Two Dimensional LC with Diode-Array-Detection & Quadrupole Time-of-Flight Detection
The mass of Earth is $6 \times 10^{24}$ kg. If Archimedes can lift 60 kg, he would need a lever with an arm ratio of $10^{23}:1$. So if the short arm is one meter long, the lever length will be $10^{23}$ meters plus one. Also, note that he would have to push the lever for $10^{20}$ meters to shift the Earth just by one millimeter.

Give me a column long enough (with enough time and enough pressure) and I can separate anything
-Bob Giuffre
What is Two Dimensional LC

The selective transfer of a fraction (or fractions) from one chromatographic column to a secondary chromatographic column for further separation
Why Two Dimensional LC

- 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)
- Trace enrichment of major compounds of interest (column focusing)
- Increased peak capacity
- Second dimension mobile phase for amenable to mass spectrometry
Principles of Two Dimensional HPLC

- Long efficient first column retains sample components in one mode
- The eluent flows through an injection loop (this first step may include peak detection for heart cutting the sample)
- The loop content is automatically injected into a 2\textsuperscript{nd}, fast column giving rise to an orthogonal separation
Road Blocks to Two Dimensional 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
Without Two Dimensional Software: Complex Gradient and Valve Switch Tables

**Capillary pump 1:**
gradient across analytical column

<table>
<thead>
<tr>
<th>Time</th>
<th>% B</th>
<th>Time</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>175</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>201.1</td>
<td>65</td>
</tr>
<tr>
<td>26.1</td>
<td>65</td>
<td>201.2</td>
<td>3</td>
</tr>
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<td>26.2</td>
<td>3</td>
<td>210</td>
<td>3</td>
</tr>
<tr>
<td>35</td>
<td>3</td>
<td>236.1</td>
<td>65</td>
</tr>
<tr>
<td>61.1</td>
<td>65</td>
<td>236.2</td>
<td>3</td>
</tr>
<tr>
<td>61.2</td>
<td>3</td>
<td>245</td>
<td>3</td>
</tr>
<tr>
<td>70</td>
<td>3</td>
<td>271.1</td>
<td>65</td>
</tr>
<tr>
<td>96.1</td>
<td>65</td>
<td>271.2</td>
<td>3</td>
</tr>
<tr>
<td>96.2</td>
<td>3</td>
<td>280</td>
<td>3</td>
</tr>
<tr>
<td>105</td>
<td>3</td>
<td>306.1</td>
<td>65</td>
</tr>
<tr>
<td>131.1</td>
<td>65</td>
<td>306.2</td>
<td>3</td>
</tr>
<tr>
<td>131.2</td>
<td>3</td>
<td>315</td>
<td>3</td>
</tr>
<tr>
<td>140</td>
<td>3</td>
<td>340</td>
<td>65</td>
</tr>
<tr>
<td>166.1</td>
<td>65</td>
<td>340.1</td>
<td>90</td>
</tr>
<tr>
<td>166.2</td>
<td>3</td>
<td>345</td>
<td>90</td>
</tr>
</tbody>
</table>

- 500 mL/min
- Run time: 345 minutes, 8.9 minutes
- Injection volume: 60 mL

**Capillary pump 2:**
gradient across SCX column

<table>
<thead>
<tr>
<th>Time</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>135</td>
<td>10</td>
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<td>200</td>
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<td>320</td>
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</tr>
<tr>
<td>320.1</td>
<td>0</td>
</tr>
<tr>
<td>350</td>
<td>0</td>
</tr>
</tbody>
</table>

- 500 mL/min
- Run time: 350 min.

**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>3</td>
<td>column 1</td>
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<tr>
<td>6</td>
<td>column 2</td>
</tr>
<tr>
<td>10</td>
<td>column 1</td>
</tr>
<tr>
<td>13</td>
<td>column 2</td>
</tr>
<tr>
<td>17</td>
<td>column 1</td>
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<td>20</td>
<td>column 2</td>
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<tr>
<td>24</td>
<td>column 1</td>
</tr>
<tr>
<td>27</td>
<td>column 2</td>
</tr>
<tr>
<td>30</td>
<td>column 1</td>
</tr>
<tr>
<td>34</td>
<td>column 2</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>30</td>
<td>Pos 1</td>
</tr>
<tr>
<td>65</td>
<td>Pos 2</td>
</tr>
<tr>
<td>135</td>
<td>Pos 1</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>
Two Approaches to Two Dimensional HPLC

- **Comprehensive**: All of the sample from the first column is ‘trapped & released’ on to the second column.

