

# **On the Advantages and Costs of Two-dimensional HPLC (2D-LC)**

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# What is Chromatography About?

## Separating Components of Mixtures

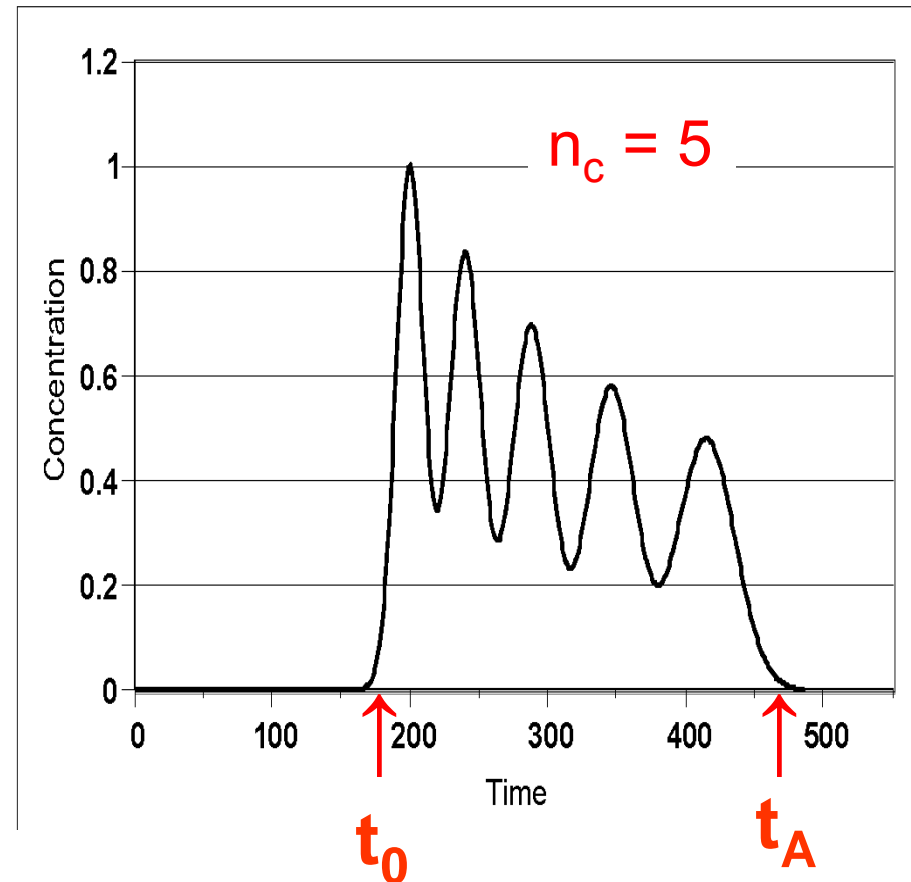
- Column efficiency:

$$N = (t_R/\sigma)^2 = 16 (t_R/w_{base})$$

- Resolution:

$$R = (t_{R,2} - t_{R,1}) / 2(\sigma_1 + \sigma_2)$$

- The Peak capacity is the number of peaks with  $R = 1$  that can be placed between  $t_0$  and  $t_A$



# Conditional Peak Capacity

- In gradient elution, the conditional peak capacity is the ratio of
  - The distance between the first and last peak
  - To the average peak width

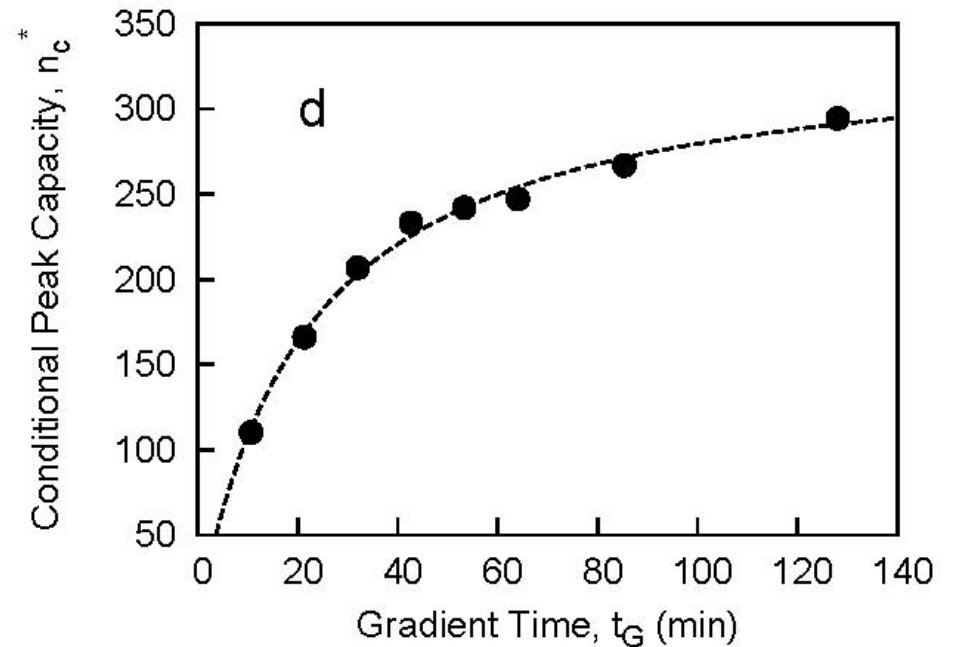
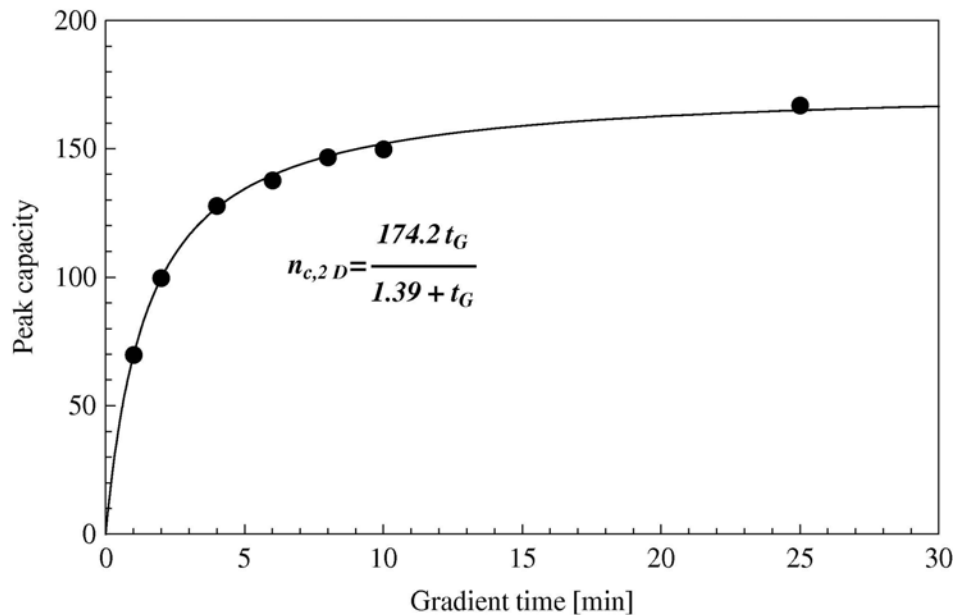
$$n_c = \frac{t_{R,n} - t_{R,1}}{W}$$

