Demystifying 2d-LC

Fast and Efficient Multi-dimensional LC
Overview

• Why two-dimensional LC?
• Setup of a 2D-LC System
• Modes of 2D-LC
  • Comprehensive 2D-LC
  • Heart-Cutting 2D-LC
  • Multiple Heart-Cutting 2D-LC
  • High Resolution Sampling 2D-LC
• Software for all 2D-LC modes
• 2D-LC Method Developments
Why Two Dimensional LC?

- Sample cleanup by removing matrix or interfering compounds
- Increase sample throughput (two separations going on at once)
- Trace enrichment of major compounds of interest (column focusing)
- *Increased peak capacity*
- *Second dimension mobile phase amenable to mass spectrometry*
- *Further resolution of a complex mixture that cannot be separated on a single mode/column*
Resolution - a measure of the ability to separate two components
A Function of Selectivity, Column Efficiency, or Retention

Resolution affects resolution most:
- Change bonded phase
- Change mobile phase

\[ R_s = \frac{N^{1/2}}{4} \cdot \frac{(\alpha-1)}{\alpha} \cdot \frac{k}{(k+1)} \]

- Plates: 5000, 10000, 15000, 20000
- Alpha: 1.10, 1.35, 1.60, 1.85, 2.1
- k: 2.0, 4.5, 7.0, 9.5, 12.0
Principles of Two Dimensional HPLC

- Long efficient first column retains and separate sample components in one chromatography mode (first dimension)
- The eluent flows through valve with injection loops (Comprehensive or Heart Cutting modes)
- The loop content is automatically introduced into a 2\textsuperscript{nd}, fast column (UHPLC) for an orthogonal separation mode
- LC chromatography in the second dimension is “fast”.
Two-dimensional LC System configuration

1D Pump  →  Autosampler  →  1D Column  →  1D Detector (optional)

→

2D Pump

Injector

2D Column

2D Detector
Two Approaches to 2D HPLC

- **Comprehensive**: All of the sample from the first column is ‘trapped & released’ on to the second column in sequential fractions throughout the first dimension run.

- **Heart-cutting**: Detector in the first dimension detects peaks to be trapped and released on to the second dimension column.
COMPREHENSIVE 2D-LC
Comprehensive 2D-LC

- The whole 1D effluent is injected onto 2D system
- (Ultra)Short 2D gradients necessary → Good data quality with fast pumps & detector
- Full („comprehensive“) 2D information!
Comprehensive 2D-LC

How it works

- Collection of effluent from the first dimension column in loop 1
- Analysis of the content from loop 2

- Collection of effluent from the first dimension column in loop 2
- Analysis of the content from loop 1
Cocurrent:
The loops are filled and eluted in the same flow direction.

Countercurrent:
The loops are filled in one flow direction and eluted backwards.
Comprehensive 2D-LC

Operating Principle

Valve switch

Loop 1

Waste

1st Dim. Column

2nd Dim. Column

2nd Dim. Pump

Loop 2
Comprehensive 2D-LC

Operating Principle

Valve switch

Loop 1

1st Dim. Column

2nd Dim. Pump

2nd Dim. Column

Loop 2

Waste
Comprehensive 2D-LC

**Operating Principle**

Valve switch

- Loop 1
  - 1st Dim. Column
  - 2nd Dim. Column
  - Waste

- Loop 2
  - 2nd Dim. Pump
Comprehensive 2D-LC

Operating Principle

1\textsuperscript{st} Dim. Column

2\textsuperscript{nd} Dim. Column

Loop 1

Waste

2\textsuperscript{nd} Dim. Pump

Loop 2
Peak capacity in comprehensive 2D-LC

The peak capacity product rule

Theoretically, the peak capacity of a comprehensive 2D-LC analysis is the product of the peak capacities of the first and second dimension:

$$n_{2D} = 1n \times 2n$$

But, this is only valid if

- The separations deployed in the first and second dimension are completely independant of each other (orthogonal)
- The separation achieved in the first dimension is maintained on transfer to the second dimension
Peak capacity in comprehensive 2D-LC

\[ P = f(\sqrt{N}) \]

