

# Increasing Peak Capacity Using the Agilent 1290 Infinity 2D-LC Solution

## Technical Overview

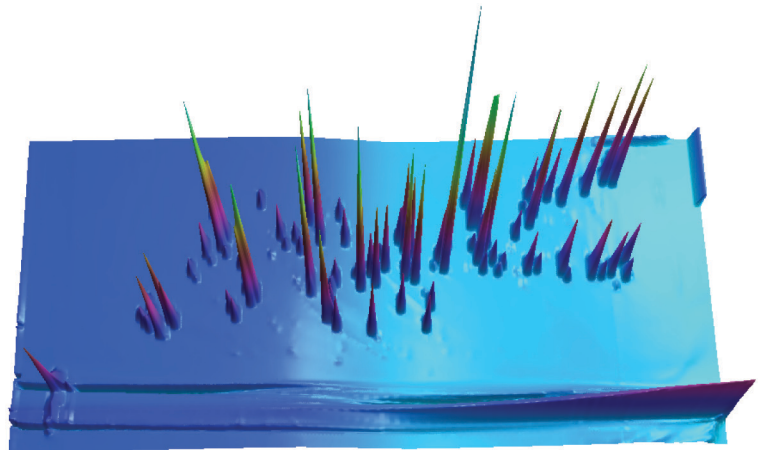
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### Abstract

Two-dimensional comprehensive LC is an effective way to significantly increase resolving power and peak capacity in liquid chromatography. This Technical Overview demonstrates that with the Agilent 1290 Infinity 2D-LC solution, the effective peak capacity can be increased by a factor of 3 to 4 compared to classical HPLC (using columns with 3.5- $\mu\text{m}$  particles), or to UHPLC (using columns with 1.8- $\mu\text{m}$  particles) while analysis time is maintained. This increase in available peak capacity significantly improves the resolving power for complex samples and, moreover, drastically reduces the probability of overlap of solute peaks.



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## Introduction

For the chromatographic analysis of complex samples, peak capacity ( $n_c$ ) is the key parameter to specify the probability and quality of the separation. This metric, introduced in 1967 by Giddings<sup>1</sup>, is defined as the number of peaks that can be separated with a certain resolution in a chromatographic run or defined time window. Peak capacity for one-dimensional gradient LC runs can easily be calculated using Equation 1, where  $t_{grad}$  is the total gradient run time, and  $\bar{w}$  is the average peak width. To calculate peak capacity for a separation with unit resolution ( $R_s = 1$ ), the peak width at 13.4 % height is commonly taken (four times the standard deviation of a peak,  $4\sigma$ ).

$$n_{c,grad} = 1 + \frac{t_{grad}}{\bar{w}}$$

Equation 1.

For most gradient analyses, there is a part of the gradient that is not populated with peaks, for example, the beginning (system and column void volume) and end of the chromatogram (column rinsing). Therefore, Equation 2 is a more truthful calculation, taking into account only the actual elution window, that is, the time interval in which all compounds of interest elute. In Equation 2,  $t_l$  and  $t_r$  are the retention times of the last and the first eluting peaks, respectively. Under optimal conditions, the compound eluting at  $t_l$  will have a retention factor ( $k$ ) of about 2, resulting in sufficient retention for good chromatography with minimal loss of separation space at the beginning of the run.

$$n_{c,ew} = 1 + \frac{t_l - t_r}{\bar{w}}$$

Equation 2.

Unfortunately, peaks are not evenly distributed along a chromatogram, but elute in a more or less random fashion determined by the sample composition and by the stationary and mobile-phase chemistries. Consequently, peak capacity does not estimate the true number of compounds that will be separated in a chromatographic run, but it is very useful to compare chromatographic methods and approaches, since it is a basic parameter in the theory of component overlap<sup>2</sup>.

Advances in column technology (smaller particles, superficially porous particles) and instrumentation (pressure capabilities, dead volume reduction) have provided means to increase peak capacity. In previous works, we have reported peak capacities of about 900–1,000 in 9 hours with HPLC equipment<sup>3</sup> and 850 in 4 hours with UHPLC equipment<sup>4</sup>.

