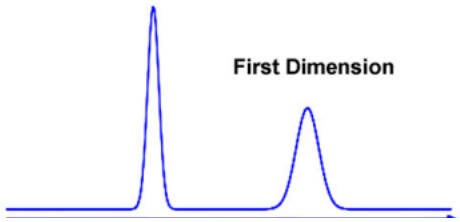
Tutorial on 2D HPLC; Requirements and instrumental implementation

. . .

What is Multidimensional HPLC?

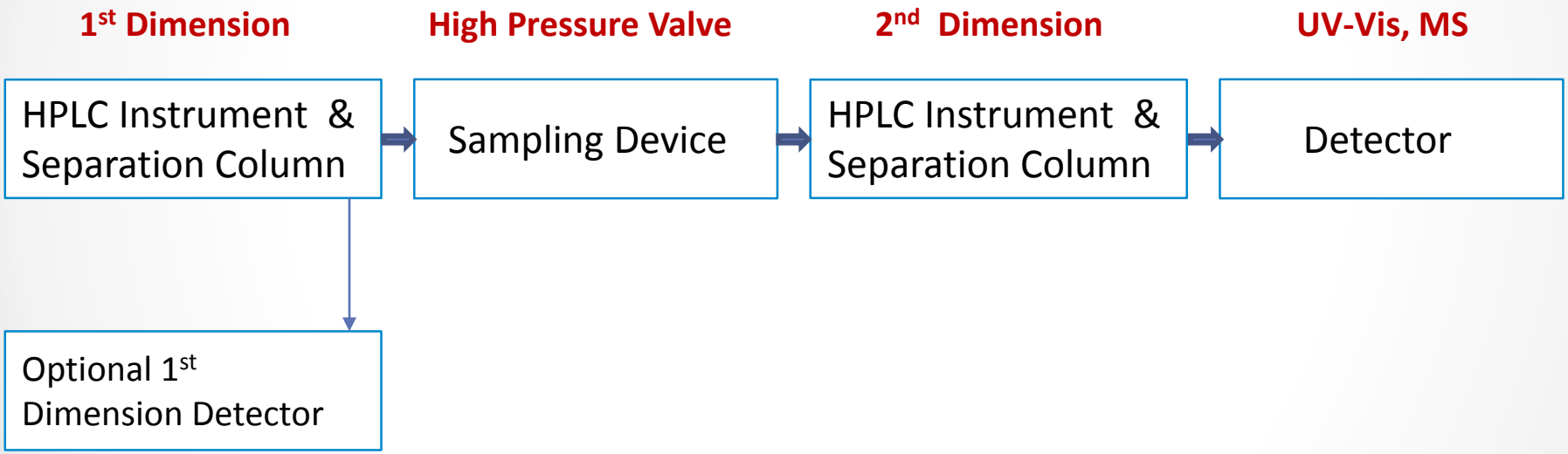


Peak capacity by the product of the number of bins

$${}^{1}Z_p * {}^{2}Z_p$$

What is Multidimensional HPLC?

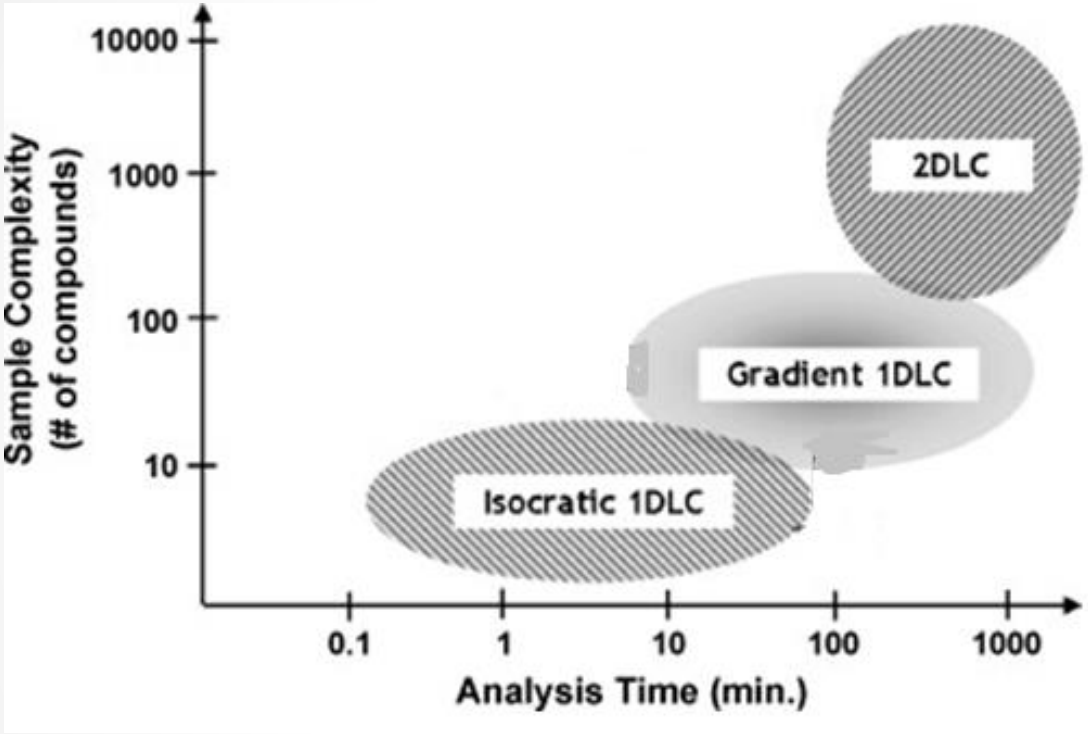
Simple Block Diagram



Applications Areas of MDLC

- Food, Beverages and Consumer Goods
  - Original Ingredients, Contamination, Proof Authenticity
- Proteomics, Metabolomics
  - Life Science Research
  - Biomarker discovery
  - Biopharmaceutical (originator or biosimilars)
- Environmental Analysis
  - Identification of Pollutants, Contaminants, Accidents
  - Polymers, Oligomers, Branching, Functional Group Analysis
- Forensics & Toxicology
  - Poison, Doping,
- Pharmaceutical Analysis
  - DMPK, metabolite identification
  - Traditional Chinese Medicine

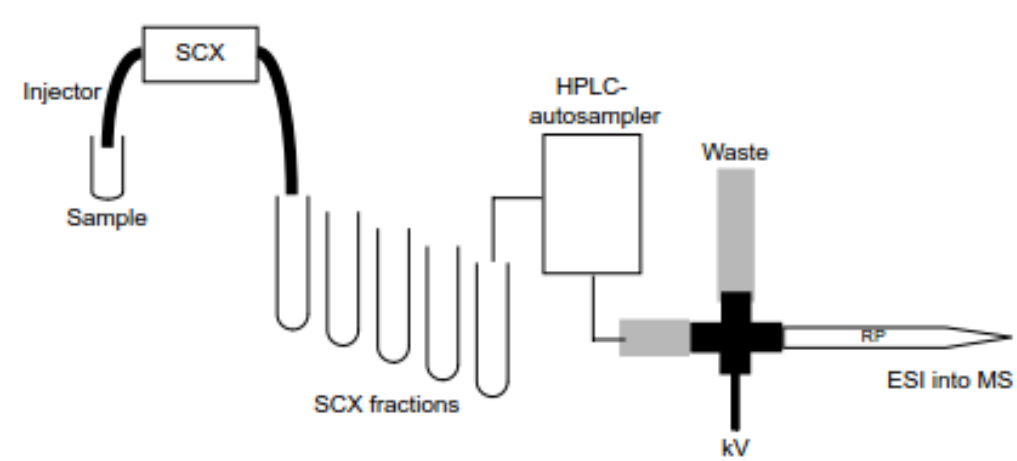
Positioning of HPLC Techniques[1,2]



Adapted from [1]Stoll, D., University of Minnesota Ph.D. Dissertation, 2007, [2]Stoll, D., *et al.*, *J. of Chrom. A*, 1168, 3 (2007)

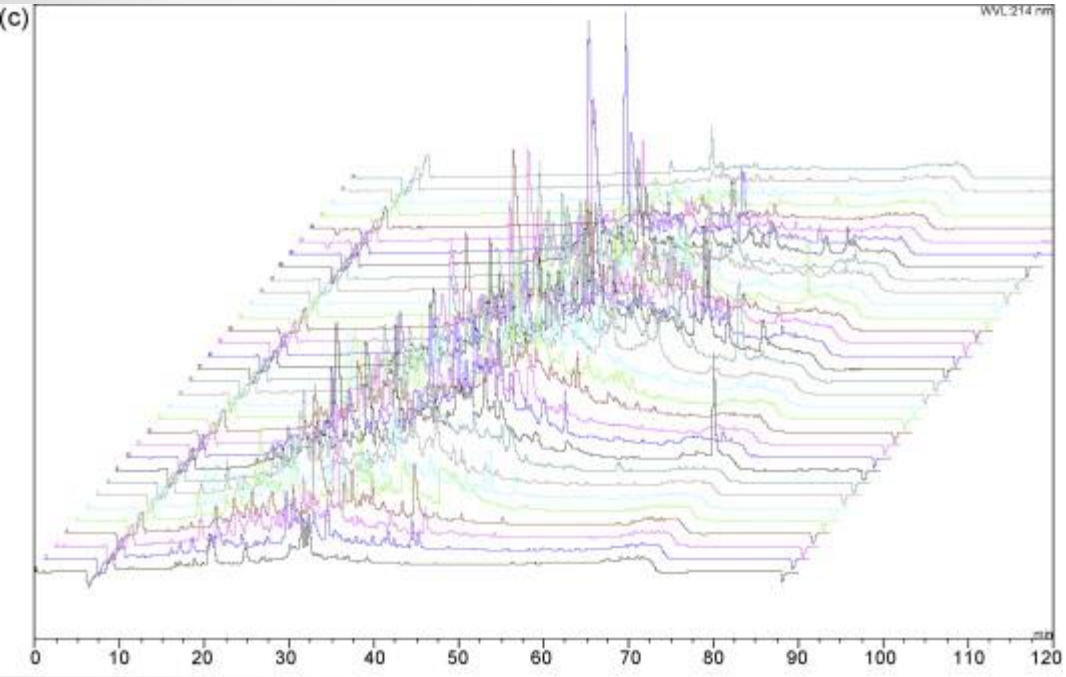
Principle Methods of 2D LC

- "Offline" methods (sequential)
  - Collect fractions from the 1st dimension separation, stored and re-injected in the 2nd dimension separation column in separate next run.



Picture taken from S.K. Swanson and M.P. Washburn, Drug Discovery Today, 10, 719 (2005)

Principle Methods of 2D LC



1st dimension:
150 mm L x 2.1 mm ID x 3.5 µm XBridge phenyl column

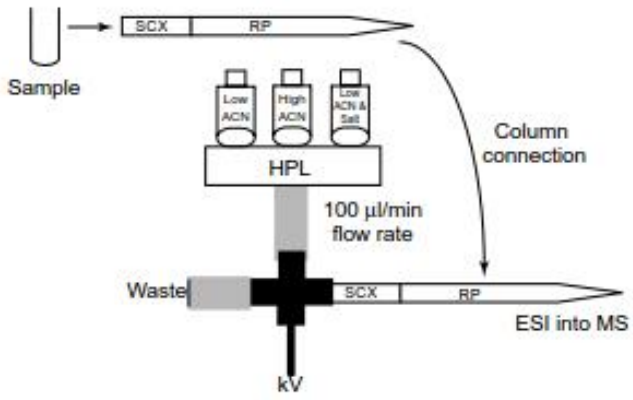
Offline fraction collection and reinjection in the **2nd dimension**:
150 x 0.075 mm, 3 µm Pepmap 100Å C18 particles

Total time required 40x2hrs!!

