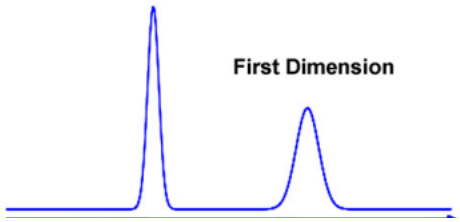
Multidimensional HPLC Tutorial Part - 1

Introduction. Basic concepts – heart-cut vs comprehensive 2D-LC. Definition of orthogonality. The sampling problem of 2D LC.

What is Multidimensional HPLC?

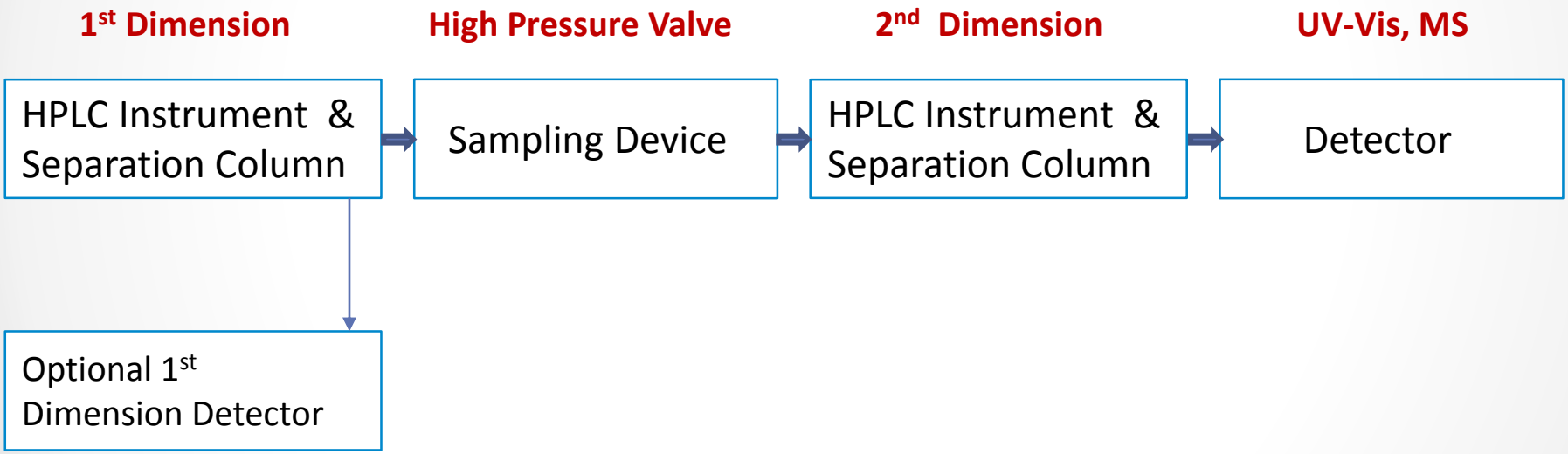


Peak capacity by the product of the number of bins

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

What is Multidimensional HPLC?

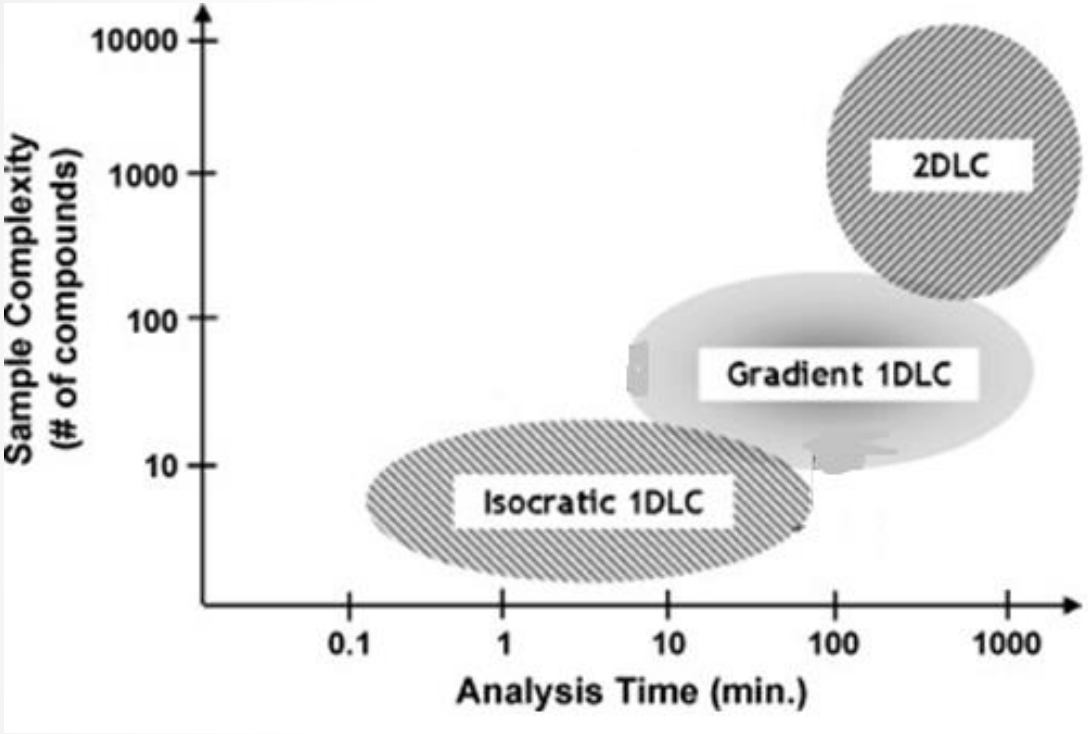
Simple Block Diagram



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]



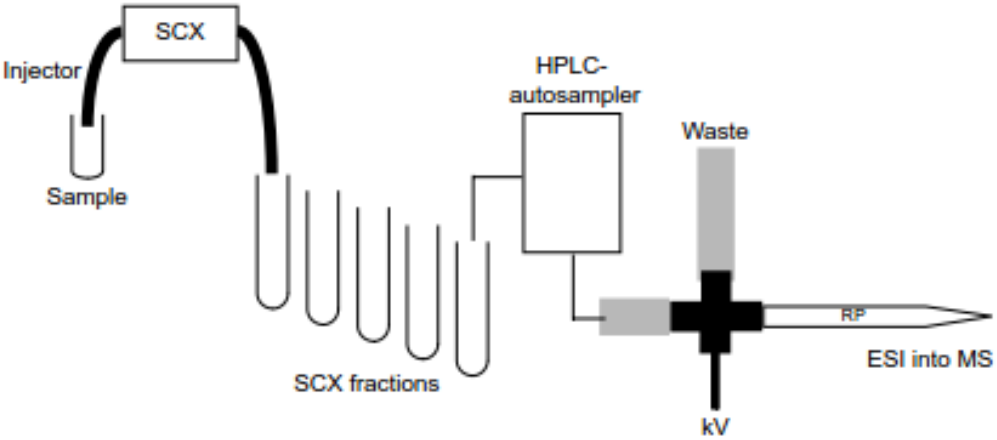
Adapted from [1]Stoll, D., University of Minnesota Ph.D. Dissertation, 2007, [2]Stoll, D., *et al.*, *J. of Chrom. A*, 1168, 3 (2007)

Important Nomenclature

- LC-LC heart-cut two-dimensional liquid chromatography
- LCxLC comprehensive two-dimensional liquid chromatography
- ^{1}D 'first dimension'; for example, ^{1}D column means 'first dimension' column
- ^{2}D 'second dimension'; same ^{2}D column means 'second dimension' column
- 1D denotes a one-dimensional system
- 2D denotes a two-dimensional system e.g. 2D LC
- $^{1}t_r$ retention time for a given peak in the first dimension
- $^{1}t_0$ "dead" time of the first dimension conditions
- ^{1}k retention factor of a given compound eluting from the first dimension column
- ^{1}N the number of theoretical plates of the first dimension column
- ^{1}w width of a peak eluting from the first dimension column
- $^{1}R_S$ resolution of a peak pair eluting from the first dimension column
- $^{1}Z_p$ peak capacity of the first dimension column
- n_c number of components in the sample

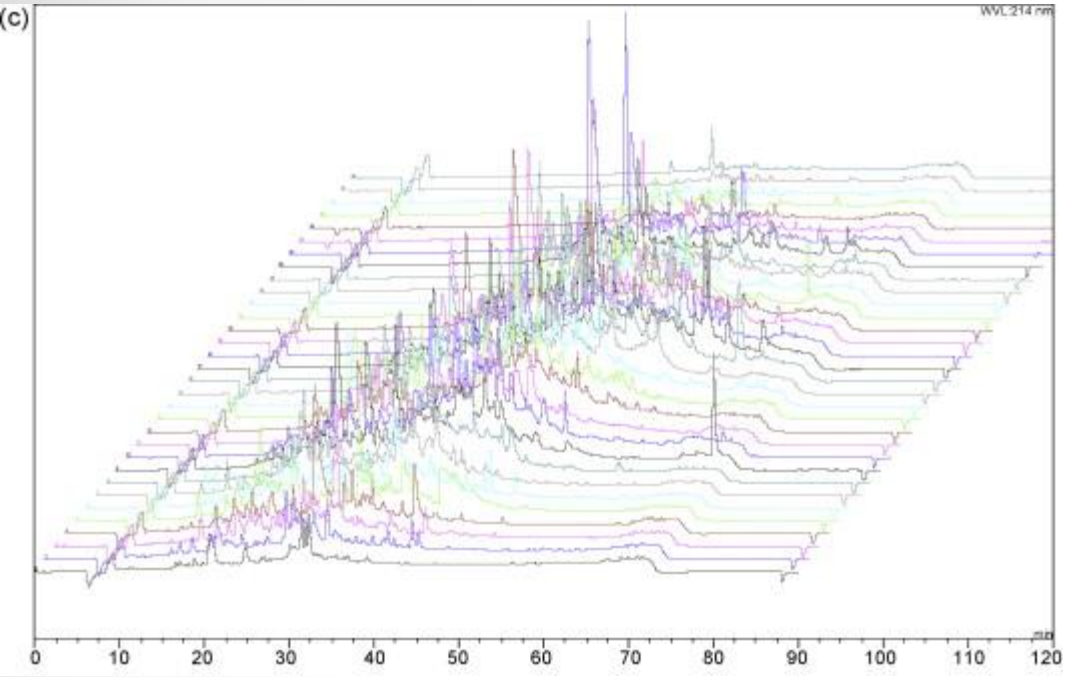
Principle Methods of 2D LC

- "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

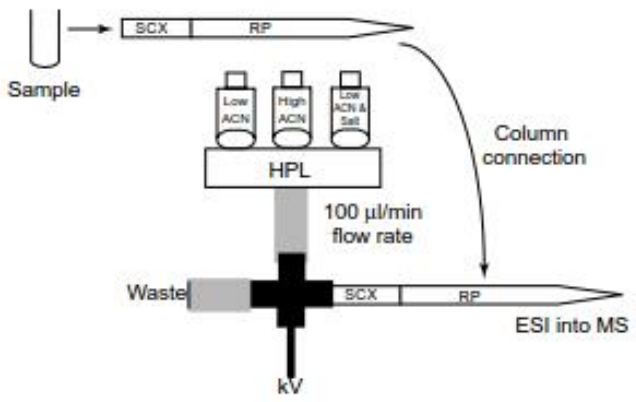
Offline fraction collection and reinjection in the **2nd dimension**:
150 x 0.075 mm, 3 µm Pepmap 100Å C18 particles

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



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

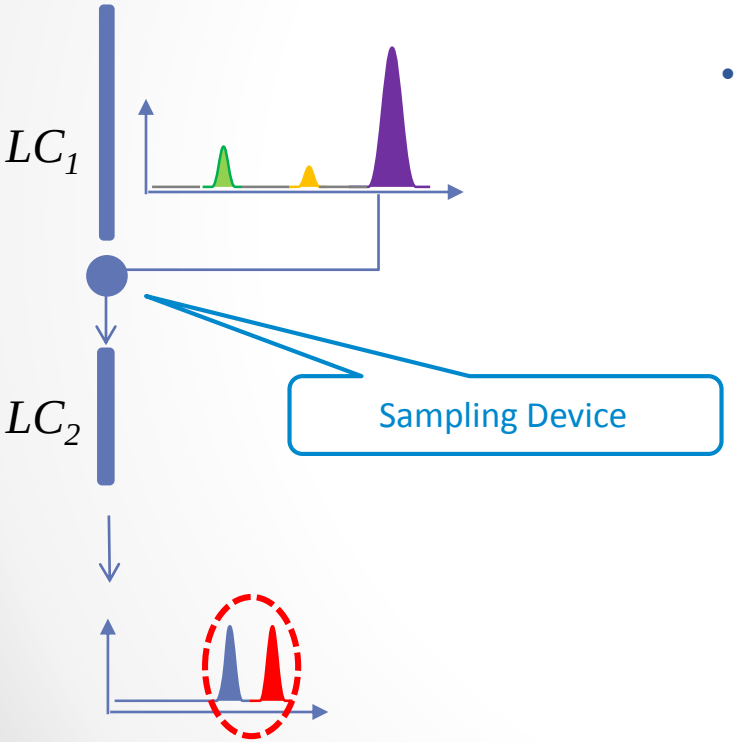
*J.R. Yates III et al., Int. J. of Mass Spectrometry 219 (2002) 245