- It is best measured with myoglobin digest which has few peptides and well resolved peaks

# How to Achieve High Peak Capacities

- Do **gradient elution** (for very complex samples)
- **Increase plate number** (but  $100 N$  gives  $10 n_c$ )
- **Increase the range of elution times**, effective in gradient elution (bottom) but much less under isocratic conditions (top)

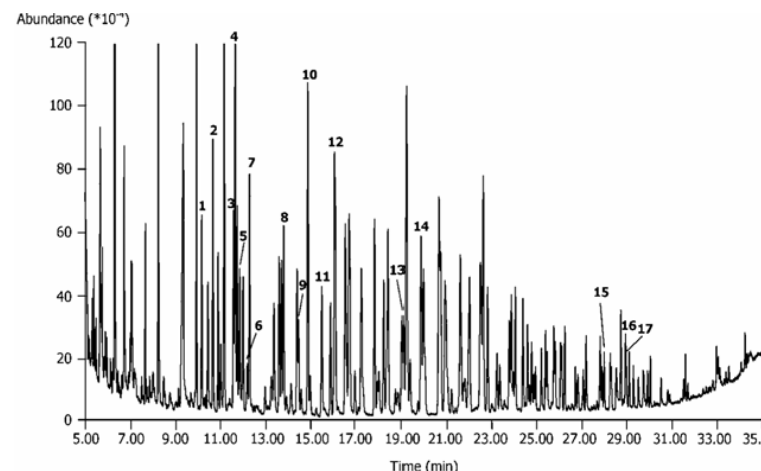
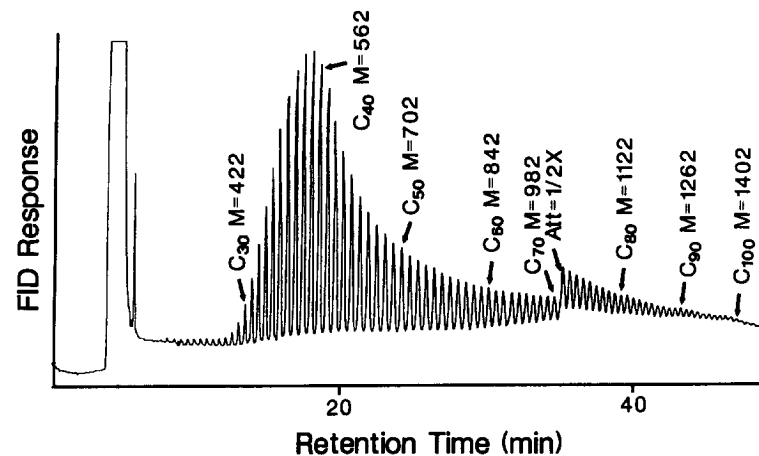
# Peak Capacity for BSA Digest



- Left: Gradient 5 to 50% ACN in H<sub>2</sub>O, 3 ml/min
- Right: Gradient 5 to 50% ACN, 0.9 ml/min
- Halo column, 5 cm, 60°C
- *Gilar, Daly, Kele, Neue, Gebler, JCA, 2004, 1061, 183*

# How Many Components can we Resolve with a Given Peak Capacity?

- Many with the proper sample (Fischer-Tropsch wax, Pressure-gradient SFC),  $n_c=100$ , 80 peaks
- Not so many with most samples (MeOH extract of salad, spiked with pesticides, Temperature program GC),  $N=120000$ ,  $n_c=400$ , 129 peaks



# Why 2D-LC?

- Because it is much faster to successively generate twice in a row an  $n_c$  peak capacity than a  $2 n_c$  peak capacity once
- But we need to use two different retention mechanisms, giving most different retention patterns for the two separations. These mechanisms are said to be orthogonal

# Peak Capacity in 2D HPLC

- The 2D peak capacity is

$${}^2n_c = {}^1n_{c,1} {}^1n_{c,2} n_F \cos \beta_{1,2}$$

- With  ${}^1n_{c,1}$  and  ${}^1n_{c,2}$  peak capacities of the columns used in the two dimensions
- $n_F$  Nobuo factor or loss of peak capacity due to backmixing during transfer
- $\cos \beta_{1,2}$  coefficient of correlation between the two retention mechanisms used