K. Sandra et al., J. Chrom. B, 877, 1019 (2009)

Principle Methods of 2D LC

- "Offline" methods (sequential)
  - Collect fractions from the 1st dimension separation, stored and re-injected in the 2nd dimension separation column later.
- "Stop-and-Go" methods e.g. MuDPIT*
  (Multi-Dimensional Protein Identification Technology)
  - One column packed with a segment of ion exchanger and a larger segment of RP-phase.
    A pulsed salt gradient in IEX displaces a fraction of the sample onto the RP-column



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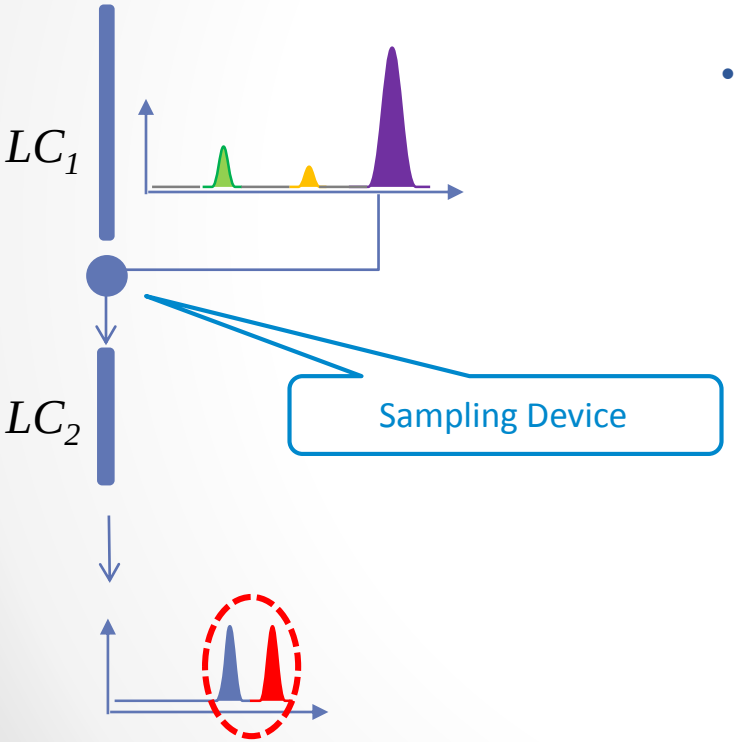
*J.R. Yates III et al., Int. J. of Mass Spectrometry 219 (2002) 245

Principle Methods of 2D LC

- "Offline" methods (sequential)
  - Collect fractions from the 1st dimension separation, stored and re-injected in the 2nd dimension separation column later.
- "Stop-and-Go" methods (e.g. Multi-Dimensional Protein Identification Technology)
  - One column packed with a segment of ion exchanger and a larger segment of RP-phase. A pulsed salt gradient in IEX displaces a fraction of the sample onto the RP-column
- "On-line" methods (parallel)
  - **Heart-cut:**
    Selected fractions from the 1st dimension separation and intermediately stored on-line and delivered on-line to the 2nd dimension separation
  - **Comprehensive:**
    Fractions are continuously taken from the eluate from the 1st dimension separation, intermediately stored on-line and delivered to the 2nd dimension separation

Principle Methods of 2D LC

Heart-cutting LC-LC



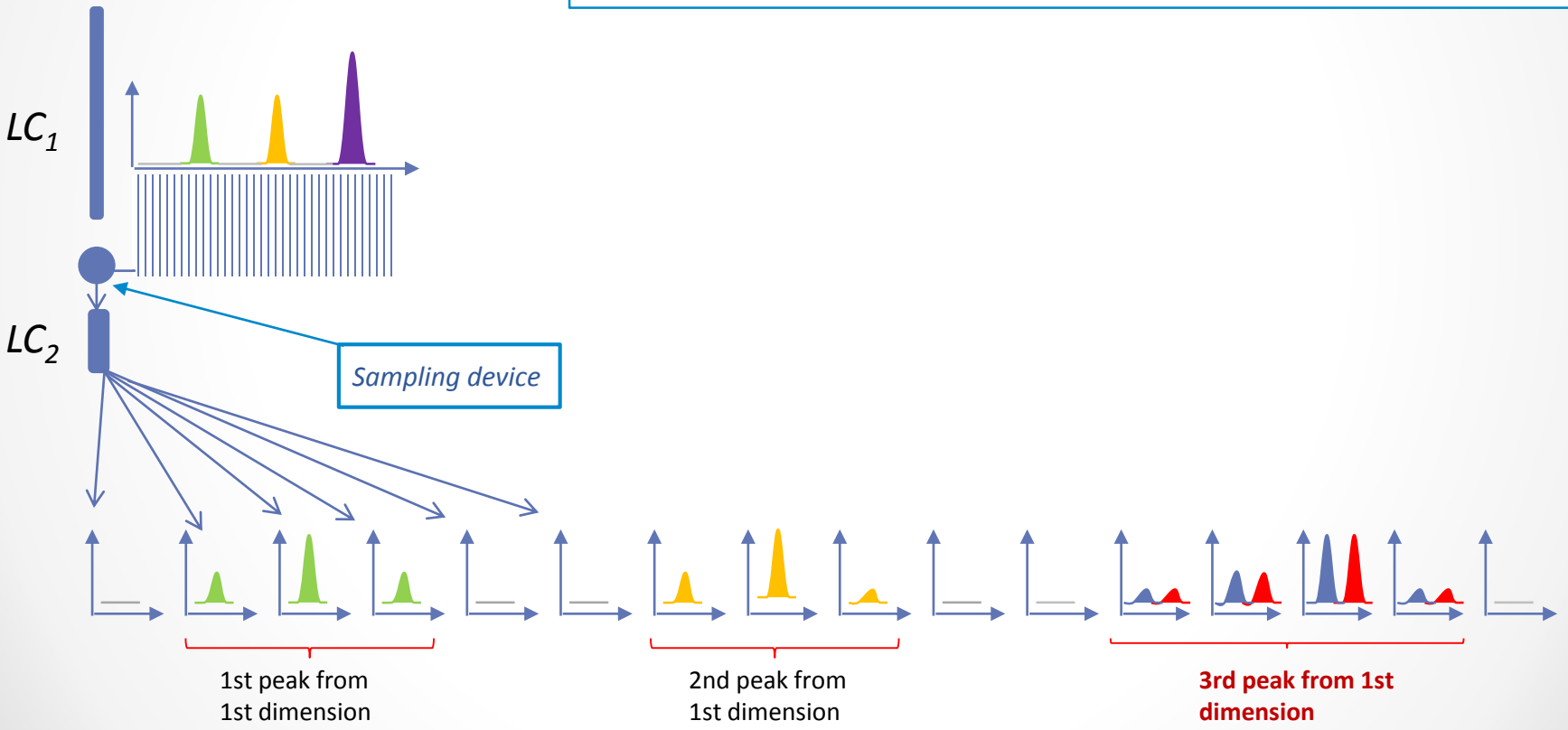
- Selected fractions of the 1st dimension separation are injected onto the 2nd dimension column
  → 1st dimension detector optional
- Long 1st dimension gradient separation possible
  → good data quality Limited information

Slide courtesy of Agilent Technologies

Principle Methods of 2D LC

Comprehensive 2D-LC

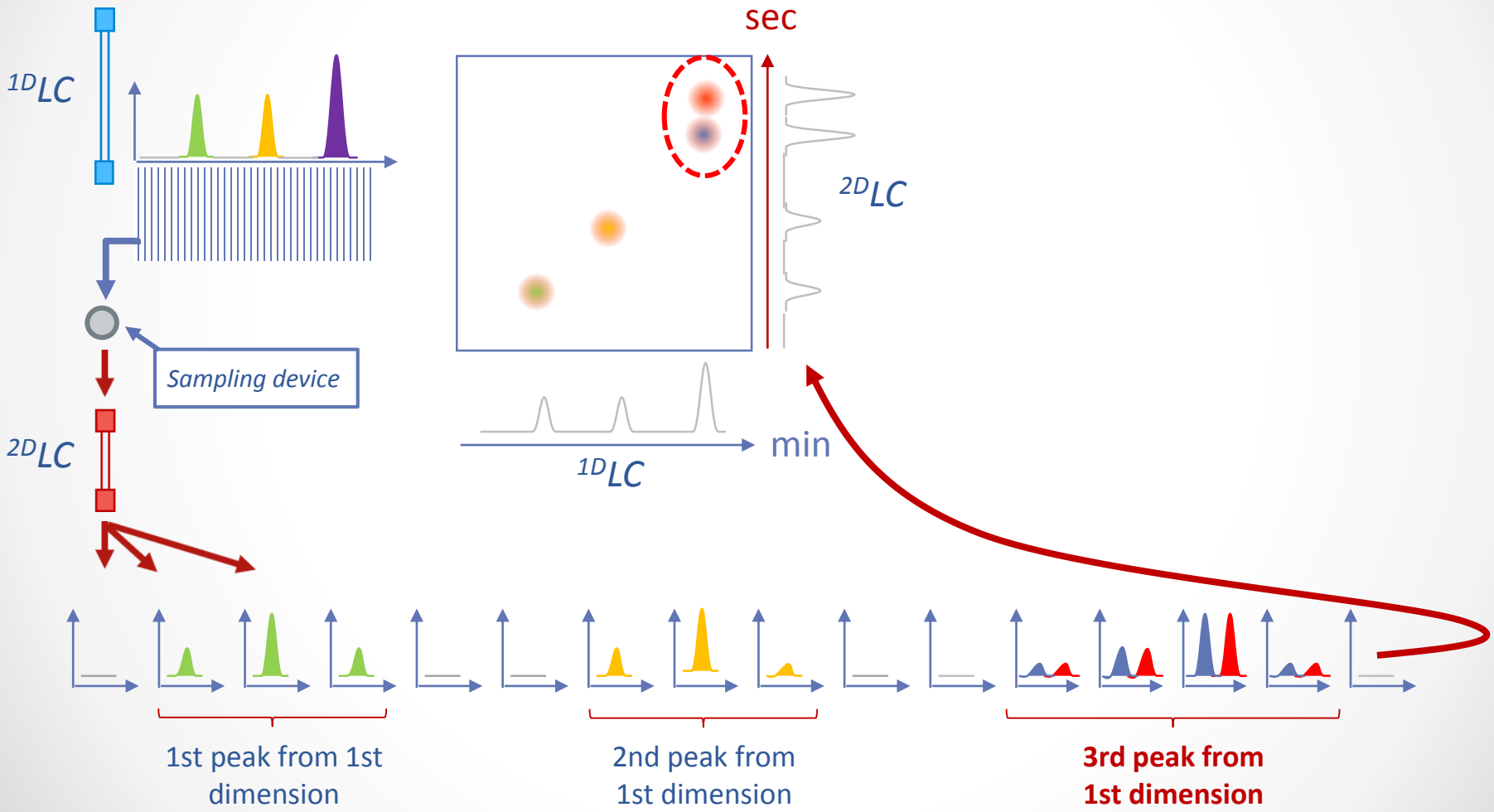
- The whole ^{1}D effluent is continuously injected onto ^{2}D column
- In the 2nd dimension (Ultra)Short 2D gradients are necessary mandating fast pumps & detector
- → Good data quality; full („comprehensive“) 2D information!



