

Simplify Complex Separations. Fast.



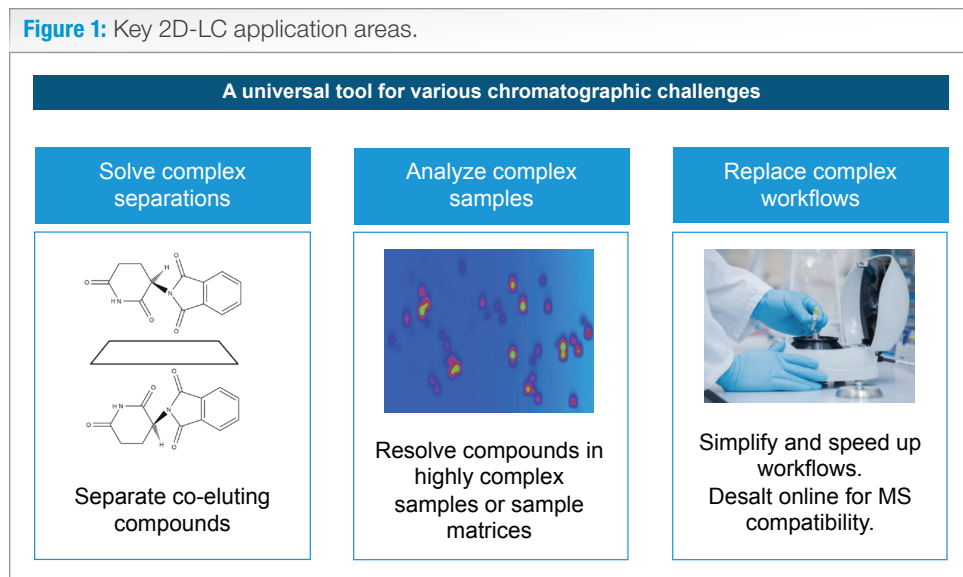
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How to use two-dimensional liquid chromatography to shorten analysis times, eliminate manual steps, and increase detection sensitivity in complicated separations.

Introduction

In conventional high-performance liquid chromatography (HPLC) or ultrahigh-performance liquid chromatography (UHPLC), co-eluting compounds are very hard to separate. In the case of chiral compounds, not even mass spectrometry (MS) can be used to distinguish them. Even though they can be separated by chiral columns, these columns do not have good selectivity for other compounds. In addition, natural substances and sample matrices in protein digests or polymeric matrices often require the separation of hundreds of compounds that take unreasonably long times. And finally, when multiple separations must be performed sequentially, the workflow involves manual sample preparations and offline fractionations that add to the complexity and length of the method. In these situations, two-dimensional liquid chromatography (2D-LC) can be used to achieve dramatic improvements in resolution and significant time savings. Additionally, 2D-LC is an excellent tool for automatic desalting of samples and for making separations involving buffers MS-compatible, thus simplifying sample preparation and handling (**Figure 1**)

Figure 1: Key 2D-LC application areas.



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Typical 2D-LC Instrument Setup and Separation Modes

A 2D-LC system is a combination of two conventional (one-dimension) LC systems (**Figure 2**).

The first instrument, or first dimension (¹D), is a normal LC system with a pump, an autosampler, and a detector. The detector is required for peak-based operations and it is otherwise optional but very useful for gathering complete two-dimensional information. In the second dimension (²D), another column and a second pump are used (i.e., different stationary and mobile phases can be applied in ²D for additional separation). Ideally, these phases are chosen so that an orthogonal separation is performed, which maximizes resolution and peak capacity. The interface between the two systems is the 2D-LC valve with its two sample loops in the most simple setup. This is the heart of the system—it handles the flow between the two dimensions and acts as an injector for the second dimension (**Figure 3**). Fully symmetric flow paths of the Agilent 2D-LC valve yield the highest area and retention time reproducibility.

A valve with just two loops creates a time dependence between the first and second dimension: As long as the second dimension is busy with the analysis, at most one more cut can be stored in the sample loop in ¹D. The Agilent InfinityLab 2D-LC Solution offers a set of two specialized multiple heart-cutting (MHC) valves with six sample loops each, which in alternate use, can intermediately store a large number of cuts arriving from the first dimension and can therefore decouple both dimensions with respect to time in a fully automated fashion.

“2D-LC is a robust technology that can enable, simplify and speed up complex separations and significantly increase detection selectivity and sensitivity. It has been proven successful in many applications, including pharma, biopharma, chemicals, food analysis, fuel analysis, and forensic toxicology. In many cases, 2D-LC can reduce analysis times by up to a factor of four.”