# Four Implementations of 2D-LC

- **On-line**. Fractions from column 1 are periodically injected on-line into column 2 and analyzed
- **Stop and Go**. Same as On-line 2D-LC but elution of the first column is stopped during each second-dimension analysis. This is **Mudpit**
- **Off-line**. Fractions eluted from column 1 are collected, stored, and some of them are analyzed on column 2
- **Column switching, heart-cutting**. The old way (not bad at all when it works)

# Comprehensive 2D-LC Definition

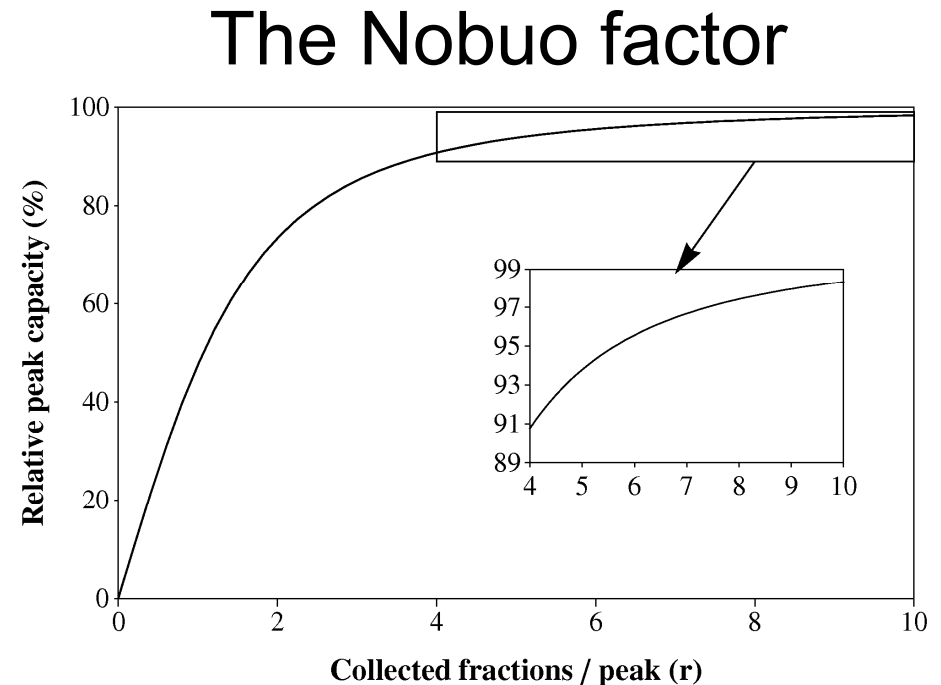
- **All** the molecules of **all** the sample components (or a large, constant aliquot of them) **migrate along the columns used in the two dimensions** of the separation and are eluted from the second column

# Principle of On-Line 2D-LC

- A long, efficient first column implements a first retention mechanism and separates the sample components after a certain retention pattern
- The eluent flows through an injection loop
- The loop content is periodically injected into a 2nd, fast column giving orthogonal separations
  - To avoid back-mixing the period must be less than  $\frac{1}{4}$  the band width of the first column
  - Detection may take place between the 2 columns
  - Several second-columns may be used in parallel