Another effective way to obtain high peak capacity, but within a more realistic time frame such as 60 minutes, is to perform comprehensive 2D-LC or LC×LC where the total sample is subjected to two different, independent separations. LC×LC is known to substantially increase peak capacity as long as the two dimensions are orthogonal and the separation obtained in the first dimension is essentially maintained upon transfer to the second dimension<sup>5,6</sup>. Although the peak capacity generated by the fast, second-dimension separation is, in general, relatively small, the impact on the overall peak capacity is significant. The theoretical peak capacity for an LC×LC analysis under ideal conditions would be the product of the peak capacities in both dimensions.

This value, however, needs to be reduced due to various factors such as lack of orthogonality and incomplete 2D separation space coverage, undersampling, and other technical factors. As a result, the peak capacity in 2D chromatography will be at best about one order of magnitude larger than its one-dimensional counterpart<sup>7,8</sup>.

This is, however, still significantly higher than the optimized one-dimensional separation for the given analysis in the same analysis time.

To illustrate this, a study was performed to evaluate the practical increase in peak capacity that can be generated with the Agilent 1290 Infinity 2D-LC solution using state-of-the-art equipment and columns. A mixture of 69 solutes was prepared, and the separations obtained by HPLC, UHPLC, and LC×LC were compared.

More information on the theoretical background of LC×LC and the technical aspects of the 1290 Infinity 2D-LC solution can be found in the Agilent Primer *Principles, Practical Implementation and Applications of Two-Dimensional Liquid Chromatography*<sup>8</sup>.

## Experimental

### Samples and sample preparation

The complete sample containing 69 compounds is a mixture of four submixtures diluted in acetonitrile.

Polyaromatic Hydrocarbons (PAHs), Mix 25, US EPA 16 (Dr. Ehrenstorfer, Augsburg, Germany)

- 16 compounds, 2,000 µg/mL in acetone/benzene
- Final concentration: 2.5 µg/mL (separate submixture in acetone)
- Acenaphthene  
Acenaphthylene  
Anthracene  
Benzo[a]anthracene  
Benzo[a]pyrene  
Benzo[b]fluoranthene  
Benzo[k]fluoranthene  
Benzo[ghi]perylene  
Chrysene  
Dibenzo[ah]anthracene  
Fluoranthene  
Fluorene  
Indeno[1,2,3-cd]pyrene  
Naphthalene  
Phenanthrene  
Pyrene

Phthalate esters, Analytes Mix 3  
(Dr. Ehrenstorfer, Augsburg, Germany)

- 17 compounds, 1,000 µg/mL in hexane
- Final concentration: 30 µg/mL (separate submixture in acetonitrile)
- Benzyl benzoate  
*Bis*(2-ethoxyethyl)phthalate  
*Bis*(2-ethylhexyl)phthalate  
*Bis*(2-methoxyethyl)phthalate  
*Bis*(2-*n*-butoxyethyl)phthalate  
*Bis*(4-methyl-2-pentyl)phthalate  
Butylbenzylphthalate  
Dimethylphthalate  
Diamyl phthalate  
Dicyclohexyl phthalate  
Diethyl phthalate  
Dihexyl phthalate  
Diisobutyl phthalate  
Dinonyl phthalate  
Di-*n*-butyl phthalate  
Di-*n*-octyl phthalate  
Hexyl-2-ethylhexyl phthalate

Phenones, RRLC Checkout Sample  
(Agilent Technologies, Waldbronn, Germany)

- 9 compounds, 100 µg/mL in water/acetonitrile
- Final concentration: 12 µg/mL (separate submixture in acetonitrile)
- Acetanilide  
Acetophenone  
Benzophenone  
Butyrophenone  
Heptanophenone  
Hexanophenone  
Octanophenone  
Propiophenone  
Valerophenone

Pesticides, Mix 34 (Dr. Ehrenstorfer, Augsburg, Germany)

- 27 compounds, 100 µg/mL in acetonitrile
- Final concentration: 40 µg/mL (separate submixture in acetonitrile)
- Atrazine  
Atrazine-desethyl  
Atrazine-desethyl desisopropyl  
Chloroxuron  
Chlorpropham  
Chlortoluron  
Crimidin  
Cyanazine  
Diuron  
Fenuron  
Isoproturon  
Linuron  
Metamitron  
Metazachlor  
Methabenzthiazuron  
Metobromuron  
Metolachlor  
Metoxuron  
Metribuzin  
Monolinuron  
Prometryn  
Propazine  
Propham  
Sebuthylazine  
Simazine  
Terbutylazine  
Terbutryne

### Instrumentation

An Agilent 1290 Infinity 2D-LC solution with the following configuration was used for the experiments.