Figure 2: Setup of a 2D-LC instrument.

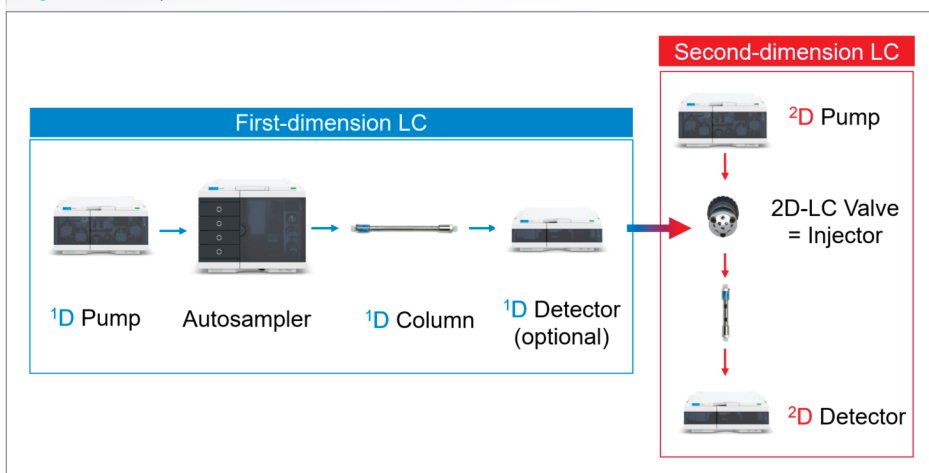


Figure 3: Switching the 2D-LC valve transfers peaks to the second dimension.

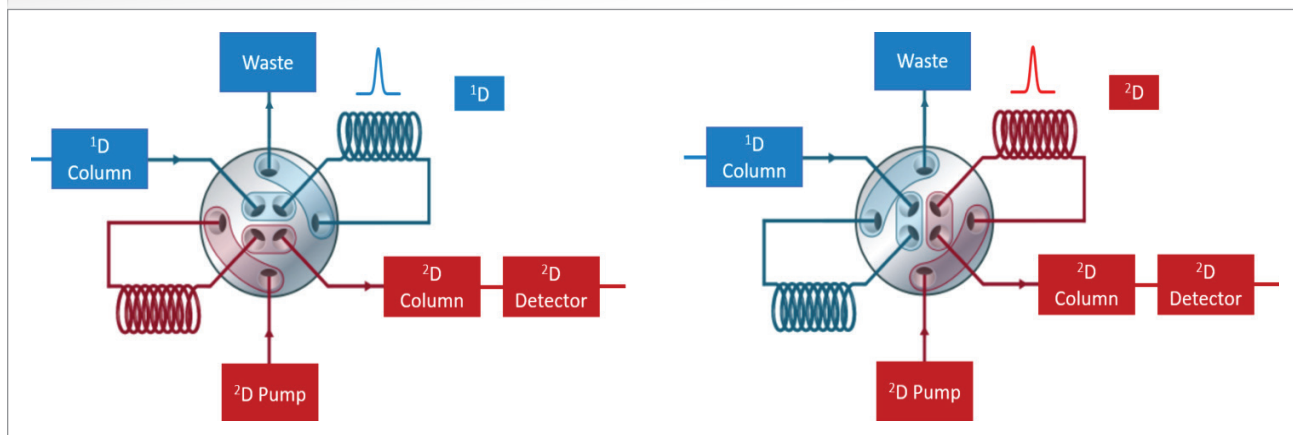


Figure 4: 2D-LC method setup in three simple steps.

The screenshot displays the software interface for setting up a 2D-LC method. It is divided into several sections:

- General settings / Advanced settings:**
 - 2D-LC Mode:** Three options are shown: Comprehensive (unselected), Heart-Cutting (selected, marked with a blue '1'), and Hilites sampling (unselected).
 - Solvents:** A: 90% (A1: 100.0% Water V.03); B: 10% (B1: 100.0% Acetonitrile V.03).
 - Flow settings:** 2D Flow: 1.00 ml/min; use idle flow: 0.00 ml/min.
- 2D gradient:** A table showing time intervals and flow rates. A blue '2' is placed over this section.

Time [min]	% B
0.00	10.00
1.25	60.00
- Sampling table:** A table with columns for Time [min], Mode, Sampling time [min], and Loop filling.

Time [min]	Mode	Sampling time [min]	Loop filling
2.72	Time based	0.07	> 300
2.79	Time based	0.07	104
2.86	Time based	0.07	100
3.35	Time based	0.07	> 300
3.42	Time based	0.07	100
3.80	Peak based	0.09	
- Operating values:** Solvent consumption table.