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

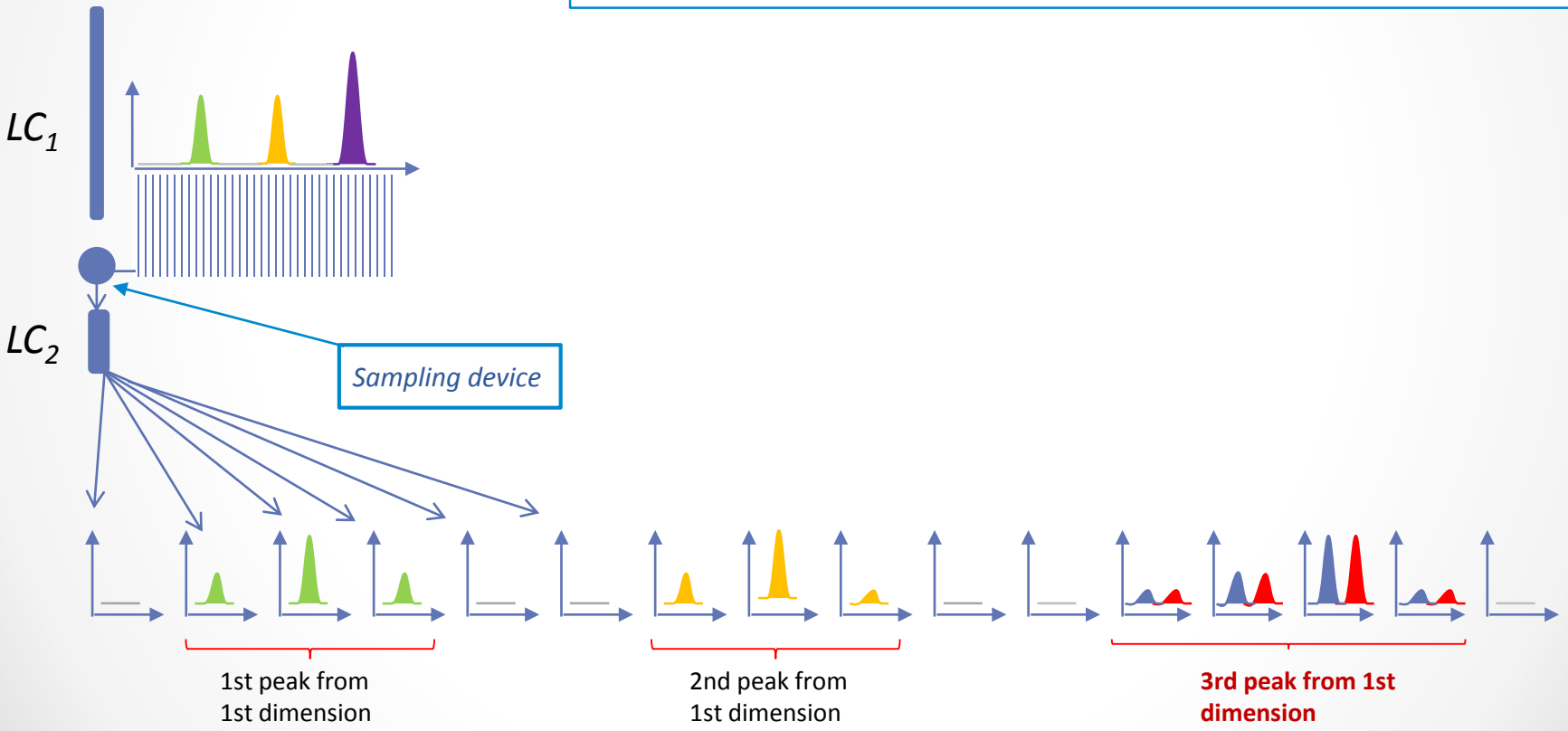


- Selected fractions of the 1st dimension separation are injected onto the 2nd dimension column
  → 1st dimension detector optional
- Long 1st dimension gradient separation possible
  → good data quality Limited information

Principle Methods of 2D LC

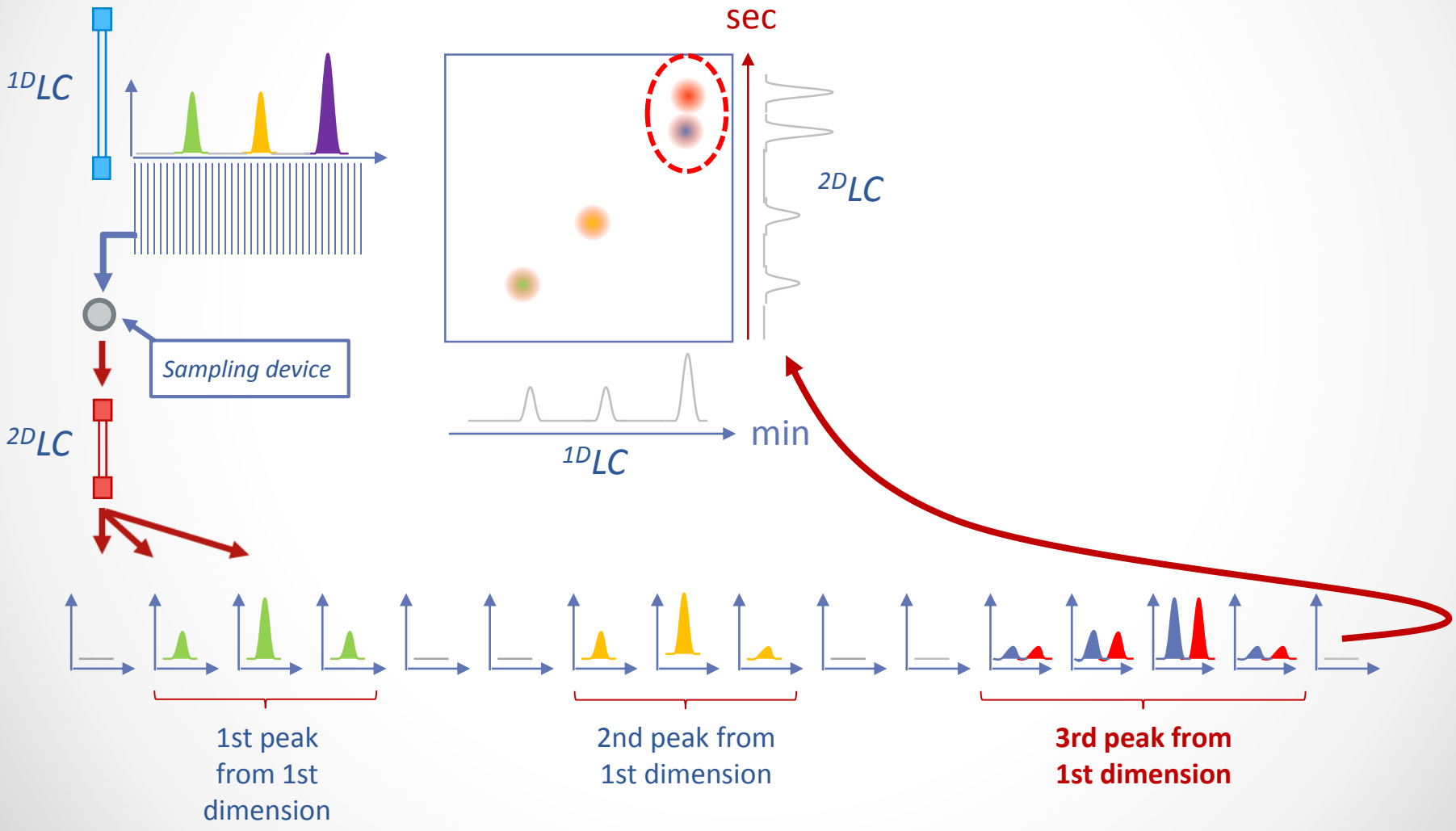
Comprehensive 2D-LC

- 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!



Principle Methods of 2D LC

Comprehensive 2D LC



Peak Capacity in 1D and 2D HPLC

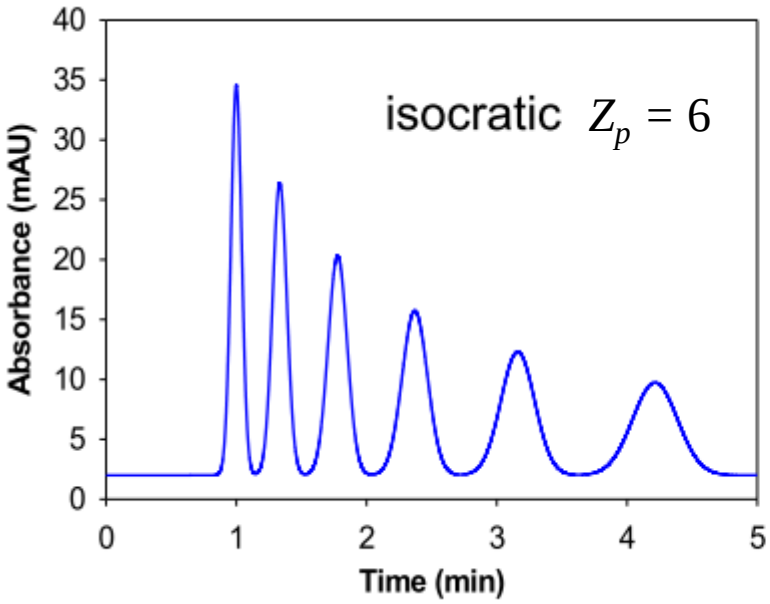
Why do Multidimensional HPLC?

- 1D HPLC does not give enough resolution to deal with complex samples (n_c >> 50)
- Sample fingerprinting, classification, identification of contaminants, source of origin determination, detection
- Essential for “non-targeted” analysis (e.g. life science research*)
- Targeted analysis to isolate solutes from a complex matrix

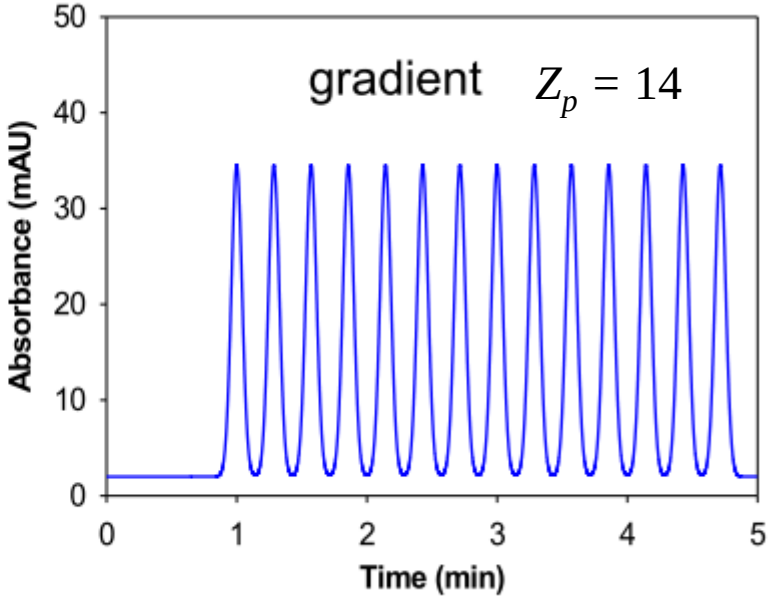
***Short anecdote**

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

Limitations

- In real sample peaks are not regularly spaced but are randomly spaced.
- Peaks are not all the same size (height) thus the resolution criterion R_s = 1 may not show two maxima which only works for equal size peaks
- Davis–Giddings Statistical Model of Peak Overlap

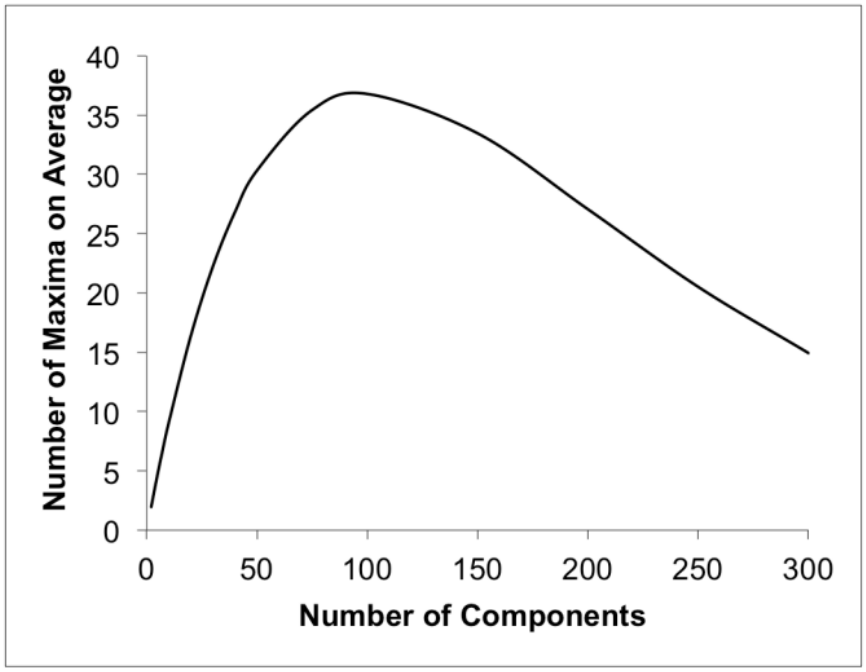
$$s = n_c \exp\left(\frac{-2n_c}{Z_p}\right)$$

n_c = # of components in the sample
s = # of pure peaks in the chromatogram

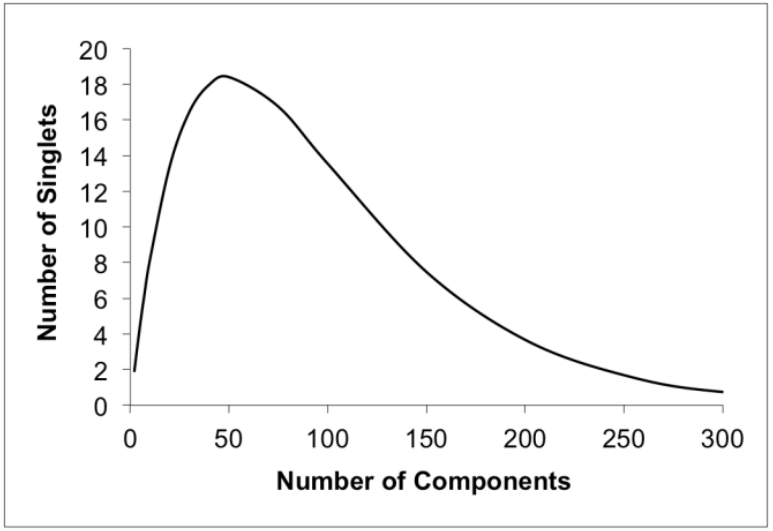
Peak Capacity (Z_p) in 1D HPLC

Davis–Giddings Statistical Model of Peak Overlap

of observed peaks (Z_p =100)



# of pure single peaks (Z_p =100)



$p_{max} = 0.37\ Z_p$

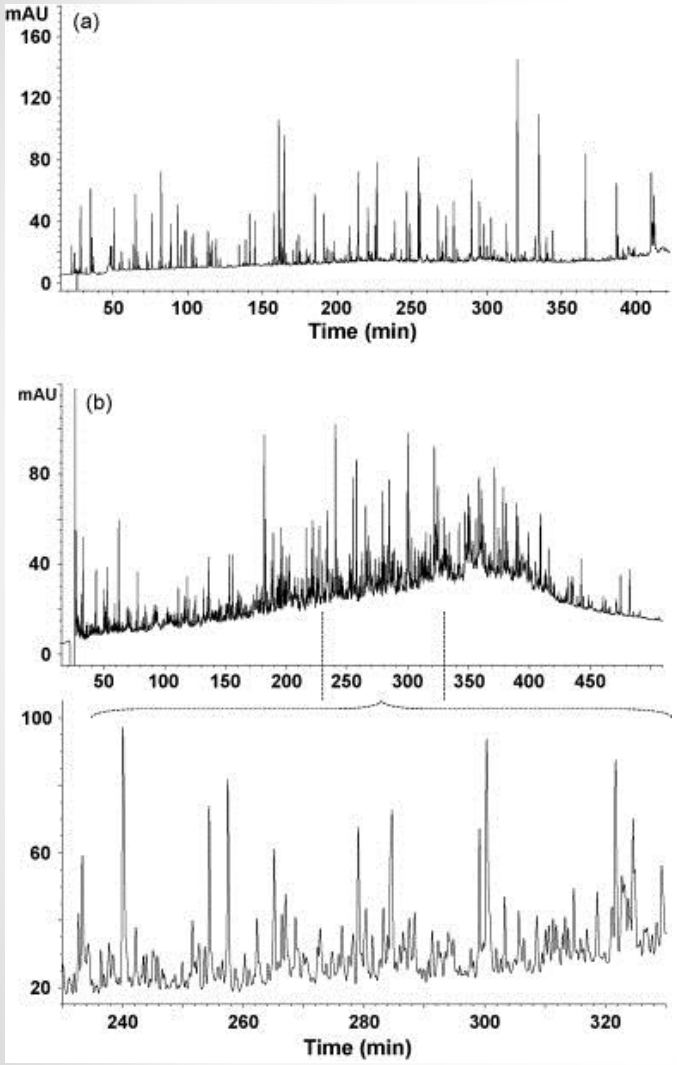
$s_{max} = 0.18\ Z_p$

Calculate

J. M. Davis and J. C. Glddings, Anal. Chem. 1983, 55, 418-424

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.