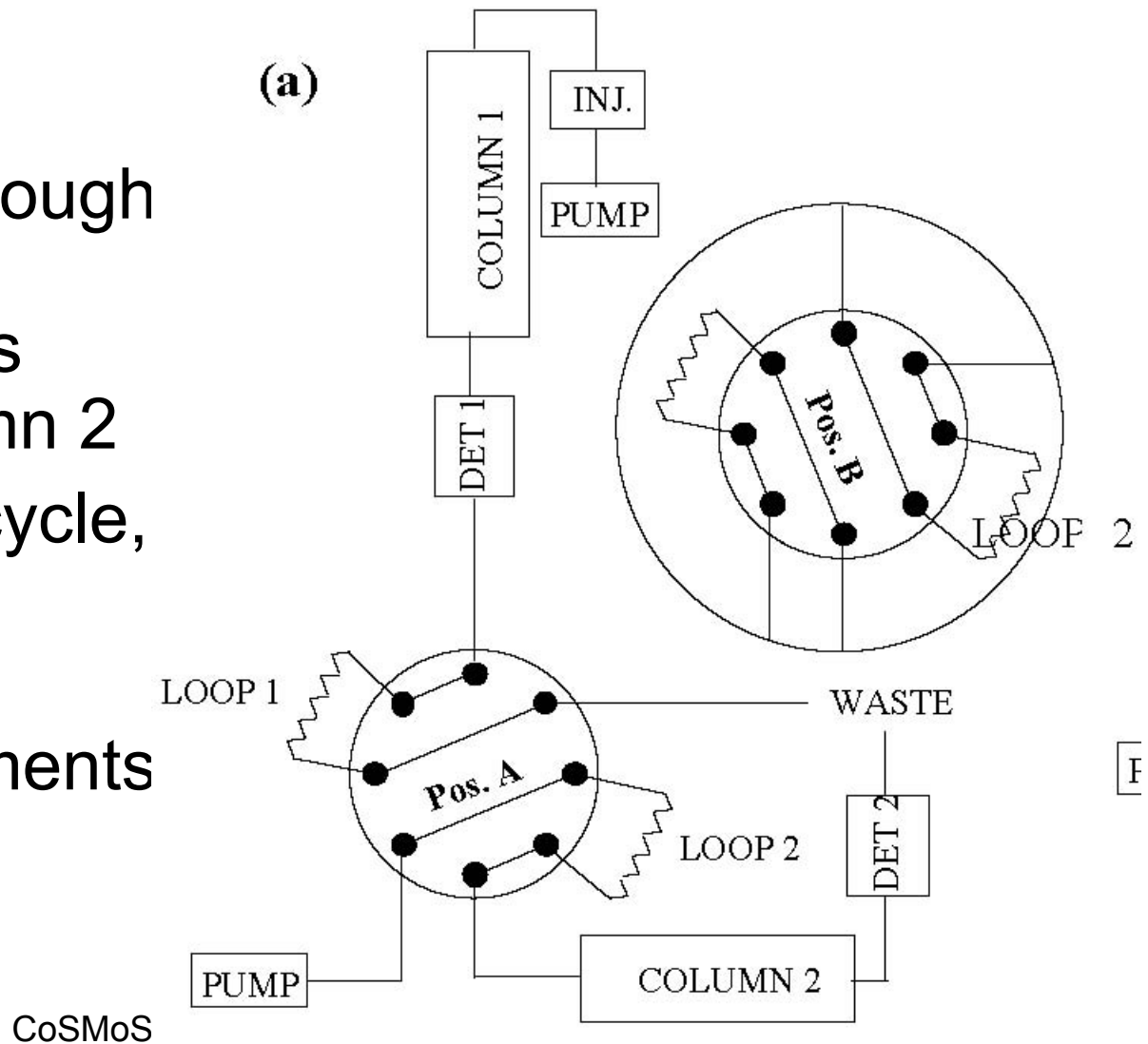
# The Nobuo Factor

- The apparent or relative peak capacity in the first dimension increases with increasing density of fraction collection ( $r$ )
- Increase proportional to  $r$  for  $r < 2$
- The gain tapers off for  $r > 3$
- **The density of fraction collection must be optimized**
- There is no reason for  $r=4$  to be best

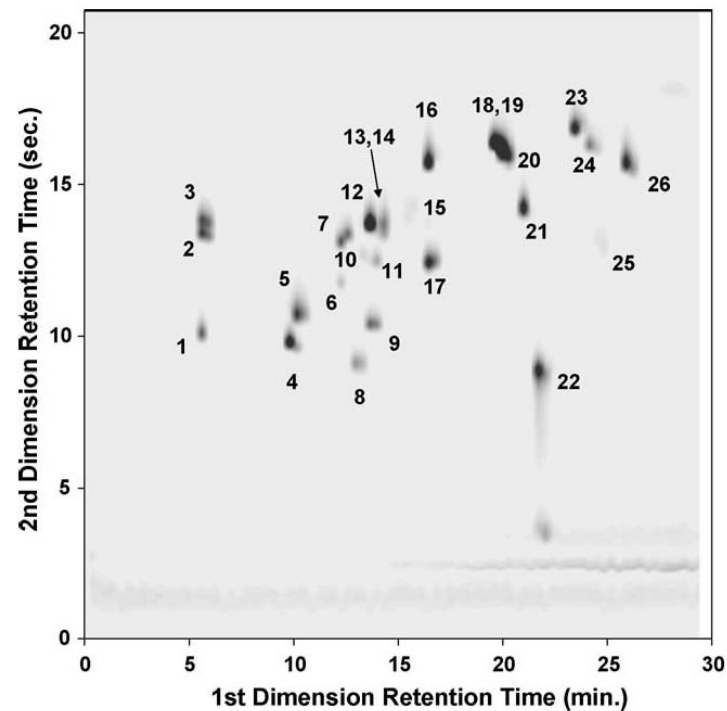
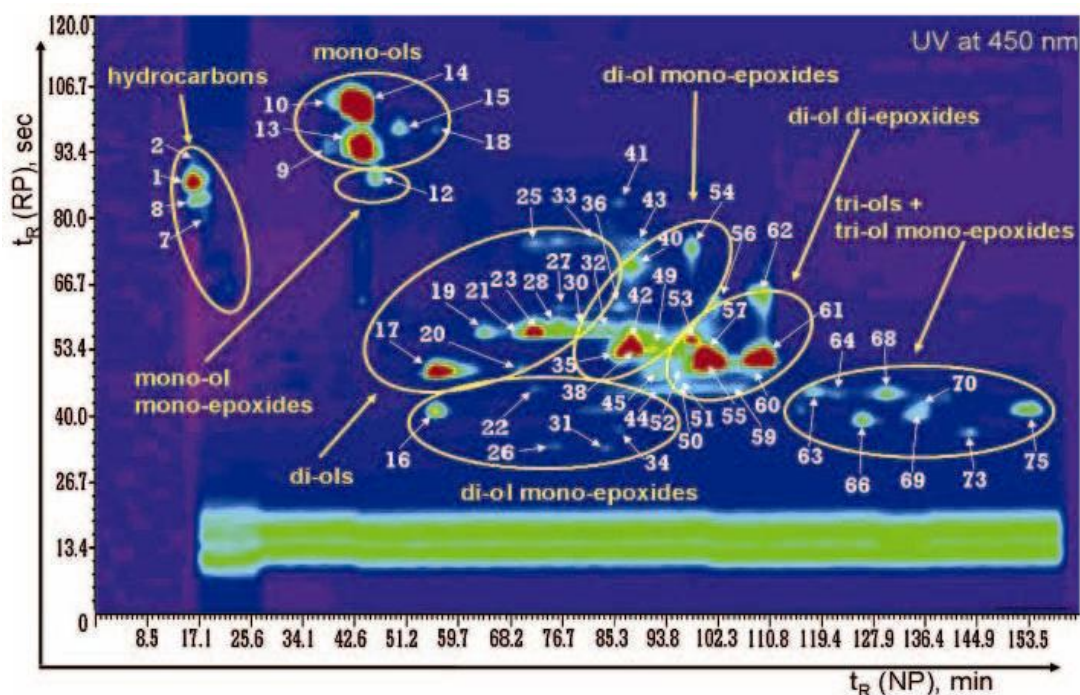