	A	B	C	D
1D Pump	3.217	6.987	0.000	0.000
2D Pump	11.274	5.732		
- Preview:** A chromatogram showing %B vs. min. A blue '3' is placed over the chromatogram. The plot shows several peaks, with vertical bars indicating the cuts taken from the 1D chromatogram.

Set up your 2D-LC method in three simple steps

1. Choose your 2D-LC mode.
2. Define your second-dimension gradient—shifted gradients help you maximize separation performance.
3. Select the areas in the first-dimension chromatogram where you want to increase resolution.

“Shifted gradients improve resolution and provide increased peak capacity and coverage of the 2D space. The data analysis software can show the 1D chromatogram with the cuts that have been taken.”

This allows flexible run times in the second dimension for increased resolution. With this setup, a 2D chromatogram can be obtained for each 1D cut, even for long run times in either dimension.

In the high-resolution sampling mode (also known as selective comprehensive mode), multiple cuts from broad peaks are taken to perform a more detailed analysis in the second dimension. This technique effectively reduces solvent transfer to the second dimension and retains the pre-separation from 1D to achieve the highest resolution in both dimensions. It is very useful for quantitative analyses.

In comprehensive mode, the entire 1D effluent is cut in equal size pieces to be analyzed in the second dimension. Only two sample loops are required; while one loop is being filled in 1D, the other is analyzed in 2D. The outcome of this mode of analysis is a two-dimensional plot of 2D versus 1D retention times, and it is very useful for complex samples.

Agilent Active Solvent Modulation (ASM) enables one to reduce interference from the 1D solvent in the 2D column. A parallel flow path uses 2D solvent to dilute the 1D solvent from the sample loop. The ASM dilution factor can be optimized from 1.5 to 5 and results in a specific solvent gradient. This leads to focusing of the sample fraction on the head of the 2D column and therefore to increased resolution and sensitivity. The best gradient is easily determined with the help of the software that controls the instrument (**Figure 4**).

Shifted gradients improve resolution and provide increased peak capacity and coverage of the 2D space. The data analysis software can show the 1D chromatogram with the cuts that have been taken. This chromatogram, or the sampling table, can be used to easily navigate between the associated 2D chromatograms. The software can also be used with single-quadrupole MS detection for displaying mass spectra and extracted ion chromatograms.

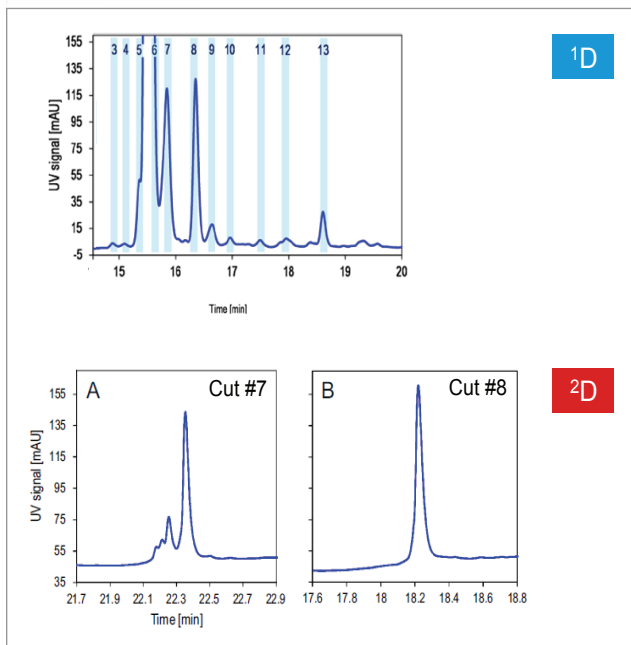
Examples of 2D-LC Separations

The chiral separation of ibuprofen, an active pharmaceutical ingredient (API) with two enantiomers, is a classic example of single heart-cutting. A C18 column in 1D separates it from impurities, and a chiral column in 2D separates its enantiomers.

Another example is the characterization of low concentrations of impurities in insulin. In this case, 1D is a reversed-phase separation using an ammonium phosphate and sodium sulfate buffer. In 2D, multiple heart cuts from 1D are not only separated, but are also desalted in preparation for MS detection (**Figure 5**).

This approach replaces the traditional offline fractionation method, significantly shortening the workflow from days to hours and enabling unattended operation.

