

Performance evaluation of the Agilent 1290 Infinity 2D-LC Solution for comprehensive two-dimensional liquid chromatography

Technical Overview



Abstract

This Technical Overview presents an example of a comprehensive setup for two-dimensional liquid chromatography (2D-LC) based on the Agilent 1290 Infinity 2D-LC Solution. The instrument configuration and the setup of the interface between the first and second chromatographic dimension is described in detail. The analysis of a standard sample with statistical evaluation of the results is shown to demonstrate the performance of the instrument.



Agilent Technologies

Introduction

The separation of complex samples can be improved by deploying a comprehensive two-dimensional liquid chromatography (LCxLC) system. In an LCxLC system, typically two pumps and two columns are used and fractions from the separation on the first column (first dimension) are continuously transferred to a second column (second dimension). This transfer is done by filling two loops alternately controlled by valve switching.

The advantage of LCxLC separation compared to a standard LC separation is the increased peak capacity due to the multiplicative behavior of the peak capacities of the first and second dimension¹. This is an idealistic model that is only valid if completely orthogonal separation mechanisms are used for the separation on the columns in the first and second dimension². This state can be approximated for separations such as ion exchange in the first and reversed phase separation in the second dimension. In reality, similar selectivities like different reversed phase selectivities are used. In this case, the peak capacity is decreased and can be explained as an accessible triangular area of the two-dimensional chromatogram used for the separation³. This can be optimized by designing complex gradients for the separation in the second dimension to increase the accessible area used for separation and herewith increasing peak capacity.

To achieve best separation performance, a complex gradient was designed for the second dimension by a specialized software tool.

Experimental

Equipment

The Agilent 1290 Infinity 2D-LC Solution used for the performance evaluation comprised the following modules:

- Two Agilent 1290 Infinity Pumps (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A) with thermostat (G1330)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316B) with 2-position/4-port duo valve (G4236A) for 2D-LC
- Agilent 1290 Infinity Diode Array Detector (G4212A) with 60-mm Agilent Max-Light flow cell (G4212-60007)

In this configuration, the first and second dimension pumps are identical. Typically, the second dimension pump must be an Agilent 1290 Infinity pump to deliver fast gradients to the second dimension column. The first dimension pump is flexible and could also be an Agilent 1260/1290 Infinity Quaternary Pump, an Agilent 1260 Infinity Binary Pump or an Agilent 1260 Infinity Capillary Pump.

Software

- Agilent OpenLAB CDS ChemStation Edition, version C.01.03 with 2D-LC add-on software
- LCxLC Software for 2D-LC data analysis from GC Image LLC, Lincoln, NE, USA

Columns

First	
dimension:	Agilent ZORBAX RRHD
	Eclipse Plus C18,
	150 × 2.1 mm, 1.8 µm

Second

dimension: Agilent ZORBAX RRHD Eclipse Plus Phenyl Hexyl, 50 × 3.0 mm, 1.8 µm

Method

First dimension pump

Solvent A:	Water + 0.1% formic acid
Solvent B:	Acetonitrile + 0.1% formic acid
Flow rate:	0.1 mL/min
Gradient:	5% B at 0 min 95% B at 30 min 95% B at 40 min
Stop time:	40 min
Post time [.]	15 min

Second dimension pump

Solvent A:	Water + 0.1% formic acid	
Solvent B:	Methanol + 0.1% formic acid	
Flow rate:	3 mL/min	
Initial gradient:	5% B at 0 min 15% B at 0.5 min 5% B at 0.51 min 5% B at 0.65 min	
Gradient modulation:	5% B at 0 min to 50% B at 30 min 15% B at 0.5 min to	
	95% B at 30 min	
	50% B at 30 min	
	5% B at 0.65 min to 50% B at 30 min	

The final second dimension gradient is shown in Figure 1. The user interface for setup of the second dimension gradient is shown and explained in Figure 2.



Figure 1

2D-LC Gradient profile.1st dimension Gradient (red): 0 min 5% B–30 min 95% B, 40 min–95% B. Stop time: 40 min. Post time: 15 min. 2nd dimension Gradient (blue): Initial Gradient: 0 min–5% B, 0.5 min–15% B, 0.51 min–5% B, 0.65 min–5% B. Gradient Modulation: 0 min 5% B to 30 min 50% B, 0.5 min 15% B to 30 min 95% B, 0.51 min 5% to 30 min 50% B.



Figure 2

Window for the set-up of the second dimension gradient in a 2D-LC separation.