Slide courtesy of Agilent Technologies

Principle Methods of 2D LC

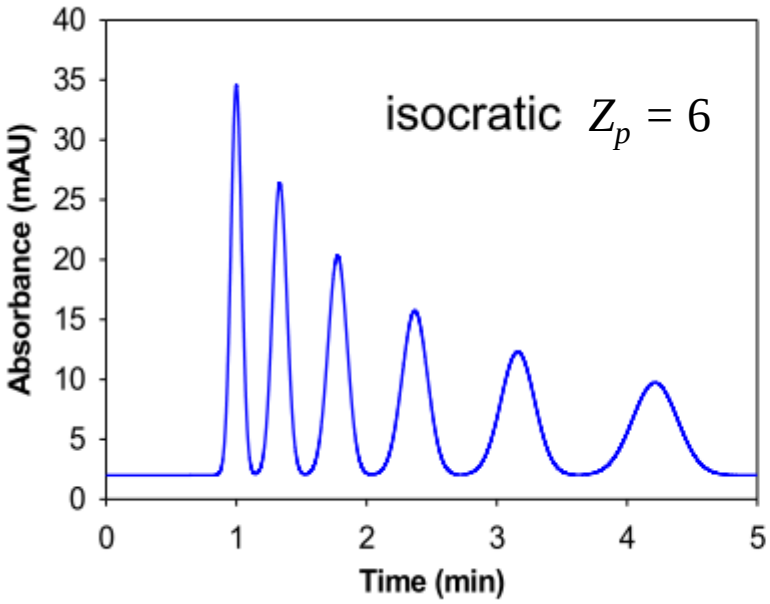
Comprehensive 2D LC



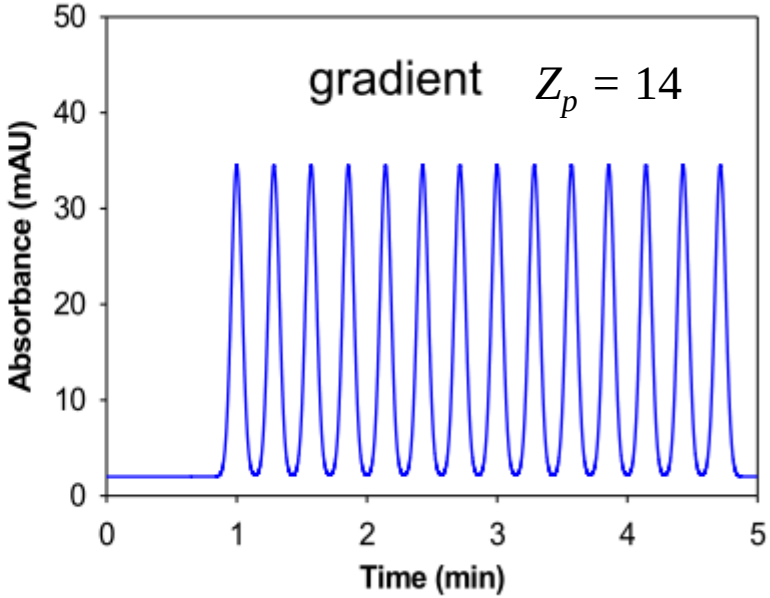
Slide courtesy of Agilent Technologies

Peak Capacity (Z_p) in 1D HPLC

of peaks separated with equal resolution



Assuming linear solvent strength gradient*



$$Z_p = \frac{\sqrt{N}}{6R_s} ln\left(1 + k_{last}\right) + 1$$

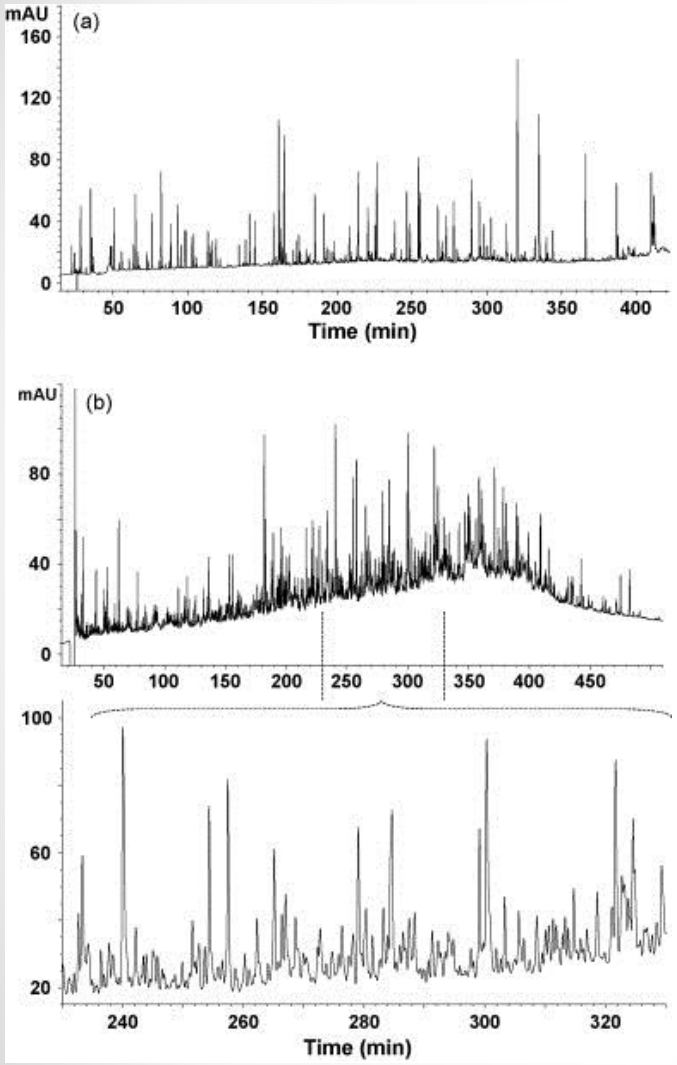
$$Z_p = 1 + \frac{t_g}{w_{av}}$$

Z_p : peak capacity
k'_{last} : retention factor of the last peak
R_s : required resolution (base line separation: $R_s \rightarrow 1.5$)

LC column, Z_p = 50, k = 10, N_{req} = calculate

Peak Capacity (Z_p) in 1D HPLC

Practical Example



BSA (a) and a depleted human serum tryptic digest (b) on 8 250× 2.1 mm ID × 5 µm Zorbax SB300-C18 columns.

Temperature 60 °C.

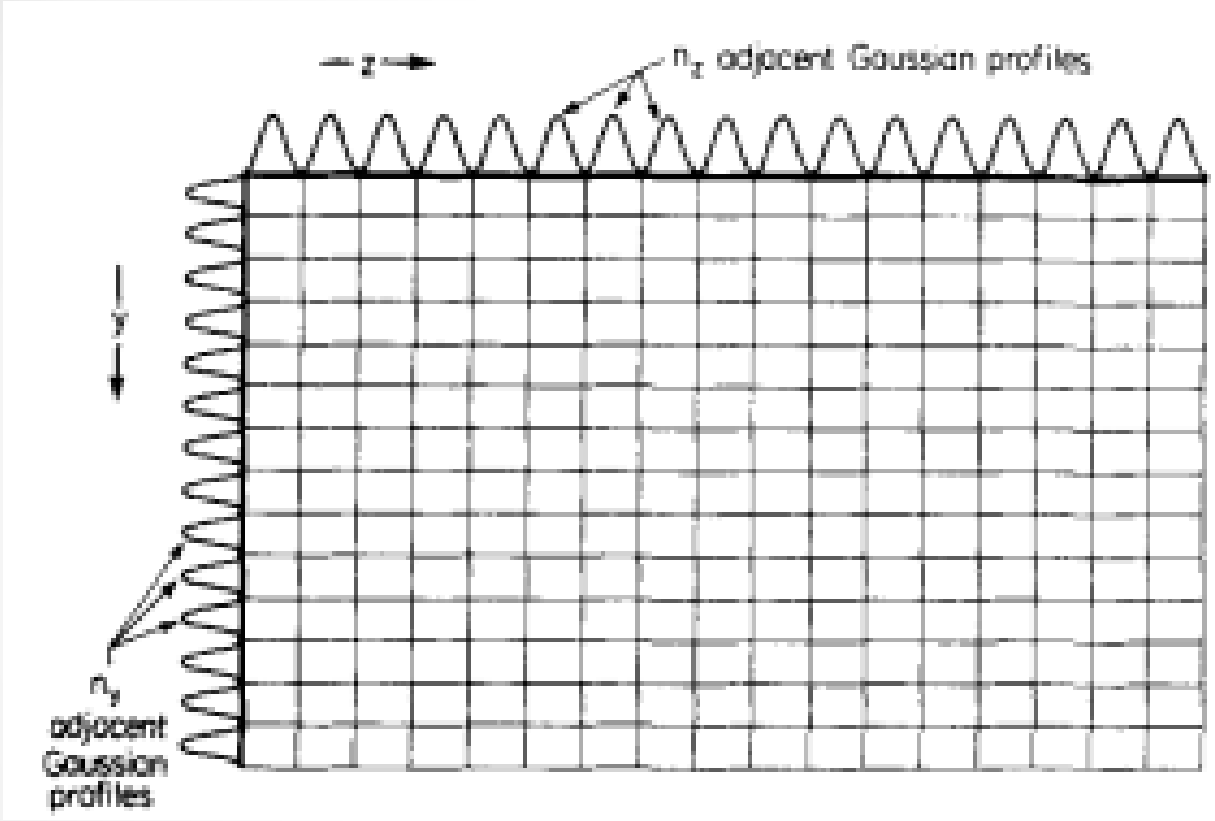
Mobile phase A 2% ACN, 0.1% TFA and mobile phase B 70% ACN, 0.1% TFA. Gradient slope 0.135% B/min, flow rate 200 µL/min.

Detection wavl. 214 nm

P. Sandra, G. Vanhoenacker, J. Sep. Sci., 30 (2007), p. 241

Peak Capacity in Comprehensive 2DLC

The geometric orthogonality concept



2nd Dimension Separation

1st Dimension Separation → $^{1}Z_p$

In this case $^{2D}Z_p$= 17x14 = 238!
For 1D separation, N_{req} = calculate

Theoretically:

$$^{2D}Z_p = {}^{1}Z_p \; x \; {}^{2}Z_p$$

The Giddings "Product Rule"

Giddings, J. C. J. High Resolut. Chromatogr. 1987, 10, 319-323

The Sampling Problem in 2D LC

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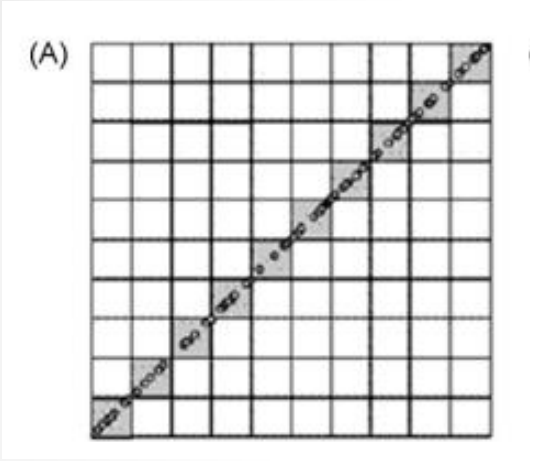
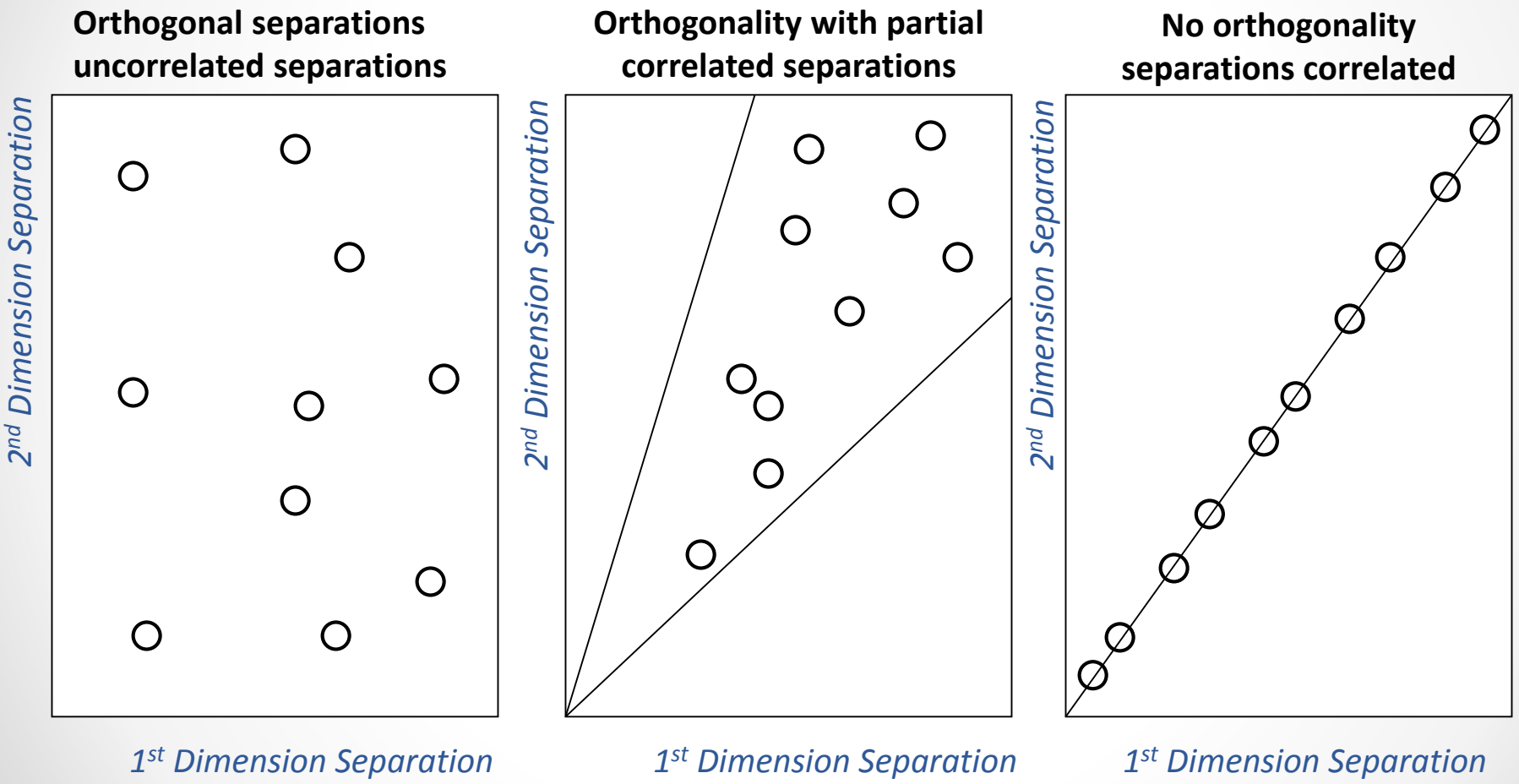
Orthogonality in Comprehensive 2DLC



(A) Non-orthogonal system, ^{1}D column is identical with ^{2}D column. Area coverage represents 10% orthogonality.

