Multidimensional HPLC Tutorial Part - 2

Requirements 1st Dimension, 2nd Dimension, Column Selection, Method Development and Examples

Requirements to the 1st Dimension Separation

Dimensions, Stat. Phase Selection, Isocratic or Gradient Elution

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

1st Dimension Separation

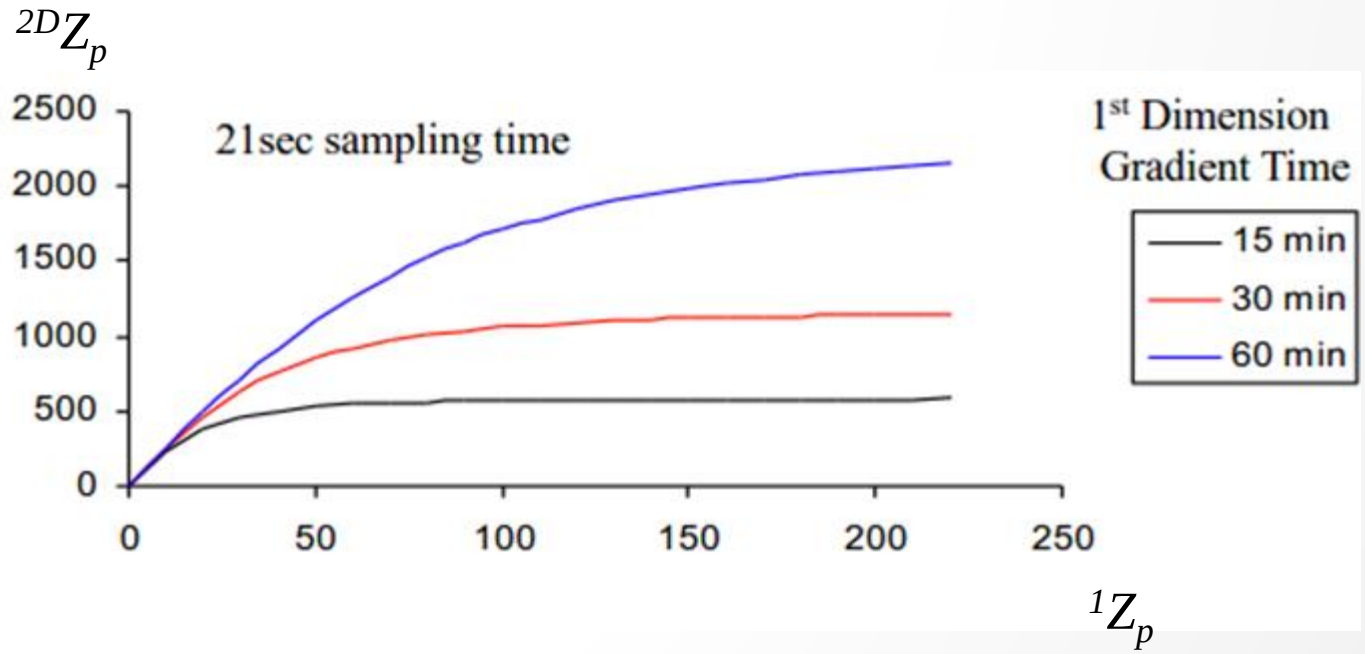
Requirements

- 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

Requirements to the 2nd Dimension Separation

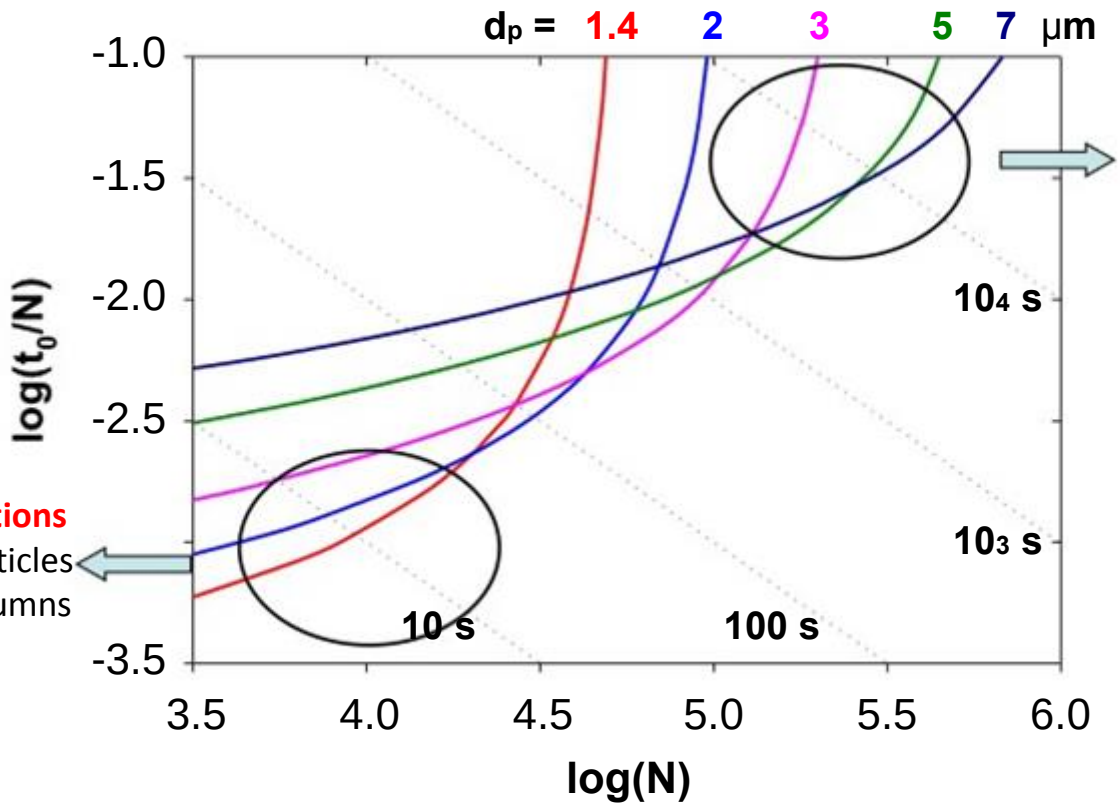
2nd Dimension Separation

Requirements

- Must be very high speed separation(UHPLC!!)
- Isocratic or gradient separation
- 2nd dimension stationary phase should provide analyte focusing
- Stable column with minimal retention time drift and have excellent longevity (e.g. at low pH, high pressure or temperature)