# Schematics of Instrument for 2D-LC

- The eluent from column 1 flows through loop 1 while the content of loop 2 is analyzed on column 2
- At the end of the cycle, loops 1 and 2 are exchanged
- Two HPLC instruments are needed

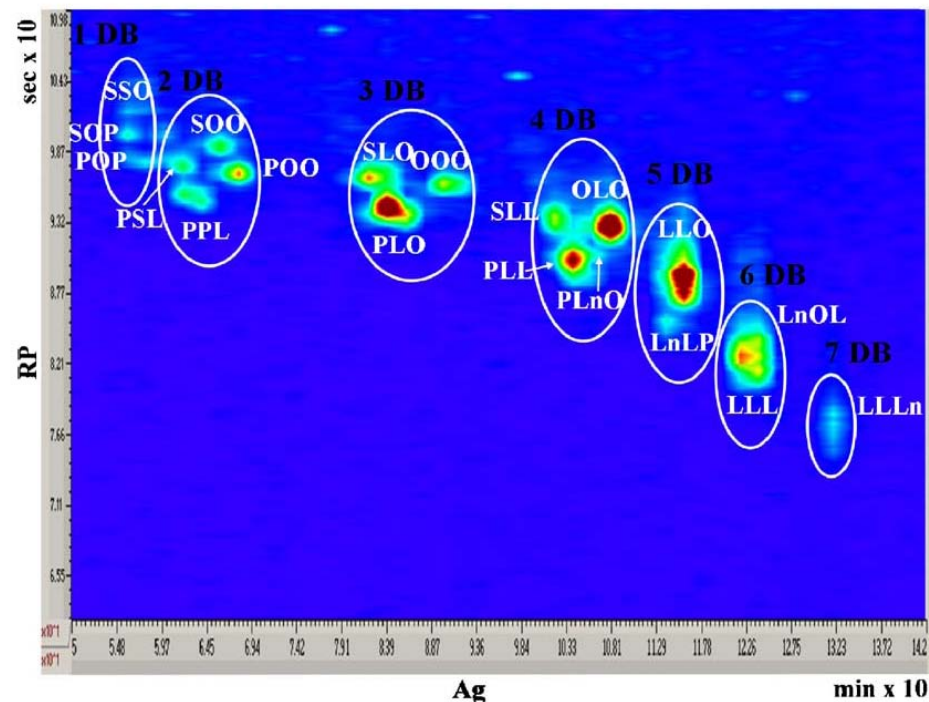
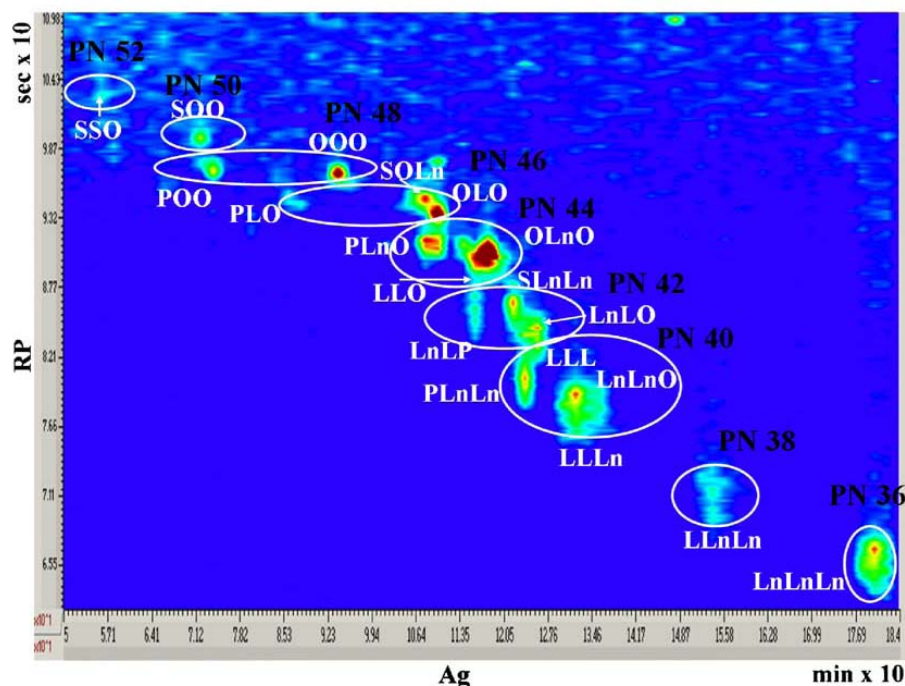


# Examples of On-Line 2D-LC



- Left, sweet orange juice, SupelcosilxChromolith.  
 $n_c=800$
- Right, mixture of indolic metabolites. Discovery HS-F5 x ZirChrom Carb; gradients of ACN in buffers.  $n_c=870$
- *Jandera, AC, 78, 7743* CoSMoS, Boston, 08/3-5/2009 *Carr, JCA, 1122, 123.*<sup>14</sup>