M. Gilar et al. Anal. Chem., 77, 6426 (2005)

Separation Space Utilization by Orthogonal and Correlated Mechanisms

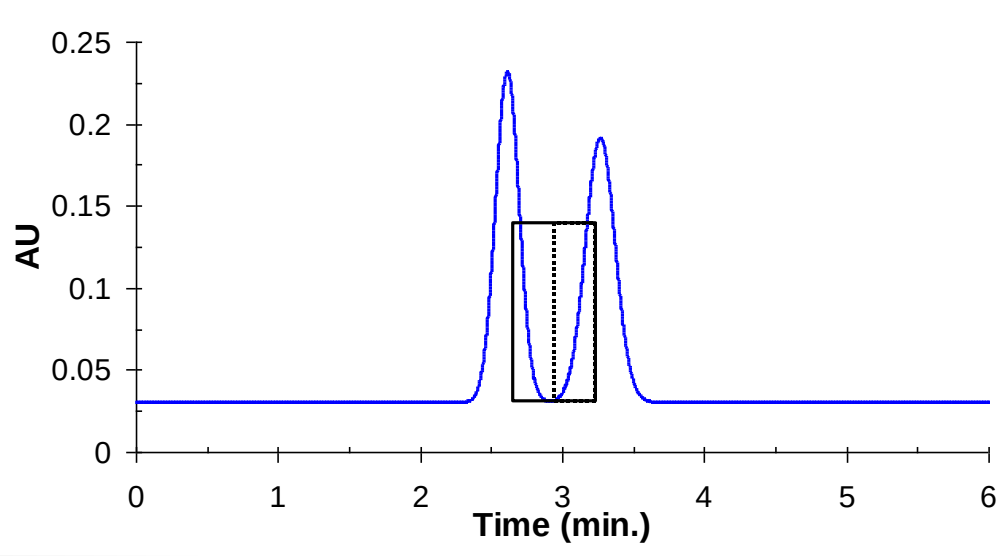


Slide courtesy of Prof. P. Carr & Dr. D. Stoll

The Undersampling Problem*

The Murphy-Schure-Foley Criterion

*Slide courtesy of Prof. Pete. Carr & Dr. Dwight Stoll



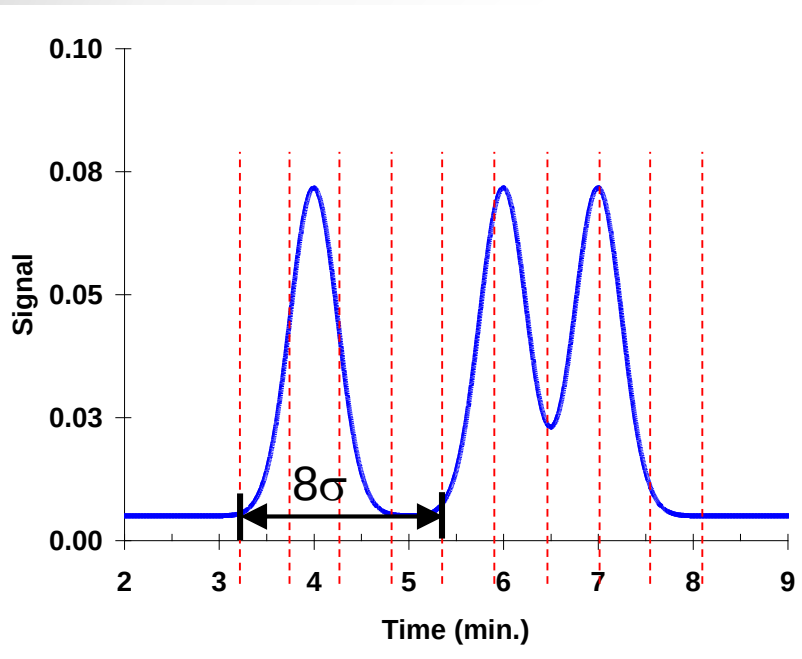
Clearly if we take a sample as indicated and inject it into a second dimension we will partially “un do “ the separation already accomplished in the first dimension.

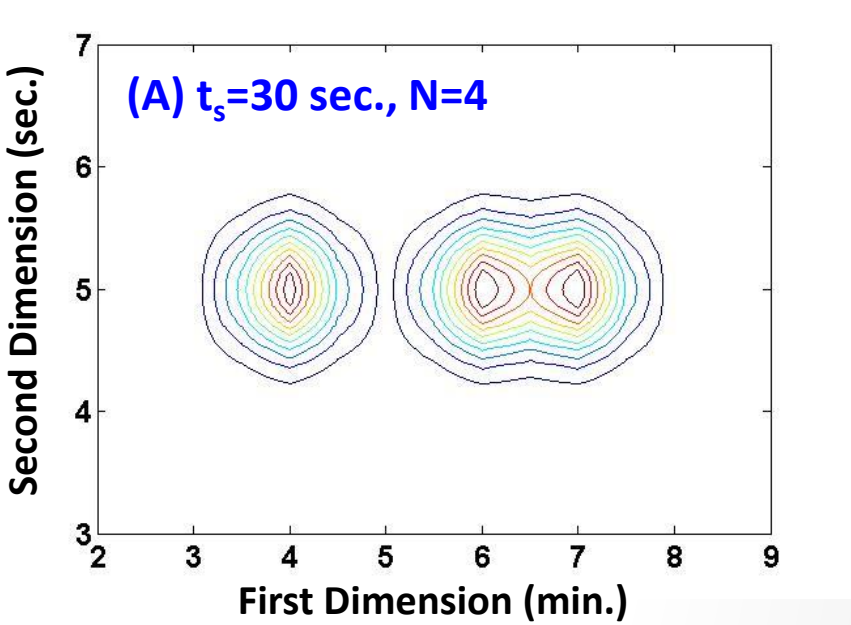
According to M-S-F **one needs to take at least 4 samples** across the 8σ base width of each first dimension peak **to minimize the effect of undersampling**.

Murphy, R. E.; M. R. Schure; J. P. Foley *Anal. Chem.*, 1998; Vol. 70, pp 1585-1594

The Undersampling Problem

The Murphy-Schure-Foley Criterion





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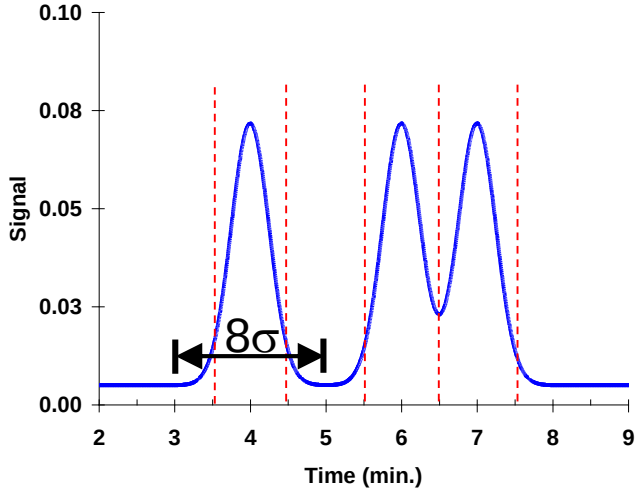