2nd Dimension Separation

Influence of Particle Size – Poppe Plot for Isocratic Separation



High efficiency separation

- Large particles
- Long columns
- Long run time

Fast separations

- Small particles
- Short columns

ΔP_{max} = 400 bar

Knox equation

A = 1.0, B = 1.5, C = 0.05

$D_m = 1 \times 10^{-5}$ cm²/sec

$\eta = 0.001$ *Pa/sec*

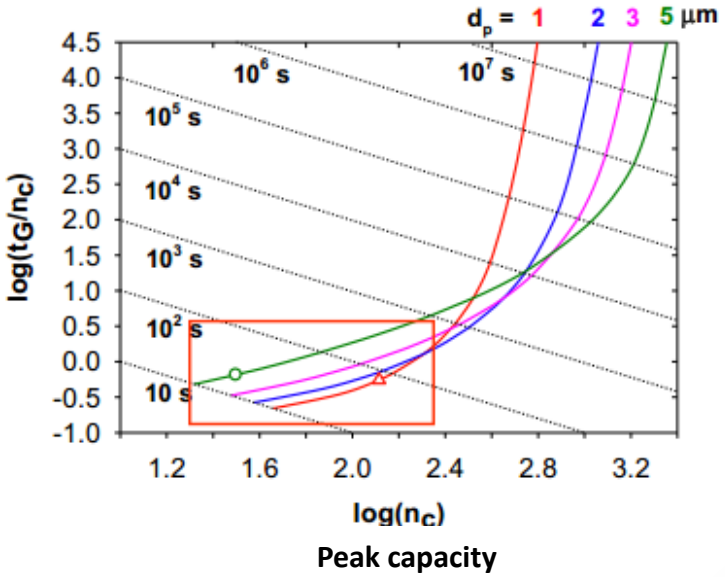
- Use columns with very small particles for ultra fast separations
- Work at ultra high pressure

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

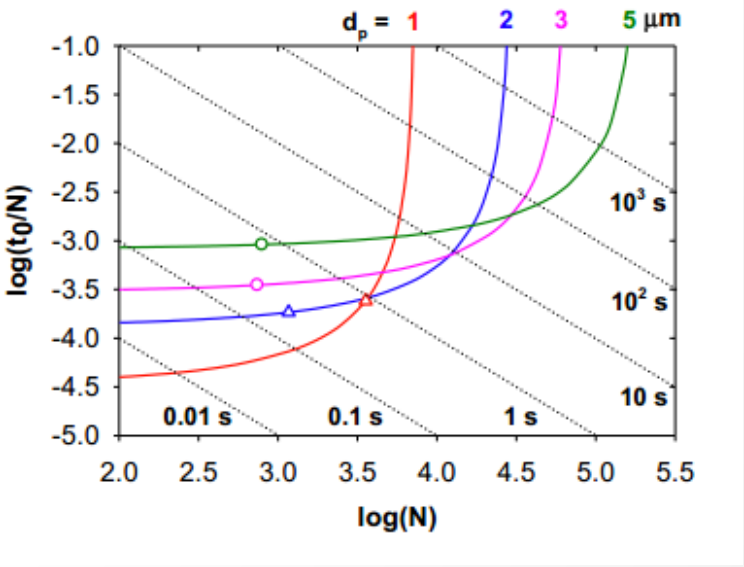
2nd Dimension Separation

Influence of Particle Size – Poppe Plot for Gradient Separation

Gradient Poppe Plot for 11 Peptides



Isocratic Poppe Plot for Alkylphenone



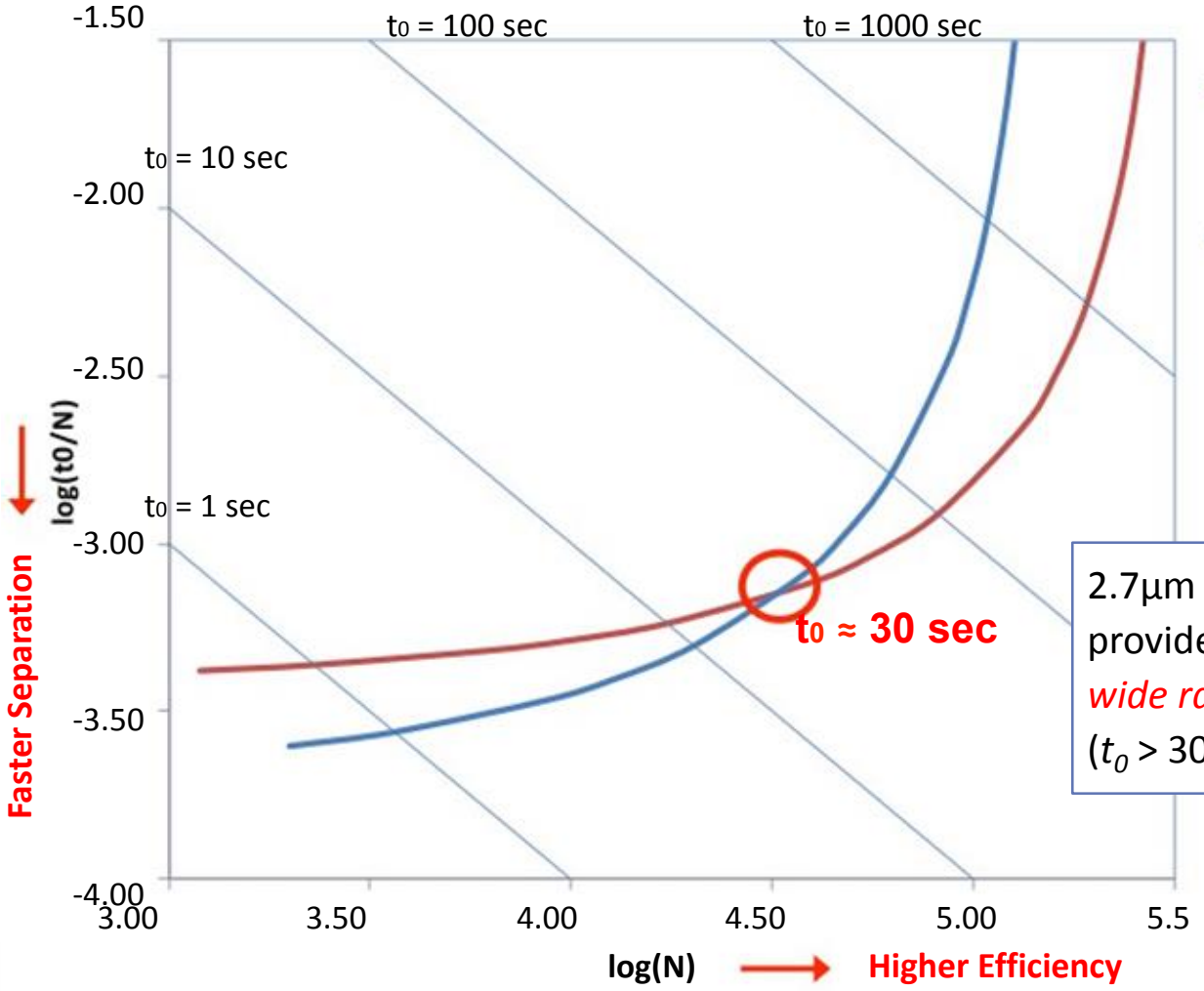
ΔP_{max} = 400 bar D_m = 1 ×10-5 cm²/s, η = 0.69 *cP*

Isocratic and gradient Poppe plots lead to qualitatively the same conclusions on effect of particle size on peak capacity

Wang, X.; Stoll, D. R.; Carr, P. W.; Schoenmakers, P. J. J. Chromatogr., A 2006, 1125, 177