**LC** + **LC**

Peak capacity = 10  \hspace{1cm}  Peak capacity = 10  \hspace{1cm}  Peak capacity = 14

**LC** × **LC**

Peak capacity = 10  \hspace{1cm}  Peak capacity = 10  \hspace{1cm}  Peak capacity = 100 !
Peak capacity as a measure of resolving power

Peak capacity is the number of peaks, which can be separated in a given time with a given resolution (Rs = 1.5)

The peak capacity can be calculated from the gradient time $t_g$ and the average peak width $\bar{w}$:

$$n = 1 + \frac{t_g}{\bar{w}}$$
The importance of peak capacity
Statistical theory of component overlap

“… using the statistical theory of peak overlap …”

“… peak resolution is severely compromised when the number of components present in a sample overrates 1/3 of the peak capacity.”


“…in order to resolve 98% of the components, the peak capacity must exceed the number of components by a factor of 100.”

Peak capacity in comprehensive 2D-LC
Importance of Orthogonality

Orthogonal separation mechanisms

Partly orthogonal separation mechanisms

Non-orthogonal separation mechanisms

\[ n_{2D} = n \times 2n \]

\[ n_{2D} < n \times 2n \]

\[ n_{2D} \ll n \times 2n \]
Peak capacity in comprehensive 2D-LC

The undersampling or remixing problem

In comprehensive 2D-LC analysis, the first-dimension effluent is collected in a loop for a certain amount of time (modulation time). Unless the collected volume of first-dimension effluent is a very small fraction of the peak volume, it is inevitable that some remixing takes place in the loop and, therefore, some resolution achieved in the first dimension is lost on transfer to the second dimension. This is called undersampling.

To minimize the remixing effects, three to four fractions should be collected across the $8\sigma$ width of a first dimension peak.
Peak capacity in comprehensive 2D-LC

The undersampling or remixing problem
Peak capacity in comprehensive 2D-LC

Fractional surface coverage

There are several approaches for determination of the fractional surface coverage.

Using the Gilar-Stoll approach, the 2D separation space is divided into bins. Then the percentage of bins that are within the perimeter of bins occupied by peaks is determined:
Peak capacity
HPLC vs. UHPLC vs. Comprehensive 2D-LC

Peak capacity increased by a factor of 3 compared to 1D UHPLC or 4 compared to 1D HPLC while maintaining the analysis time!

1D
- Elution window: 58.8 min
- $\bar{w}$ (4σ): 0.2306 min
- Peak capacity: 258

2D
- Elution window: 24 sec
- $\bar{w}$ (4σ): 1.4 sec
- Peak capacity: 17

2D-LC theoretical peak capacity: 4330

Fractional surface coverage $f$: 75%

Undersampling correction factor $\beta$: 4.1

$\Rightarrow$ Practical peak capacity: about 800

Technical Overview 5991-6123EN
Comprehensive 2D-LC Generation of 2D and 3D plots using Imaging Software

1. Modulation

Raw 2D chromatogram (at second column outlet)

Second-dimension chromatograms stacked side by side
Comprehensive 2D-LC

Generation of 2D and 3D plots
Calibrations May Be Done with Imaging Software Using ‘Blobs’ (Cones)
Calibration May Be Generated from Multiple Blobs

Response Vs. Concentration for Caffeine

Calibration Table for Caffeine

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.000000</td>
<td>5849561.82981</td>
</tr>
<tr>
<td>10.00000</td>
<td>608875.13394</td>
</tr>
<tr>
<td>2.00000</td>
<td>140641.53676</td>
</tr>
<tr>
<td>50.00000</td>
<td>2918411.05514</td>
</tr>
</tbody>
</table>
HEART-CUTTING TWO-DIMENSIONAL LC
Two-dimensional LC System configuration

1D Pump → Autosampler → 1D Column → 1D Detector

2D Pump → Injector → 2D Column → 2D Detector
Why heart cutting two-dimensional LC?