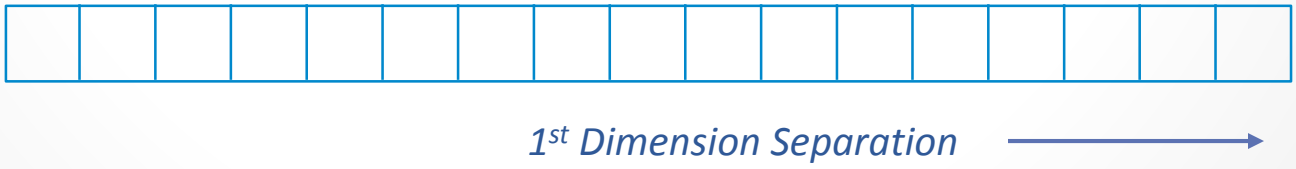
Mobile phase A 2% ACN, 0.1% TFA and mobile phase B 70% ACN, 0.1% TFA. Gradient slope 0.135% B/min, flow rate 200 µL/min.

Detection wavl. 214 nm

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

Peak Capacity in Comprehensive 2DLC

The geometric orthogonality concept



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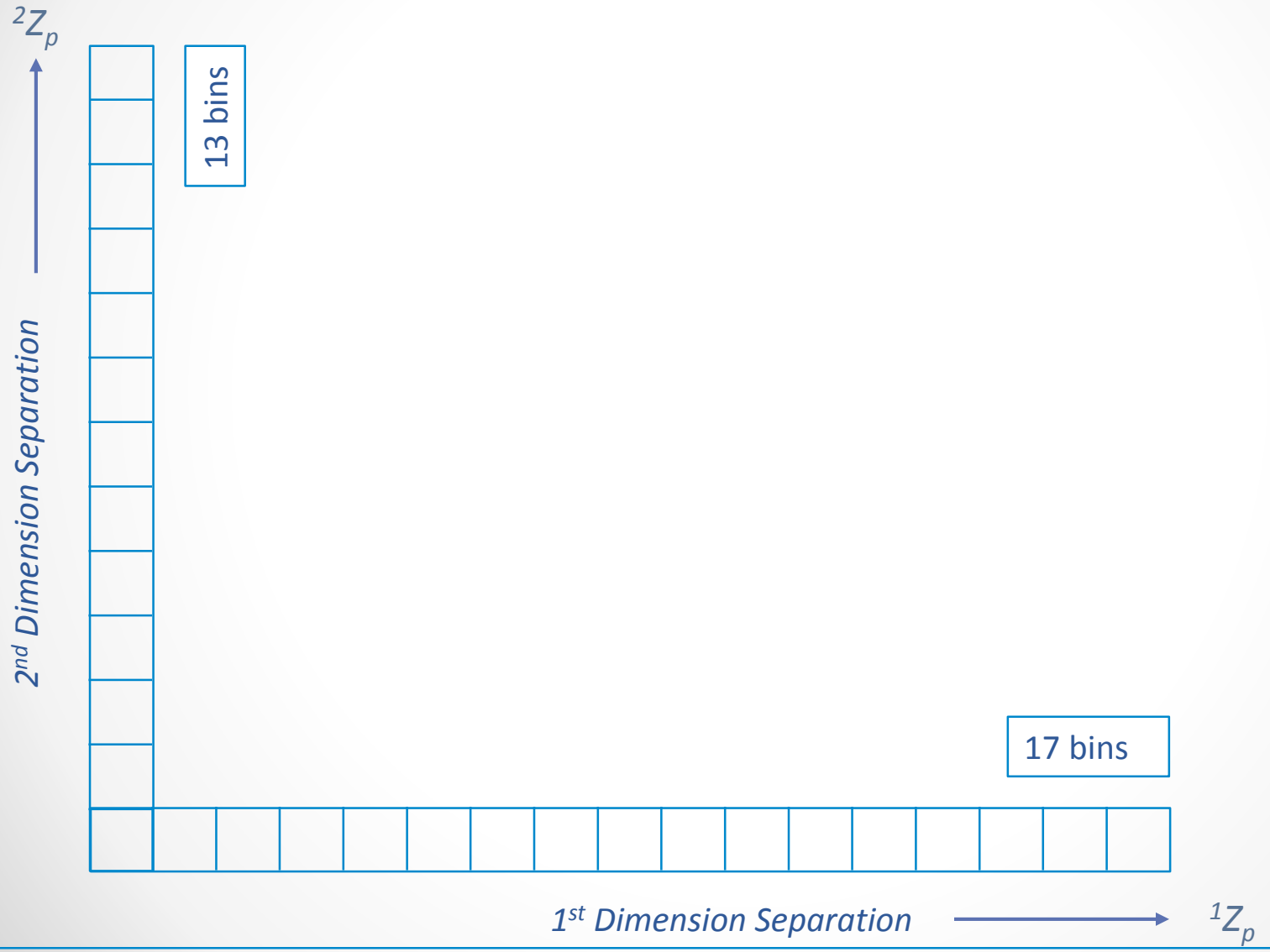

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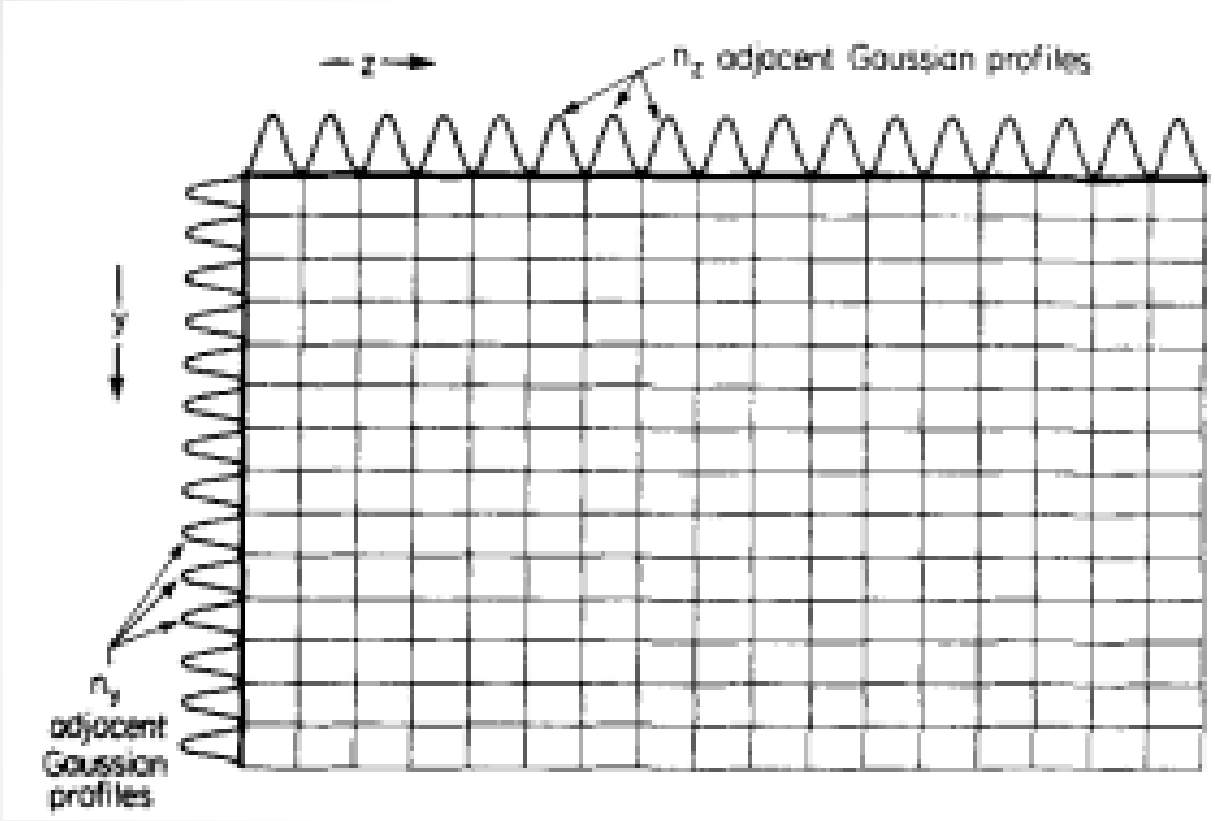
The geometric orthogonality concept



2nd Dimension Separation

1st Dimension Separation → $^{1}Z_p$

Theoretically:

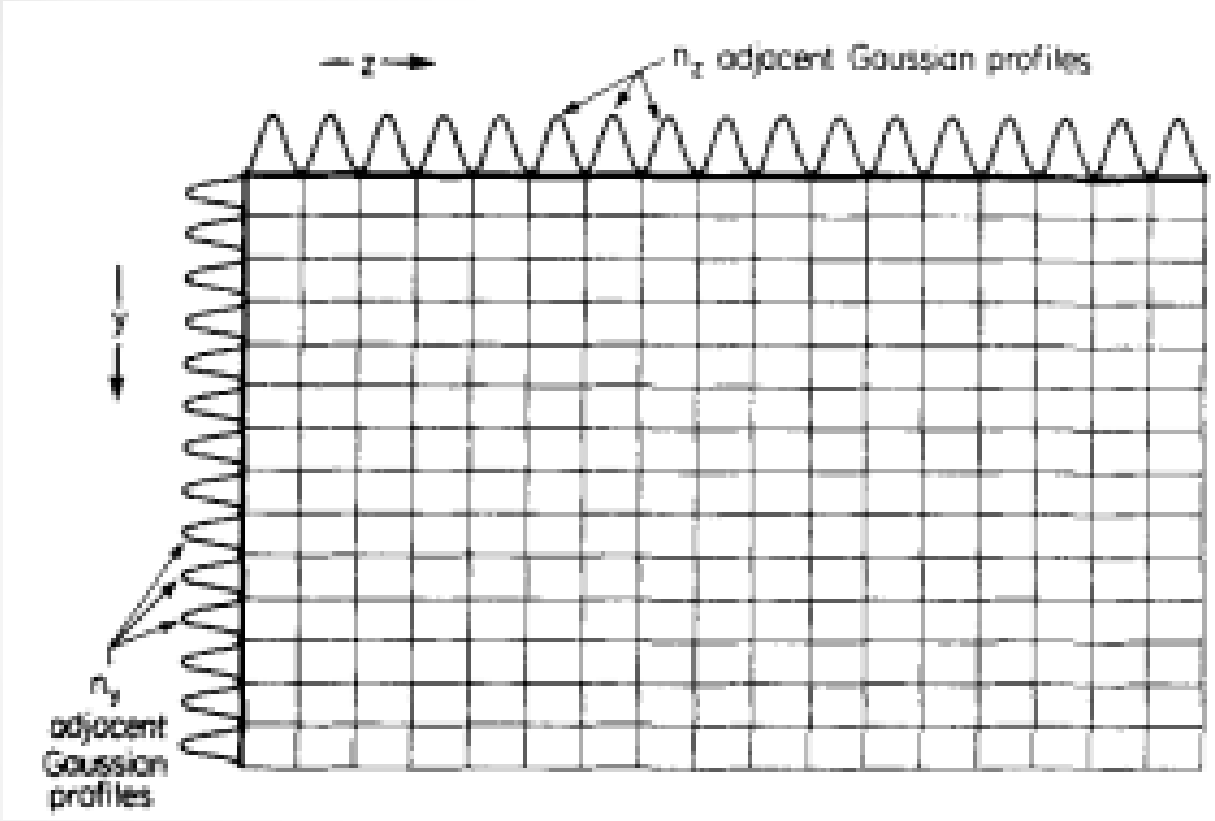
$$^{2D}Z_p = {}^{1}Z_p \; x \; {}^{2}Z_p$$

The Giddings "Product Rule"

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

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

Peak Capacity in Comprehensive 2DLC

Giddings Criteria for the Product Rule

- **ORTHOGONALITY:**
  "First, the components of a mixture are subjected to two or more separation steps in which their displacements depend on different factors."
  - The retention of the sample solutes must be controlled by two (or more) different physical-chemical properties and the two separation systems must separate the species by different mechanisms. The retention of the component is describe by two or more retention times (by 2 dimensions)
- **SAMPLING:**
  "The second criterion is that when two components are substantially separated in any single step, they remain separated until the completion of the separation step."
  - Width of the sample (in time or volume units) from first separation
  - Once the solutes are separated there must be no remixing (peak broadening) induced by doing the second separation

J.C. Giddings in "Multidimensional Separations", H. J. Cortes (ed.), vol. 50 in Chromatographic Science Series, Marcel Dekker, 1990.