- Agilent 1290 Infinity Binary Pump (first dimension) (G4220A)
- Agilent 1290 Infinity Binary Pump (second dimension) (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostat (G1330A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector with standard flow cell (G4212A)
- Agilent 1290 Infinity Valve Drive (G1170A)
- Agilent 2 position/4-port duo-valve for 2D-LC (G4236A)

### Software

- Agilent OpenLAB CDS ChemStation Edition Software (revision C.01.07) with 1290 Infinity 2D-LC software (revision A.01.02)
- GC Image LC×LC Edition Software for 2D-LC data analysis (GC Image, LLC., Lincoln, NE, USA)

## Results and Discussion

### Comparison of one-dimensional HPLC and UHPLC

Since efficiency in LC is inversely proportional to the particle size, reducing the particle diameter leads to reduced peak widths and, thus, higher peak capacity. However, smaller particles also generate a higher pressure-drop across the column, and UHPLC systems are required to run analyses with these small particle (sub-2  $\mu\text{m}$ ) columns. The Agilent 1290 Infinity LC system does not only provide the necessary power to operate sub-2- $\mu\text{m}$  columns, dispersion is also minimized due to an optimized flow-path with minimal delay and dead volume. It has been demonstrated that this optimized UHPLC system, in combination with small particle columns, results in a higher peak capacities compared to peak capacities that can be generated on classic HPLC instrumentation with standard particle HPLC columns<sup>4</sup>.

To be able to make a fair comparison between HPLC and UHPLC columns, the tests performed in this study were generated on the same 1290 Infinity LC system with the same configuration and system volumes. Figure 1 shows the result of a one-dimensional separation of the 69-component test mix on both columns using a flow rate of 0.1 mL/min. From the chromatograms, it is obvious that peaks are narrower on the UHPLC column compared to the column packed with 3.5- $\mu\text{m}$  particles. Consequently, the resolution and peak capacity on this column are higher. This is, for instance, clearly illustrated by the separation of the peaks eluting, in the window between 44 and 46 minutes (between labeled peaks 6 and 7).

### 1D-LC Method

Separation conditions	
Column for HPLC	Agilent ZORBAX RRHD Bonus-RP, 2.1 $\times$ 150 mm, 3.5 $\mu\text{m}$ (p/n 863700-901)
Column for UHPLC	Agilent ZORBAX RRHD Bonus-RP, 2.1 $\times$ 150 mm, 1.8 $\mu\text{m}$ (p/n 859768-901)
Solvent A	Water
Solvent B	Methanol
Flow rate	Varied from 100 to 450 $\mu\text{L}/\text{min}$
Gradient	0–60 minutes: 30–100 %B 60–75 minutes: 100 %B
Column temperature	25 $^{\circ}\text{C}$
Detection	
Wavelength	Signal 220/10 nm, reference 400/100 nm
Data rate	40 Hz
Injection*	
Volume	Adapted to flow rate (combined with equal volume of water with injection program) Flow rate 100 $\mu\text{L}/\text{min}$ : 1.0 $\mu\text{L}$ (combined with equal volume of water) Flow rate 150 $\mu\text{L}/\text{min}$ : 1.5 $\mu\text{L}$ (combined with equal volume of water) Flow rate 200 $\mu\text{L}/\text{min}$ : 2.0 $\mu\text{L}$ (combined with equal volume of water) Flow rate 250 $\mu\text{L}/\text{min}$ : 2.5 $\mu\text{L}$ (combined with equal volume of water) Flow rate 300 $\mu\text{L}/\text{min}$ : 3.0 $\mu\text{L}$ (combined with equal volume of water) Flow rate 350 $\mu\text{L}/\text{min}$ : 3.5 $\mu\text{L}$ (combined with equal volume of water) Flow rate 400 $\mu\text{L}/\text{min}$ : 4.0 $\mu\text{L}$ (combined with equal volume of water) Flow rate 450 $\mu\text{L}/\text{min}$ : 4.5 $\mu\text{L}$ (combined with equal volume of water)
Needle wash	5 seconds flush port (methanol/acetonitrile 50/50)
Injector temperature	4 $^{\circ}\text{C}$

\*The samples were injected with a water plug to avoid peak broadening or splitting due to the strong injection solvent.