- **Heart-cutting**: Detector in the first dimension allows components only to be trapped and released on to the second column.
2D-LC - Difference between comprehensive 2D-LC and heart-cutting 2D-LC

Comprehensive 2D-LC (LCxLC):

The complete effluent of the first column will be injected to the second column and will be analyzed 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.
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 separation 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.
Overview of Agilent Approach

- Offers both comprehensive 2DLC and heart-cutting 2DLC
- Unique features like automatically shifted gradients or peak-triggered operation
- Highest focus on simplest but still highly flexible 2DLC method set-up
- Highest flexibility on hardware set-up
  - Different pumps, autosamplers and detectors supported
  - Detectors at different positions (after 1st dimension column, after 2nd dim. column, at waste-line)
  - Different valve set-up possibilities supported
- High performance data analysis
Hardware – Module-flexibility

1. Dimension

Typically "lower-end" pump, e.g. quaternary

Autosampler

Optional Detector for peak detection/heart cutting Non-destructive

For 1st dimension chromatogram

2. Dimension

One or two 1290 Infinity TCC

‘high-end’ pump, e.g. 1290 binary capable of ballistic gradients

Any HPLC compatible detector

Second dimension detector(s)

Mass Spectrometer

Autosampler Optional Detector for peak detection/heart cutting Non-destructive

For 1st dimension chromatogram
Hardware – Module-flexibility

1. Dimension

- 1290 Infinity Binary Pump
- 1290 Infinity Autosampler or 1260 HiP Autosampler
- 1260 Infinity Capillary Pump
- 1260 Infinity Autosampler
- Optional 1260/1290 Infinity Detector

For 1st dimension chromatogram and peak-triggering

One or two 1290 Infinity TCC

2. Dimension

- 1290 Infinity Binary Pump (mandatory!)
- Optional 1260/1290 Infinity Detector
- 1260/1290 Infinity detector
- To monitor waste-line

1260/1290 Infinity Series

The Measure of Confidence

Agilent Technologies
Two Dimensional Schematic with Heart Cutting/Peak Detection

First Dimen Pump → AutoSampler → First Dimen Column → First Dimen Detector (heart cutting) non-destructive → Switch Valve → Second Dimen Column → Second Dimen Detector (nondestructive or destructive MS)

First dimension detector optional for comprehensive mode
<table>
<thead>
<tr>
<th>Mode Combination*</th>
<th>Application</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>Column Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEC &amp; RP</td>
<td>PROTEOMICS</td>
<td>Orthogonality</td>
<td>Low Peak Capacity</td>
<td>AGILENT BIO-IEX; ZORBAX STABLEBOND, ECLIPSE, EXTEND, BONUS RP, POROSHELL</td>
</tr>
<tr>
<td>SEC &amp; RP</td>
<td>POLYMERS</td>
<td>Orthogonality</td>
<td>Low Peak Capacity</td>
<td>AGILENT PL-GEL (VARIOUS BEDS); ZORBAX STABLEBOND, ECLIPSE, EXTEND, BONUS RP, POROSHELL</td>
</tr>
<tr>
<td>NP &amp; RP</td>
<td>PHARMACEUTICALS</td>
<td>Orthogonality</td>
<td>Solvent compatibility, limited</td>
<td>ZORBAX CYANO, AMINO, SIL; ZORBAX STABLEBOND, ECLIPSE, EXTEND, BONUS RP, POROSHELL</td>
</tr>
<tr>
<td></td>
<td>METABOLOMICS</td>
<td></td>
<td>application</td>
<td></td>
</tr>
<tr>
<td>RP &amp; RP</td>
<td>PHARMACEUTICALS</td>
<td>Miscible Solvents, broadest applications, fast speed, gradient on both dimensions, highest peak capacity</td>
<td>Strongly depends on column choice of mobile phase choice</td>
<td>ZORBAX STABLEBOND, ECLIPSE, EXTEND, BONUS RP, POROSHELL</td>
</tr>
<tr>
<td></td>
<td>METABOLOMICS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFFINITY &amp; RP</td>
<td>PROTEOMICS</td>
<td>Orthogonality</td>
<td>Low Peak Capacity, Off-line</td>
<td>AGILENT MULTIPLE AFFINITY REMOVAL COLUMNS</td>
</tr>
<tr>
<td>SEC &amp; NP</td>
<td>POLYMERS</td>
<td>Orthogonality</td>
<td>Low Peak Capacity</td>
<td>AGILENT PL-GEL (VARIOUS BEDS); ZORBAX CYANO, AMINO, SIL</td>
</tr>
<tr>
<td>SEC &amp; IEC</td>
<td>PROTEOMICS</td>
<td>Orthogonality</td>
<td>Low Peak Capacity</td>
<td>AGILENT BIO-SEC; AGILENT BIO-IEX</td>
</tr>
</tbody>
</table>