- Increased peak capacity
- Further resolution of a complex mixture that cannot be separated on a single column
- Sample cleanup by removing matrix or interfering compounds
- Increase sample throughput (two separations going on at once)
Heart-Cutting Workflow with Dual Loop
First Cut Sent to $^2$D

1D

Cut: #1

20 min
Limit of Heart-Cutting Workflow with Dual Loop
Missed Next Two Peaks

First cut is being analyzed
Next two peaks are missed
Limit of Heart-Cutting Workflow with Dual Loop Limited Volume

1D
Cut: #1
#2
20 min

2D
4.5 min
4.5 min
Limit of (Single) Heart-Cutting

- Skipped interesting peaks during 2D cycle.
Limit of (Single) Heart-Cutting

- Skipped interesting peaks during 2D cycle.
- There may be an additional co-eluting peak located at a different position than that where the heart cut was made.
New Multiple Heart-Cutting (MHC) Solution

From Dual Loop to Multiple Heart Cut

Loop-1

Loop-2

Deck with 6 loops

One of the loops replaced by 6-column selector valve fitted with 6 loops:

Parking deck cluster offering 7 sampling positions.
Dual-Deck MultipleHeart-Cut
Unmatched Multiple Heart-Cutting 2D-LC Usability

Smart Valve-Loop Setup with 6 or 12 loops → 2D-LC valve + one or two 6/14 valves

Pre-aligned loop-valve kits, just add to the existing 2D-LC system

Online status monitoring
New Multiple Heart-Cutting (MHC) Solution

From dual loop to MHC

Deck with 6 loops

One of the loops replaced with selector valve fitted with 6 loops
Parking deck cluster offering 7 sampling positions.

Both loops replaced with two selector valves fitted with 6 loops each
Parking deck cluster offering 12 sampling positions.
New Multiple Heart-Cutting (MHC) Solution

Different view for better illustration

Deck A

Deck B

Loop-1

Loop-6

1D-column

2D-column

2D-pump

Waste

Deck A

Deck B

Agilent Technologies
Multiple heart-cutting 2D-LC

- Several parts of the 1D effluent are injected onto 2D system
- Long 2D gradients possible, therefore, high resolution is 2D possible
- 2D information for multiple 1D peaks
High resolution sampling 2D-LC

- Multiple consecutive heart-cuts across a 1D peak or region of interest
- Long 2D gradients possible
- Detailed 2D information for a certain 1D peak
- Quantification of compounds coeluting in 1D
(Multiple) Heart-cutting can be performed either **time-based** or **peak-based**.

- **Time-based** means that heart-cut times are defined in a timetable. This timetable can be constructed according to the first dimension retention time of peaks in a reference chromatogram.

- **Peak-based** means that heart-cutting is triggered by the first dimension detector.

---

**Time-based**

**Peak-based**

Peak-based operation

In peak-based operation, heart-cutting is triggered upon peak detection at the first dimension detector. Parameters for peak detection are set similar to integrator settings by threshold and/or slope.
Peak-based operation
Triggering and sampling time

In peak-based operation the valve switch occurs:

- if the sampling time has elapsed (sampling time controls cut position!)
- or if the signal falls below threshold/slope, whichever comes first!
1290 Infinity II 2D-LC Solution with Multiple Heart-Cutting
High Resolution Sampling

Where to take the cut?
High Resolution Heart-Cutting

- Variables: snip time, number of snips
1290 Infinity II 2D-LC Solution

Sample complexity

API impurity profiling

Heart-cutting 2D-LC

Complex formulations

Multiple heart-cutting 2D-LC

Bio-pharmaceuticals

Comprehensive 2D-LC

Natural products, biological samples....