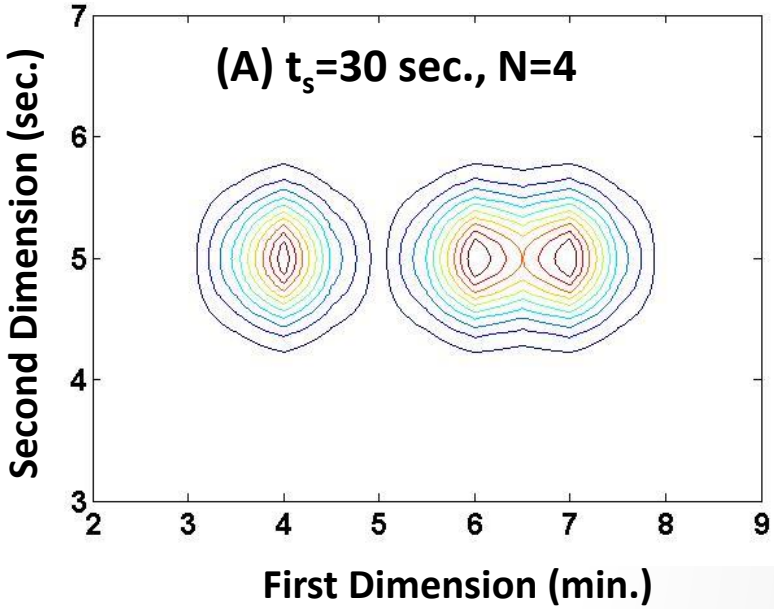

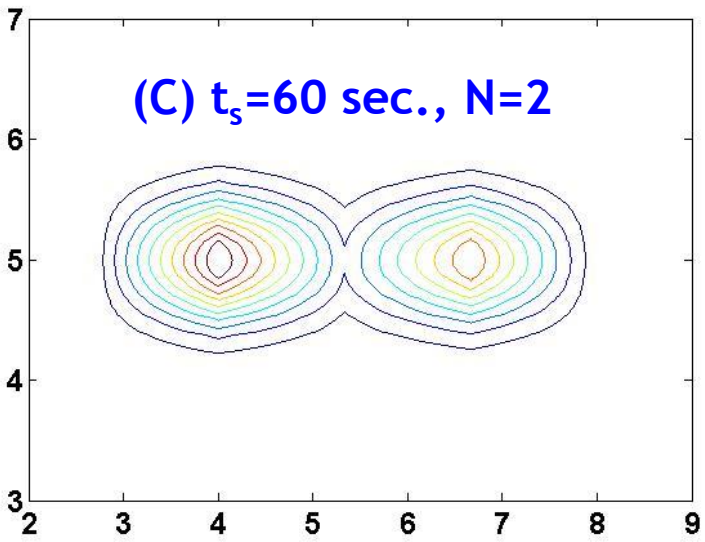

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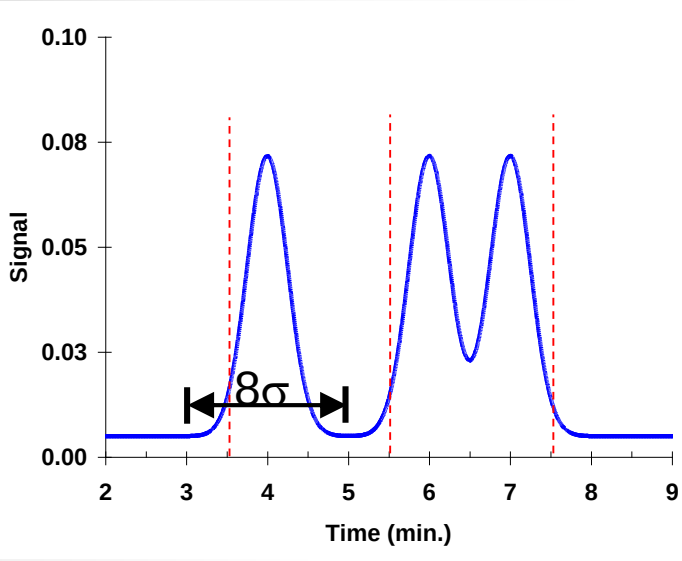
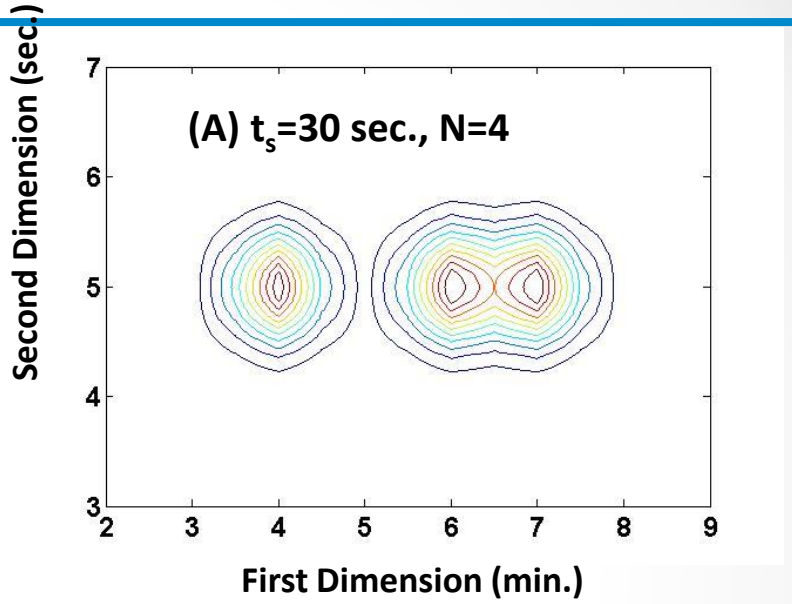
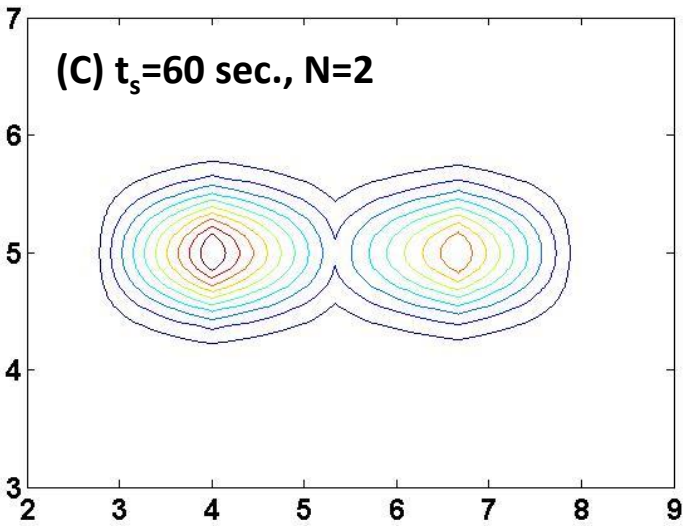
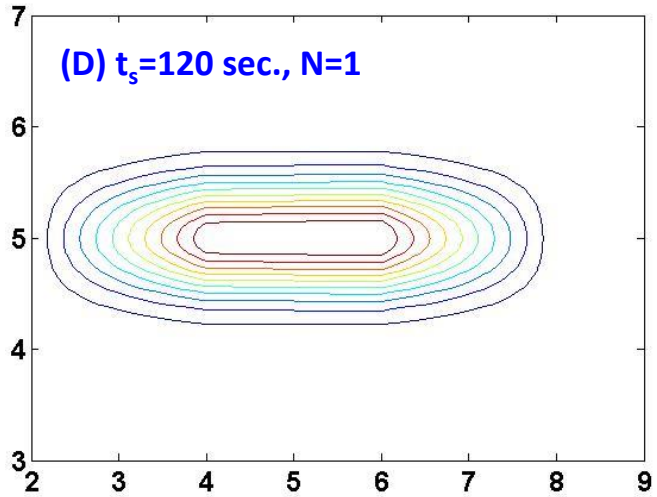




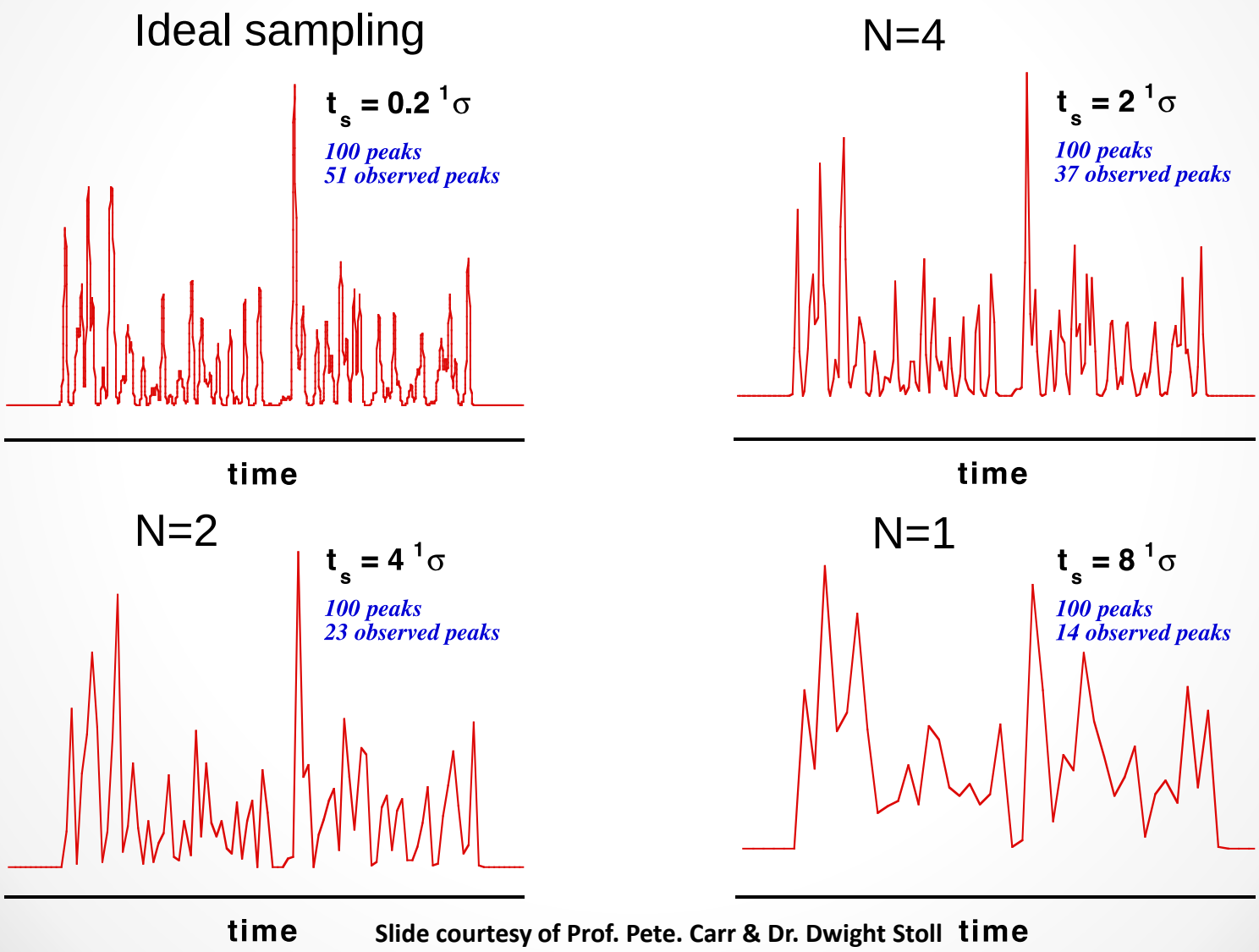




Slide courtesy of Prof. Pete. Carr & Dr. Dwight Stoll

The Undersampling Problem

Alternative View of Undersampling the First Dimension



Slide courtesy of Prof. Pete. Carr & Dr. Dwight Stoll

Peak Capacity in Comprehensive 2DLC

Implications of <β>

- We want to make the sampling time short.
- In LC x LC ${}^1t_{sample} = {}^2t_{cycle}$
- Prefer ${}^1t_{sample} < {}^2t_{cycle}$ (under fill the sample loop!)
- ${}^2t_{cycle} = {}^2t_{gradient} + {}^2t_{re\text{-}equilibration}$
- Don't make ${}^1t_{sample}$ too short since 2D separation peak capacity decreases if ${}^2t_{gradient}$ decreases
- Clearly there is an optimum range in t_{sample} (${}^2t_{cycle}$)

Slide courtesy of Prof. P. Carr & Dr. D. Stoll

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Slide courtesy of Prof. P. Carr & Dr. D. Stoll

Practical Implementation for 2D HPLC

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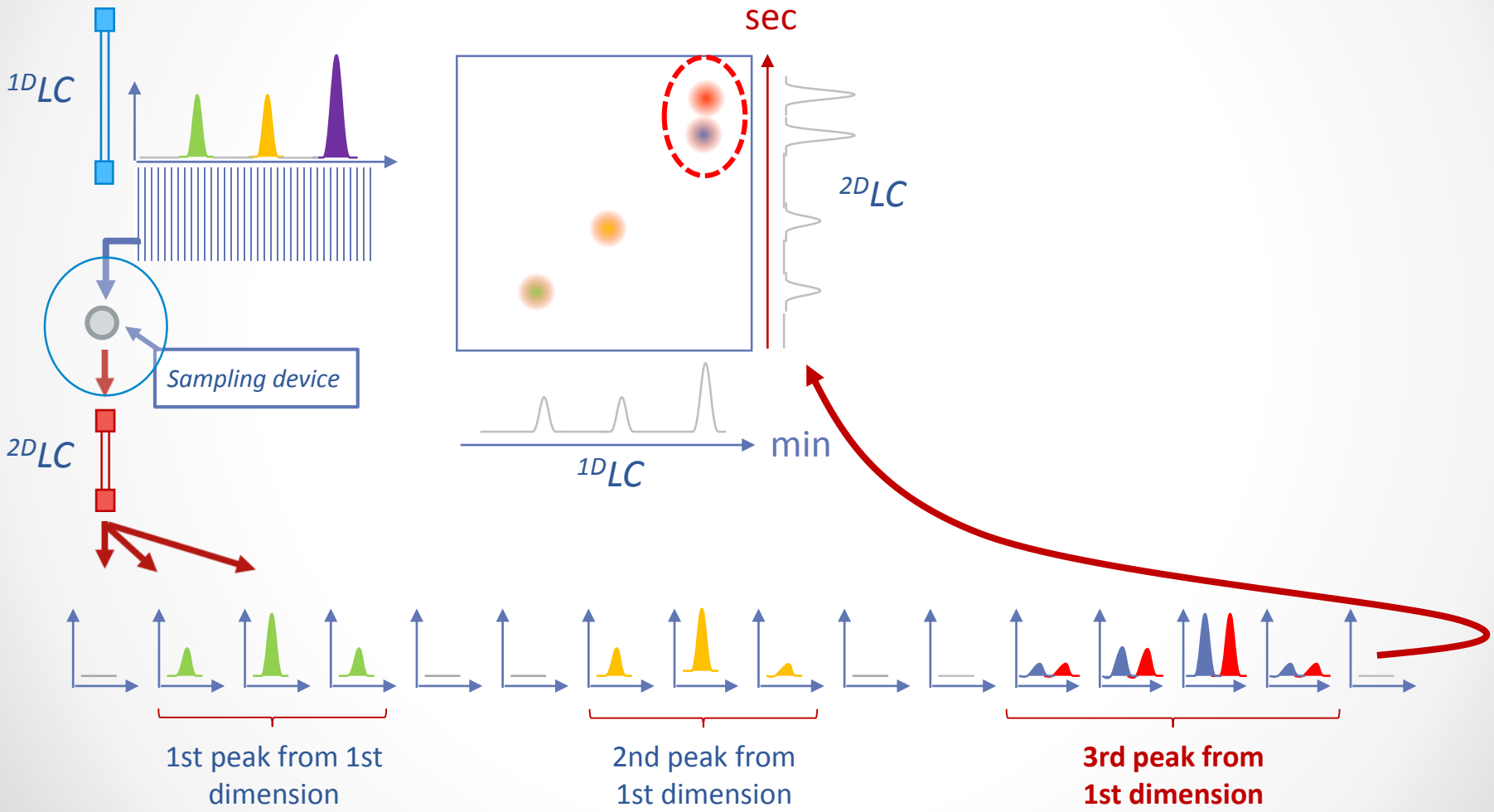
Sampling Device, Column Selection