2nd Dimension Separation

Sub-2-µm Totally Porous vs Superficially Porous Particles

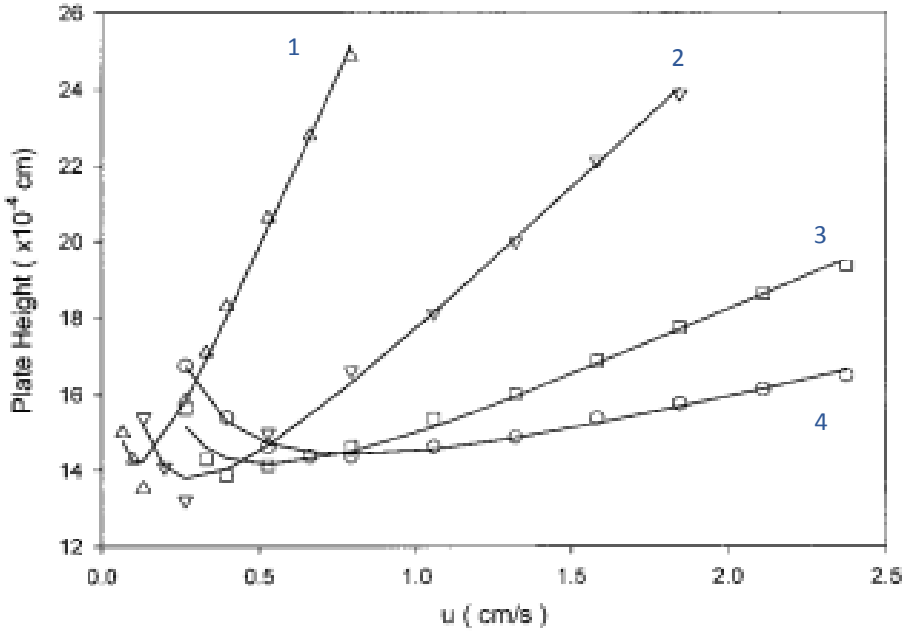


2.7µm superficially porous particles provide best separation in *a very wide range of analysis time* (t_0 > 30 sec, or t_R > 3 min)

Wang et al J. Chromatogr. A 2012, 1228, 72-88

2nd Dimension Separation

Effect of Temperature*



Higher temperature

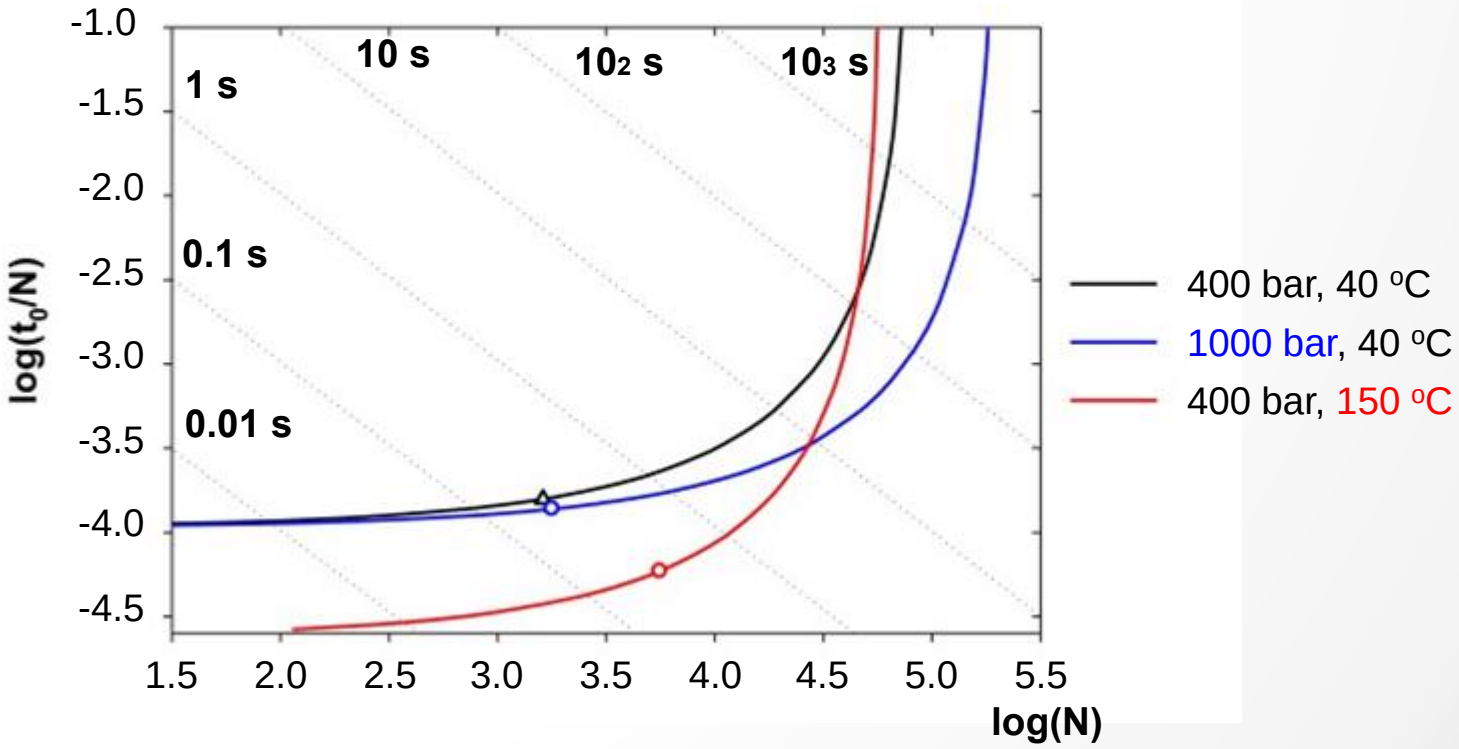
- $u_{0,opt}$ increases
- C-term flattens

Plate height vs linear velocity at various temperatures for well-retained solutes;
1, 25 °C (decanophenone, k 12.2)
2, 80 °C (dodecanophenone, k 7.39)
3, 120 °C (tetradecanophenone, k 12.3)
4, 150 °C (tetradecanophenone, k') 7.00).

***P. Carr et al., Anal. Chem. 2000, 72, 1253-1262**

2nd Dimension Separation

Effect of Temperature



Higher temperature is more advantageous than pressure increase!

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

2nd Dimension Separation

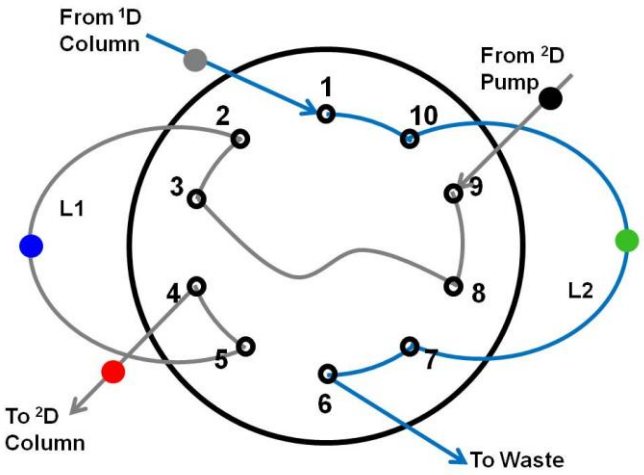
Column Technology Options

Characteristics	Advantages	Disadvantages
1. Totally porous particles < 2µm	• Low HETP → short columns • Many commercial sources	• Requires UHPLC equipment, expensive • Frictional heating in the 2nd dimension separation
2. Superficially porous particles > 2 µm	• Low HETP and low back pressure • Compatible with standard HPLC equipment • Many commercial sources	• Requires UHPLC equipment Expensive • Lower phase ratio → less retention
3. Monoliths	• Medium HETP and very low back pressure ➔ fast • Compatible with standard HPLC equipment	• Low max. pressure rating • Less retentive • Few suppliers
4. High Temperature LC	• Low back pressure Reduction of HETP Improved peak shape	• Requires special heater • Stability of packing and solutes • Selectivity changes with temperature