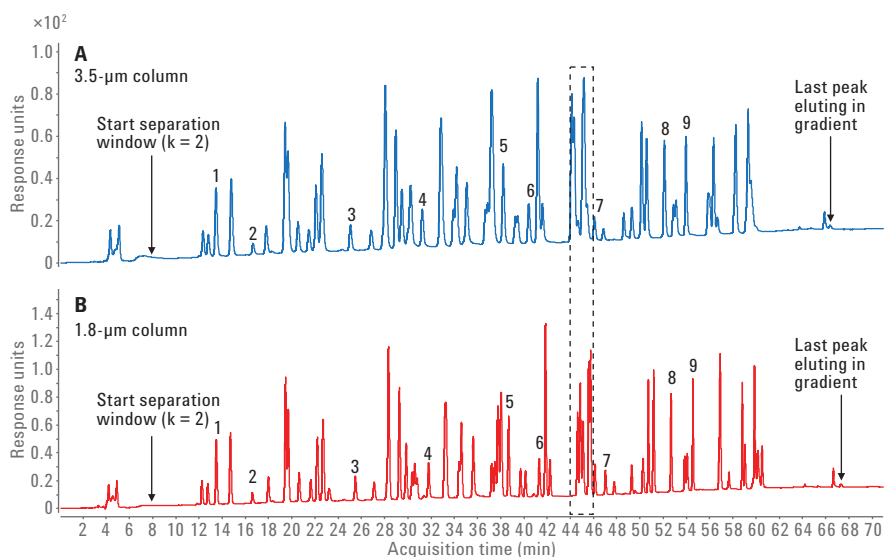


Figure 1. Comparison of one-dimensional (1D-LC) separation on 3.5- $\mu\text{m}$  column (HPLC) and 1.8- $\mu\text{m}$  column (UHPLC) under identical conditions. Flow rate is 0.1 mL/min. For other conditions, see section on methods. The labeled peaks are the reference peaks used in the peak capacity calculation (1, 3, and 4 are phenones, 2 and 5 are pesticides, 6 and 7 are PAHs, and 8 and 9 are phthalate esters).

## 2D-LC Method

First dimension	
Column	Agilent ZORBAX RRHD Bonus-RP, 2.1 × 150 mm, 1.8 μm (p/n 859768-901)
Solvent A	Water
Solvent B	Methanol
Flow rate	100 μL/min
Gradient	0–60 minutes: 30–100 %B 60–70 minutes: 100 %B Post-time: 10 minutes at 30 %B
Column Temperature	25 °C
Second dimension	
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 3.0 × 50 mm, 1.8 μm (p/n 959757-302)
Solvent A	Water
Solvent B	Acetonitrile
Flow rate	2 mL/min
Idle Flow rate	0.3 mL/min
Initial gradient	12 %B at 0 minutes 18 %B at 0.35 minutes 18 %B at 0.40 minutes 12 %B at 0.41 minutes
Gradient modulation	12 %B at 0 minutes to 100 %B at 70 minutes 18 %B at 0.35 minutes to 100 %B at 60 minutes
Column Temperature	45 °C
Modulation	
Loops	Two 60 μL loops, co-current configuration
Modulation time	0.50 minutes
Injection*	
Volume	5 μL (combined with 5 μL water with injection program)
Needle wash	5 seconds flush port (methanol/acetonitrile 50/50)
Injector temperature	4 °C
Detection	
Wavelength	Signal 220/10 nm, reference 400/100 nm
Data rate	80 Hz

\*The samples were injected with a water plug to avoid peak broadening or splitting due to the strong injection solvent.

Table 1. Performance of the one-dimensional separation at various flow rates on an HPLC (3.5-μm particle) column and a UHPLC (1.8-μm particle) column, respectively. For conditions, see section on methods.  $\bar{w}$  is the average peak width at 13.4 % height.  $n_c$  is the peak capacity in the selected window ( $k = 2$  until last eluting peak).

