Separation Improvements with 2D LC

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Pittcon 2013
Philadelphia, Pa
March 18, 2013
Poster 550-3P

Agilent Technologies

Introduction

The separation of complex samples can be improved by using 2D LC with two different types of columns. The main advantage of 2D LC compared to conventional single dimension chromatography with a single column is the increase in peak capacity or resolution. But this is maximized only as long as the separations are completely orthogonal. However in the real world this othogonality is often only partial due to available column choices or difficulties in solvent/mobile phase compatibility. In this work, a wide range of superficially porous columns are evaluated to determine best available othogonality and can include normal phase, reversed-phase and HILIC column choices. These are then used to separate complex pharmaceutical and environmental mixtures using a 2D LC solution

Overview

The analysis of impurities is an important part of the development process in chemical industries. Due to the fact that impurities are structurally similar to the main compound it can often be difficult to separate them chromatographically. Of particular interest are early eluting polar compounds, as they are often biologically active. A solution to this challenge can be the use of the Agilent 1290 Infinity 2D-LC. In the heart cutting experiment the peak of interest is sampled from the first column and eluted into a loop capillary. This is then transferred by a switching valve to a second dimension column. In this way co-eluting peaks can be resolved. In this work the importance of the second dimension stationary phase is demonstrated. A wide range of revered phase columns are evaluated including C18, Cyano, Stable Bond AQ, Phenyl-Hexyl and Bonus RP. Additional work is planned using alternative solvents. A constant second dimension gradient is chosen, similar to the mobile phase of the first dimension. The peak is sampled using a counter-current capillary filling, (Fill direction and de-fill direction are opposed, compressing the peak). The peak is sampled using a precise time sampling, reproducible chromatography on the first dimension is critical. The heart cutting experiment requires no additional software for visualization of the data,

Equipment

Equipment:
The Agilent 1290 Infinity 2D-LC consisted of the following modules:

- Agilent G1329A 1200 Auto-sampler
- Agilent G1311A 1200 series Quaternary Pump (400 bar)
- Agilent G1315C 1200 Diode Array Detector (1st dimension detector)
- Agilent G4220A 1290 Infinity Pump
- Agilent G1316C 1290 Infinity Thermostatted Column Compartment (TCC) with a 2-position/4-port –duo valve (G4236A) for 2D-LC
- Agilent G4212A Infinity Diode Array Detector (2nd dimension detector).
- Open Lab version C 1.04 with 2D control software.

- The first three components were recycled from an older system and were integrated with the 1290 Infinity Pump, 1290 Infinity DAD, 1290 Infinity TCC and valve by upgrading firmware.

The following columns are utilized in this work:

Agilent Poroshell 120 EC-C18 2.1 x 100 mm 2.7 µm 695775-902

Agilent Poroshell 120 SB-C18 3 x 50 mm 2.7 µm 689975-302

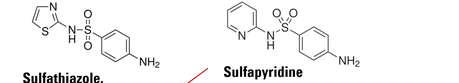
Agilent Poroshell 120 Bonus RP 3 x 100 mm 2.7 µm 695968-301

Agilent Poroshell 120 Phenyl Hexyl 3 x 50 mm 2.7 µm 699975-312

Agilent Poroshell 120 SB-AQ 3 x 50 mm 2.7 µm 689975-314

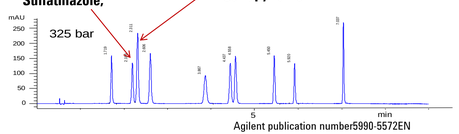
Agilent Poroshell 120 EC-CN 3 x 50 mm 2.7 µm 699975-305

A mixture of Sulfa Drugs were used in this work as model compounds. These materials were found to be stable in solution over several weeks. These compounds can be separated using a Poroshell 120 EC-C18 and a formic acid gradient,, but for this work two compounds are purposefully co-eluted. Peaks 8 and 9 are also purposefully co-eluted, but not addressed further in this work.



Sulfathiazole,

Sulfapyridine

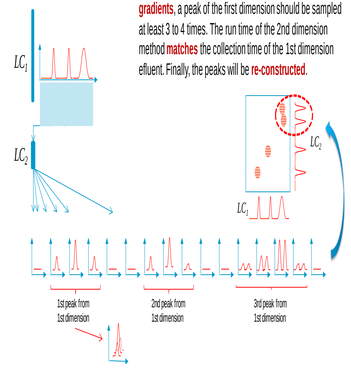


Agilent publication number5990-5572EN

Types of 2D LC Experiments

Comprehensive 2D-LC (LCxLC):

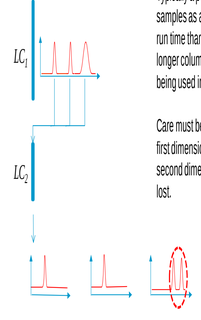
The complete effluent of the first column will be injected to the second column and will be analyized with very fast gradients, a peak of the first dimension should be sampled at least 3 to 4 times. The run time of the 2nd dimension method matches the collection time of the 1st dimension effluent. Finally, the peaks will be re-constructed.