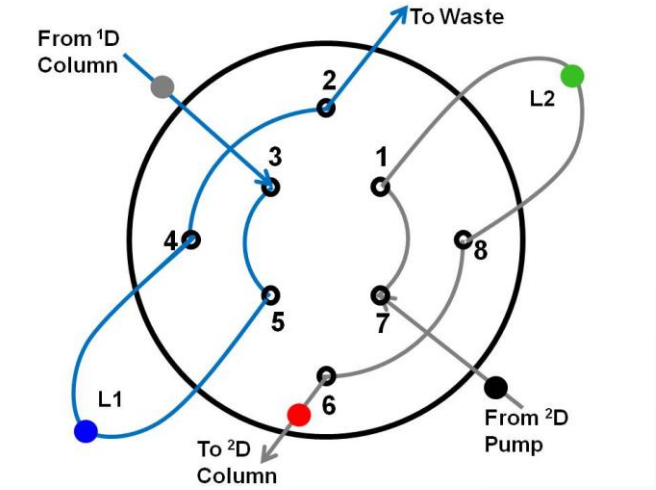
Slide courtesy of Prof. P. Carr & Dr. D. Stoll

2nd Dimension Separation

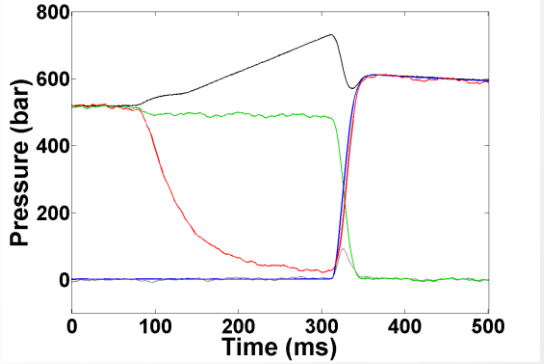
Column Stability



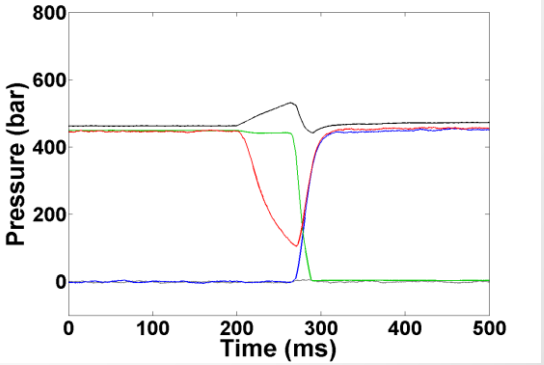
pressure sensors



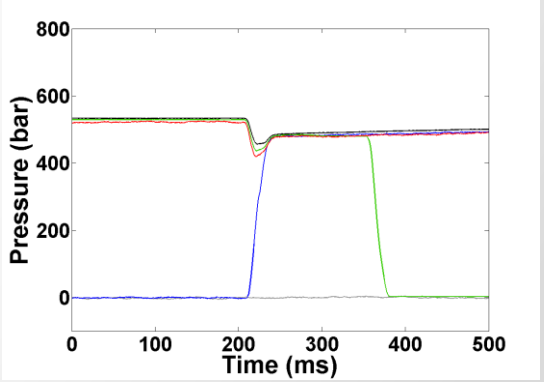
Electrical Actuated
Standard 10 port valve



Pneumatic Actuated
Standard 10 port valve



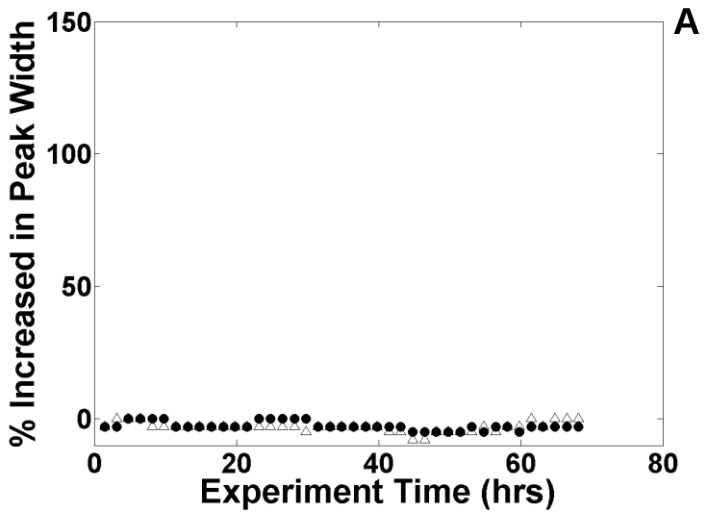
Agilent Proprietary
Duo valve

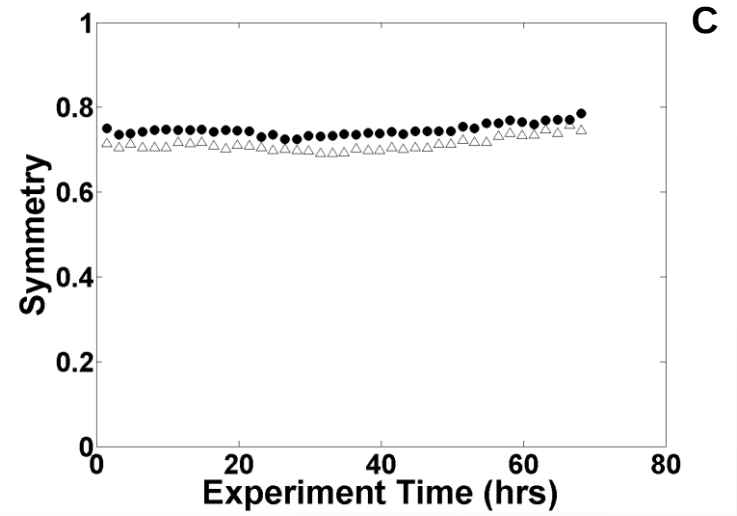


Prof. Pete. Carr & Dr. Dwight Stoll submitted for publication

2nd Dimension Separation

Column Stability





Electrically actuated 8-port/2-position valve.
Superficially porous C18 columns
Test solutes nitropentane (●) and di-propyl phthalate (Δ)

Prof. Pete. Carr & Dr. Dwight Stoll submitted for publication

2^{nd} Dimension Separation

Sample Zone Focusing

The Problem – Typical 2D-LC conditions involve relatively large injections of ^{1}D effluent into the ^{2}D column

Example:

^{1}D Flow Rate = 200 µL/min
Sampling (Modulation) Time = 20 s
Volume Injected to ^{2}D Column = 67 µL

If ^{2}D Column = 30 mm x 2.1 mm i.d (Zorbax), then:
^2V_m = 55 µL, and ^{2}Vinj./^2V_m ~ 1!