Flow rate (μL/min)	Practical elution window (min)	3.5 μm		1.8 μm	
		$\bar{w}$ (4s, min)	$n_c$	$\bar{w}$ (4s, min)	$n_c$
450	56.7			0.1885	301
400	56.8			0.1871	304
350	57.0	0.2675	212	0.1871	305
300	57.3	0.2692	212	0.1976	291
250	57.7	0.2718	212	0.2035	283
200	58.1	0.2800	206	0.2101	276
150	58.4	0.2966	195	0.2196	265
100	58.8	0.3270	177	0.2306	258

The analysis of the test mixture was also carried out on both columns using different flow rates. The injection volumes were adjusted accordingly to exclude effect of lower sample loading relative to a higher flow rate on the peak width. Peak capacities were calculated using Equation 2. The average peak width was taken for a selection of nine compounds, and the useful separation window ( $k = 2$  to last eluting peak in the gradient) was taken into account, not the complete gradient time. Due to the gradient setup, there is some separation space available before the first and after the last eluting component of the test mix. In a real sample, this space could be occupied with analytes. Table 1 summarizes the results.

Table 1 gives a good indication of the possibilities and limitations of the HPLC and UHPLC analyses. The influence of flow rate is rather small. On the 3.5-μm column, the peak capacity ranges from 180 to 210, and on the 1.8-μm column from 260 to 300.

Even at the optimal flow rate using the UHPLC column, the generated peak capacity of 305 results in a probability of only 64 % that detected peaks correspond to a single solute (about one-third of the 69 solutes will, thus, not be resolved). Visual inspection of the UHPLC chromatogram in Figure 1 indicates that about 60 to 70 % of the peaks are separated with resolution equal or higher than one, which corresponds well with theoretical prediction<sup>2</sup>. To have a good chance to separate all compounds (probability greater than 98 %), a peak capacity of over 6,900 would be required. It is clear that this will not be possible with this setup. Obviously, analytical conditions (stationary phase, mobile phase, temperature, and so on) can be changed in order to change selectivity for the compounds at hand, but the probability that compounds will be separated in one single run remains equally low.

## Two-dimensional LC×LC

The 150-mm column packed with 1.8- $\mu\text{m}$  particles (UHPLC) and operated at 0.1 mL/min was then used as a first-dimension column in an LC×LC method. Using the 1290 Infinity 2D-LC solution with a 2 position/4-port duo-valve for 2D-LC equipped with two 60- $\mu\text{L}$  sample loops, 50- $\mu\text{L}$  fractions of the first-dimension separation were transferred to the second dimension. The modulation time was 0.5 minutes. The second-dimension column was an Agilent ZORBAX Eclipse Plus C18 RRHD column thermostatted at 45 °C. In the second dimension, compounds were eluted with fast water/acetonitrile gradients of which the starting composition and gradient span were changed along the run (constantly shifted elution mode). Figure 2 shows a schematic of the valve configuration and the setup of the 2D-LC method.

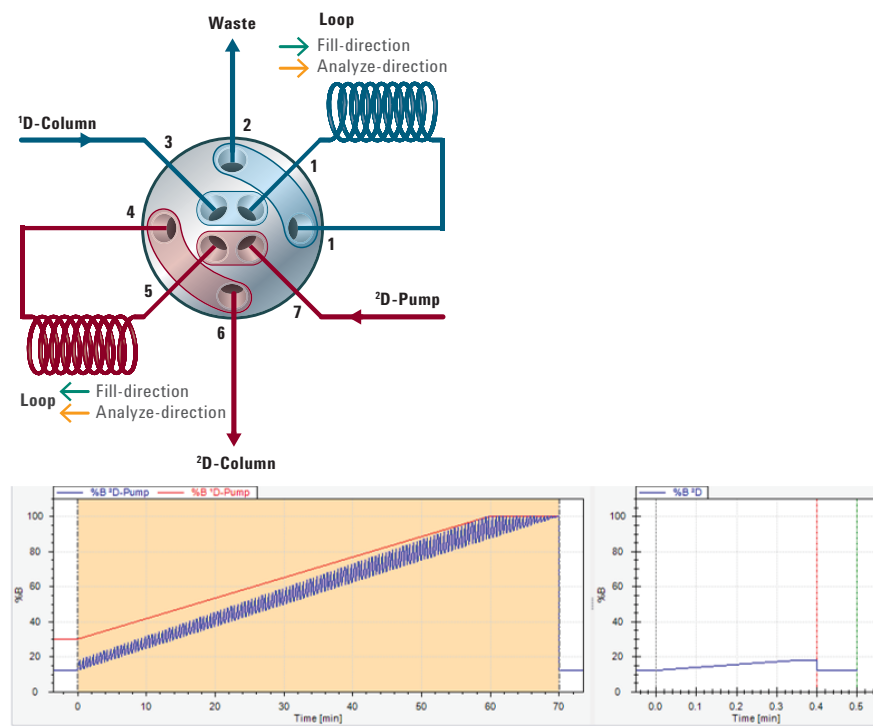


Figure 2 Configuration of the 2 position/4-port duo-valve and setup of the 2D-LC method. The red line is the first-dimension gradient, the blue line is the second-dimension gradient.