Requirements to Achieve Theoretical Peak Capacity in Comprehensive 2DLC

1. **ORTHOGONALITY** of separation mechanisms – This is a requirement imposed mostly on the **stationary phase** chemistry.
2. Peaks must cover **ENTIRE** separations "space".
3. Separation gained in one dimension cannot be diminished by separation in the other dimension. Must sample **FAST!**

$$ {}^{2D}Z_p = {}^{1}Z_p \times {}^{2}Z_p \times \frac{1}{\langle \beta \rangle} \times f_{coverage} $$

Under Sampling Correction

Orthogonality

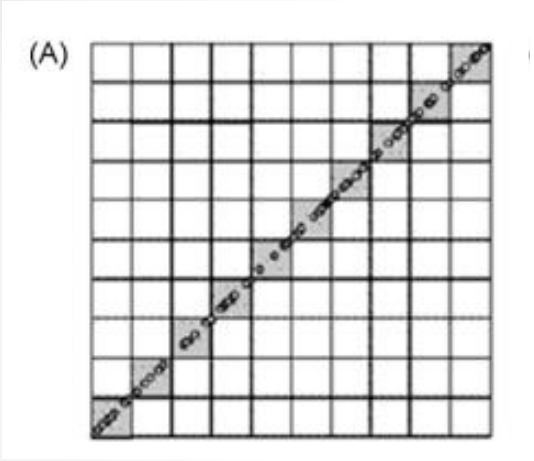
What is the most important factor?
How can we improve it?

Davis, J. M.; Stoll, D., R.; Carr, P. W. *Anal. Chem.* 2008, 80(2), 461-473

Giddings, J. C. *Multidimensional Chromatography: Techniques and Applications*; Marcel Dekker: New York, 1990

Orthogonality in 2D LC

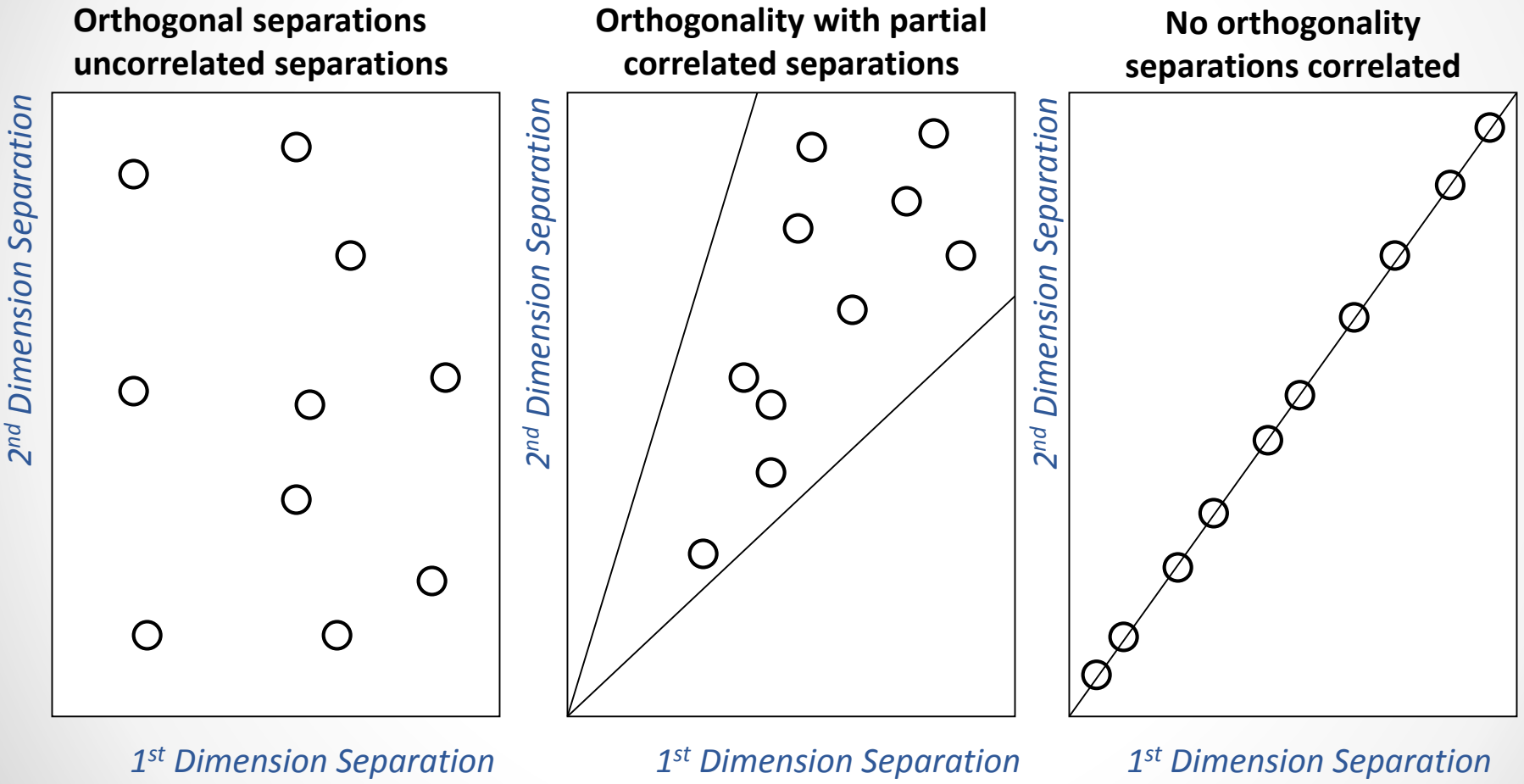
Orthogonality in Comprehensive 2DLC



(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)

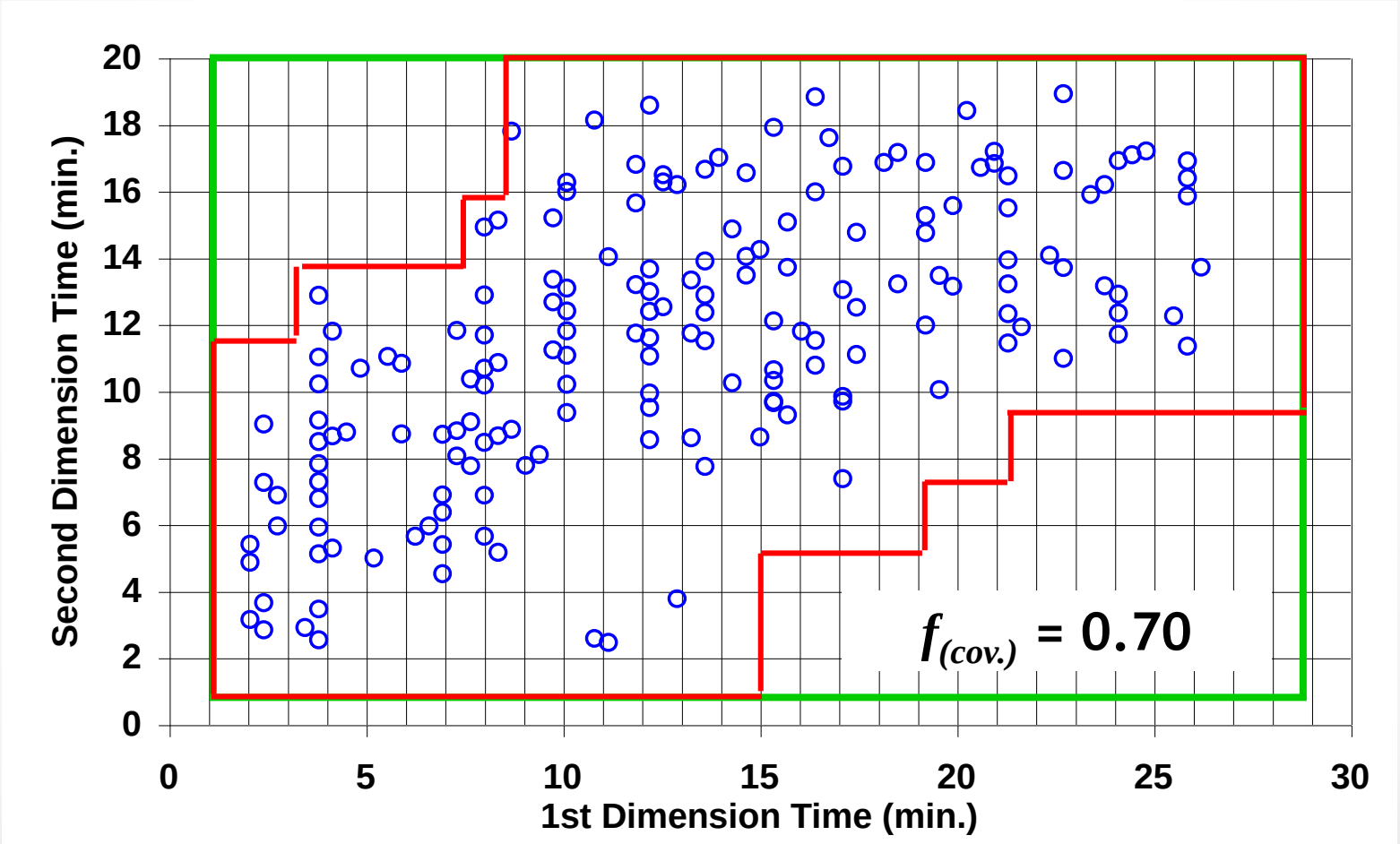
Separation Space Utilization by Orthogonal and Correlated Mechanisms



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

Fractional Utilization of 2D Space

(Stoll modified Gilar method)



Stoll, D.R., et al. *Anal. Chem*. 2008, 80, 268-278
Gilar, M. et al. *Anal. Chem.* 2005, *77*, 6426-6434.

Problems with Coverage Estimation Methods

- Correlation coefficients don't measure available space.
- Stoll-Gilar type methods are subjective and depend strongly on grid size.
- Many other methods are complex and have critical parameters.

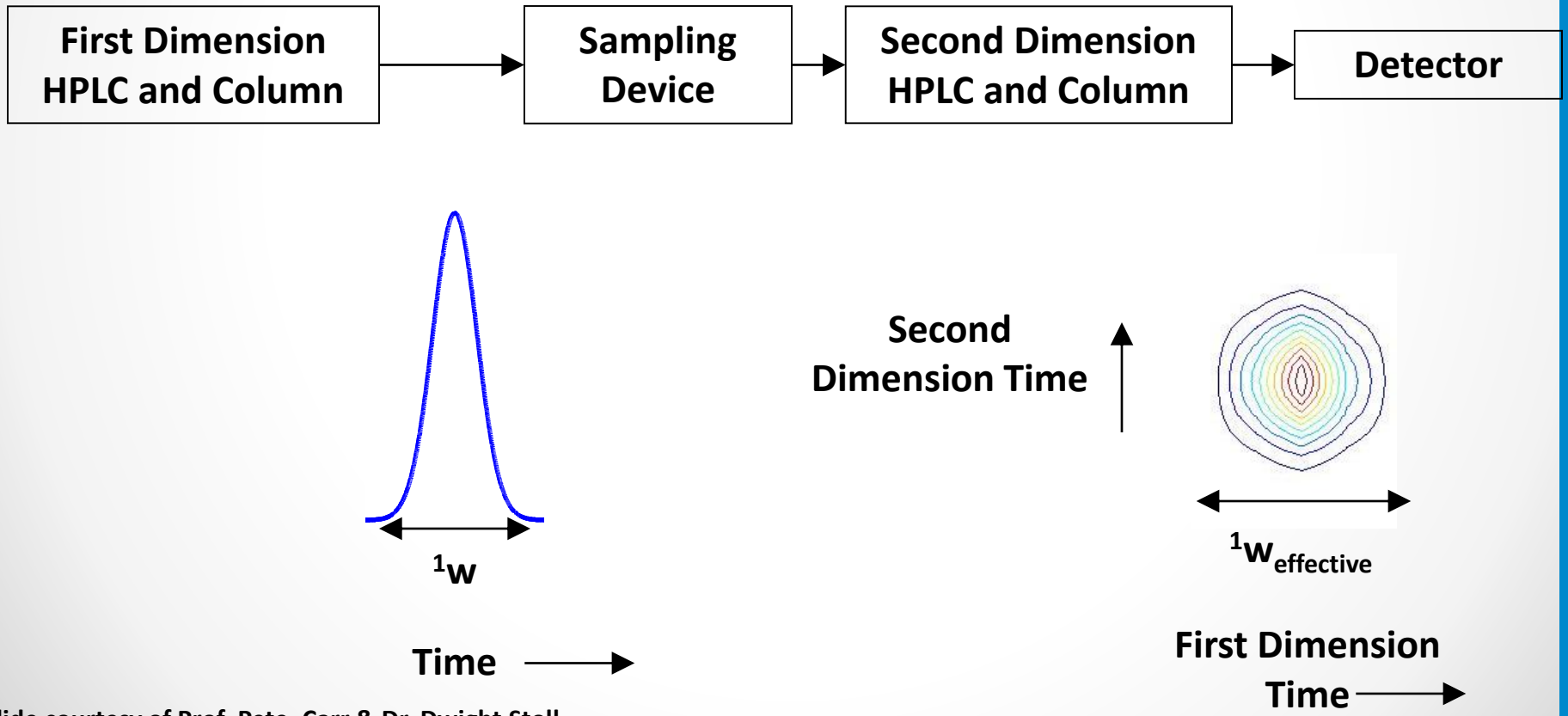
*For an overview, see the excellent comparison paper of Gilar and Schure, *et al*.

M. Gilar, J. Fridrich, M.R. Schure, A. Jaworski, Comparison of Orthogonality Estimation Methods for the Two-Dimensional Separations of Peptides, *Analytical Chemistry*. 84 (**2012**) 8722–8732.

The Sampling Problem in 2D LC

Giddings's Second Criterion

The width of a peak observed in a 2D chromatogram in the direction of the first dimension axis after sampling is effectively broader than the width of the peak that elutes from the first dimension column before sampling.