The solvent strength coming from the first dimension < solvent strength in the second dimension

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

2nd Dimension Separation

Sample Zone Focusing

	Approach		Disadvantage	Advantage
1)	Inject less ^{1}D effluent into ^{2}D column	A. B. C.	Decreases detection sensitivity Requires either low ^{1}D flow or flow splitting (1^{st} dimension detector!) Sample time is reduced	Relatively easy to implement
2)	Use larger ^{2}D column volume	A. B.	Decreases detection sensitivity Decreases ^{2}D speed -> peak capacity	Easy to implement
3)	Use more retentive column in the second dimension compared to the ^{1}D column	A.	Only possible when the chemistry of the analytes allows	Easy to implement
4)	Adjust solvent strength prior to the 2^{nd} dimension separation.	A. B.	Requires additional hardware More variables to consider in method development	Very effective when it works

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

2nd Dimension Separation

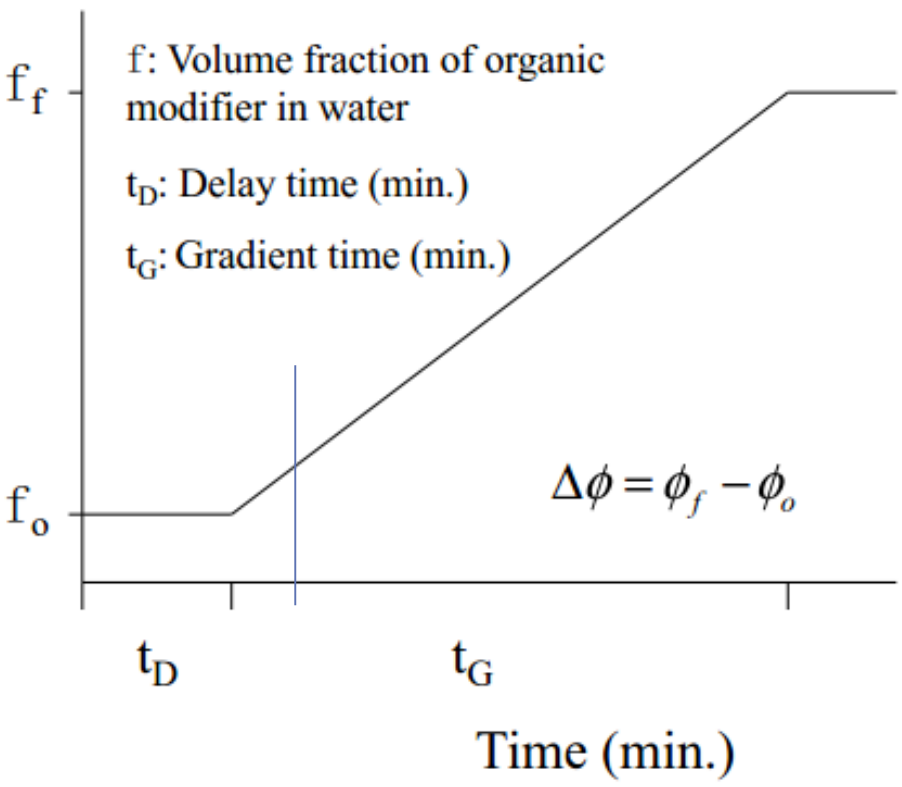
Cycle Time

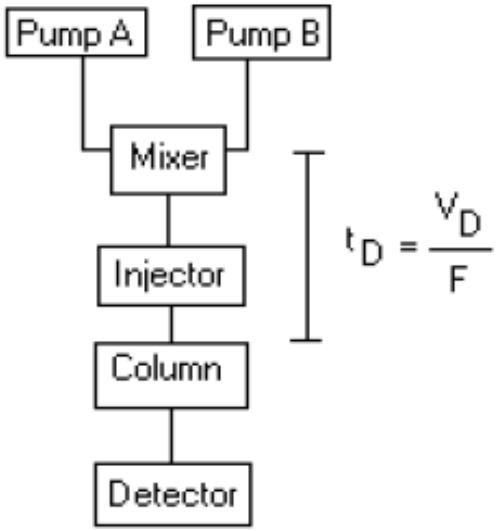
- We want to make the sampling time short
- In LC x LC $^1t_{sample} = {}^2t_{cycle}$
- Prefer $^1t_{sample} < {}^2t_{cycle}$ (under fill the sample loop!)
- $^2t_{cycle} = {}^2t_{gradient} + {}^2t_{re\text{-}equilibration}$
- So we don't want to make $^1t_{sample}$ too short since 2D separation peak capacity decreases if $^2t_{gradient}$ decreases
- ➔High Flow Rate and High Pressure Separation

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

2nd Dimension Separation

Basics of Gradient Elution





V_D: dwell volume (mL)

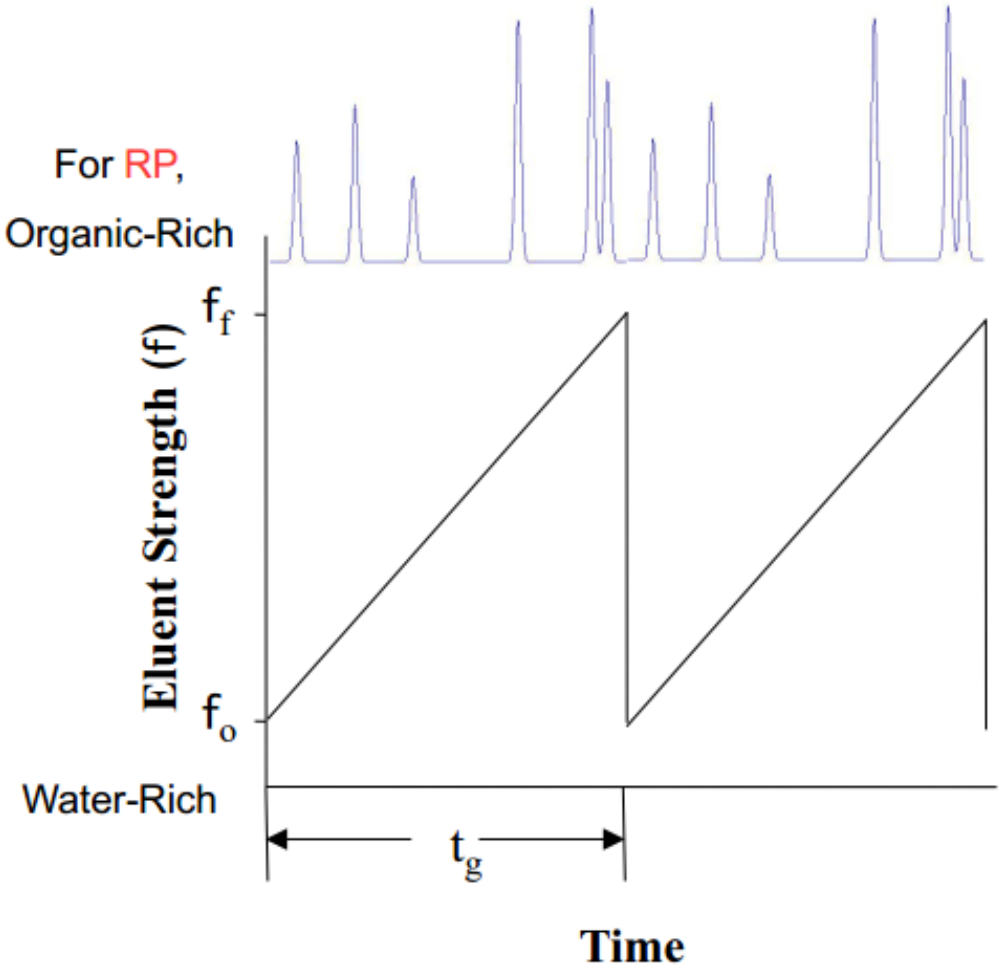
F: flow rate (mL/min.)