Heart-cutting 2D-LC (LC-LC):

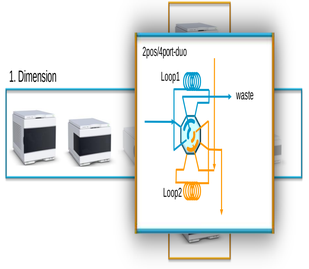
Only parts of the effluent of the first column – the peaks eluted from the 1st dimension column - will be injected to the second column.
Typically a peak from the first dimension will be samples as a whole and a gradient with a longer run time than the collection time will be used. Also longer columns with higher seperation efficiency are being used in as 2nd dimension column.

Care must be taken if peaks are eluting from the first dimension column when a gradient on the second dimension is still running – this peak will be lost.



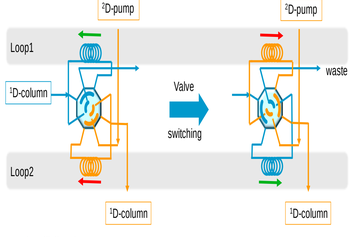
Valves

Hardware – Valves, uniqueness and flexibility



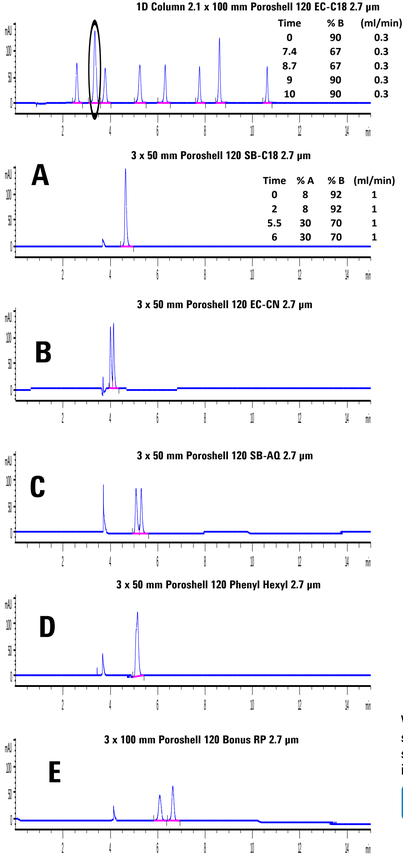
New Agilent-only special 2D-LC-valve. Single valve with fully symmetric flow-paths and symmetric fill/flush-out behavior. Only valve that allows countercurrent flush-out of loops.

Advantages of the new 2pos/4port-duo valve



All flow paths are equal (no additional bridging loops)
Symmetric contercurrent fill/defill of loops (reducing band-spread)
All in one valve (no synchronization, costs)

Results and Discussion



Bonus RP offers very unique selectivity

- Bonus RP is one of the most different phases to C18 column
- F value in Hydrophobic Subtraction Model is 255 (relative to Eclipse Plus C18)

Column	Phase	H	S	A	B	C(2.8)	C(7.0)	Type	Fs
Zorbax Eclipse Plus C18	C18	1.030	0.007	-0.072	-0.020	-0.004	0.020		0.0
Zorbax Bonus RP	EP	0.654	0.107	-1.046	0.373	-2.971	-1.103	EP	254.5
Zorbax C18	C18	1.089	0.055	0.474	0.060	1.489	1.566	A	125.6
Zorbax C8	C8	0.974	-0.041	0.216	0.176	0.974	1.051	A	86.4
Zorbax 300A SB-C18	C18	0.905	-0.050	0.045	0.043	0.254	0.701	B	24.2
Zorbax SB-CN	CN	0.502	-0.108	-0.224	0.042	-0.146	1.047	CN	20.4
Zorbax SB-Phenyl	Phenyl	0.623	-0.161	0.065	0.038	0.033	1.089	phenyl	20.1
Zorbax SB-AQ	EP	0.593	-0.120	-0.083	0.038	-0.136	0.736	EP	19.5

USP column equivalence PQRI database

The first dimension separation is carried out using a Poroshell 120 ECC-18 column. In the 2D chromatogram A, a Poroshell 120 SB-C18. is used. In this example chromatogram the peak is retained but no additional separation is achieved. This is not surprising as both columns are C18 phases. No effect on the parent chromatogram is caused by the sampling Chromatogram B is generated using a Poroshell 120 EC-CN column. In this chromatogram, the peaks are not retained as long column A. However, in this case the peaks are separated. This is most likely due to the π-π interactions between the analytes and the stationary phase. Chromatogram C(Poroshell 120 SB-AQ) shows an even greater degree of separation. than either of the previous chromatograms. Chromatogram D is generated using a Poroshell 120 Phenyl Hexyl phase. The analyte peaks in this chromatogram are When used in acetonitrile π-π interactions between the analytes and the stationary phase. Can be overwhelmed but at low organic content this is not the case. However since the column has a long aliphatic linker it can also have hydrophobic character similar to a C18. The final 2D chromatogram (E) is generated with a Poroshell 120 Bonus RP column. As shown above in the PQRI table the Bonus RP column is one of the most dissimilar phases when compared to a C18 phase. As can be seen even on this shallow gradient the peak shape is good and the peaks are well resolved.

Conclusions

When picking a 2D column for the second dimension, choose something that is dissimilar to the D1 phase, Resources such as the PQRI data base is a good guide. Future work will include use of Methanol, different buffers and HILIC.

Acknowledgements

I wish to thank Bob Giuffre, Klaus Witt, Edgar Naegele and Dale Connor for their helpful discussions and assistance in producing this work.