Although both dimensions use a reversed-phase LC mechanism, the difference in column chemistry, column temperature, and solvent type result in acceptable orthogonality as can be seen in Figure 3, showing the LC×LC plots for the 69-solute mixture and the four submixtures. The additional possibility of modeling the second-dimension gradient along the analysis also helps to improve the separation.

The peak capacity for the second dimension separation can be calculated in the same manner as for the one-dimensional runs. With an average peak width, at 13.4 % peak height, of 0.0237 minutes (about 1.4 seconds), and a useful elution window of 0.4 minutes (24 seconds), the resulting peak capacity in the second dimension is 16.8. The theoretical peak capacity for this LC×LC system under ideal conditions (that is, equal to the product of peak capacities in both dimensions) would, thus, be 4,330 ( $n_{c,D1} = 258.1$  and  $n_{c,D2} = 16.8$ ). If that would be obtained, the probability that a peak is a single compound would be more than 95 %. However, this value cannot be obtained in practice due to undersampling and incomplete orthogonality (limited 2D separation space coverage because the spots are not randomly distributed across the contour plot).

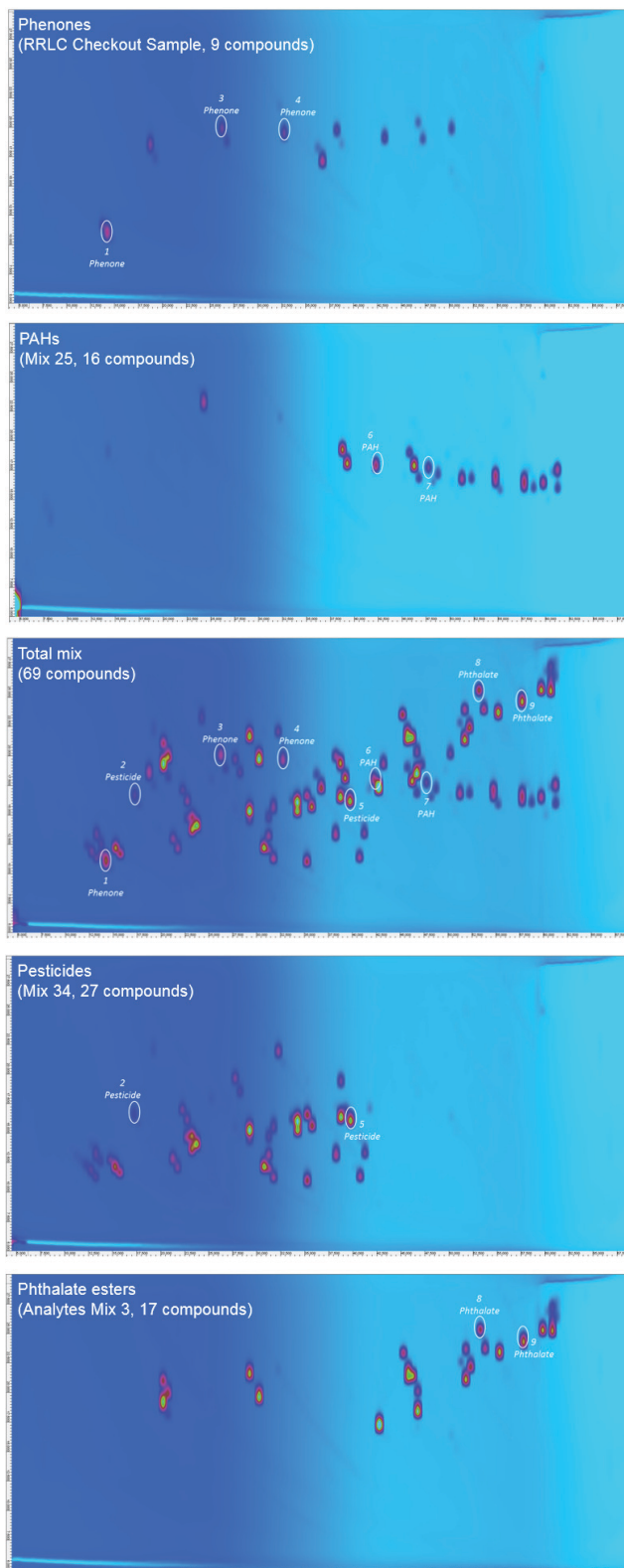


Figure 3 LC×LC contour plots of all submixtures and the total mixture. The labeled peaks are the reference peaks used in the peak capacity calculation.

Since a reversed-phase principle is used in the first and second dimension, there is some retention correlation between both separations. Consequently, the spots are scattered along a diagonal line in the contour plot, and parts of the separation space become unavailable<sup>9</sup>. Only the effective area of the plot is useful, so this needs to be corrected for using a coverage factor. The advantage of partly correlated dimensions is that calculating the coverage is relatively simple. An approach to estimate the effective