* Stoll et al, Jchrom A, 1168 (2007) 3-43
Close-up of valve plumbing

Note: One TCC may be used with 10 port/two position valve with 1D column and 2D column at different temperatures.
Close-up of valve plumbing

Note: One TCC may be used with 10 port/two position valve with 1D column and 2D column at different temperatures.
Hardware – Valves, uniqueness and flexibility

1. Dimension

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

All flow paths are equal (no additional bridging loops)
Symmetric counter-current fill/defill of loops (reducing band-spread)
All in one valve (no synchronization, costs)
Flow Diagram of the new 2pos/4port-duo valve
Heart-Cutting Valve Position One: Waiting for Peak to Arrive
Heart-Cutting Valve Position Two: Trapping a Peak
Heart-Cutting Valve Position One: Eluting Peak onto Second Dimension Column
ChemStation Dashboard:

All modules in one dashboard can be relabeled individually, e.g. "BinPump-1st-Dim"
2D-LC
- supported 2D-LC modes

comprehensive LCxLC

standard
or
Time-/Peak-triggered

complex/unknown samples: biopharma, food, polymers....

Heart-cutting LC-LC

Time-triggered
or
Peak-triggered

Known samples/improving confidence: pharma, methdev...
2D-LC
- supported 2D-gradients to improve resolution

• standard repeating with start- and end-time

• constantly shifted %B_{2D}

• constantly shifted %B_{2D} and shifted \Delta %B_{2D}

• Any combination
Effects of different time-segments

1D Method run time

1D Gradient/
1D Chromatogram

2D Time segments

2D Pump flow rate
Solvent saving!!!

Valve toggling
Increase life time!

2D Gradients

%B (1D)

F (2D)

2D Pump flow rate

Idle flow rate

%B (2D)

1D Method pre-run time

1D Method post-run time

System standby

Time-triggered

Peak-triggered

Idle
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 UI
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
Example: graphical editing of a gradient shift
- replace editing of large timetables by a few mouse operations -

1. Use context menu to enter the editing mode

2. Timetable entries are marked with circles

3. Draw a straight line by dragging the mouse to a new %B value at a specified runtime of the 1st dimension

4. When releasing the mouse, a new TT entry is made and the gradient rollout is automatically updated

5. Repeat step 3 + 4 with other TT entries

6. Insert / Delete shift points (mouse cursor and context menu changes near to a shift line)
Phenolic Antioxidants in Beverages

2D-Chromatogram

First dimension
- Column: ZORBAX Eclipes Plus C18 2.1 x 150 mm, 1.8 μm
- Mobile phase A: Water + 0.1 % formic acid
- Mobile phase B: Methanol + 0.1 % formic acid
- Gradient: at 0 min 5%B, at 30 min 95%B, at 40 min 95%B
- Flow rate: 0.1 mL/min
- Col.temp.: 25 °C
- Inj. volume: 5 µL