Comprehensive 2D-LC/(IMS)-MS/MS
COMBINATIONS OF SEPARATION MODES AND METHOD DEVELOPMENT
### Overview on separation techniques

<table>
<thead>
<tr>
<th>Separation technique</th>
<th>Stationary phase</th>
<th>Mobile phase</th>
<th>Types of analytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reversed phase chromatography (RP)</td>
<td>C18, C8, C4, C3</td>
<td>Mixtures from organic solvents and water or buffer</td>
<td>Non-polar to mid-polar organic compounds</td>
</tr>
<tr>
<td>Normal phase chromatography (NP)</td>
<td>Silica, amino, cyano, diol</td>
<td>Organic solvents</td>
<td>Organic compounds with polar groups</td>
</tr>
<tr>
<td>Hydrophilic interaction liquid chromatography (HILIC)</td>
<td>Silica, amino, cyano, diol and special phases (zwitterionic)</td>
<td>&gt;50-60 % Acetonitrile with aqueous buffer</td>
<td>Highly polar compounds</td>
</tr>
<tr>
<td>Size exclusion chromatography (SEC)</td>
<td>Porous particles</td>
<td>Organic or aqueous solvents</td>
<td>Synthetic polymers, biopolymers</td>
</tr>
<tr>
<td>Ion exchange chromatography (IEX)</td>
<td>SCX, WCX, SAX, WAX</td>
<td>Buffer</td>
<td>Ionic compounds</td>
</tr>
</tbody>
</table>
Separation Techniques
Reversed phase chromatography

- Separation based on partitioning between stationary and mobile phase; increasing retention with increasing hydrophobicity

- Stationary phase: non-polar (e.g. C18, C8, C4, C3)

- Mobile phase: aqueous, relatively polar (e.g. mixtures of water/buffer with acetonitrile or methanol)

- Widely applied for non-polar to mid-polar compounds
Separation Techniques

Normal phase chromatography

• Separation based on affinity for a polar stationary phase; retention e.g. due to hydrogen bonds, dipol-dipol-interactions

• Stationary phase: polar (e.g. silica, amino, cyano, diol)
• Mobile phase: non-polar, non-aqueous (e.g. hexane, heptane)
• Separation of compounds with polar groups, e.g. fatty acid methyl esters
Separation Techniques
Hydrophilic interaction liquid chromatography

- Separation based on partitioning between the eluent and the adsorbed water layer as well as on ion exchange e.g. with silanol groups

- Stationary phase: hydrophobic (e.g. silica, amino, cyano, diol and special phases (zwitterionic))

- Mobile phase: >50-60 % acetonitrile with aqueous buffer (formate, acetate), gradient from 100 % ACN to 50 %ACN

- Separation of highly polar compounds, e.g. amino acids, carbohydrates, nucleosides
Separation Techniques

Size exclusion chromatography

• Separation of molecules in solution based on their size
• Column filled with porous particles; isocratic analysis

• No interaction of sample with the packing material, only diffusion into and out of pores
• Used e.g. for fractionation of proteins and molecular weight determination of polymers
Separation Techniques
Ion exchange chromatography

• Separation based on ionic interactions

SCX (Strong cation exchange) –SO$_3$H
WCX (Weak cation exchange) –COOH
SAX (Strong anion exchange) –N(CH$_3$)$_3$
WAX (Weak anion exchange) –N(C$_2$H$_5$)$_2$

• Stationary phase: polymer with charged sites
• Mobile phase: salt or pH-gradient
• Separation of charged molecules, e.g. proteins, nucleotides, organic acids
## Combinations of Separation Mechanisms Orthogonality and Compatibility

<table>
<thead>
<tr>
<th>Separation</th>
<th>Orthogonality</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEX/RP</td>
<td>+++</td>
<td>Mainly for peptides/proteins, compatible mobile phases</td>
</tr>
<tr>
<td>SEC/RP</td>
<td>+++</td>
<td>Mainly for peptides/proteins, compatible mobile phases</td>
</tr>
<tr>
<td>NP/RP</td>
<td>+++</td>
<td>Incompatible mobile phases</td>
</tr>
<tr>
<td>HILIC/RP</td>
<td>++</td>
<td>Difficult to interface, not suitable for many analytes</td>
</tr>
<tr>
<td>RP/RP</td>
<td>+</td>
<td>Orthogonality low, compatible mobile phases, easy to interface</td>
</tr>
</tbody>
</table>
Combinations of Separation Mechanisms
RPLC in both dimensions