area (surface coverage,  $f_{coverage}$ ) is shown in Figure 4 for the RPLC×RPLC separation of the 69 solutes using the conditions described above. The calculated surface coverage is about 75 %, which is very good considering the similarity between both retention mechanisms. The ability to change the second gradient during the analysis using the 1290 Infinity 2D-LC solution is of the utmost importance<sup>10</sup>.

The LC×LC separation should be considered a three-step process:

1. First dimension LC analysis
2. Sampling of the first dimension
3. Second dimension LC analysis of the sampled fraction<sup>11</sup>

To avoid severe loss of resolution between peaks, the first dimension should be sampled at least three times over the  $8\sigma$  peak width, so the modulation time should be about  $2.5\sigma$  or below. With the analysis at hand, this would implicate that the modulation time should be less than 9 seconds using an UHPLC separation in first dimension and below 12 seconds using a classical HPLC separation in the first dimension. With the current state-of-the-art equipment such modulation speed is not easy to achieve and, in general, modulation times are in the order of 20 to 60 seconds. Consequently, the first dimension is

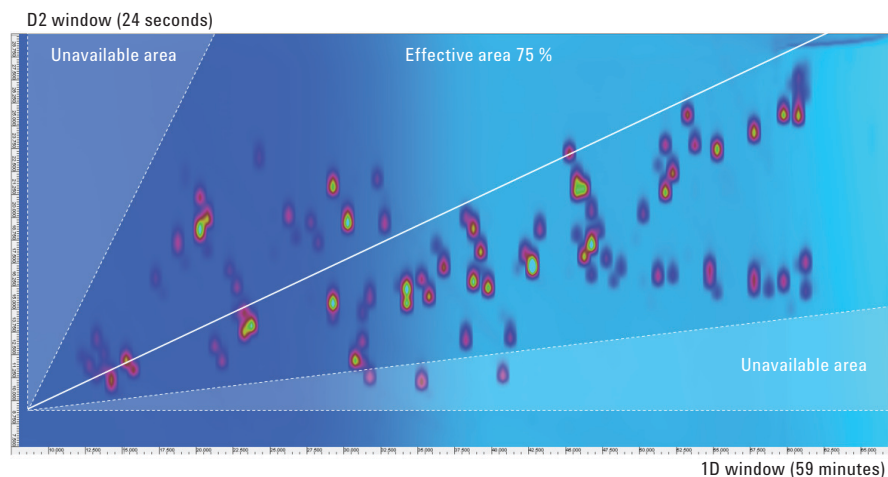


Figure 4 LC×LC contour plots of the total mix indicating the effective and unavailable separation spaces.

significantly undersampled and this has a significant impact on the overall LC×LC peak capacity. In this particular case, the undersampling is obvious since a total of 118 modulations are carried out over the effective separation window for which a first dimension peak capacity of 258 (177 for HPLC) was calculated (see

Table 1). To illustrate this, the modulation frequency (represented as vertical lines) is overlaid with the first dimension separation for, respectively, a classical HPLC and an UHPLC separation in Figure 5 (elution window between 25 and 55 minutes is shown).