Figure 5: First dimension: Separation of insulin with a 40 mM phosphate buffer. Create 13 cuts for 2D analysis. Second dimension: Upon further separation, four isomers are exhibited in cut #7. The second dimension is also used for making this buffer-based separation compatible with Q-TOF detection by online desalting.



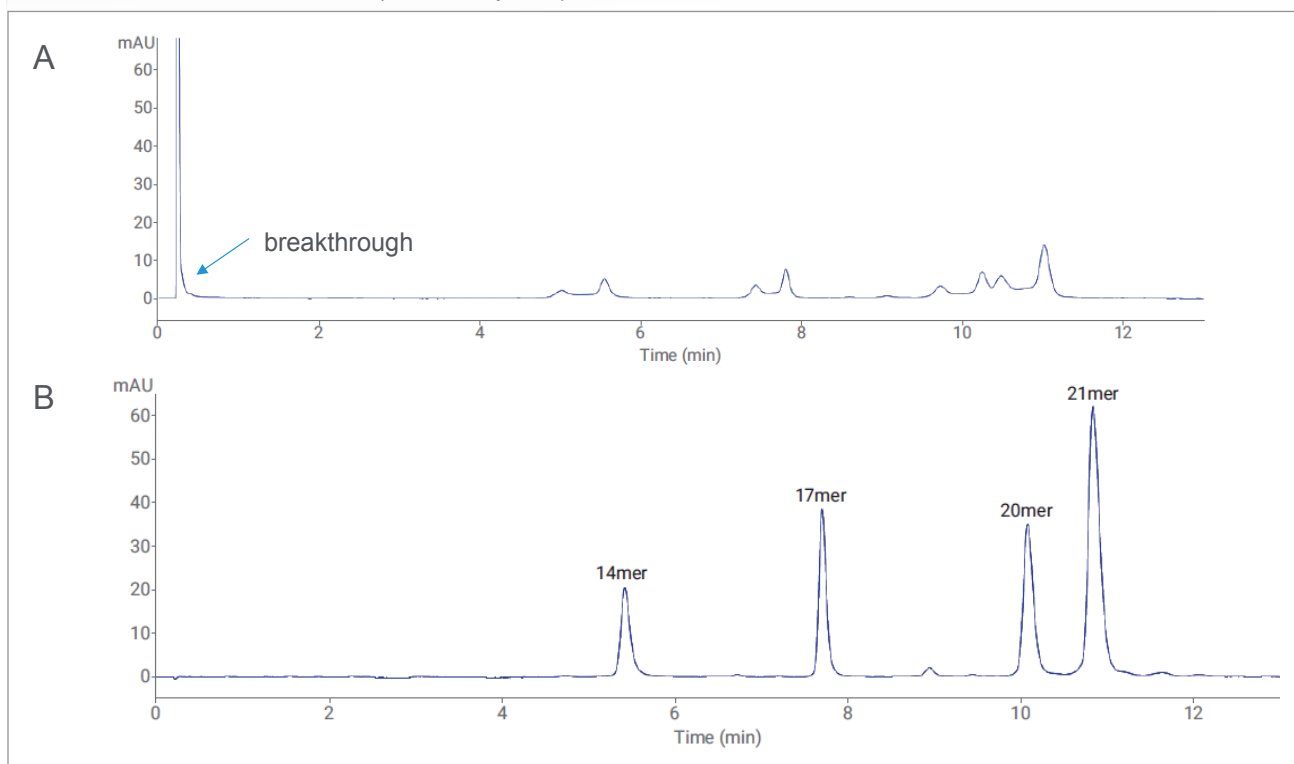
2D-LC can also be used to eliminate the need for manual purification of oligonucleotides. In the traditional anion exchange approach, the oligonucleotide fractions contain high levels of salts, such as sodium chloride, after separation. These high salt levels must be removed before analyzing the fractions by IP-RPLC (ion-pairing-reversed-phase chromatography), as they prevent the oligonucleotides from participating in ion pairing and this would lead to changes in retention time, peak distortion, and breakthrough. The desalting is usually done manually by centrifugation and adds considerable time to the analysis. In contrast, using an Agilent PLRP-S column in 1D, the oligonucleotides elute in one peak, which is then transferred to the second dimension for analysis by IP-RPLC (**Figure 6**).

By eliminating the manual desalting step, the workflow speed is increased significantly, reducing the analysis time from 96 to 26 minutes, while delivering good retention time precision in both dimensions.

A pharmacokinetic study of a drug candidate and its 16 metabolites is yet another example of how 2D-LC can simplify and expedite a complex separation. The original process involved six 1D methods taking 150 minutes to complete; switching to a 2D method cut the analysis time to just 35 minutes.