Advantages of using RPLC in both dimensions are:

- Wide applicability and high efficiency of RP separations
- Compatibility with mass spectrometric detection (RP in the second dimension)
- Good compatibility between the first and second dimension
- Availability of stationary phases with different selectivity

When developing a 2D-LC method with RP in both dimensions, a pair of columns with different selectivity should be selected. One approach for classifying RP columns according to their selectivity is the Hydrophobic Subtraction Model (HSM).
Combinations of Separation Mechanisms

RPLC: Hydrophobic Subtraction Model

Hydrophobic Subtraction Model:

- Describes the interactions between RP columns and different analytes according to hydrophobicity, steric hindrance, acidity, basicity and ion-exchange capacity

- Calculation of a column selectivity factor ($F_s$) as a metric for the selectivity differences between two RP columns

- Characteristics of more than 600 RP columns are available as a web-based database (www.hplccolumns.org)

- Keep in mind that retention and selectivity not only depend on the column, but also on the mobile phase (organic solvent, pH) and the temperature
Considerations for method development
Transfer of 1D effluent to 2D

Maintaining the resolution achieved in 1D:

- The volume of 1D effluent that is transferred to 2D determines to what extent remixing of sample components separated in 1D occurs in the sample loops.
- For multiple heart-cutting, loops are typically overfilled, so that the volume transferred equals the loop size (40 µL).
- Using high resolution sampling, smaller volumes of 1D effluent can be transferred to 2D. In this case, less remixing occurs.
Considerations for method development
Transfer of 1D effluent to 2D

Effect of transfer of 1D effluent on the 2D chromatography:

- The transfer of 1D effluent to 2D equals an injection to the 2D column with the volume transferred being the injection volume!
- The type and volume of solvent (1D effluent) transferred to 2D influences the 2D chromatography.
- It is beneficial, if the solvent (1D effluent) transferred has a low elution power in 2D. In this case, a high bandwidth suppression at the head of the 2D column is achieved.
Road Blocks to 2D Chromatography

- Typically first dimension gradient is a long, slow gradient followed by rapid, repeated gradients on the second dimension so a very low delay volume pump is needed capable of ballistic gradients.
- Difficult to coordinate timing between first and second dimension gradient.
- Difficult to coordinate valve timing between first and second dimension.
- Difficult to coordinate heart-cutting first dimension detector with trapping valve.
- Any changes to one time table necessitates changes to all the other tables.
Complex Gradient and Valve Switch Tables

<table>
<thead>
<tr>
<th>Time</th>
<th>% B</th>
<th>Time</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>175</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>201.1</td>
<td>65</td>
</tr>
<tr>
<td>135</td>
<td>10</td>
<td>201.2</td>
<td>3</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td>210</td>
<td>3</td>
</tr>
<tr>
<td>230</td>
<td>30</td>
<td>236.1</td>
<td>65</td>
</tr>
<tr>
<td>280</td>
<td>50</td>
<td>236.2</td>
<td>3</td>
</tr>
<tr>
<td>295</td>
<td>50</td>
<td>245</td>
<td>3</td>
</tr>
<tr>
<td>320</td>
<td>100</td>
<td>271.1</td>
<td>65</td>
</tr>
<tr>
<td>320.1</td>
<td>0</td>
<td>271.2</td>
<td>3</td>
</tr>
<tr>
<td>350</td>
<td>0</td>
<td>280</td>
<td>3</td>
</tr>
<tr>
<td>350</td>
<td>0</td>
<td>306.1</td>
<td>65</td>
</tr>
<tr>
<td>360.1</td>
<td>0</td>
<td>306.2</td>
<td>3</td>
</tr>
<tr>
<td>360.1</td>
<td>0</td>
<td>315</td>
<td>3</td>
</tr>
<tr>
<td>360.1</td>
<td>0</td>
<td>340</td>
<td>65</td>
</tr>
<tr>
<td>360.1</td>
<td>0</td>
<td>340.1</td>
<td>90</td>
</tr>
<tr>
<td>360.1</td>
<td>0</td>
<td>345</td>
<td>90</td>
</tr>
</tbody>
</table>