The delay volume is a unique and important property of the instrument and a BIG PROBLEM for the second dimension of 2DLC

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

2nd Dimension Separation

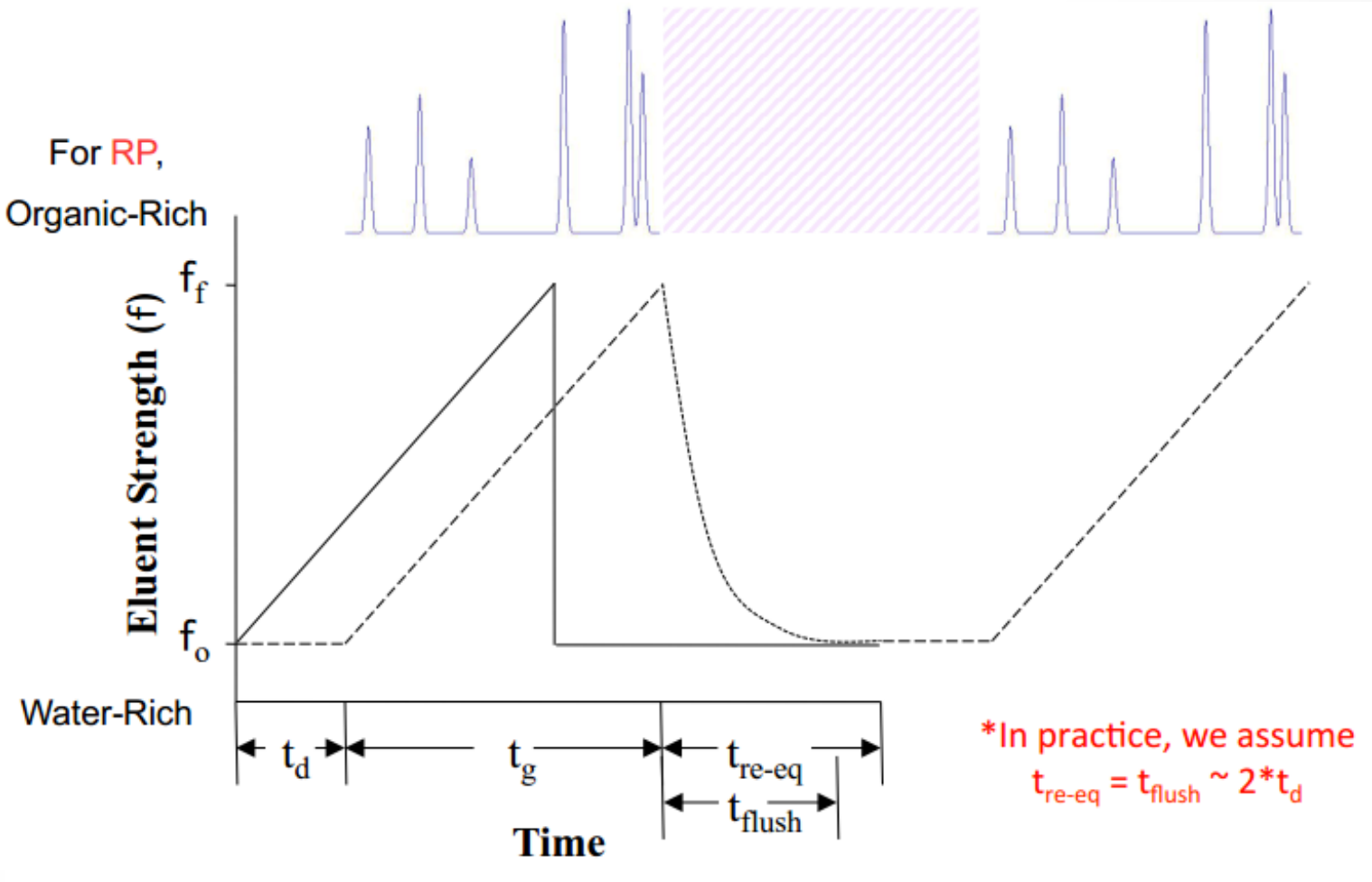
Ideal Situation



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

2nd Dimension Separation

Real Situation



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

2nd Dimension Separation

Calculation of Cycle Time

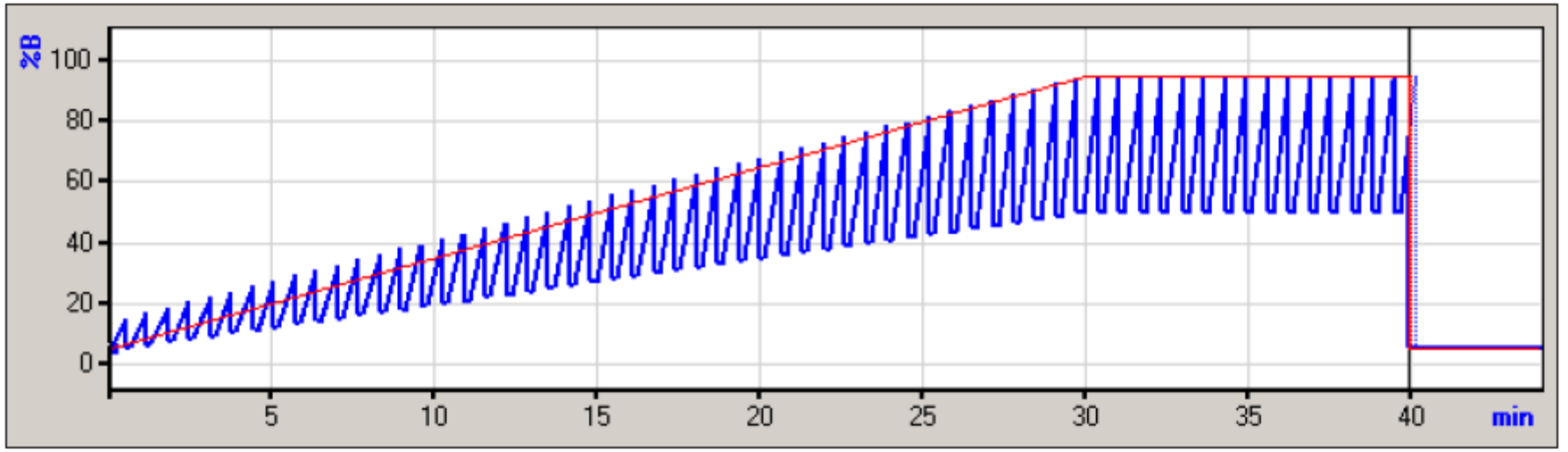
$$t_{cycle} = t_d + t_{gradient} + t_{re-equilibrate} = t_d + t_{gradient} + 2.V_{2Dcolumn}.F$$

	Gradient Delay volume (V_d, mL)	Flow Rate (mL)	Gradient Delay Time (t_d)	Analysis Time
Legacy Pumps (e.g., HP1100)	1.0	0.25	4 min.	30 min.
	1.0	3.0	20 s	60 s
New Pumps (e.g., 1290, Acquity)	0.1	0.25	24 s	30 min.
	0.1	3.0	2 s	30 s

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

2nd Dimension Separation

Optimize Gradient Separation



Adapt start composition of 2nd dimension gradient separation!!

