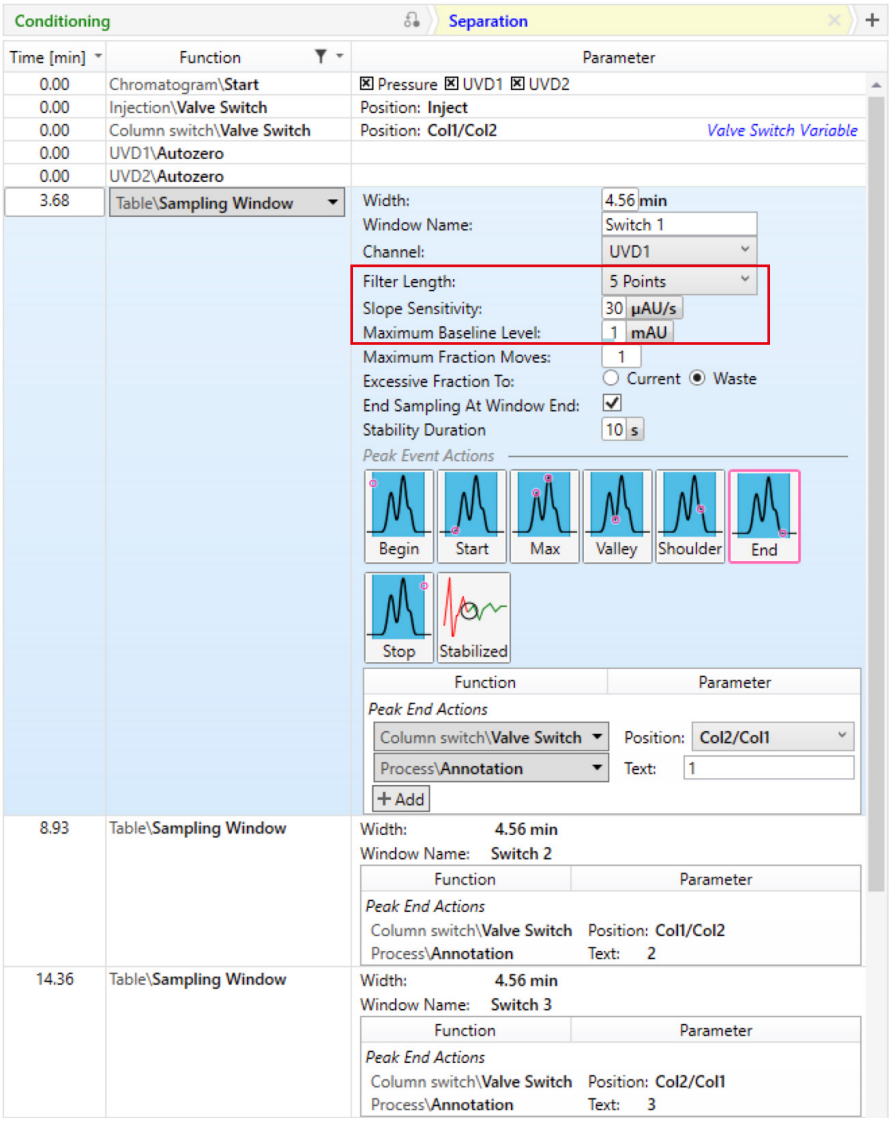

Fig. 5 Method visualization from PurityChrom® 6; red rectangle - Filter Length, Slope Sensitivity and Maximum Baseline Level to adapt peak windows.

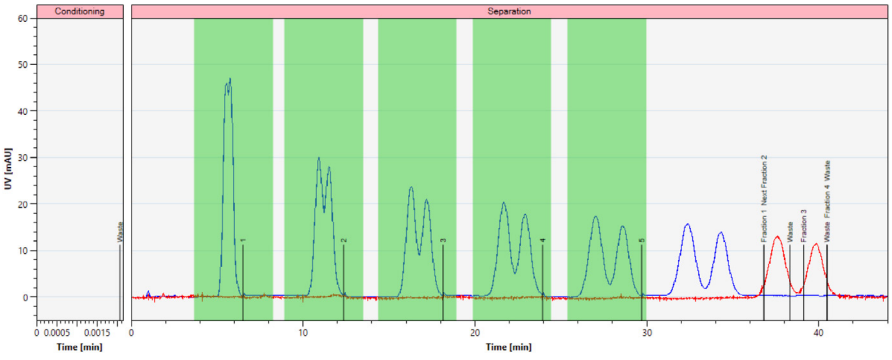

Fig. 6 Chromatogram of alternative pumping recycling with switch automatization. Blue – UV 1, red – UV 2; 3.5 ml/min; 100 µl injection of standard solution 0.2 mg/ml Stevioside and Rebaudioside A

The advantage of using the "Sampling windows" function is its flexibility to recognize the peaks, even when the peaks shape change or measurements are not fully reproducible. To validate how flexible and robust the automatic valve switching is, different concentrations of Stevioside and Rebaudioside A solution were injected while the Sampling Windows kept the same integration parameters **(Fig. 7)**. The automatic recycling method worked with solutions until four times higher concentrations.