Sampling Device for LCxLC

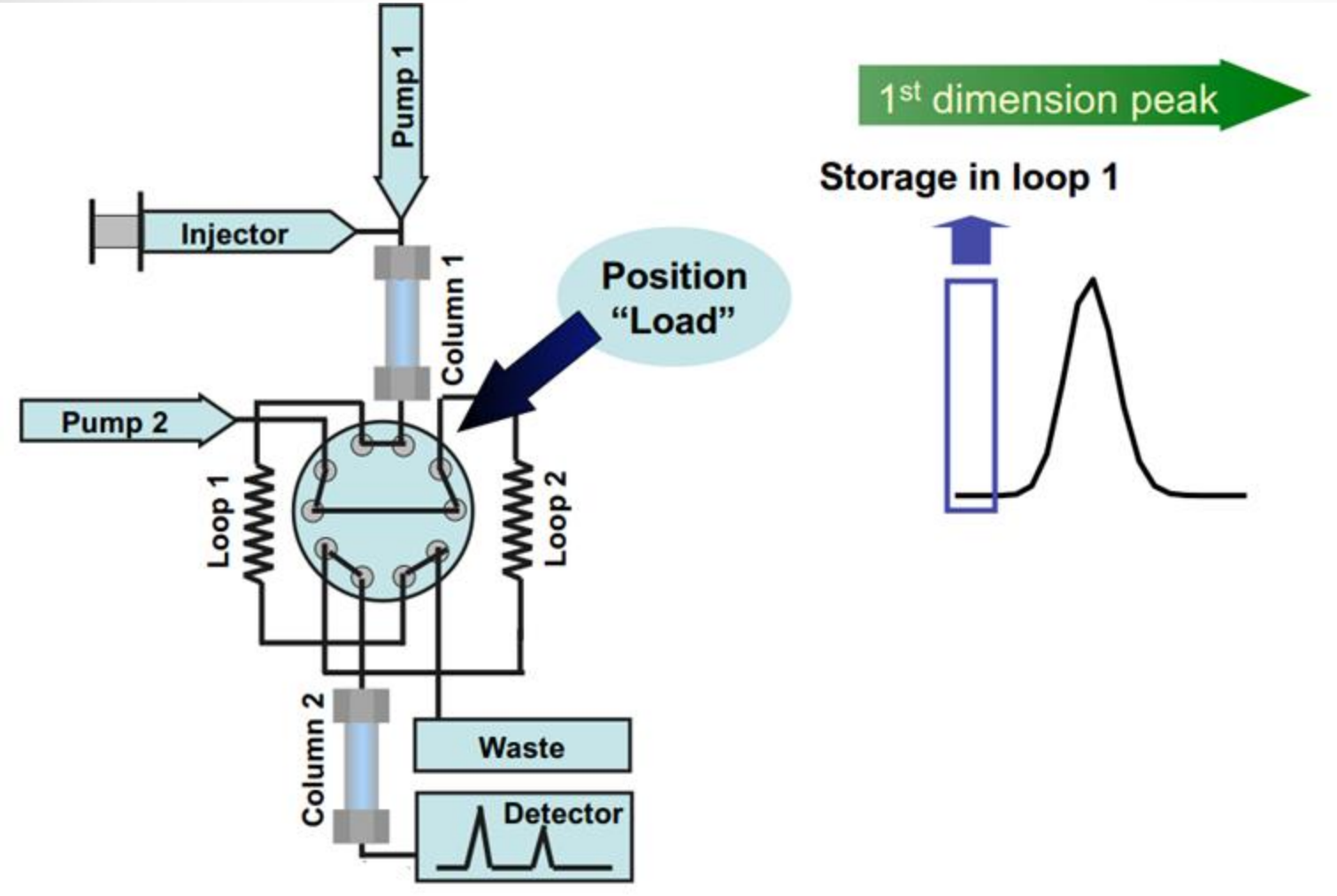
First-In First-Out (FIF0) Configuration



Slide courtesy of Agilent Technologies

Sampling Device for LCxLC

First-In First-Out (FIFO) Configuration (10 port, 2 position valve)



Slide courtesy of Prof. P. Schoenmakers

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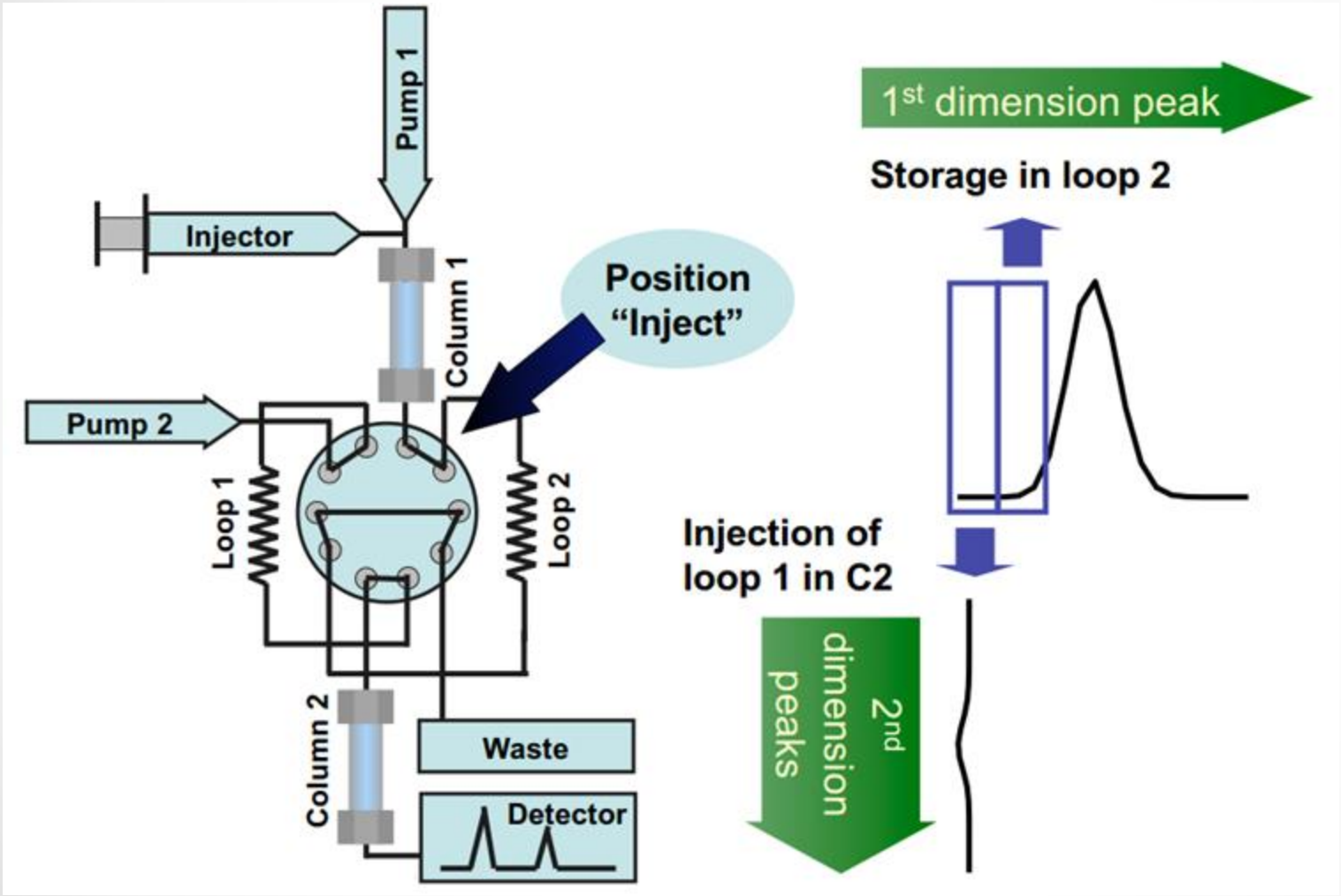

Slide courtesy of Prof. P. Schoenmakers

Sampling Device for LCxLC

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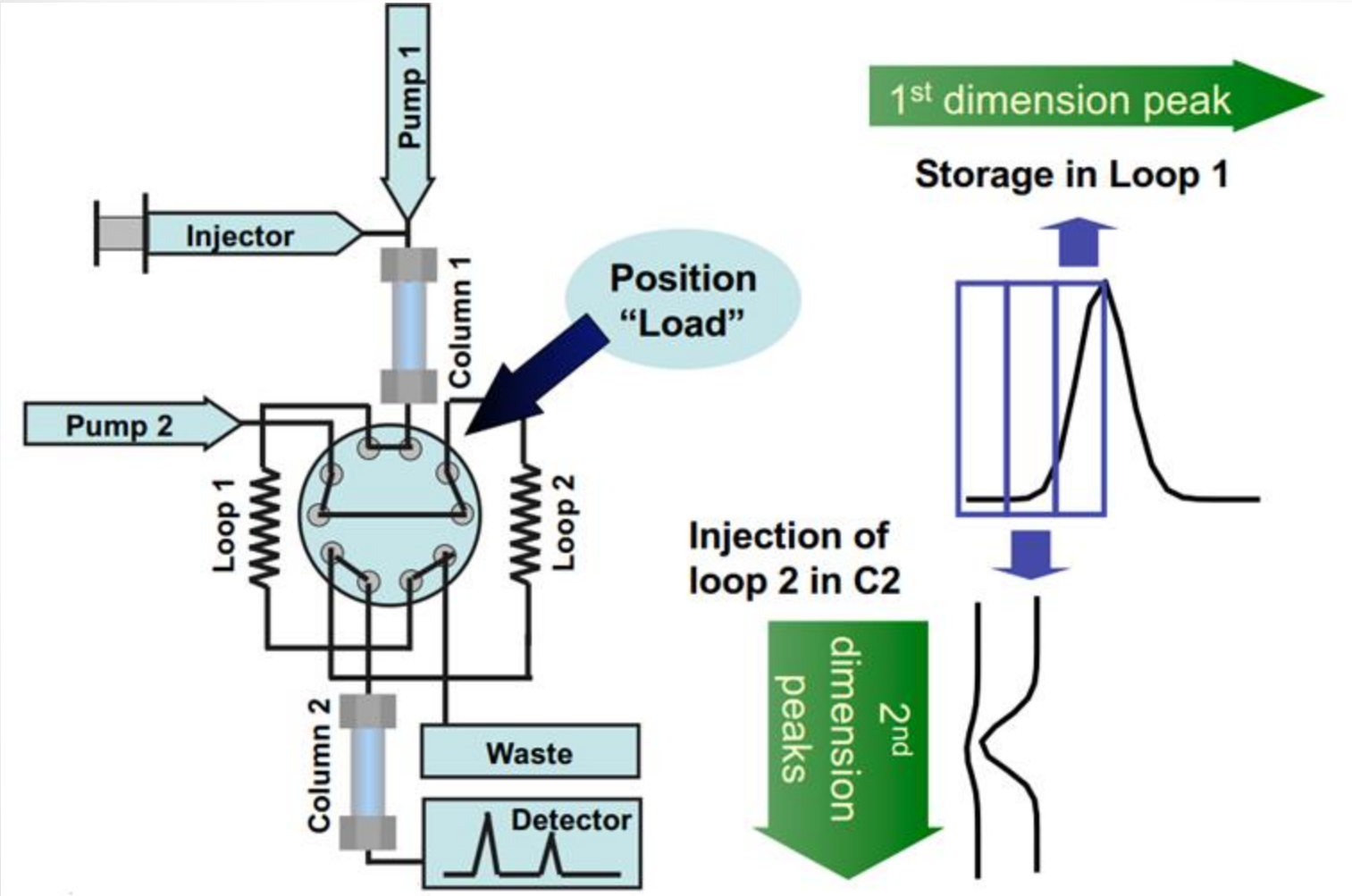
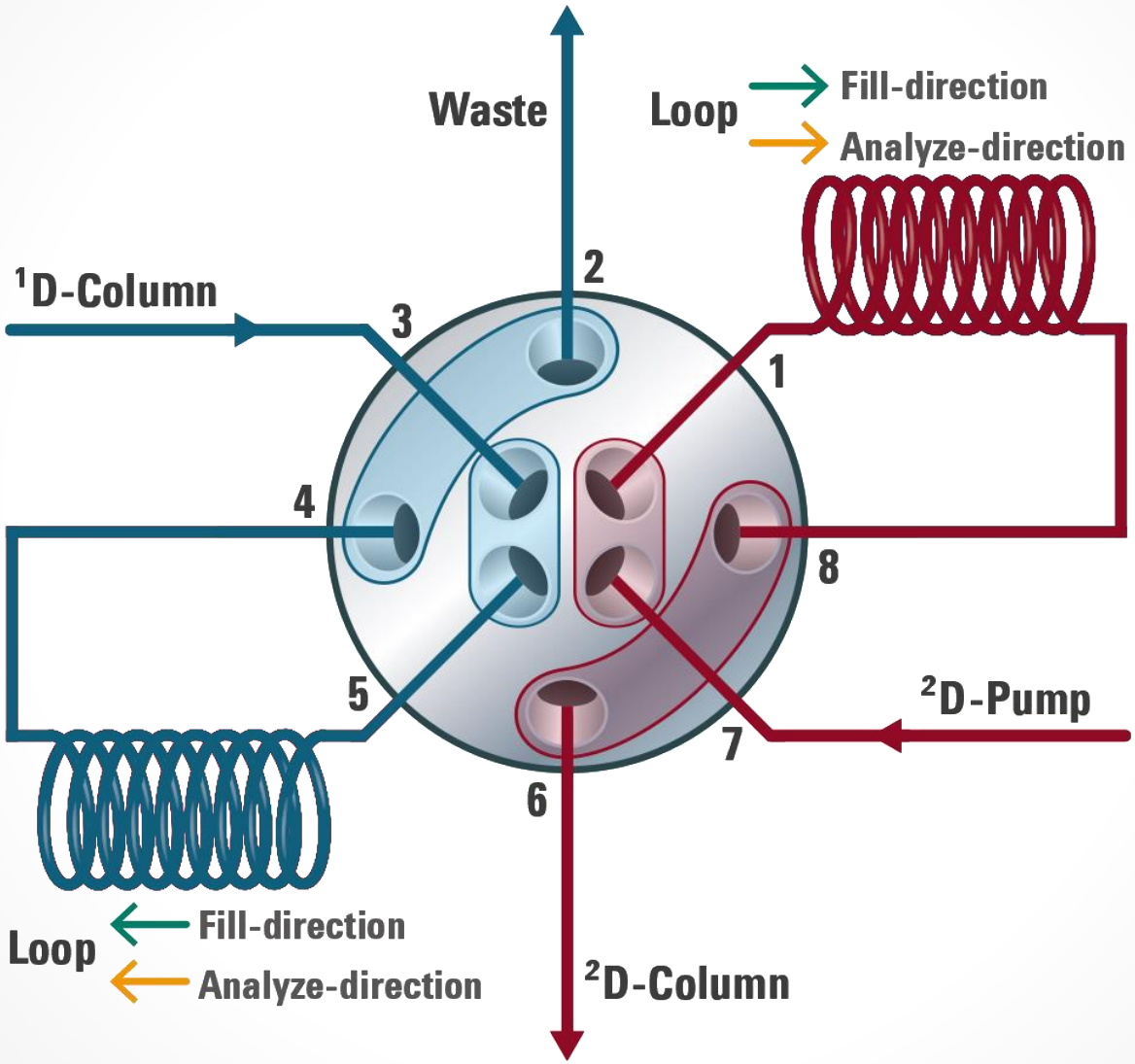