- A) Nested table for the set-up of the 2D-LC gradient and gradient shift.
- B) Table for the set-up of time, and peak-based triggering events to start 2D-LC separation.
- C) Graphic display of the 2D-LC gradient according to the set-up in the 2D-LC gradient table A and B. The gradient can be changed by drag and drop of the points of the curve in this graphical interface. The changed values are written back to the gradient table.
- D) Graphical display of a single gradient snip in the second dimension which is repeated by the modulation rate and shifted as given in the gradient table.
- E) Calculations which help to set-up the 2D-LC separation experiment from currently used LC parameters.

Thermostatted column compartment

- First dimension column at left side at 25 °C.
- Second dimension column at right side at 60 °C.
- Two 80 µL loops are connected to the 2-position/4-port duo valve and are located at the left side. The valve is switched automatically after each second dimension modulation cycle. The complete plumbing is shown in Figure 3. In this case, the loops are used in a countercurrent manner (the loops are filled and eluted from different sides).

Autosampler

- Injection volume: 5 µL
- Sample temperature: 8 °C
- Needle wash: 6 s in methanol

Diode array detector

- Wavelength: 260/4 nm, Ref. 360/16 nm
- Slit: 4 nm
- · Data rate: 80 Hz
- Flow cell: 60 mm Max-Light
 flow cell

Chemicals

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



Figure 3

2D-LC with 2-position/4-port duo valve interface configuration, green arrow (\rightarrow) Fill-direction, red arrow (\rightarrow) Analyze-direction, (LCxLC, countercurrent).

RRLC Checkout Sample containing acetophenone, propiophenone, butyrophenone, valerophenone, hexanophenone, heptanophenone, octanophenone, benzophenone and acetanilide was purchased from Agilent Technologies (p/n 5188-6529). Gradient Evaluation Mix containing uracil, phenol, methyl paraben, ethyl paraben, propyl paraben, butyl paraben, and heptyl paraben was purchased from Sigma-Aldrich (catalog no. 48271). Reversed Phase Test Mix containing uracil, phenol, N,N-Dimethyl-m-toluamide and toluene was purchased from Sigma-Aldrich (catalog no. 47641-U). Sulfamethazine (Stock solution: 100 µg/mL, catalog no. S6256) and 2-hydroxy quinoline (Stock solution: $100 \,\mu g/mL$; catalog no. 270873) were purchased from Sigma-Aldrich.

Sample

The standards were mixed to a 2D-LC test sample as following:

- RRLC Checkout Sample: 100 µL
- Gradient Evaluation Mix: 800 µL
- Reversed Phase Test Mix: 200 μL
- Sulfamethazine: 100 µL
- 2-hydroxy quinoline: 100 µL

Results and discussion

The maximum peak capacity in a two-dimensional LC separation can only be achieved in cases where the separation mechanisms of the two dimensions are truly orthogonal. If they are similar, only a triangular range of the separation area is used. This becomes especially important when two reversed phase separations are combined in a 2D-LC experiment such as separation on a C18 phase for standard separation and a hexylphenyl phase for selective separation of aromatic compounds. Typically, the separated compounds are narrowly distributed around a diagonal line in the separation diagram (Figure 4.1).

This is especially true when a simple gradient is used for the second dimension. The separation in the second dimension can be optimized for an improvement in separation by a modification of the second dimension gradient. This is done with the special 2D-LC software tool for gradient setup. In the first step (Figures 4.2 and 4.3) the second dimension gradient maximum is adapted to the first dimension maximum at the respective time. This opens the separation angle and improves separation, but the compounds lose their focus and show dispersion along the separation. To avoid this and to move them into the middle of the separation, the starting composition of the gradient is also moved to a higher organic level during the separation in the second dimension (Figure 4.4) which achieves optimum focusing, area and separation.

In the optimized separation, all compounds were clearly separated in the second dimension, even compounds co-eluting from the first dimension (Figure 5).



Figure 4

Optimization of the 2nd dimension gradient. Red line in bottom pictures shows the 1st dimension gradient and the blue line the repeating 2nd dimension gradient.

- 1) 2nd dimension gradient repeats between 5% and 95% organic
- 2) First adjustment of the second dimension gradient to improve separation by lower organic in the second dimension for the compounds of higher polarity
- Second adjustment to the same maximum organic composition as given from the first dimension at each top of the 2nd dimension gradient
- 4) Increase in organic starting composition during the runtime to improve separation and focus the compounds by better usage of separation time in the 2nd dimension



Figure 5

Comparison of peaks eluting at 21.45 minutes from the first dimension separated acceding to the second dimension gradient shown in Figure 3.1 (A) and 3.4 (B)

- A) Peaks of compound 9 and 10 eluting at 27.78 sec and 29.51 sec
- B) Peaks of compound 9 and 10 eluting at 22.65 sec and 26.30 sec

The compounds could be detected by the 2D-LC data analysis software and are annotated as red circles (Figure 6). The retention times in the first and second dimension are given in Table 1. The compound number 1 is uracil, which shows the dead time of 4.55 minutes for the first dimension and 8.32 seconds for the second dimension. Compound number 20 is octanophenone eluting at the end of the separation at 33.80 minutes/ 27.94 seconds.