Slide courtesy of Agilent Technologies

Column Selection for 2D LC

2D LC Column Selection

Considerations

Molecular structure of the analytes:

- Functional groups determine hydrophobicity, polarity and H-bond donor or acceptor
- Are there ionizable groups?
- Permanent charges or zwitterions?
- Molecular weight, size & shape?

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

2D LC Column Selection

Is RPxRP a good choice?

- RPLC is the most frequently used mode of HPLC
  - Wide availability, familiarity
  - RPLC is compatible with polar water, soluble (bio)molecules
  - Normally high plate counts and peak capacity (esp. in gradient mode)
  - Different brand RP columns will behave “orthogonal”
  - Retention of ionizable molecules will change strongly with pH of the eluent
- Eluents used in normal phase polar LC are incompatible with eluents used in RPLC
- Ion exchange will only separate ions of one type (anions or cations) and has relatively low plate counts
  - IECxRP for proteomics (offline method and also MUDPit)
- SEC is good for high MW solutes but has low peak capacity
  - SECxRP method (see Method Development section)
- HILIC has good solvent compliance for subsequent RP separation
  - HILICxRP method (see Method Development section)

Adapted from slide of Prof. P. Carr & Dr. D. Stoll

2D LC Column Selection

Mode Combinations

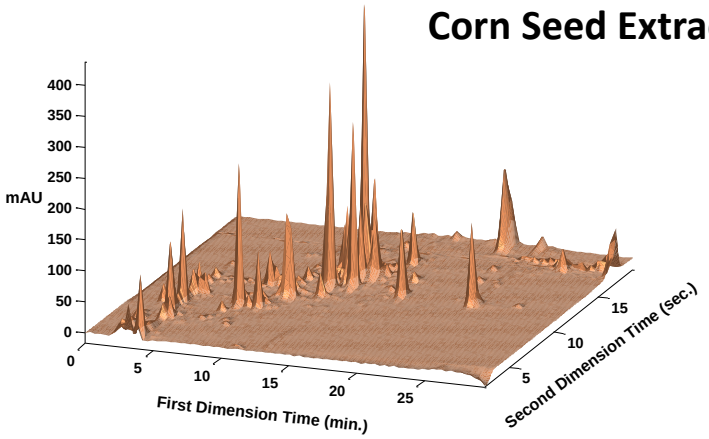
Mode	IEC x RP[1]	SEC x RP[2]	NP x RP[3]	RP x RP[4]	HILIC x RP[5]	HILIC x HILIC[6]	AC x RP[7]	SEC x NP[8]	SEC x IEC[9]
Orthogonality	++	++	++	+	+	-	++	+	+
Peak Capacity	+	+	+	++	+	+	-	-	--
Peak Capacity/time	-	--	+	++	+	+	-	--	--
Solvent Compatibility	+	+	--	++	+	++	+	+	+
Applicability	+	+	-	++	+	-	+	-	-
Score	4	3	1	9	5	2	2	-2	-3

Stoll, D. R.; Li, X.; Wang, X.; Carr, P. W.; Porter, S. E. G.; Rutan, S. C. *Journal of Chromatography A*. 2007, 1168, 3–43.

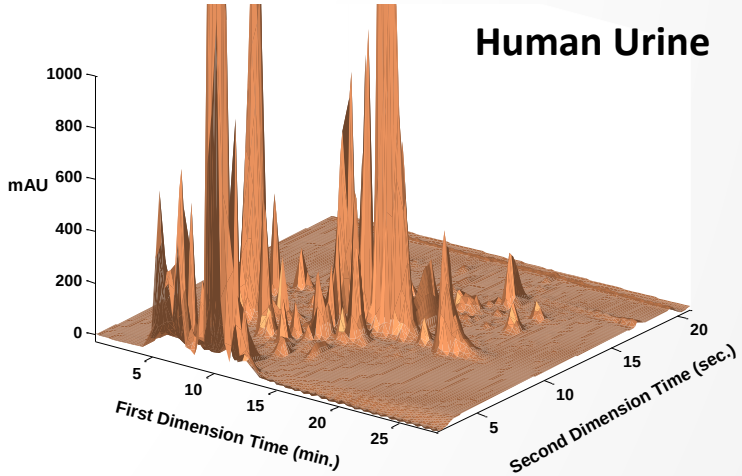
2D LC Column Selection

Is RPxRP a good choice?