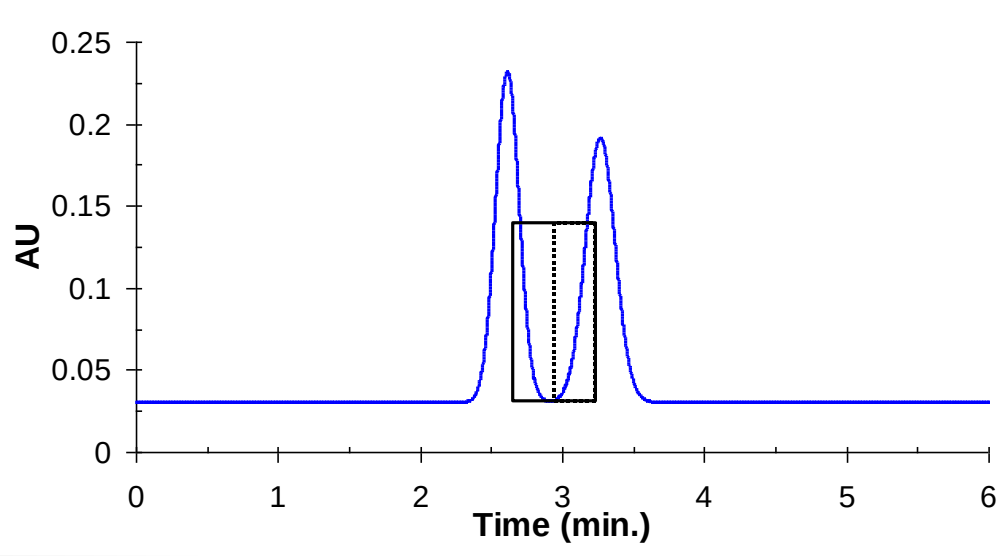


Slide courtesy of Prof. Pete. Carr & Dr. Dwight Stoll

The Undersampling Problem*

The Murphy-Schure-Foley Criterion

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



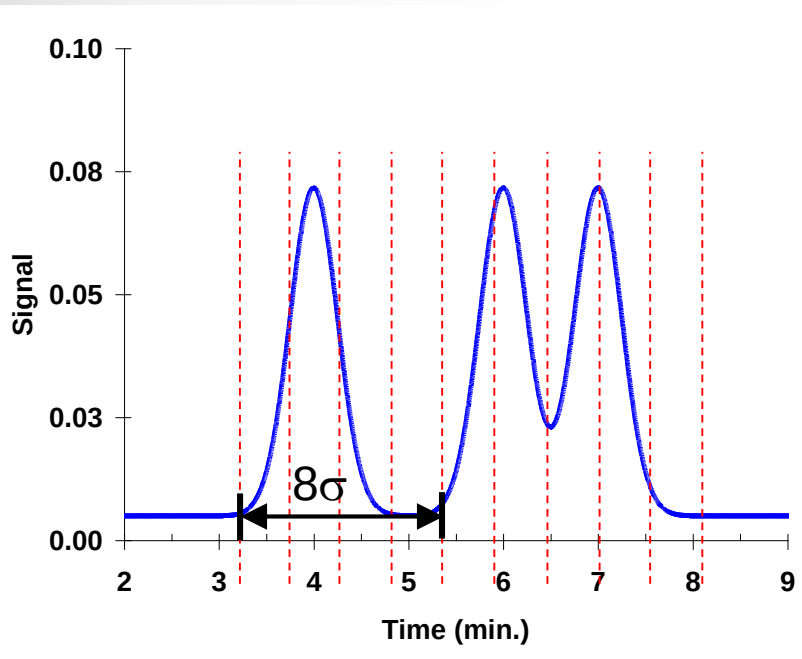
Clearly if we take a sample as indicated and inject it into a second dimension we will partially “un do “ the separation already accomplished in the first dimension.

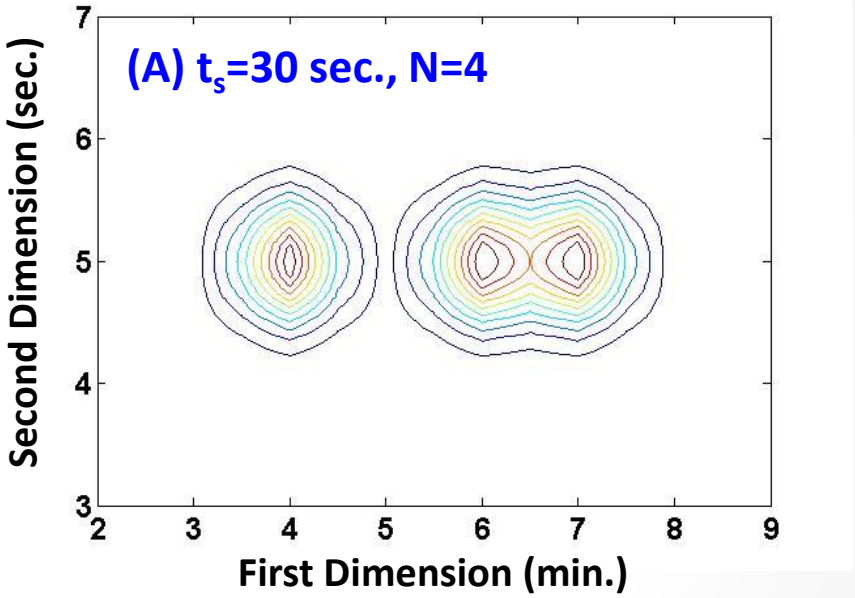
According to M-S-F **one needs to take at least 4 samples** across the 8σ base width of each first dimension peak **to minimize the effect of undersampling**.

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

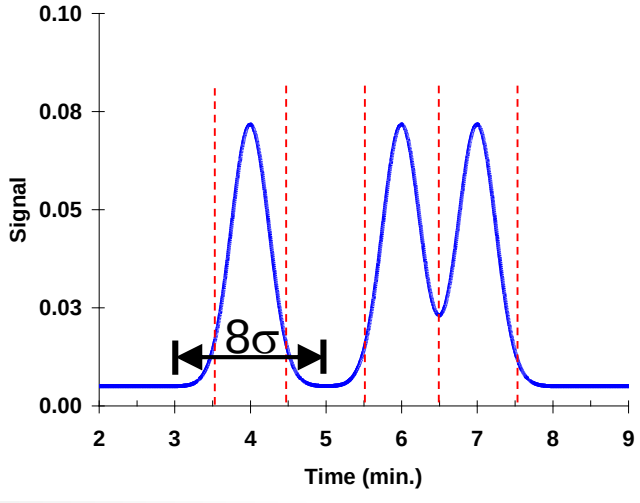


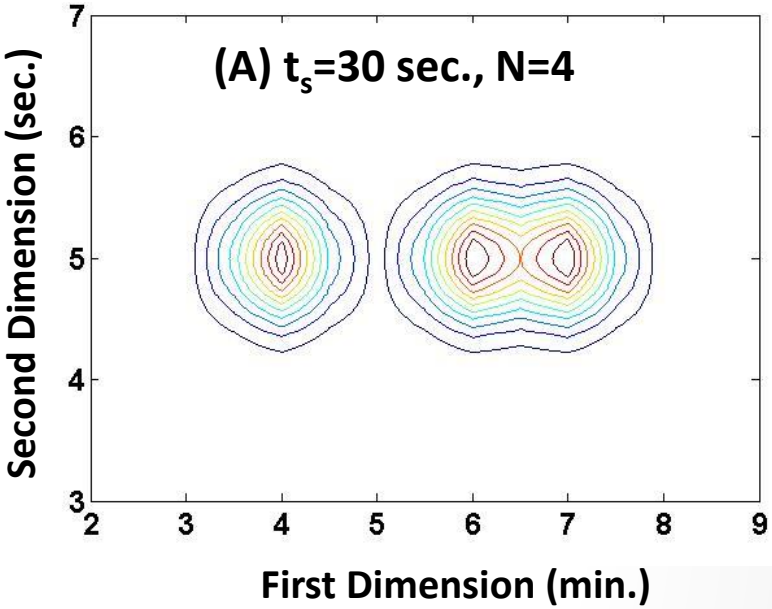


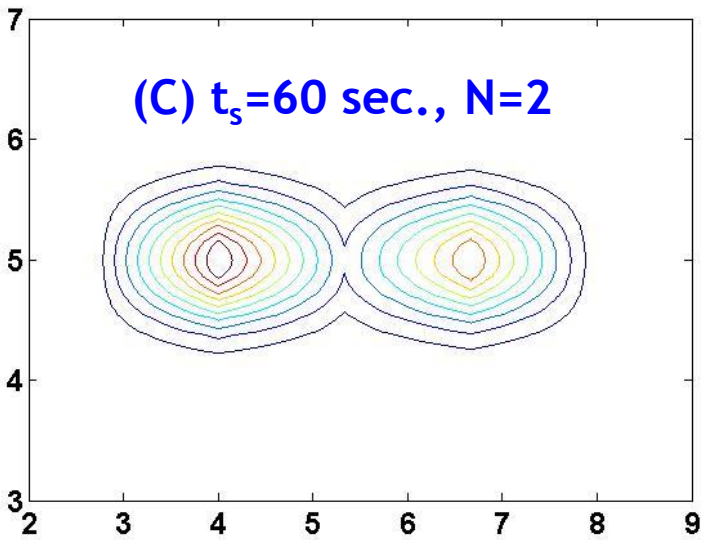
Slide courtesy of Prof. Pete. Carr & Dr. Dwight Stoll

The Undersampling Problem

The Murphy-Schure-Foley Criterion







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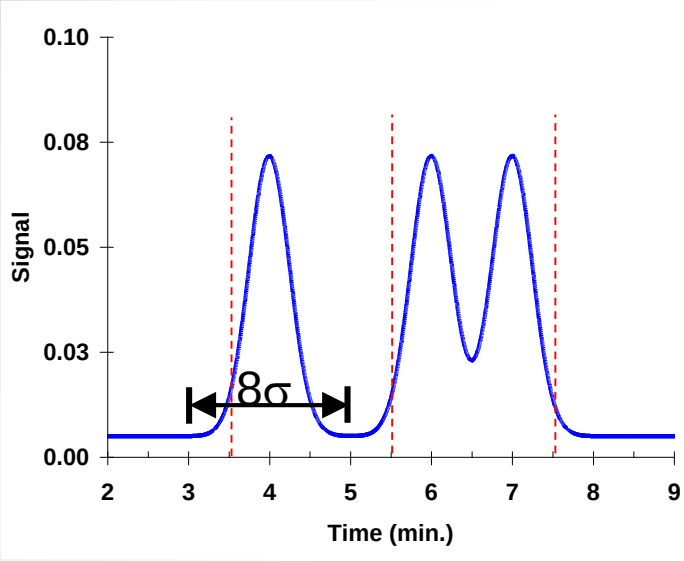
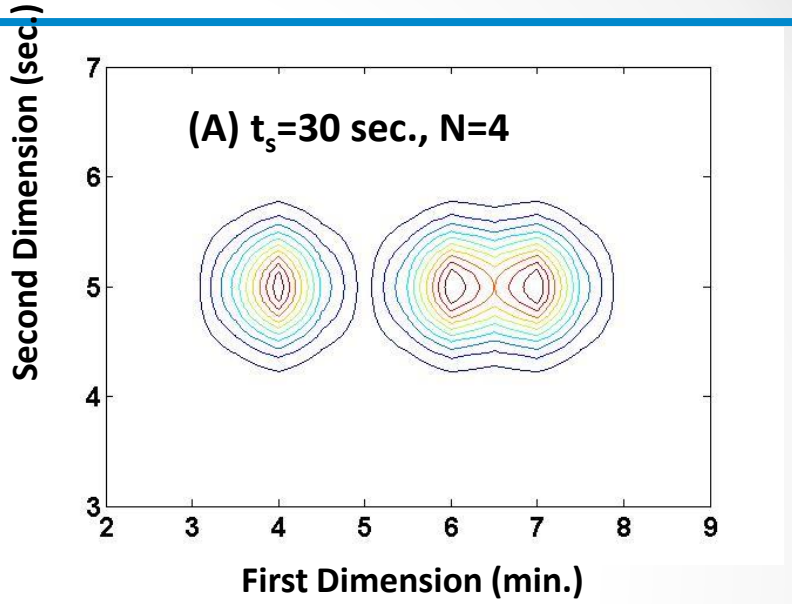
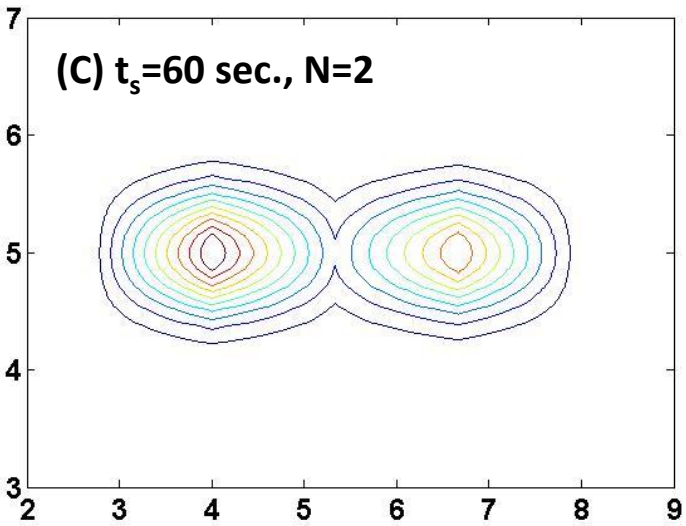
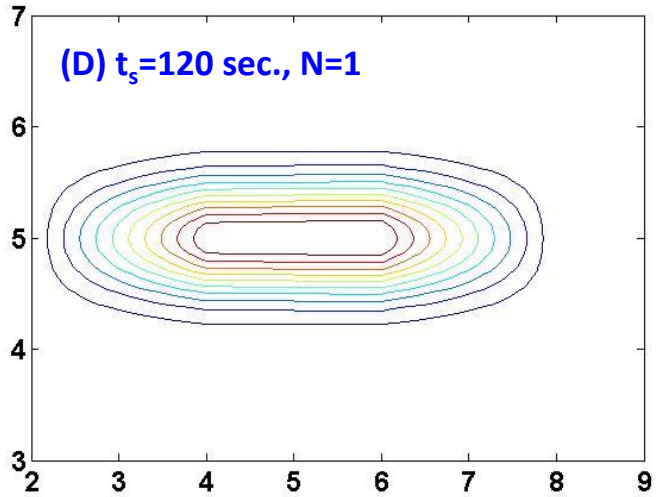
The Undersampling Problem