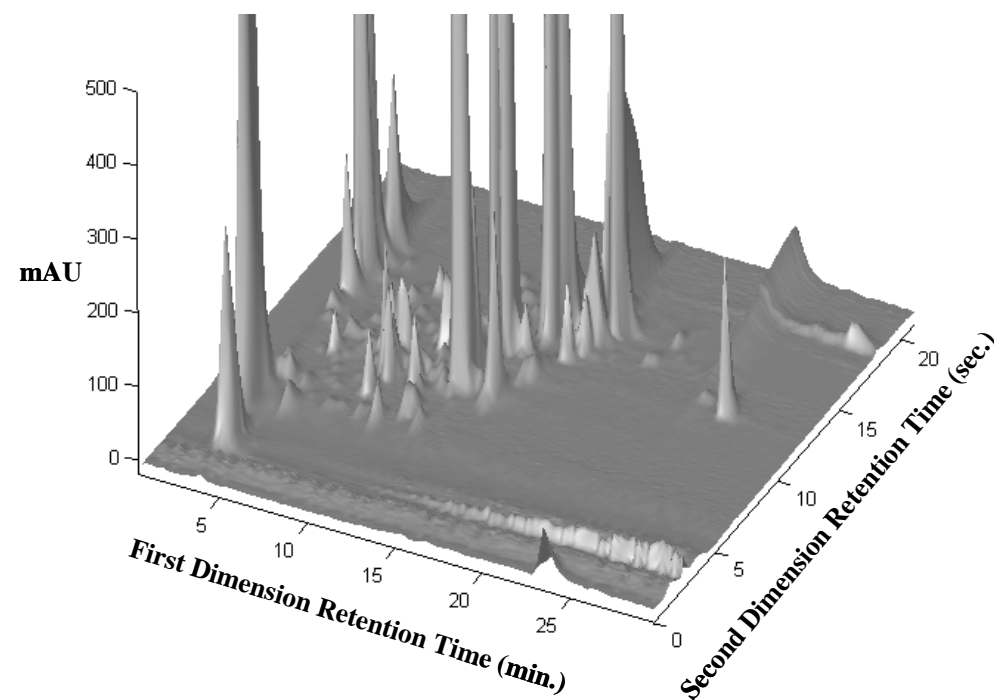
# Examples of On-Line 2D-LC



- Separation of triglycerides, soybean and linseed oils
- 1<sup>st</sup> column, silica+1 M Ag<sup>+</sup>NO<sub>3</sub><sup>-</sup>, C<sub>6</sub>H<sub>14</sub> 0.7 / 0.9% ACN
- 2<sup>nd</sup> column, Chromolith-C<sub>18</sub>, i-PrOH+ACN, 4 ml/min

# Example of LCxLC Separation

- **First separation:**
  - $-\text{C}_3\text{H}_6-\text{C}_6\text{F}_5$  bonded silica
  - Aqueous  $\text{PO}_4/\text{ClO}_4$  buffer with 0 to 70% ACN gradient
- **Second separation:**
  - Zirchrom-Carb
  - Aqueous  $\text{ClO}_4$  buffer with a 0 to 70% ACN gradient
- *P.W. Carr, Pittcon 2005*

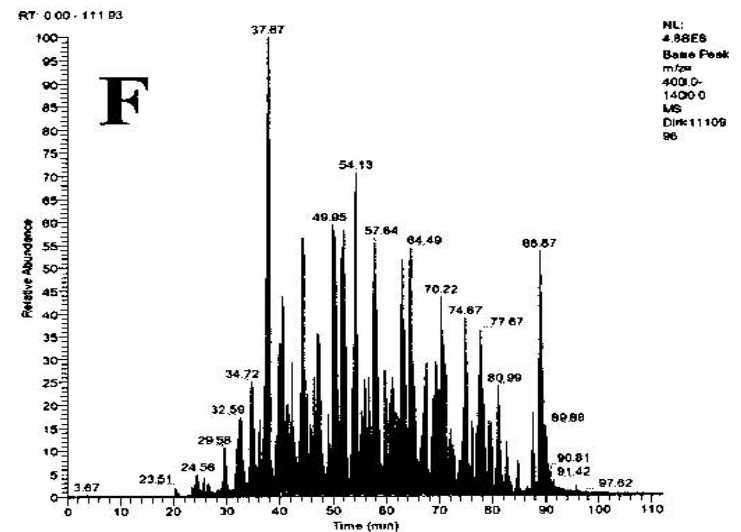
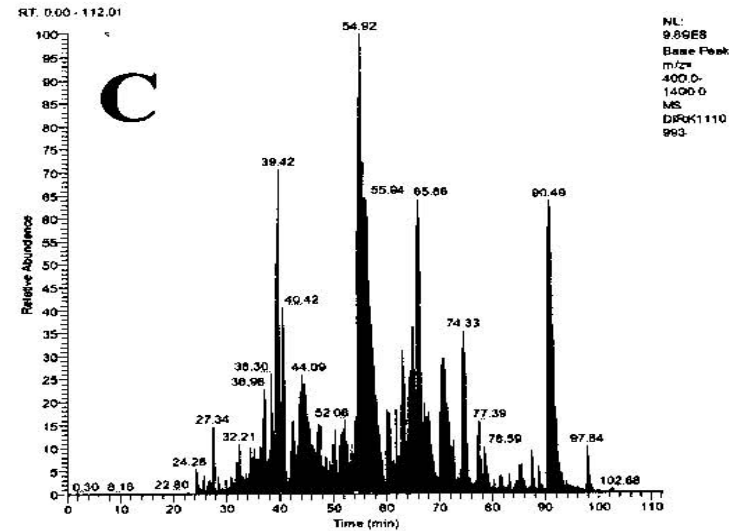


# Stop-and-Go 2D-LC

- Eluent from column 1 is stored in column 2 for a certain time
- Then, elution from column 1 is stopped and the fraction stored on column 2 is analyzed
- The process is resumed until all the sample is eluted from columns 1 and 2
- This is the **Mudpit** analytical process

# Principle of MUDpit 2

- 1<sup>st</sup> dimension, SCX, steps gradient of  $\text{NH}_4\text{Cl}$  in ACN/water, w/ steps of ACN and  $\text{NH}_4\text{Cl}$
- 2<sup>nd</sup> dimension, RPLC, gradient of ACN in water
- Analyses of 2 out of 15 SCX fractions



# Principle of Off-Line 2D-LC

- The first column eluent is collected and stored as a continuous series of fractions
- These fractions are stored and analyzed on a second column
- The parameters of both columns and the collection frequency are all independent, so they all must be optimized
- One instrument, a fraction collector, an automatic sampling system are needed