Capillary pump 2:
Gradient across analytical column

<table>
<thead>
<tr>
<th>Time</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>201.1</td>
<td>65</td>
</tr>
<tr>
<td>201.2</td>
<td>3</td>
</tr>
<tr>
<td>210</td>
<td>3</td>
</tr>
<tr>
<td>236.1</td>
<td>65</td>
</tr>
<tr>
<td>236.2</td>
<td>3</td>
</tr>
<tr>
<td>245</td>
<td>3</td>
</tr>
<tr>
<td>271.1</td>
<td>65</td>
</tr>
<tr>
<td>271.2</td>
<td>3</td>
</tr>
<tr>
<td>280</td>
<td>3</td>
</tr>
<tr>
<td>306.1</td>
<td>90</td>
</tr>
<tr>
<td>345</td>
<td>90</td>
</tr>
</tbody>
</table>

6-port valve: timetable

<table>
<thead>
<tr>
<th>Time</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>column 2</td>
</tr>
<tr>
<td>26.1</td>
<td>column 1</td>
</tr>
<tr>
<td>35</td>
<td>column 2</td>
</tr>
<tr>
<td>61.1</td>
<td>column 1</td>
</tr>
<tr>
<td>70</td>
<td>column 2</td>
</tr>
<tr>
<td>96.1</td>
<td>column 1</td>
</tr>
<tr>
<td>105</td>
<td>column 2</td>
</tr>
<tr>
<td>131.1</td>
<td>column 1</td>
</tr>
<tr>
<td>140</td>
<td>column 2</td>
</tr>
<tr>
<td>166.1</td>
<td>column 1</td>
</tr>
<tr>
<td>175</td>
<td>column 2</td>
</tr>
<tr>
<td>201.1</td>
<td>column 1</td>
</tr>
<tr>
<td>210</td>
<td>column 2</td>
</tr>
<tr>
<td>236.1</td>
<td>column 1</td>
</tr>
<tr>
<td>245</td>
<td>column 2</td>
</tr>
<tr>
<td>271.1</td>
<td>column 1</td>
</tr>
<tr>
<td>280</td>
<td>column 2</td>
</tr>
<tr>
<td>306.1</td>
<td>column 1</td>
</tr>
<tr>
<td>315</td>
<td>column 2</td>
</tr>
<tr>
<td>345</td>
<td>column 1</td>
</tr>
</tbody>
</table>

10-port valve: timetable

<table>
<thead>
<tr>
<th>Time</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Pos 1</td>
</tr>
<tr>
<td>5</td>
<td>Pos 2</td>
</tr>
<tr>
<td>10</td>
<td>Pos 1</td>
</tr>
<tr>
<td>10</td>
<td>Pos 2</td>
</tr>
<tr>
<td>135</td>
<td>Pos 2</td>
</tr>
<tr>
<td>170</td>
<td>Pos 1</td>
</tr>
<tr>
<td>205</td>
<td>Pos 2</td>
</tr>
<tr>
<td>240</td>
<td>Pos 1</td>
</tr>
<tr>
<td>275</td>
<td>Pos 2</td>
</tr>
<tr>
<td>310</td>
<td>Pos 1</td>
</tr>
</tbody>
</table>
Agilent 2D-LC Add-On Software

ChemStation Dashboard:

All modules in one dashboard can be relabeled individually, e.g. "BinPump-1st-Dim"
2D-LC System Configuration
“One screen for the entire system”

Define 1D / 2D pump
Define detector in the second dimension
Define peak detector (optional)