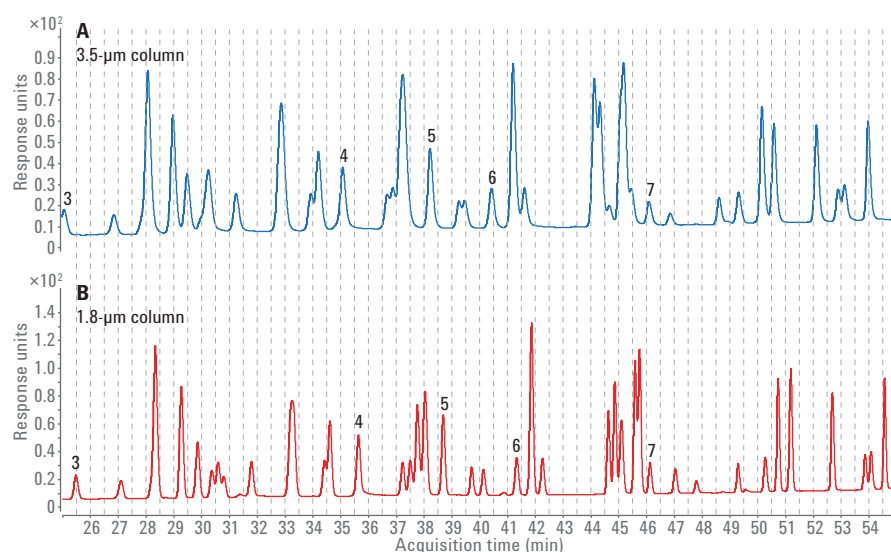


Figure 5 First-dimension separation on 3.5 and 1.8-µm columns (Agilent ZORBAX RRHD Bonus-RP, 2.1 × 150 mm, 0.1 mL/min) with indication of the modulation intervals.



The influence of undersampling can be measured by the undersampling correction factor ( $\beta$ ) calculated according to Equation 3<sup>11</sup>.

$$\beta = \sqrt{1 + 3.35 \left( \frac{t_{c,D2} n_{c,D1}}{t_{g,D1}} \right)^2}$$

Equation 3.

Here,  $t_{c,D2}$  is the second dimension cycle time (modulation time),  $t_{c,D1}$  is the first dimension peak capacity, and  $t_{g,D1}$  is the first dimension gradient time. The theoretical peak capacity should be divided by this factor to correct for undersampling. Since the peaks are narrower in the first dimension, the influence of undersampling is more dramatic in the case of UHPLC, reducing the peak capacity by a factor of 4.

The formula for calculating the effective LC×LC peak capacity ( $n'_{c,2D}$ ) can be written as in Equation 4.

$$n'_{c,2D} = \frac{n_{c,D1} n_{c,D2}}{\beta} f_{\text{coverage}}$$

Equation 4.

For the LC×LC conditions used here (both HPLC and UHPLC), the effective peak capacity is about 800. This is about four times higher than a one-dimensional classical HPLC separation, and almost three times higher than the one-dimensional UHPLC analysis. The fact that there is hardly any difference in peak capacity when UHPLC or HPLC are used in the first dimension is due to this undersampling phenomenon, and in complete accordance with the findings of Carr and colleagues<sup>11</sup>.

Consequently, for the separation of the 69-component mixture used in this study, the probability that a peak is a single compound is increased from 64 to 84 %. This might seem low, but is quite significant in practical work.

## Conclusion

A comparison was made between one-dimensional HPLC, one-dimensional UHPLC, and two-dimensional LC×LC analyses using the same columns and instrument. The Agilent 1290 Infinity 2D-LC solution enabled significant increase of peak capacity in LC×LC separations. For a test mixture containing 69 compounds, the peak capacities, calculated based on the practical separation window, for a 60-minute gradient at optimum flow rate were determined.

- 210 for one-dimensional HPLC (250 to 300  $\mu\text{L}/\text{min}$ )
- 300 for one-dimensional UHPLC (350 to 450  $\mu\text{L}/\text{min}$ )
- 800 for two-dimensional LC×LC (with first dimension flow rate at 100  $\mu\text{L}/\text{min}$ )

The increase of peak capacity by a factor of 3 (compared to UHPLC) to 4 (compared to HPLC) significantly increases the number of solutes that are separated in complex samples, and significantly reduces the number of solute overlap.

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