As shown in **Fig. 7 B**, injecting a 0.8 mg/ml standard solution, the fifth switch was missing as the last peak was not automatically recognized. If peaks integration doesn´t end completely inside of Sampling Windows, the prevention of the switch of the valve happens due to non-recognition of peaks end. Therefore, considering higher concentrations than used in the manual switch chromatogram, the parameters of the Peak Windows needed to be adapted as the peak shapes changed.

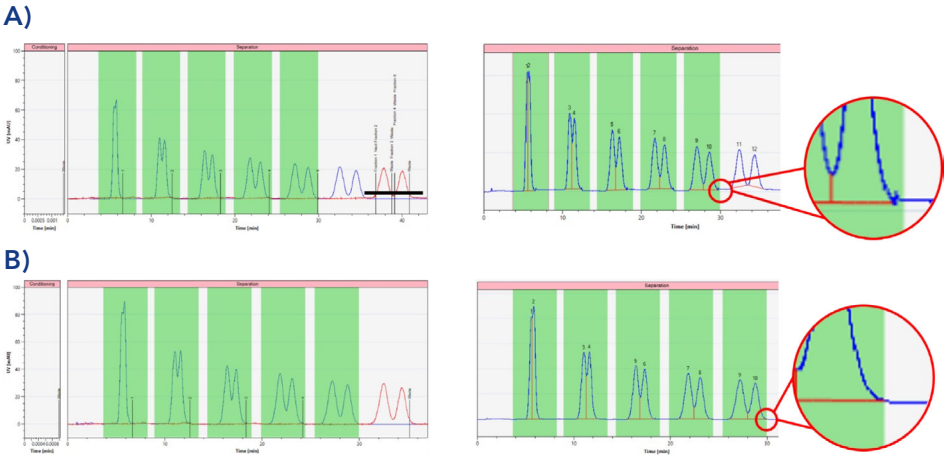

Fig. 7 A left: Chromatogram of alternative pumping recycling with switch automatization; **right:** integration of the chromatogram; standard solution of 0.2 mg/ml Stevioside and Rebaudioside A. **B left:** Chromatogram of alternative pumping recycling with switch automatization; **right:** integration of chromatogram; standard solution of 0.8 mg/ml Stevioside and Rebaudioside A.

CONCLUSION

Starting from the simple close loop recycling system, recycling chromatography was improved by introducing the alternative pumping recycling process. This improvement avoids pumping the target peaks through the pump and therefore increased the resolution of the target peaks. As this system has only one UV detector, the determination of peak separation needs several runs, making the method development time consuming. To further improve the alternative pumping recycling system, a second UV detector was integrated into the system. This second UV detector, placed between the two columns, monitors the process in real time enabling method development in one run. Moreover, the software PurityChrom® 6 allows with the function "Sampling Windows" an automatized recognition of the peaks once a base method is developed. This automatization tolerates changes in the sample concentration and peak shape to a certain degree making the method more robust.

The presented method can be used either in analytical or preparative chromatography. Here the application was shown for a semi-preparative set-up.

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MATERIAL AND METHODS

Tab. 1 Instrument: KNAUER recycling system

Instrument	Description	Article No.
Pump	AZURA® P 6.1L, HPG 50 ml pump ceramic head	APH68FB
Assistant 1	AZURA Assistant ASM 2.2L	AY00592
	Left module: valve drive VU4.1	EWA04
	Middle module: UVD 2.1S	EDA03XA
Injection Valve	2-position valve, 6 ports, sst, 1/16"	AVD26AE
Assistant 2	AZURA Assistant ASM 2.2L	AY00592
	Left module: valve drive VU4.1	EWA04
	Middle module: UVD 2.1S	EDA03XA
Column switch Valve	2-position valve, 8 ports, sst. 1/16"	AVD36AE
Flow cell (UVD 1)	Semi-preparative 3 mm UV Flow Cell 3 mm path length, 1/16", 2 µl volume, 300 bar, sst	A4044
Flow cell (UVD 2)	Semi-preparative 3 mm UV Flow Cell 3 mm path length, 1/16", 2 µl volume, 300 bar, sst	A4044
Columns	2 x Eurospher II C18, 10 µm, 100 Å, 150 x 8 mm	15GE181E2N
Capillaries	PEEK 1/16", 0.5 mm	A2525
Software	PurityChrom® 6 Full License	A2681

Tab. 2 Recycling method

Parameter	Value
Flow rate	3.5 ml/min
Data rate	2 Hz
Temperature	Ambient
Injection volume	100 µl
Wavelength	210 nm
Mob. Phase A	H_2O
Mob. Phase B	Acetonitrile
Isocratic	30% B

REFERENCES

[1] A. Seidel-Morgenstern and G. Guiochon, Theoretical Study of Recycling in Preparative Chromatography, AIChE Journal May 1993, Vol.39, No.5.

[2] Menke J., Monks K. Recycle your peaks – A comparison of two recycling methods App. Note VTN0007.; https://www.knauer.net/de/recycle-your-peaks-a-comparison-of-two-recycling-methods/a39722