Figure 6

Detection of all used compounds in the optimized 2D-LC separation. The compounds are separated in the second dimension, even compounds which are co-eluting from the first dimension in the same time segment are separated in the second dimension. The detected compounds are annotated by round red circles. The numbers correspond to the following list:

 uracil, 2) sulfamethazine, 3) 2-hydroxy quinoline, 4) acetanilide, 5) phenol, 6) methyl paraben, 7) acetophenone, 8) ethyl paraben, 9) propyl paraben, 10) N,N-Dimethyl-m-toluamide, 11) propiophenone, 12) butyl paraben, 13) butyrophenone, 14) toluene, 15) benzophenone, 16) valerophenone, 17) hexanophenone, 18) heptyl paraben, 19) heptanophenone, 20) octanophenone

	Compound	RT I (min)	RT II (sec)
1	Uracil	4.55	8.32
2	Sulfamethazine	13.00	16.71
3	2-Hydroxy quinoline	13.00	20.06
4	Acetanilide	14.30	17.61
5	Phenol	15.60	14.56
6	Methyl paraben	16.25	20.95
7	Acetophenone	18.85	20.58
8	Ethyl paraben	18.85	22.88
9	Propyl paraben	21.45	22.65
10	N,N-Diethyl-m-toluamide	21.45	26.38
11	Propiophenone	22.75	19.81
12	Butyl paraben	23.40	23.71
13	Butyrophenone	24.05	21.65
14	Toluene	26.00	19.57
15	Benzophenone	26.00	22.21
16	Valerophenone	27.95	21.28
17	Hexanophenone	29.90	22.95
18	Heptyl paraben	29.90	26.62
19	Heptanophenone	31.85	26.89
20	Octanophenone	33.80	27.94

Table 1

Compounds in the 2D-LC test mixture.

RT I (min): Retention time in the first dimension in minutes

RT II (sec): Retention time in the second dimension in seconds

The complete separation could be displayed in a three-dimensional plot (Figure 7). This plot shows the first dimension separation, achieved in 40 minutes, from the left to the right side with the overlay of the first dimension peaks at the back side. The second dimension separation, achieved in 39 seconds, goes from the front side to the backside and the overlay of all second dimension peaks could be found at the right side. This shows that some peaks are co-eluting from the first dimension and separation is done in the second dimension. In particular, this is shown for the peaks eluting at 24.050, 26.000, and 27.950 minutes from the first dimension column (Figure 8). Figures 7 and 8 show that the compounds eluting at 26.000 minutes from the first dimension are separated in the second dimension at 19 seconds and 22 seconds. The other traces at 24.050 and 27.950 minutes do not contain more than one peak.





3-dimensional plot of sample separation from the 2D-LC run. The first dimension separation (40 minutes) is shown from the left to the right. The overlay of 1st dimension peaks is shown on the back side. The second dimension separation (39 seconds) is shown from the front side to the backside. The overlay of all 2nd dimension peaks is shown on the right side.





To determine the precision of retention times and peak volumes, the sample was injected 10 times (Figure 9 and Figure 10). A precision for the retention time in the first dimension could not be determined because the equivalent of the eluent from the first dimension that was transferred to the second dimension is 0.65 minutes. The retention time precision in the second dimension separation is below 1% RSD for 15 compounds out of the set of 20 compounds and always below 2% RSD (Figure 9). For the peak volume, the RSD is below 1% for eight compounds, between 1% and 2% for eight compounds and above for four compounds but never above 3% RSD (Figure 10).

Conclusion

This Technical Overview shows the easy setup of the Agilent Infinity 2D-LC solution comprising at least one Agilent 1290 Infinity LC pump with the 2-position/4-port duo valve for 2D-LC in the column compartment. The use of the 2D-LC software add-on for Agilent OpenLAB CDS **ChemStation Edition is demonstrated** by optimizing the method in an RPxRP separation. Finally, the system performance is demonstrated by a statistical evaluation of retention times in the second dimension and the peak volumes achieved from specialized LCxLC data analysis software.

References

1.

J.C. Giddings, *Anal. Chem.*, 56 (**1984**) 1258A.

2.

J.C. Giddings, *J. Chromatogr. A*, 703 (**1995**) 3.

3.

Z. Liu, D.G. Patterson Jr., M.L. Lee, Anal. Chem., 67 (**1995**) 3840.









Figure 10

Relative standard deviation (RSD [%]) of peak volume

www.agilent.com/chem/2D-LC

© Agilent Technologies, Inc., 2012 Published in USA, August 1, 2012 Publication Number 5991-0138EN



Agilent Technologies