The Murphy-Schure-Foley Criterion









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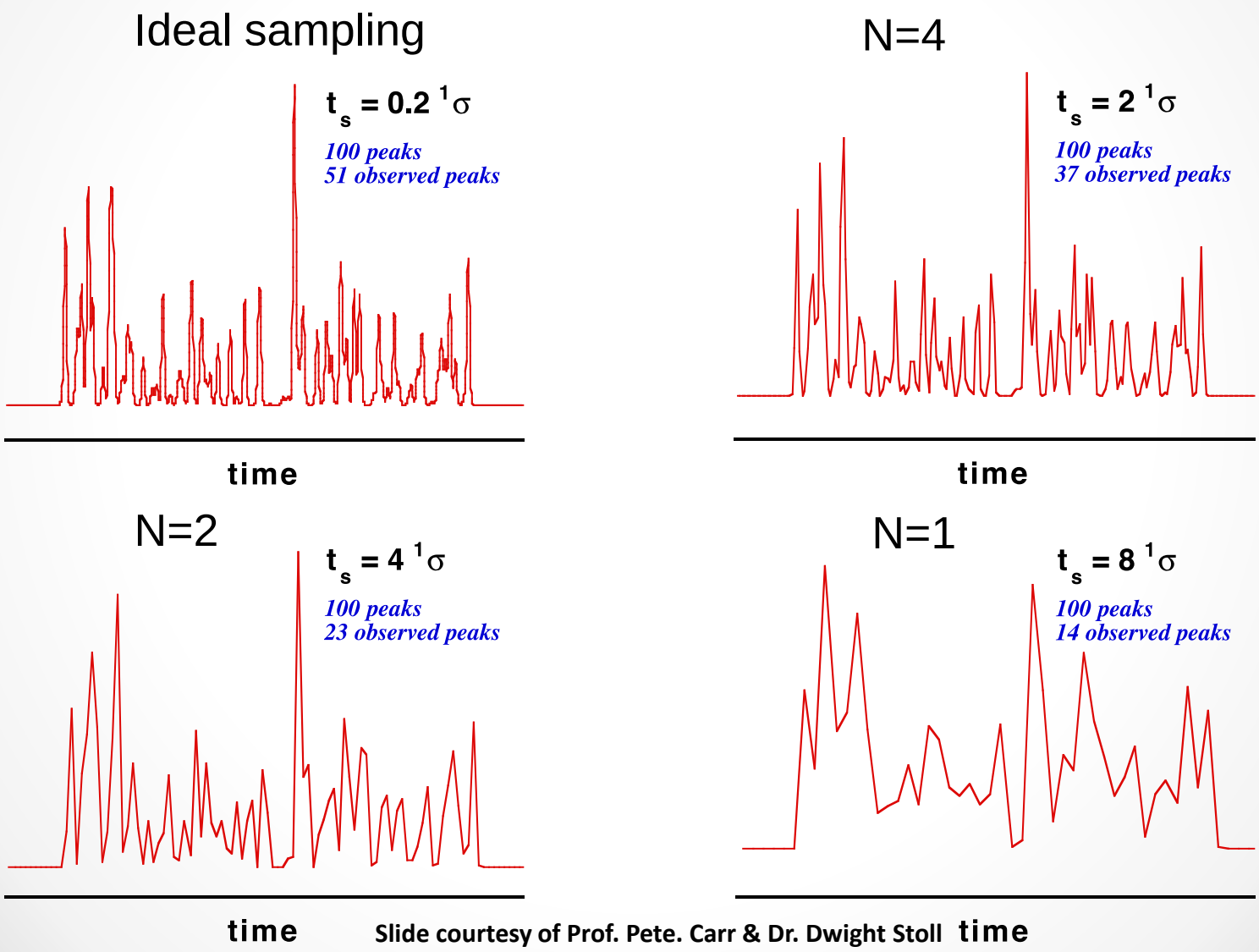
The Undersampling Problem

Alternative View of Undersampling the First Dimension



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

Peak Capacity in Comprehensive 2DLC

"Effective" Peak Capacity

$$^{2D}Z_p = {}^{1}Z_p \times {}^{2}Z_p \times \frac{1}{\langle\beta\rangle} \times f_{coverage}$$

$$<\beta>= \sqrt{\left(1+0.214.\left(\frac{t_s}{<{}^{1}\sigma>}\right)^2\right)}$$

Under Sampling Correction

Orthogonality

t_s sampling time, $^{1}\sigma$ average width 1st dimension peaks

1. <β> average correction for under sampling*
2. $f_{coverage}$ corrects for incomplete use of the separation space.

What is the most important factor?
How can we improve it?

***D.R. Stoll et al., Anal. Chem. 2008, 80, 268-278; Davis, J. M. Stoll, D., R. Carr, P. W. *Anal. Chem.* 2008, 80(2), 461-473; Giddings, J. C. *Multidimensional Chromatography: Techniques and Applications*; Marcel Dekker: New York, 1990**
Slide courtesy of Prof. P. Carr & Dr. D.R.Stoll

Peak Capacity in Comprehensive 2DLC

Example

Assume first dimension is 30 minutes long and has Z_p= 100, therefore $^1w(4\sigma)$ = 0.3 min. If we sample at a rate of N = 4 samples/8σ we must take a sample every 0.15 minutes (= **9 seconds**) and complete a second dimension chromatogram every 9 seconds.

1. Even so we will loose **27%** of the ideally available peak capacity.

2. Very fast ^{2}D separations are needed in second dimension separation.

$$^{2D}Z_p = {}^1Z_p \times {}^2Z_p \times \frac{1}{\langle\beta\rangle} \times f_{coverage}$$

$$<\beta>=\sqrt{\left(1+0.214.\left(\frac{t_s}{<^1\sigma>}\right)^2\right)}$$

Further reading:

K. Horie et al., *Analytical Chemistry*. **2007**, *79*, 3764–3770.
J Seeley, J. *Journal of Chromatography A*. **2002**, *962*, 21–27.
L.M. Blumberg, *Journal of Separation Science*. **2008**, *31*, 3358–3365.
L.M. Blumberg, et al., P. *Journal of Chromatography A*. **2008**, *1188*, 2–16.

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} ($^2t_{cycle}$)

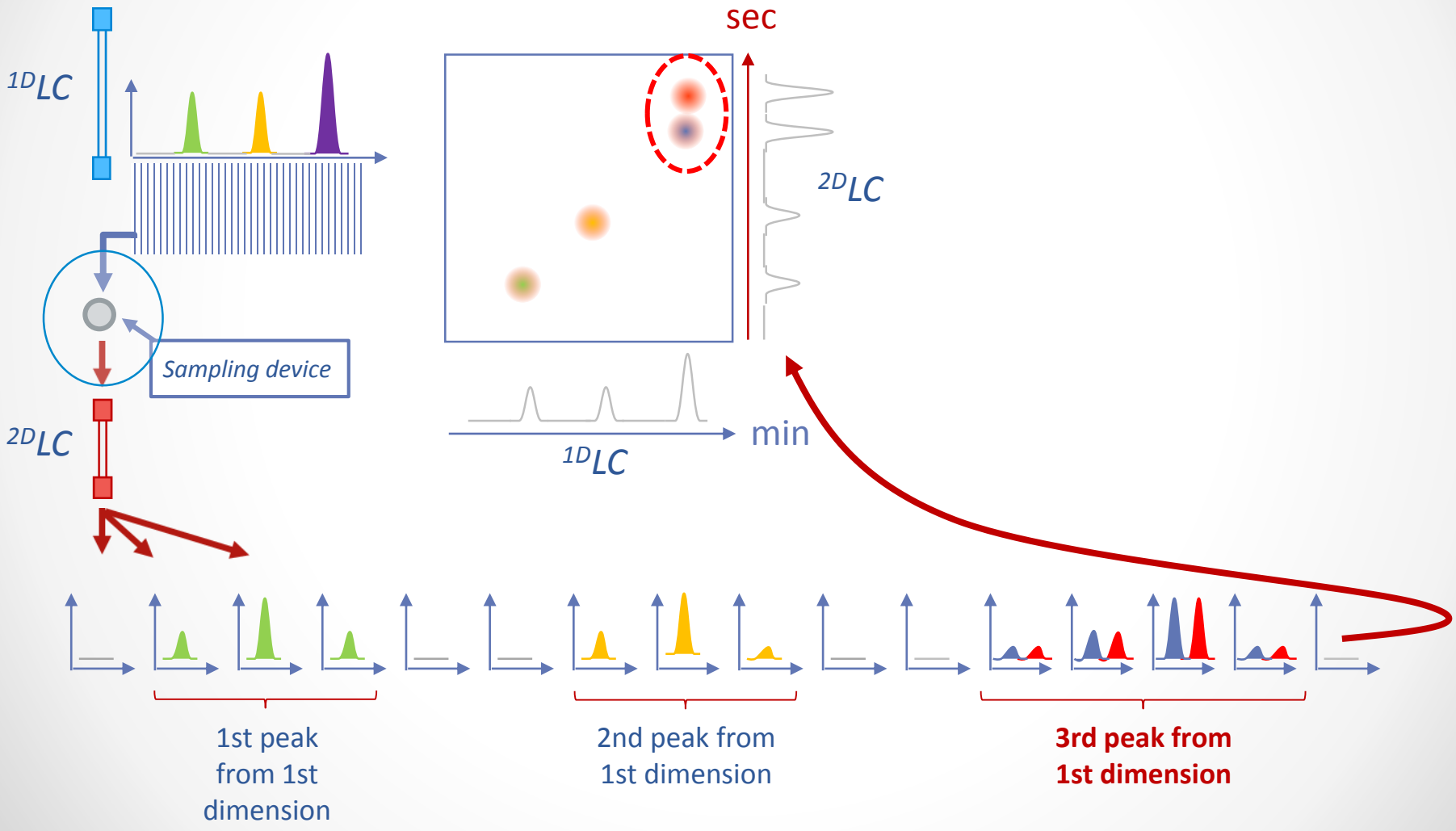
Slide courtesy of Prof. P. Carr & Dr. D. Stoll