Slide courtesy of Prof. P. Schoenmakers

Sampling Device for LCxLC

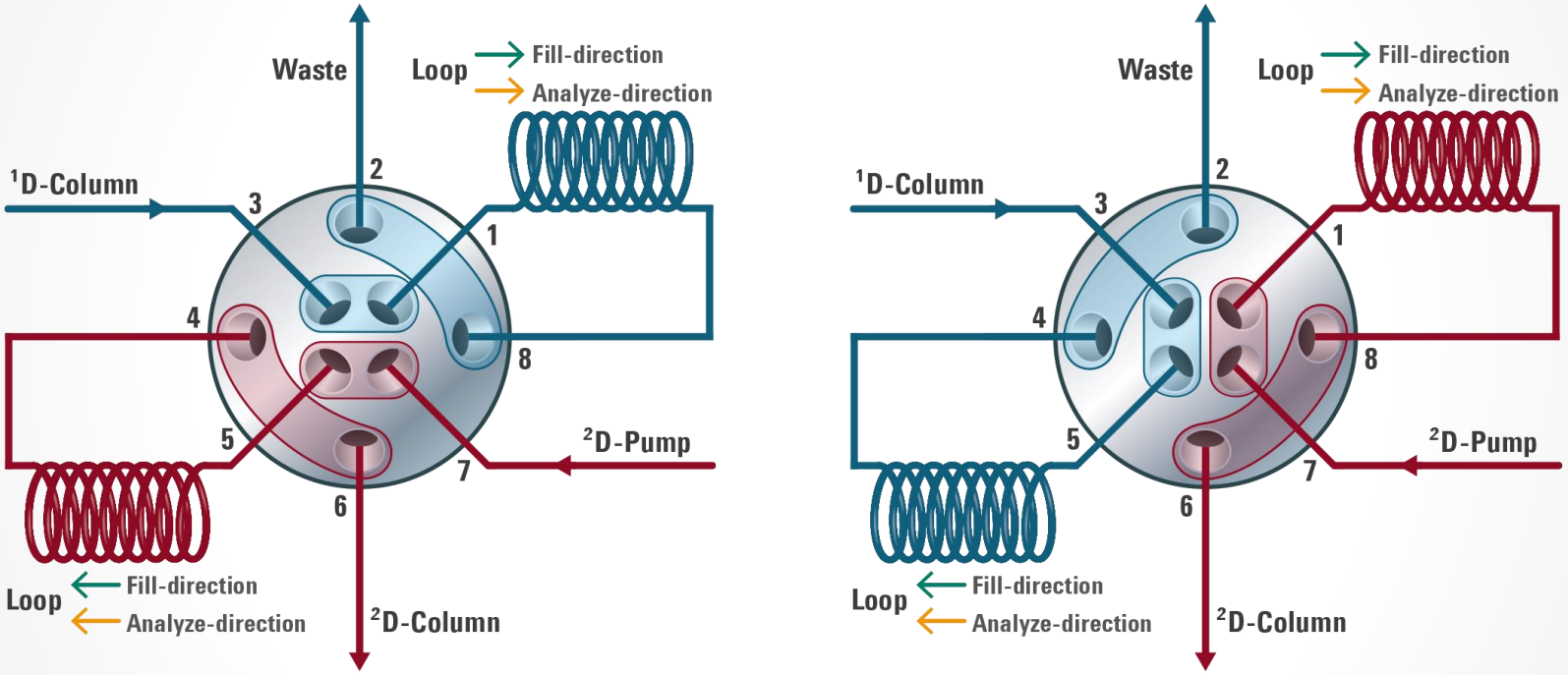
2x4 port, 2 position valve (Agilent Technologies Duo Valve)



Slide courtesy of Agilent Technologies

Sampling Device for LCxLC

2x 4 port, 2 position valve, co-current mode (Agilent Technologies Duo Valve)

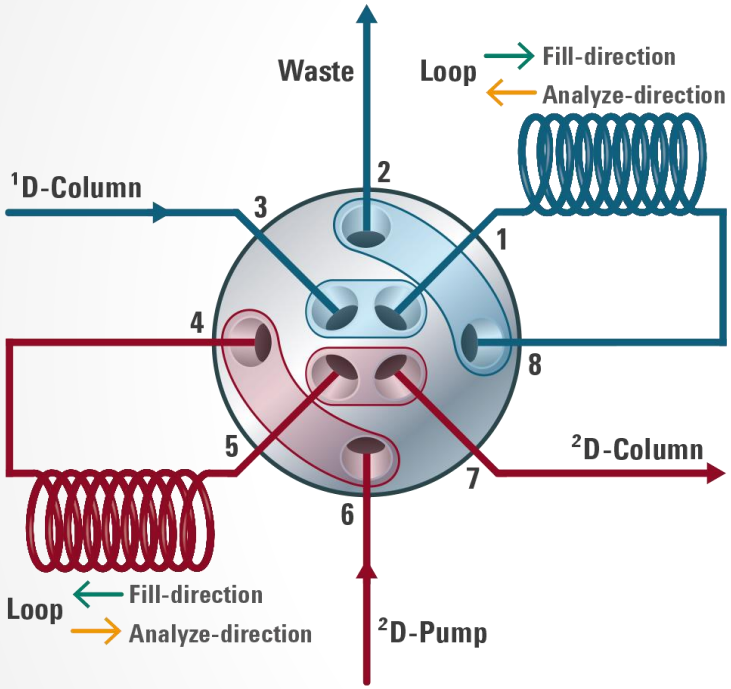


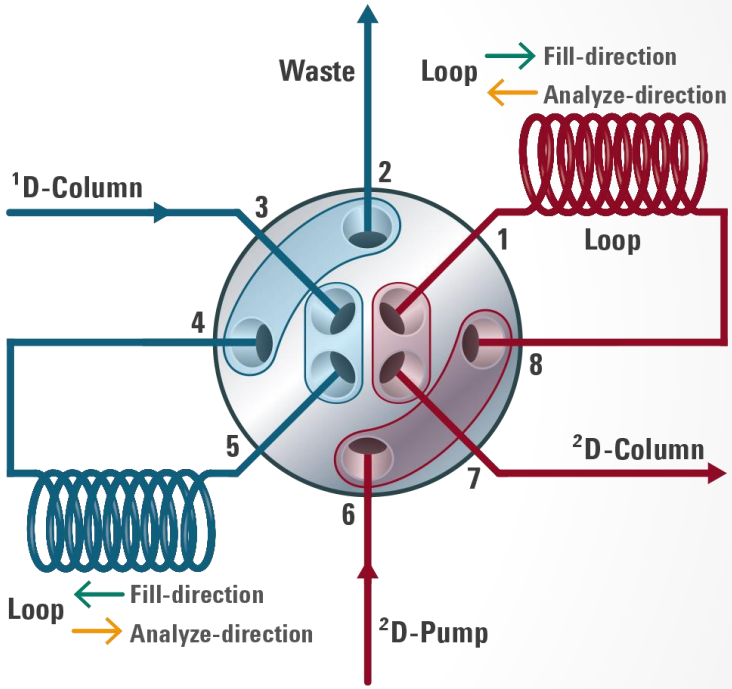
First-In-First-Out (FIFO)

Slide courtesy of Agilent Technologies

Sampling Device for LCxLC

2x 4 port, 2 position valve, counter-current mode (Agilent Technologies Duo Valve)





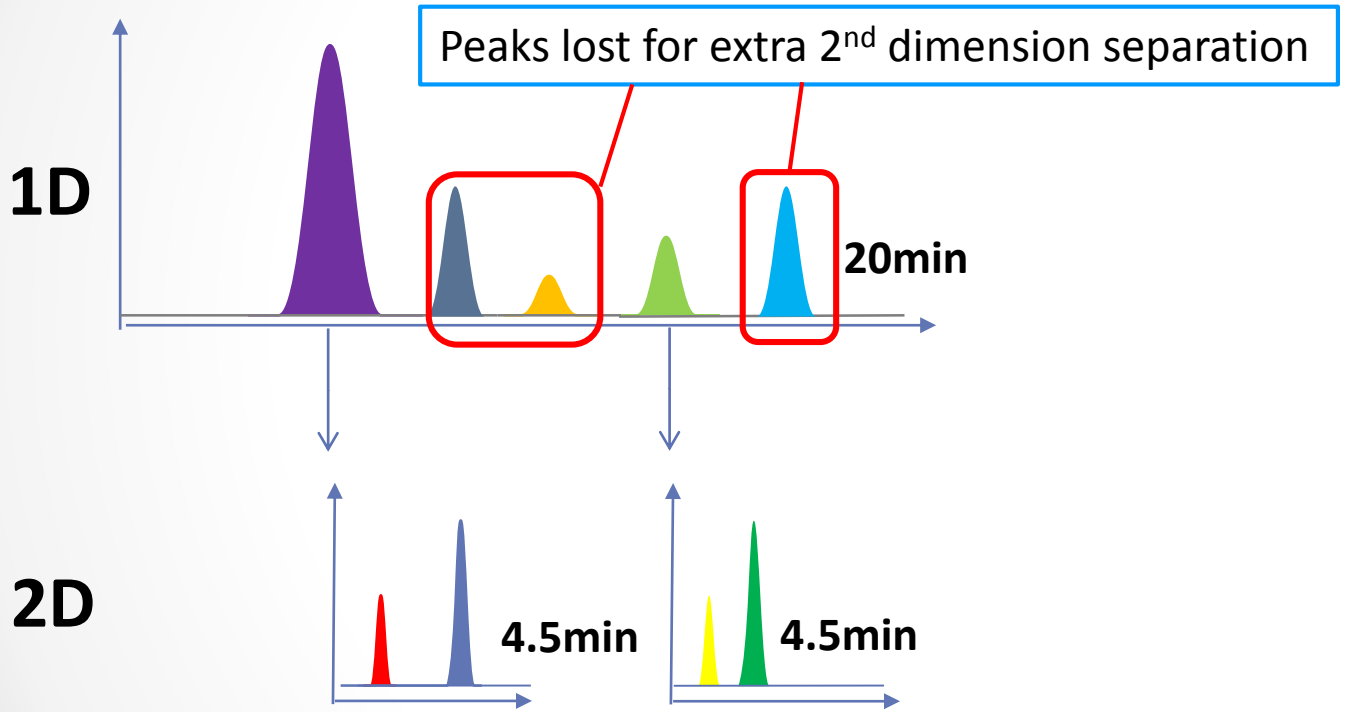
First-In-Last-Out (FIFO)

Counter-Current Mode: connections on port 6 and 7 reversed!