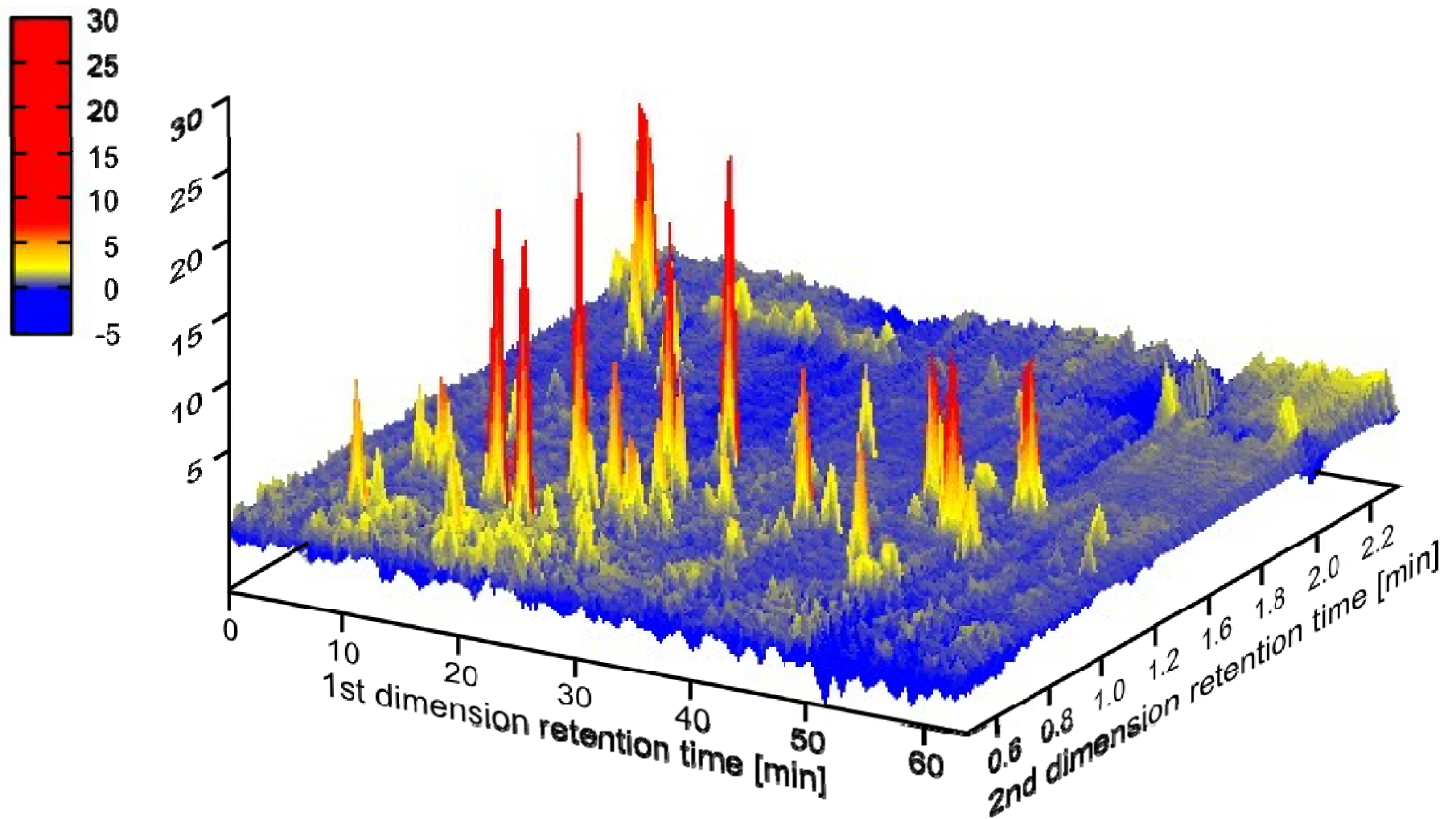
# Experimental Conditions

- **Instrument:** Agilent HPLC 1100
- **First dimension separation:** Partisphere SCX, 110x4.6 mm, 5  $\mu\text{m}$  particles, aryl- $\text{SO}_3^-$  groups, room T. Eluent 95/5  $\text{H}_2\text{O}/\text{ACN}+0.5\%$   $\text{CH}_3\text{-CO}_2\text{H}$  and linear gradient of KCl.
- **Second dimension separation:** Halo, 50x4.6 mm, 2.7  $\mu\text{m}$  particles, 60°C. Eluent 5 to 50% ACN in  $\text{H}_2\text{O} + 0.5\%$  TFA, 3 mL/min.

# Experimental Conditions (2)

- Fractions (160  $\mu\text{l}$ ) of SCX eluent collected every 20s and stored. Total, **180 fractions**
- The peak capacity was  ${}^1n_{c,1} = 54$
- The Nobuo factor is  $n_F = 0.9$
- Aliquots (50% of each of these fractions, i.e., 80  $\mu\text{l}$ ) were injected on the Halo column
- Gradient analyses, 4 min, peak capacity  ${}^1n_{c,2} = 150$