Practical Implementation for 2D HPLC

Sampling Device, Column Selection

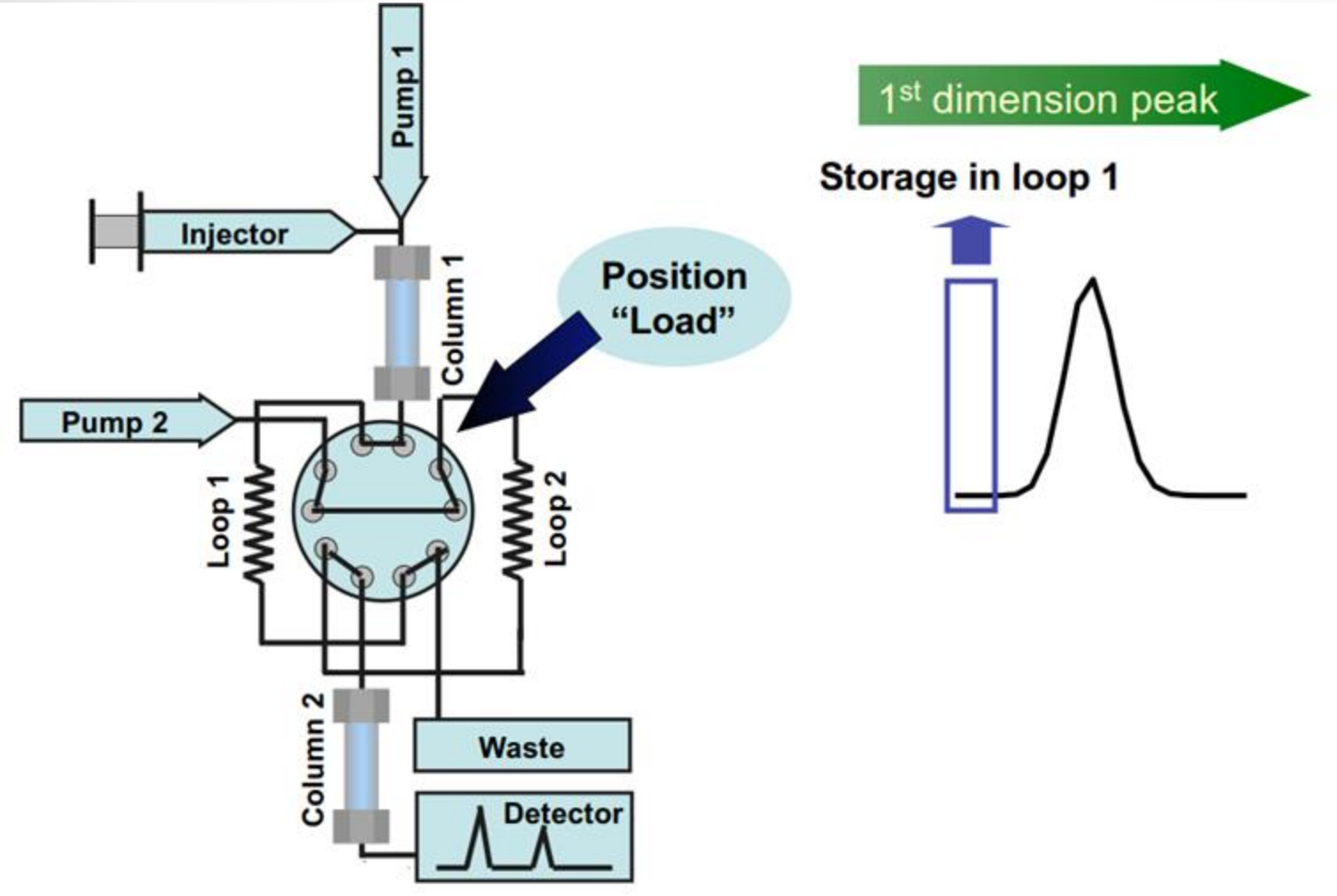
Sampling Device for LCxLC

First-In First-Out (FIF0) Configuration



Sampling Device for LCxLC

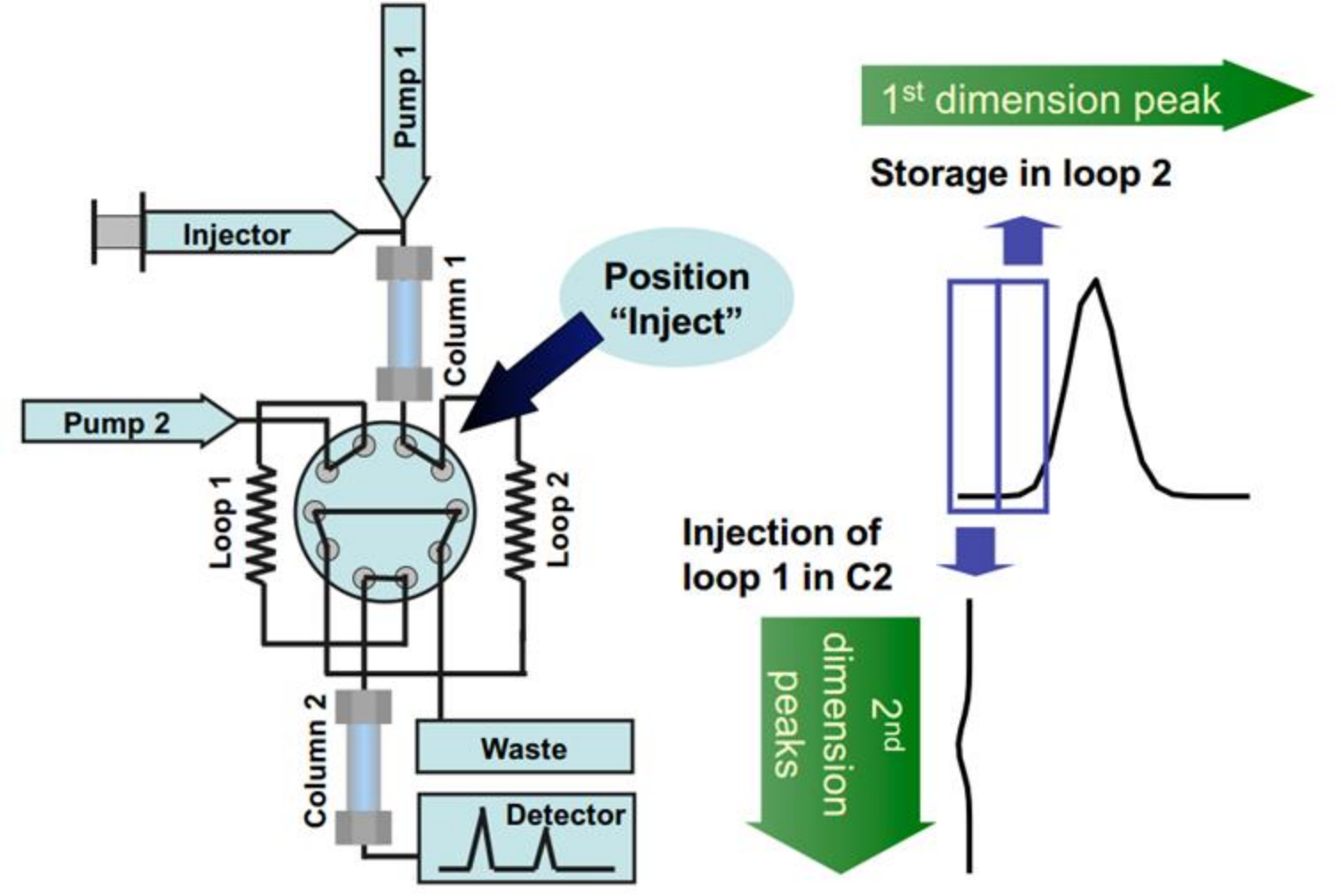
First-In First-Out (FIFO) Configuration (10 port, 2 position valve)



Slide courtesy of Prof. P. Schoenmakers

Sampling Device for LCxLC

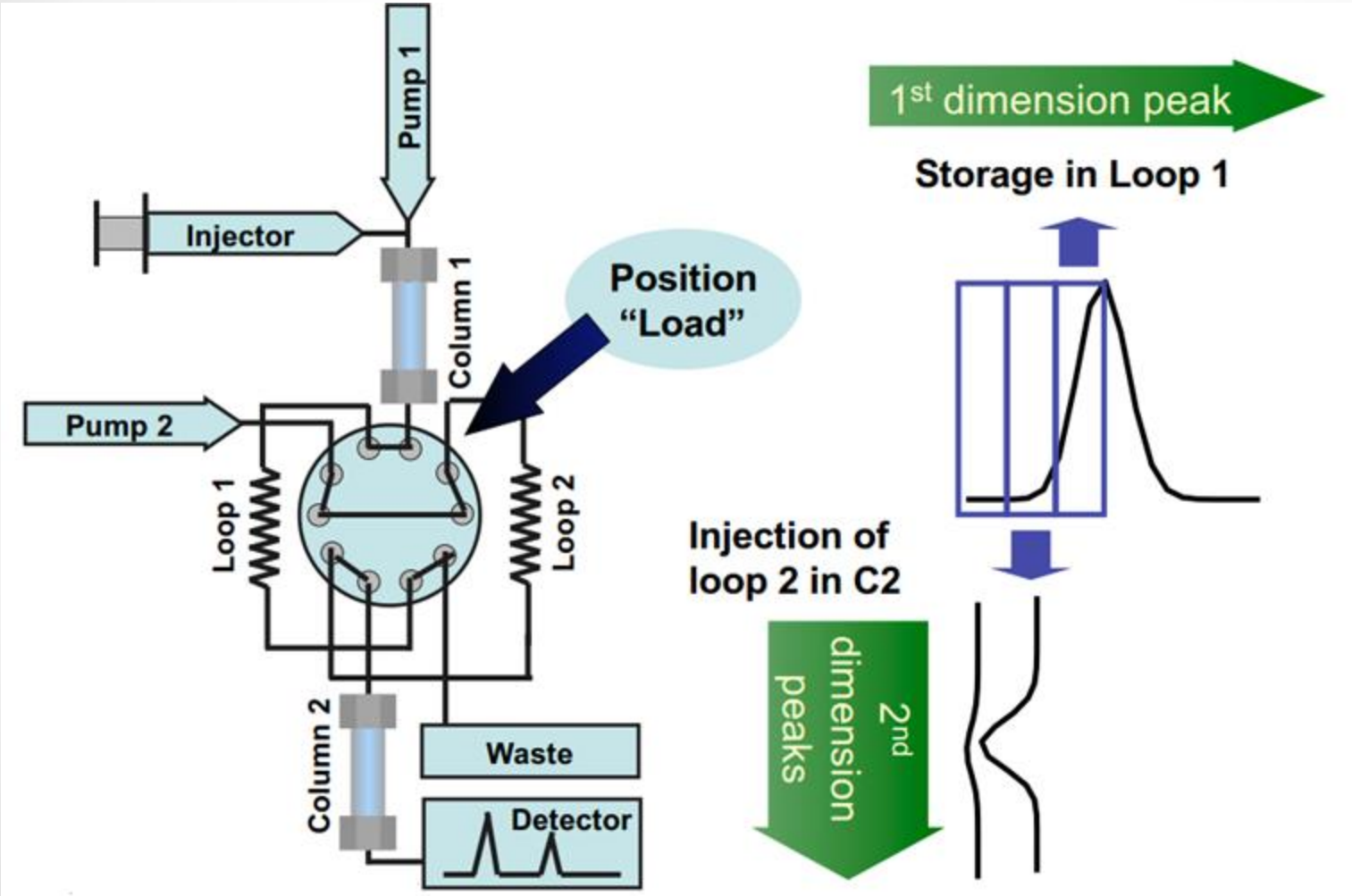
First-In First-Out (FIFO) Configuration(10 port, 2 position valve)



Slide courtesy of Prof. P. Schoenmakers

Sampling Device for LCxLC

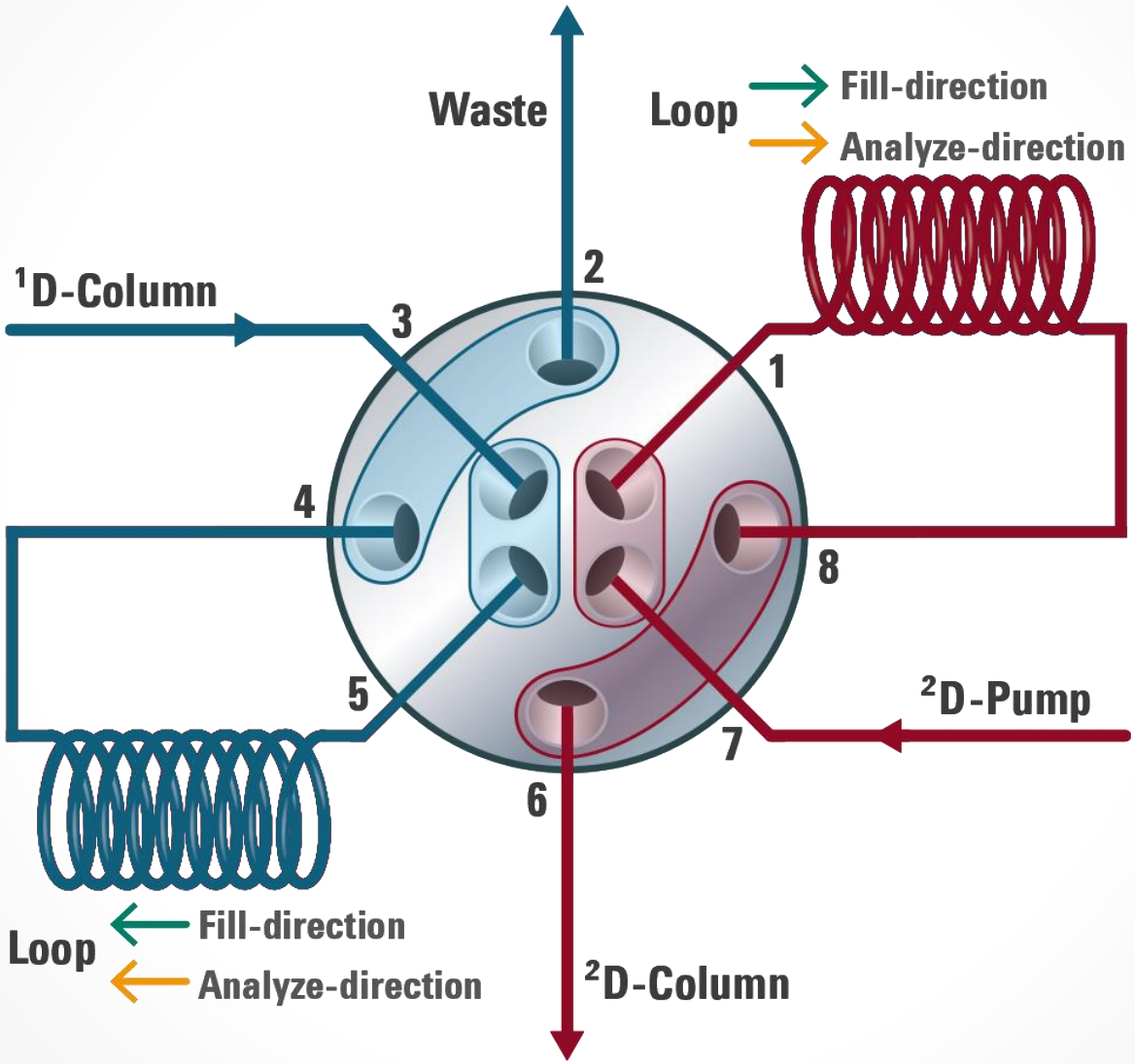
First-In First-Out (FIFO) Configuration (10 port, 2 position valve)



Slide courtesy of Prof. P. Schoenmakers

Sampling Device for LCxLC

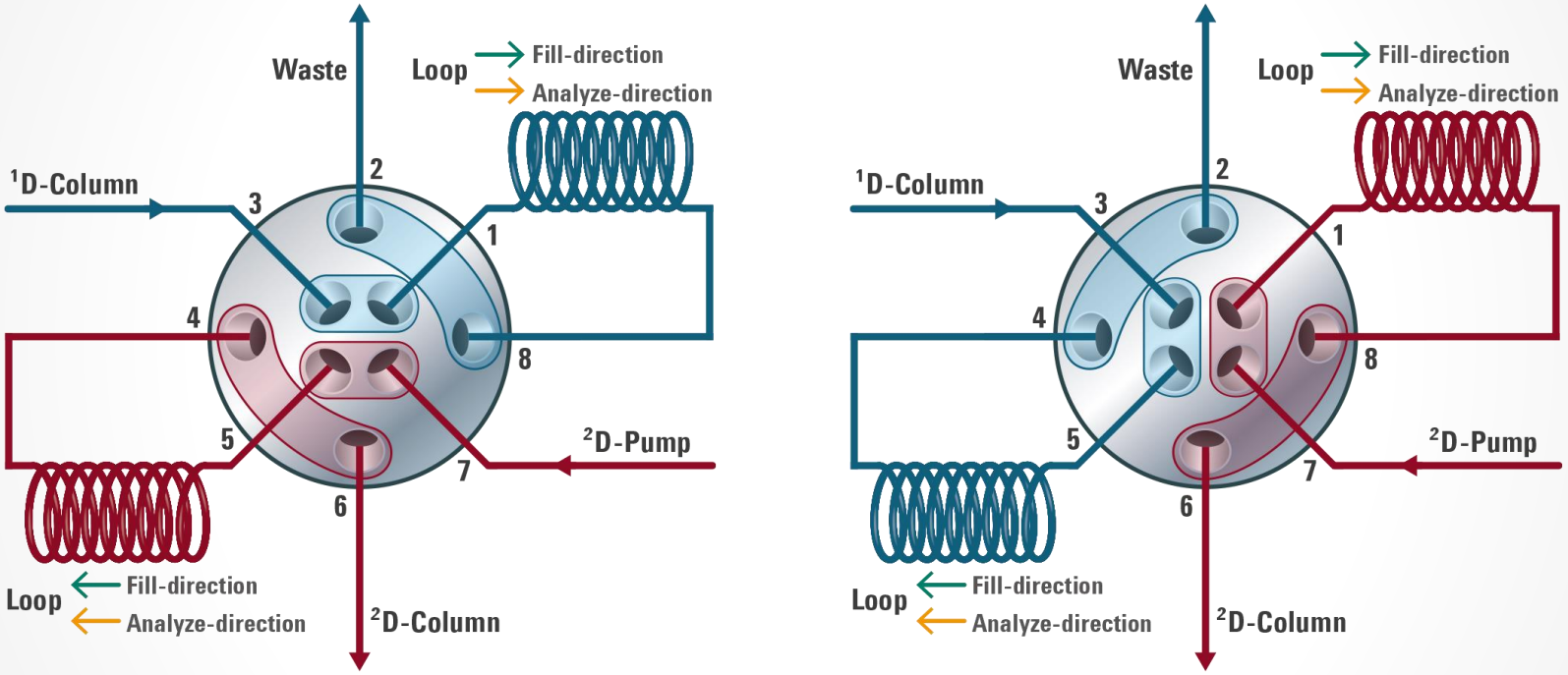
2x4 port, 2 position valve (Agilent Technologies Duo Valve)