Slide courtesy of Agilent Technologies

Sampling Device for LC-LC (Heart-Cut)

Long Analysis Time of 2nd Dimension Separation



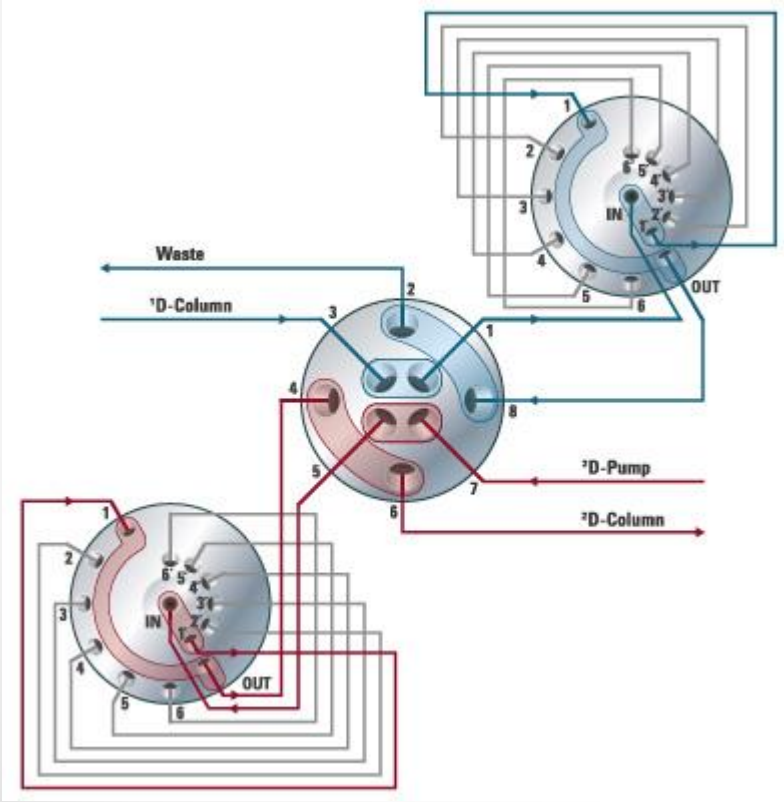
Heart-cutting Data Viewer

Slide courtesy of Agilent Technologies

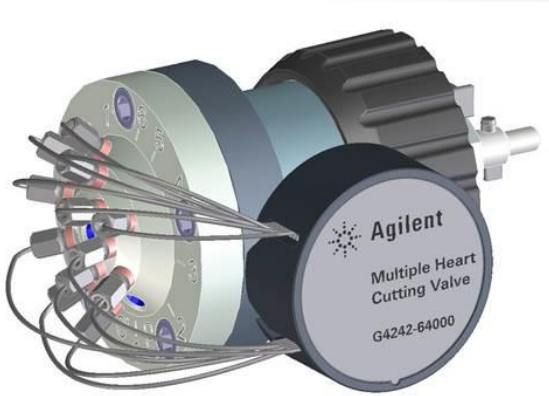
Sampling Device for LC-LC (Heart-Cut)

Agilent Multiple Heart-Cutting 2D-LC

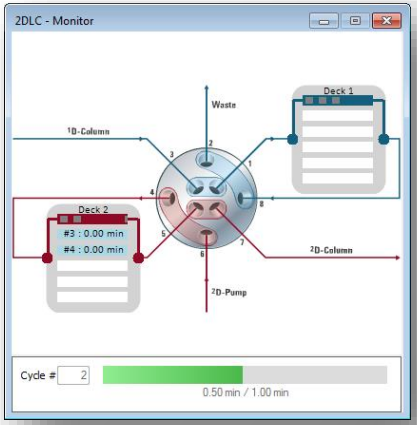
Smart Valve-Loop Setup with 12 loops
→ **2D-LC valve + two 6/14 valves**



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



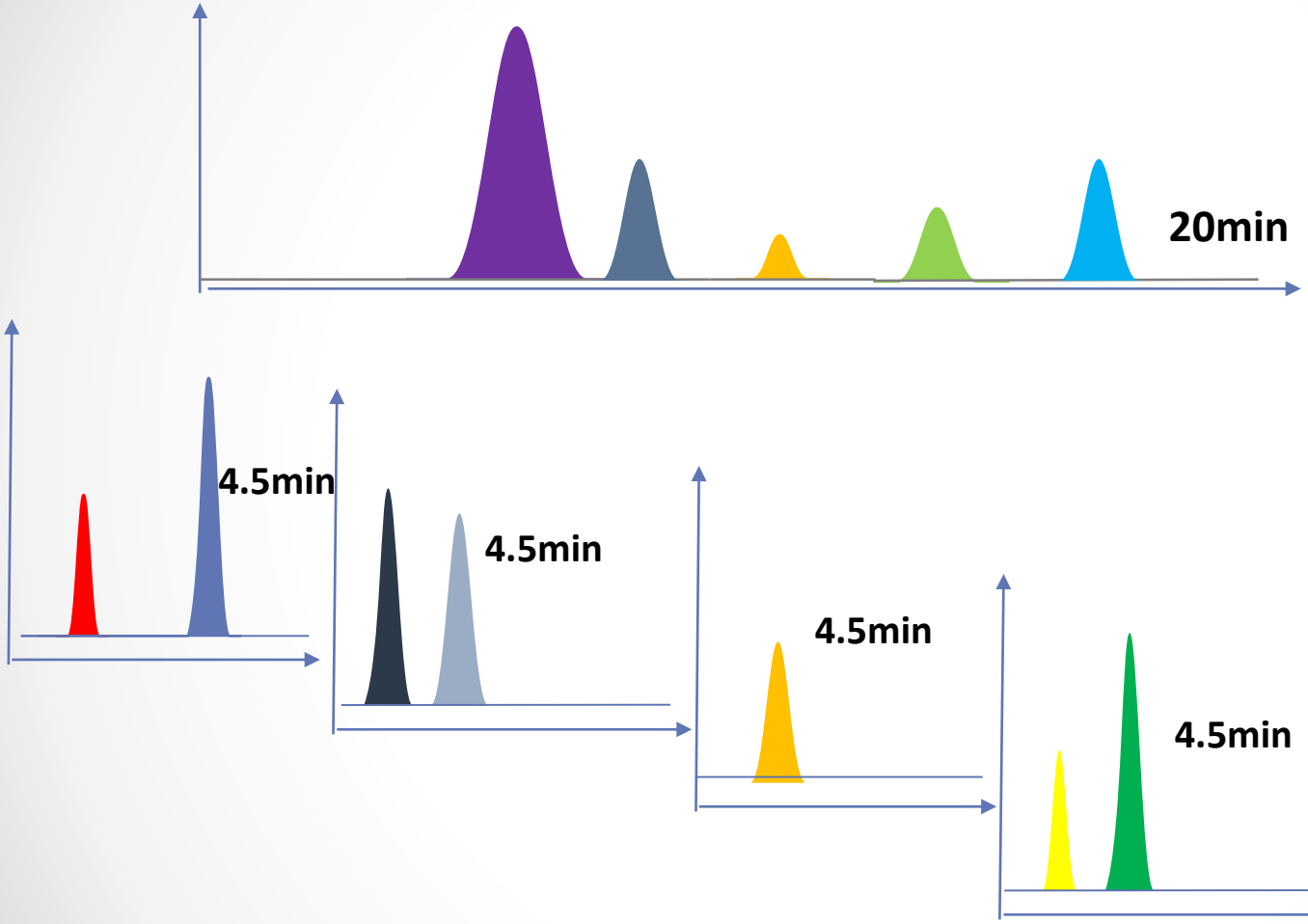
Online status monitoring

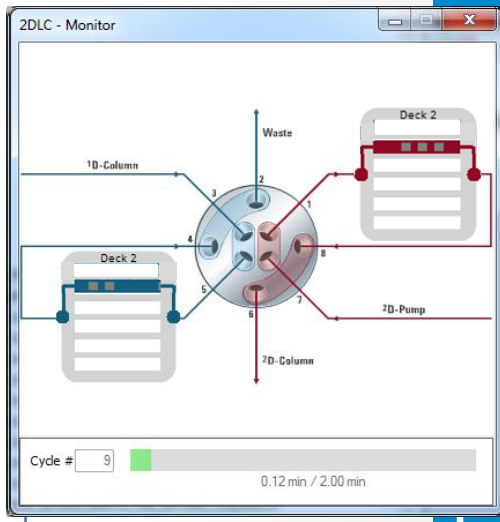


Slide courtesy of Agilent Technologies

Sampling Device for LC-LC (Heart-Cut)

Agilent Multiple Heart-Cutting 2D-LC





1st dimension detection recommended in case of unknown sample composition!!

Slide courtesy of Agilent Technologies

Peak Capacity in Comprehensive 2DLC

Implications of <β>

- We want to make the sampling time short.
- In LC x LC ${}^1t_{sample} = {}^2t_{cycle}$
- Prefer ${}^1t_{sample} < {}^2t_{cycle}$ (under fill the sample loop!)
- ${}^2t_{cycle} = {}^2t_{gradient} + {}^2t_{re\text{-}equilibration}$
- Don't make ${}^1t_{sample}$ too short since 2D separation peak capacity decreases if ${}^2t_{gradient}$ decreases
- Clearly there is an optimum range in t_{sample} (${}^2t_{cycle}$)

Slide courtesy of Prof. P. Carr & Dr. D. Stoll

Requirements to the 1st Dimension Separation

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Dimensions, Stat. Phase Selection, Isocratic or Gradient Elution

1st Dimension Separation

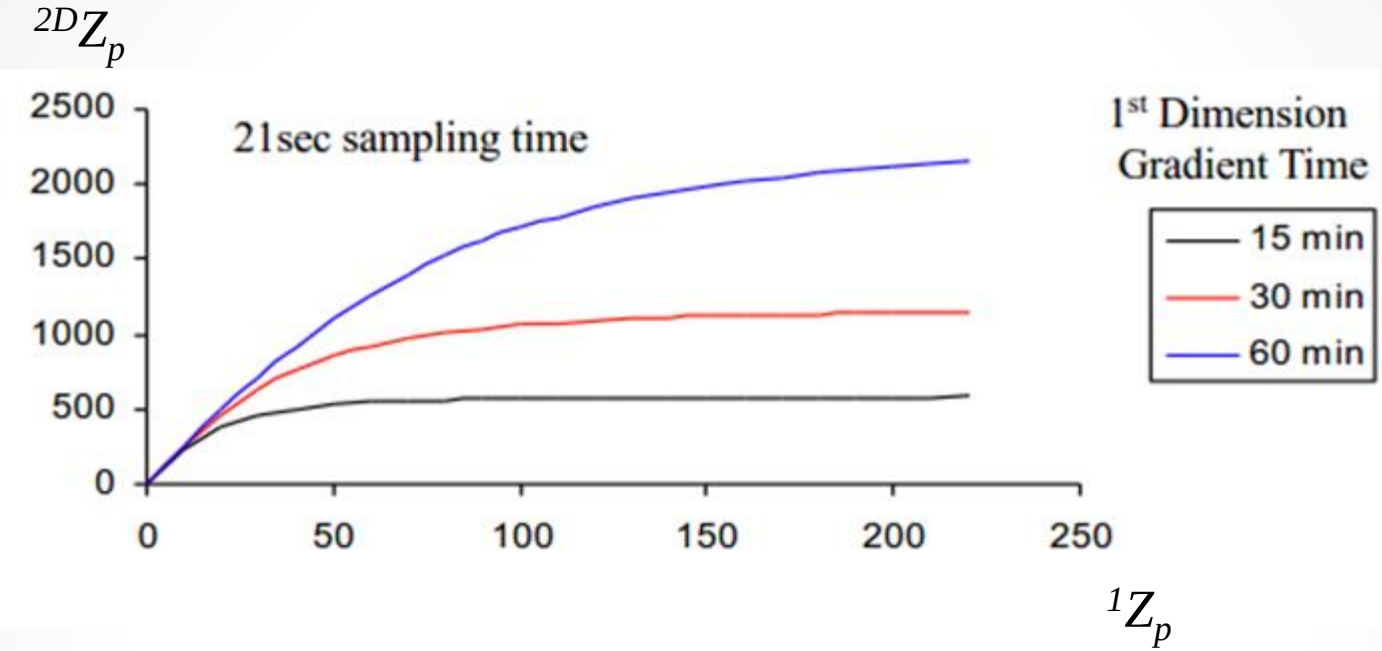
Requirements

- Narrow and long columns are preferred
- Use low flow rate where possible
  - 1D Flow Rate = 200 µL/min, Sampling Time = 20 s
    ➔ Volume Injected to 2D Column = 67 µL
- Use stationary phase that can tolerate extreme conditions (e.g. low or high pH)
- Isocratic separation or use a slow gradient separation
  - Peak width in isocratic separation is not constant; may lead to under sampling early and over sampling late in the chromatogram

Slide courtesy of Prof. P. Carr & Dr. D. Stoll

Peak Capacity in Comprehensive 2DLC

Influence of 1st dimension gradient steepness



L.W. Potts, D.R. Stoll, X. Li, P.W. Carr J. Chrom. A (2010), 1217, 5700-5709

End of Part 1

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