Select the valve(s) to be used for 2D-LC injection
Select a possible valve / loop configuration
Graphical representation of the selected valve / loop configuration:
- Flow path 1D & 2D
- Animated valve switching
Method User Interface
2D-LC specific parameters of the 2D-pump

Select the 2D-LC mode: comprehensive / heart-cutting

Define repetition of 2nd dimension gradient (Modulation time)

Define the gradient of the 2nd dimension

Show rollout of gradient in the 2nd dim over the runtime of the 1st dimension

Graphical editing of gradient shift

Solvent & Flow-Settings

Define time window(s) where the selected 2DLC mode is active

Operation values, warnings

Close-up of 2D-gradient

Access to standard method UI of the pump
Heart-cutting 2D-LC
Impurity Analysis of a Production Sample

Increased probability to detect unknown impurities in a known sample, e.g. in QA/QC auf pharmaceutical compounds, agrochemicals or fine chemicals.

• Analysis of impurities in pharmaceutical compounds and fine chemicals is an important part of the development and production process.

• Impurities are often structurally similar to the main compound, which can make it difficult to separate them chromatographically.

• The impurities can co-elute with each other or with the main compound. In the worst case, separation could be impossible with the chosen stationary/mobile phase combination.

Data from Agilent Application Note 5991-0834EN
Impurity Analysis of a Production Sample (QA/QC)

First dimension – do we see all impurities?

Column: ZORBAX RRHD Eclipse Plus C18 2.1 x 150 mm, 1.8 μm
Mobile phase A: Water, 0.1 % formic acid
Mobile phase B: Acetonitrile, 0.1 % formic acid
Gradient: at 0 min 5%B, at 30 min 95%B
Flow rate: 0.2 mL/min
Col.temp.: 25 °C
Inj. volume: 3 μL
Data acq. (DAD): 254/4 nm (ref. 360/16 nm)
Impurity Analysis of a Production Sample (QA/QC)

Heart-cut of main peak between 20.75 and 21.00 min

Column: ZORBAX Eclipse Plus C18 2.1 x 150 mm, 1.8 μm
M. phase A: Water, 0.1 % formic acid
M. phase B: Acetonitrile, 0.1 % formic acid
Gradient: at 0 min 5%B, at 30 min 95%B
Flow rate: 0.2 mL/min
Col. temp.: 25 °C
Inj. volume: 3 μL
DAD: 254/4 nm (ref. 360/16 nm), 20 Hz, 10 mm path length

Impurity G hidden under main compound in 1-D analysis!
Impurity Analysis of a Production Sample (QA/QC) Peak Capacity and Probability to Separate All Peaks

The probability that a sixth, unknown compound will be separated from the five compounds in the chromatogram is only 69%!

Peak capacity: 32

$t_l = 28$ min
$t_r = 6$ min
$w = 0.71$ min

5 compounds
(+ unknown comp.)

Probability: 69%
m = 6
Heart-cutting 2D-LC
Achiral-Chiral 2D-LC Analysis of Pharmaceutical Substances

Determination of achiral impurities and enantiomeric excess in a single run. Chiral column in the second dimension.

- ICH guideline Q3A (R2): Impurities at or above 0.05 % in new drug substances need to be reported
- Enantiomers of chiral drugs: Often differences in pharmacokinetic behavior and pharmacological activity
- Heart-cutting 2D-LC: Simultaneous impurity analysis and separation of enantiomers in one analysis

Data from Agilent Application Note 5991-4664EN
Achiral-chiral 2D-LC of Pharmaceutical Substances
Ibuprofen (racemic)

1st Dim: C18

2nd Dim: Chiral column
Achiral-chiral 2D-LC of Pharmaceutical Substances
Main compound R-Thalidomide with trace S-Thalidomide

Enantiomeric excess (ee) of R-Thalidomide: 98.1 %

1st Dim: C18

2nd Dim: Chiral column
Further reading

2D-LC Primer
Available online

Thank you for your attention!