# Map of BSA tryptic digest

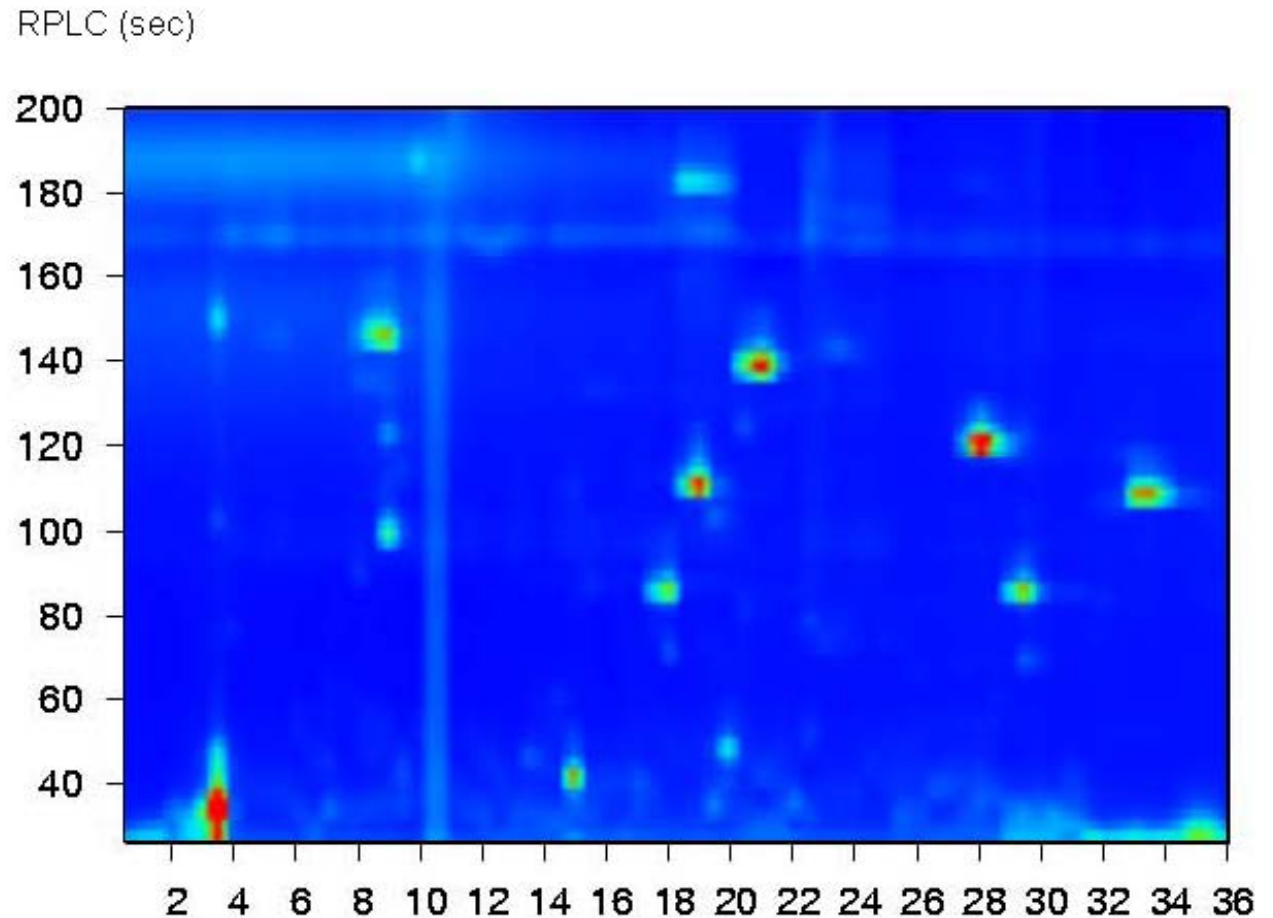


# Final results

- The total peak capacity is  ${}^2n_c=8,100 \times 0.9=7300$
- The cycle time was 9 min
- The analysis time  $9 \times 180=1620$  min= $27$  hours
- It could be reduced by half by regenerating faster the 2<sup>nd</sup> column after each gradient
- Alternate: Cycle time: 4 min; Fractions = 120
- ${}^1n_{c,1}=30$ ;  ${}^1n_{c,2}=120$ ;  ${}^2n_c = 3600$
- Analysis time:  $4 \times 120=480$  min= $8$  hours

# Off-Line Separation of Peptide Digest of Myoglobin

- 1st D, SCX,  
5cm 80 cuts  
 $n_c=50$
- 2nd D, Halo  
5cm,  $n_c=50$
- Total  $n_c=2500$
- Analysis time  
 $200 \times 80 = 16000$   
 $s = 4.4$  hours



# Which 2D-LC to Choose?

- **On-Line**
  - Fast ( $t_A = {}^1t$ ) but efficiency limited ( ${}^2n_c \ll {}^1n_c$ )
- **Stop and Go**
  - Slow ( $t_A = {}^1t + j {}^2t$ )
  - Efficiency maximum if  ${}^2n_c < {}^1n_c$
- **Off-line**
  - Slow ( $t_A = {}^1t + j {}^2t$ ,  $j$  large). Time  $\approx$  days or weeks
  - Enormous potential peak capacity  $\approx$  a million?

# Advantages and cost of 2D-LC

## • Advantages

- Straightforward to implement and use
- Large increase of the peak capacity
- Easy to couple with MS or MS/MS

## • Drawbacks

- Need to find two columns with independent retention mechanisms
- Optimization is complex
- High dilution, high LOD
- MS data system overflows

For a given resolution, the analysis time is much smaller in 2D than in 1D

# Conclusion

- Optimization may permit the achievement of very large total peak capacities within a reasonable time ( ${}^2n_c = 16\ 000$  in 24 h)
- It also permits the achievement of very fast and powerful analyses ( ${}^2n_c = 1\ 000$  in less than 1 h)
- All this is possible only if two independent retention mechanisms are available

# Warning

- For practical purposes, the contributions of  ${}^1n_{c,1}$  and  ${}^1n_{c,2}$  to  ${}^2n_c$  are not equivalent.
- There are cases in which a very large  ${}^2n_c$  is useful, no matter what are  ${}^1n_{c,1}$  and  ${}^1n_{c,2}$ .
- There are other cases in which a larger  ${}^1n_{c,1}$  or a larger  ${}^1n_{c,2}$  will not improve the separation. Example: separations of fatty acids or of triglycerides