Slide courtesy of Agilent Technologies

Sampling Device for LCxLC

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

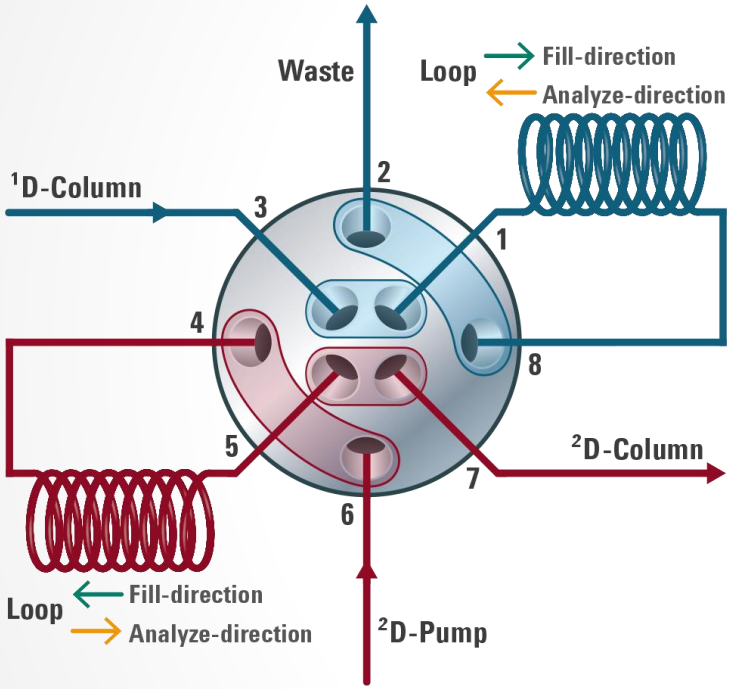


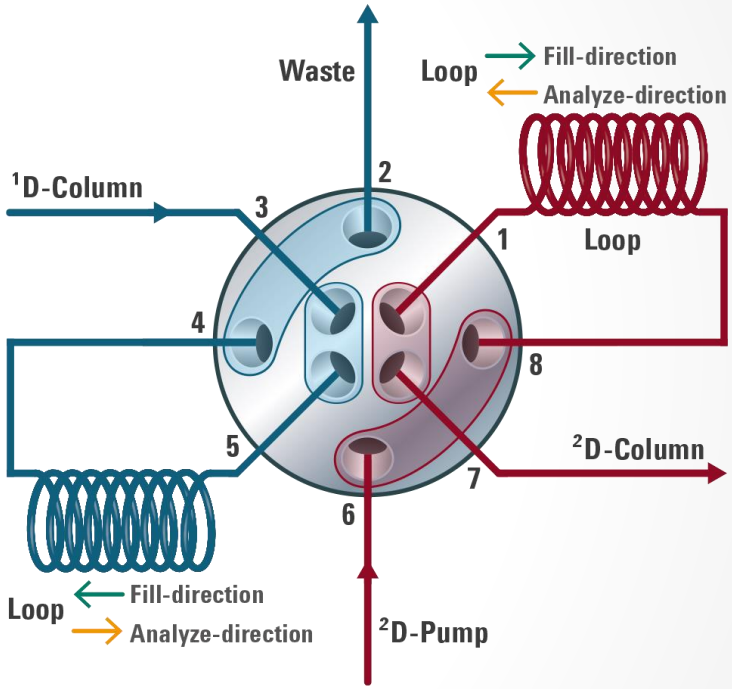
First-In-First-Out (FIFO)

Slide courtesy of Agilent Technologies

Sampling Device for LCxLC

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





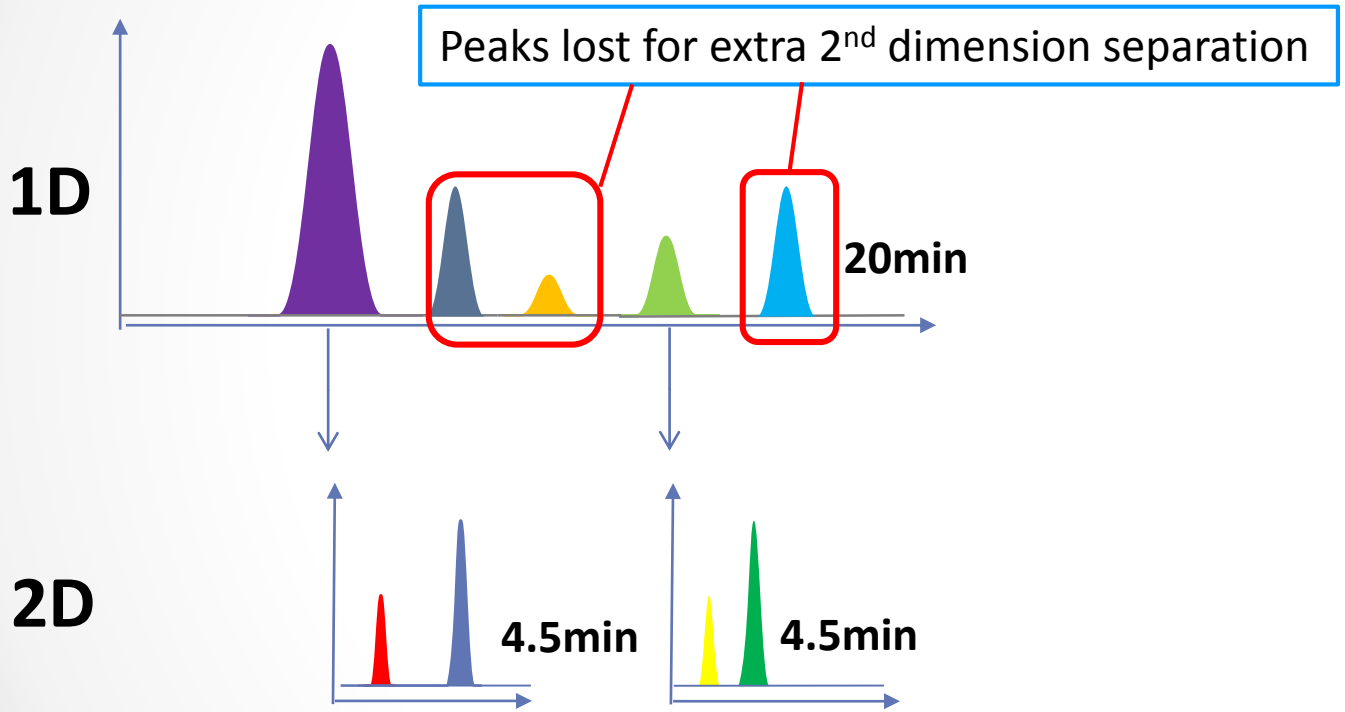
First-In-Last-Out (FIFO)

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



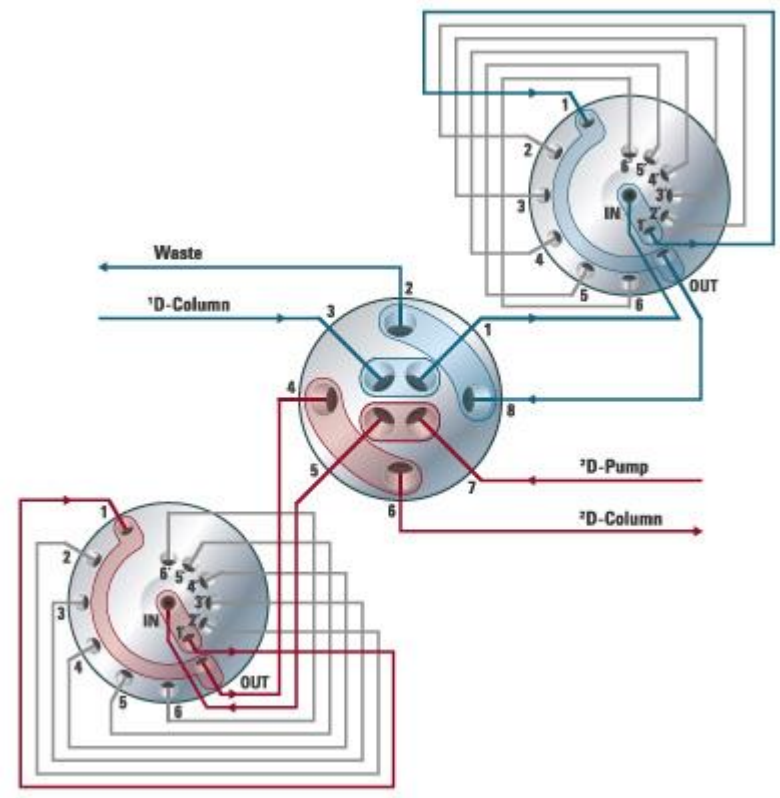
Heart-cutting Data Viewer

Slide courtesy of Agilent Technologies

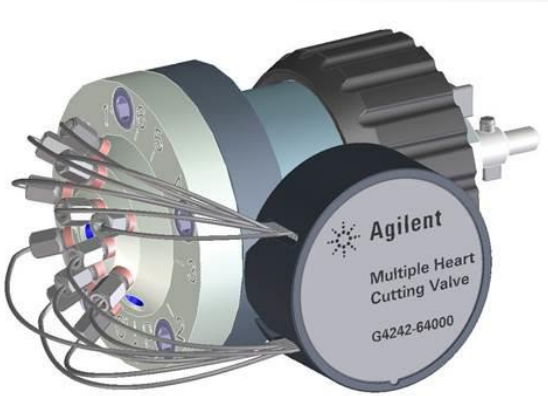
Sampling Device for LC-LC (Heart-Cut)

Agilent Multiple Heart-Cutting 2D-LC

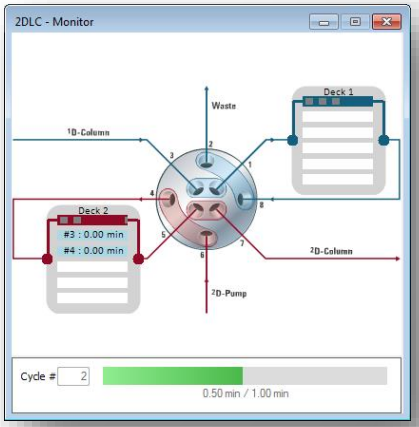
Smart Valve-Loop Setup with 12 loops
→ **2D-LC valve + two 6/14 valves**



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



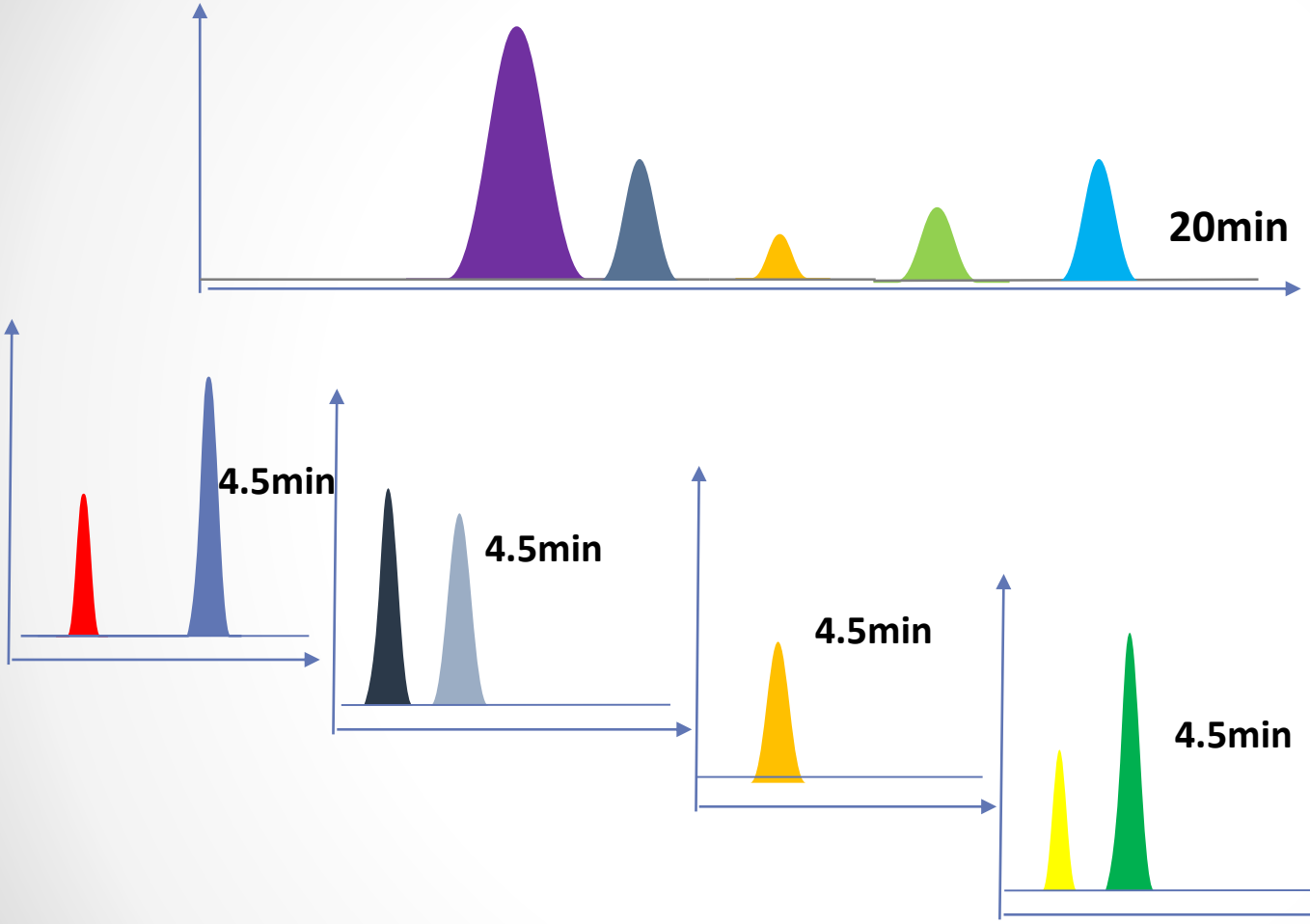
Online status monitoring

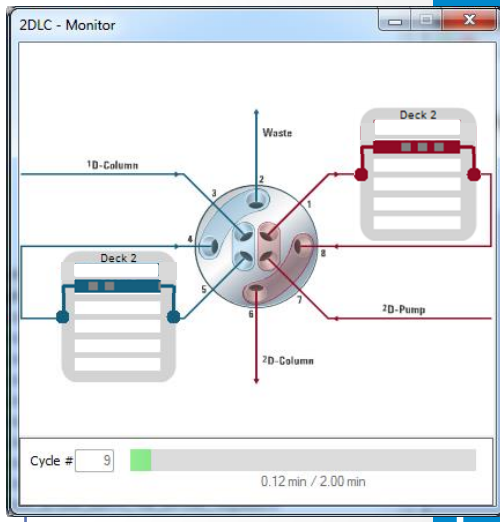


Slide courtesy of Agilent Technologies

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

End of Part 1