Second dimension
- Column: ZORBAX Eclipes Plus Phenyl Hexyl 3 x 50 mm, 1.8 μm
- Mobile phase A: Water + 0.1 % formic acid
- Mobile phase B: Methanol + 0.1 % formic acid
- Initial gradient: at 0 min 5%B, at 0.5 min 15%B, at 0.51 min 5%B
- Flow rate: 3 mL/min
- Col.temp.: 60 °C
- Data acq. (DAD): 260/4 nm, Max-Light high-sens. flow cell (60 mm path)

No: Name:
1. Gallic acid
2. Esclulin
3. 3,4 HO Benzoic acid
4. HO Phenacetic acid
5. 6,7 HO Coumarin
6. HO Benzoic acid
7. Syringic acid
8. Rutin
9. Naringin
10. Coumaric acid
11. Hesperidin
12. Ferulic acid
13. Myricetin
14. Morin
15. Resveratrol
16. Salicylic acid
17. Luteolin
18. Quercetin
19. Kaempferol
20. Apigenin
21. Naringenin
22. Hesperetin
23. 7 HO Flavone
24. Pinosylvin
25. Chrysin
26. Flavone
Phenolic Antioxidants in Beverages

Retention time and peak volume precision

Retention time precision

- Retention time RSD typically better than 0.6%

Peak volume precision

- Peak volume RSD typically better than 3%.
Phenolic Antioxidants in Beverages

Quantification of resveratrol in Merlot red wine

Calibration of 2D-LC quantification for resveratrol, 1 – 50 µg/mL

Found in Merlot red wine: 4.5 µg/mL, 4.5 mg/L
Application

Taxanes from Taxus extract

- Taxol® (*Paclitaxel*) shows *excellent antitumor activity* against breast and lung cancer.

- It was first *isolated in 1971 from Taxus brevifolia*. The content is very low and synthesis very difficult due to complex structure.

- Today it is produced by *semi-synthesis from more abundant precursors* like 10-Deacetylbaccatin-III, Baccatin-III and cephalomannine (Taxol B).
Taxanes from *Taxus* extract

*Standard mix with full gradient in second dimension*
Taxanes from *Taxus* extract

*Standard mix with shifted gradient in second dimension*
Application

Components of beer samples

Analysis of two different commercially available Japanese beer samples with different separation modes

System:
1st dim Pump: 1260 Infinity Binary Pump
AutoSampler: 1290 Infinity Autosampler
2nd dim. Pump: 1290 Infinity Binary Pump
TCC: 1290 Infinity Therm. Column Comp. w. 2DLC valve
Detector: 1290 Infinity Diode-Array Detector G4212A,
Data acquisition: OpenLAB ChemStation Edition w. 2D-LC Add-on
Data analysis: LC image software from GC image LLC

<table>
<thead>
<tr>
<th>Experiments</th>
<th>1st dimension</th>
<th>2nd dimension</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SEC</td>
<td>RP (C18)</td>
</tr>
<tr>
<td>2.</td>
<td>IEX</td>
<td>RP (C18)</td>
</tr>
<tr>
<td>3.</td>
<td>RP (C18)</td>
<td>RP (Phenyl)</td>
</tr>
</tbody>
</table>

From WikiCommons, File:Selection of Japanese beer.jpg
Application

*Components of beer samples*

Analysis of two different commercially available beer samples with different separation modes

1st dimension - SEC  2nd dimension - Reverse phase (C18)
Application

Components of beer samples

Analysis of two different commercially available beer samples with different separation modes

1\textsuperscript{st} dimension - ion exchange  \hspace{1cm} 2\textsuperscript{nd} dimension – Reverse phase (C18)

Beer A

Beer B
Application

Components of beer samples

Analysis of two different commercially available beer samples with different separation modes

1st dimension - Reverse Phase (C18)  2nd dimension - Reverse Phase (Phenyl)
Summary

- Two Dimensional HPLC can elucidate complex samples in an automated mode
- Agilent solution allows easy programming to develop and modify two dimensional setups
- Visualization tools allows for fast interrogation of data sets