Finally, using comprehensive 2D-LC to analyze bitter components in different types of beer, Agilent researchers

Figure 6: A: IP-RPLC analysis of oligonucleotides in 1M NaCl showing breakthrough, bad resolution and sensitivity. B: Good resolution and sensitivity can be achieved through manual desalting or automatic desalting in the first dimension (not shown) of a two-dimensional separation with 75% less time needed and increased reproducibility compared with the manual workflow.



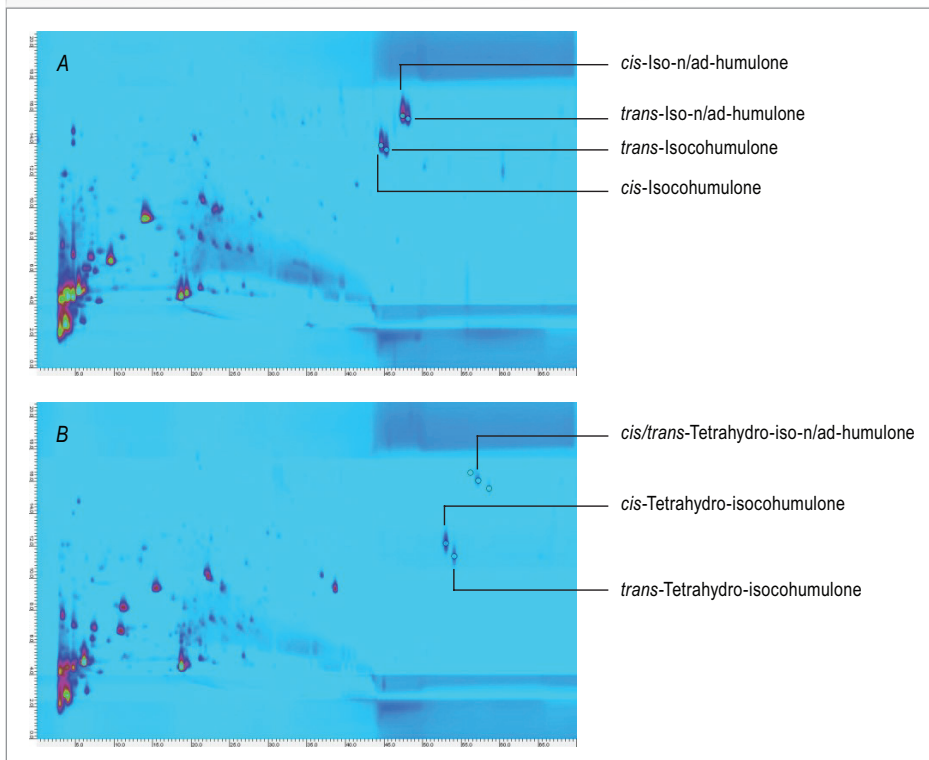
were able to show the presence of reduced isochumulones in American beer. These compounds enhance the light and foam stability of beer but are banned in German beer by law (**Figure 7**).

Conclusion

2D-LC is a robust technique that can enable, simplify and speed up complex separations and significantly increase detection selectivity and sensitivity. It has been proven successful in many applications, including pharma, biopharma, chemicals, food analysis, fuel analysis, and forensic toxicology. In many cases, 2D-LC can reduce analysis times by up to a factor of four. Key benefits of the Agilent InfinityLab 2D-LC Solution include:

- The Agilent InfinityLab 2D-LC Solution is very flexible for (multiple) heart-cutting, high-resolution sampling and comprehensive 2D-LC measurements.
- Agilent OpenLab 2D-LC Software allows setting up and developing 2D methods easily.
- A refined, but intuitive user interface allows powerful qualitative and quantitative data analysis including MS data.
- Agilent's dedicated 2D-LC valves provide the highest reproducibility of retention times and areas in both dimensions.
- Multiple heart-cutting valves allow parking multiple ¹D peaks and analyzing them in ²D for sufficient time and resolution.
- The ASM valve reduces the effects of ¹D solvents for better ²D separation.

Figure 7: 2D-LC analysis of bitter components in beer.



Agilent has compiled a comprehensive collection of 2D-LC applications, including examples of chemical and food analysis, lipidomics and metabolomics, herbal medicine analysis, and the combination of achiral and chiral separations. Agilent also offers dedicated 2D-LC training courses for beginners and experienced chromatographers.

To learn more about Agilent InfinityLab 2D-LC Solutions, please visit www.agilent.com/chem/2D-LC

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