Corn Seed Extract

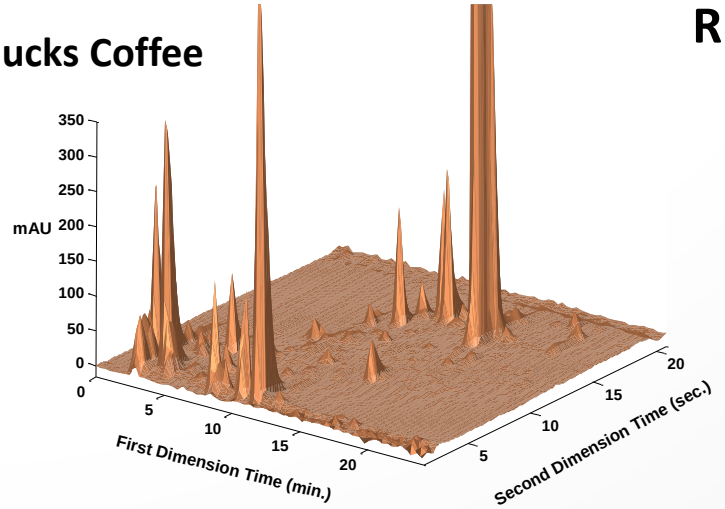


Human Urine

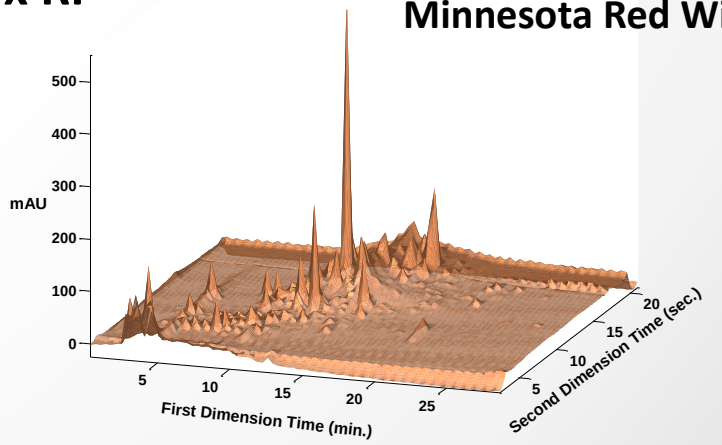


RP x RP

Starbucks Coffee



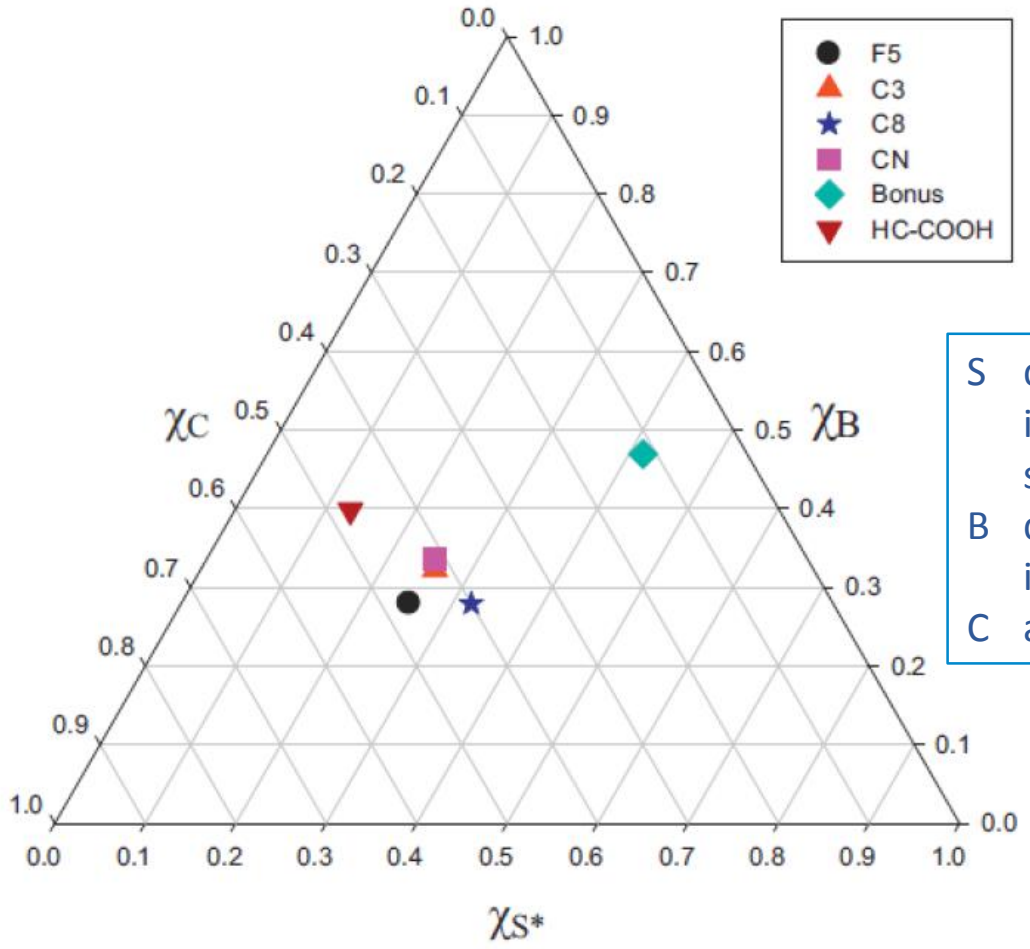
Minnesota Red Wine-2006



Stoll, D. R.; Li, X.; Wang, X.; Carr, P. W.; Porter, S. E. G.; Rutan, S. C. *Journal of Chromatography A*. 2007, *1168*, 3–43.

2D LC Column Selection

Orthogonality of RP phases



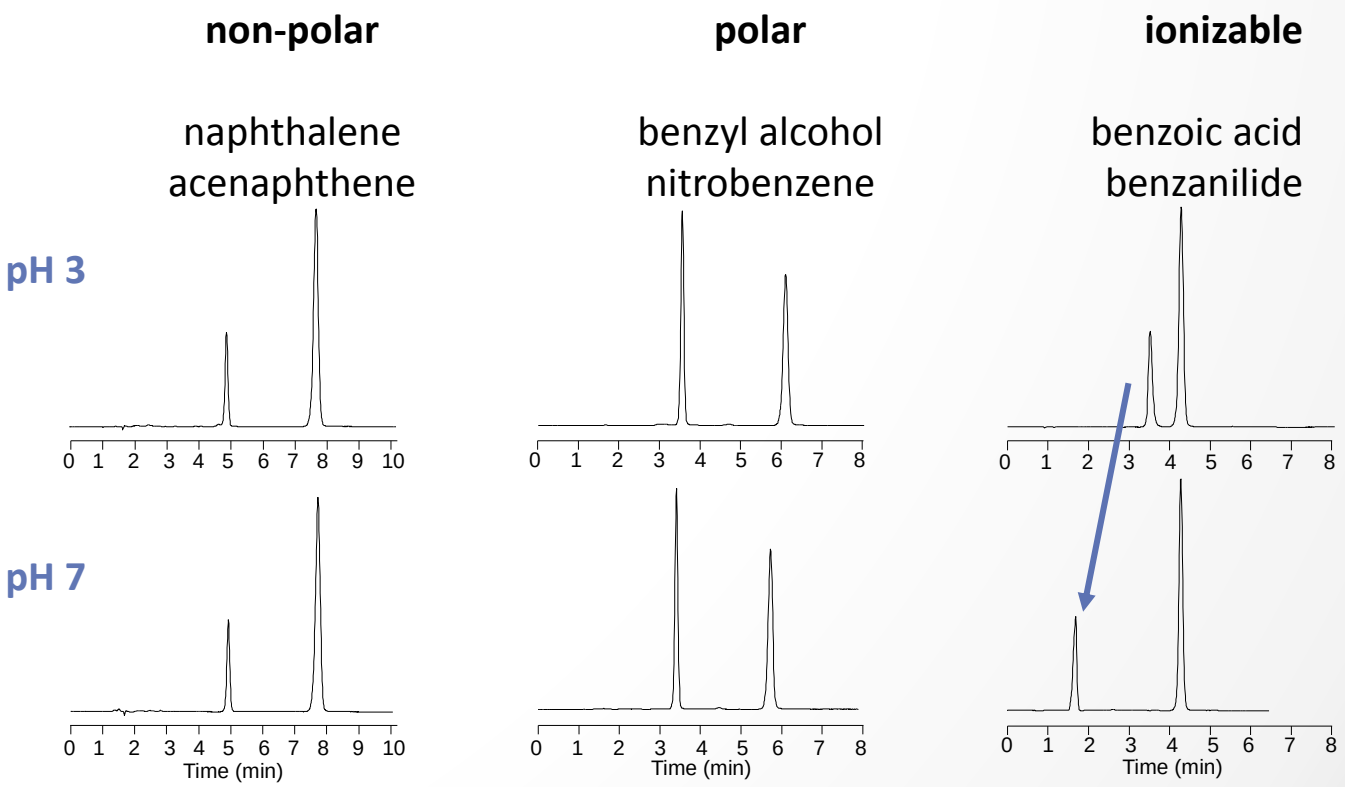
F5: Discovery HS-F5
C3: Zorbax 300SB-C3;
C8 Zorbax 300SB-C8
CN: Zorbax SB-CN
Bonus: Zorbax Bonus-RP
HC–COOH: home-made HC–COOH

S describes hydrophobic interaction between test sample solutes and stationary phase
B designates hydrogen bonding interactions
C acid/base interactions

Carr, P.W. et al., J. of Chrom. A, (2011) 1218, 6675–6687

2D LC Column Selection

Orthogonality in Dependence of Mobile Phase pH*



*Slide courtesy Jason Link, Agilent Technologies

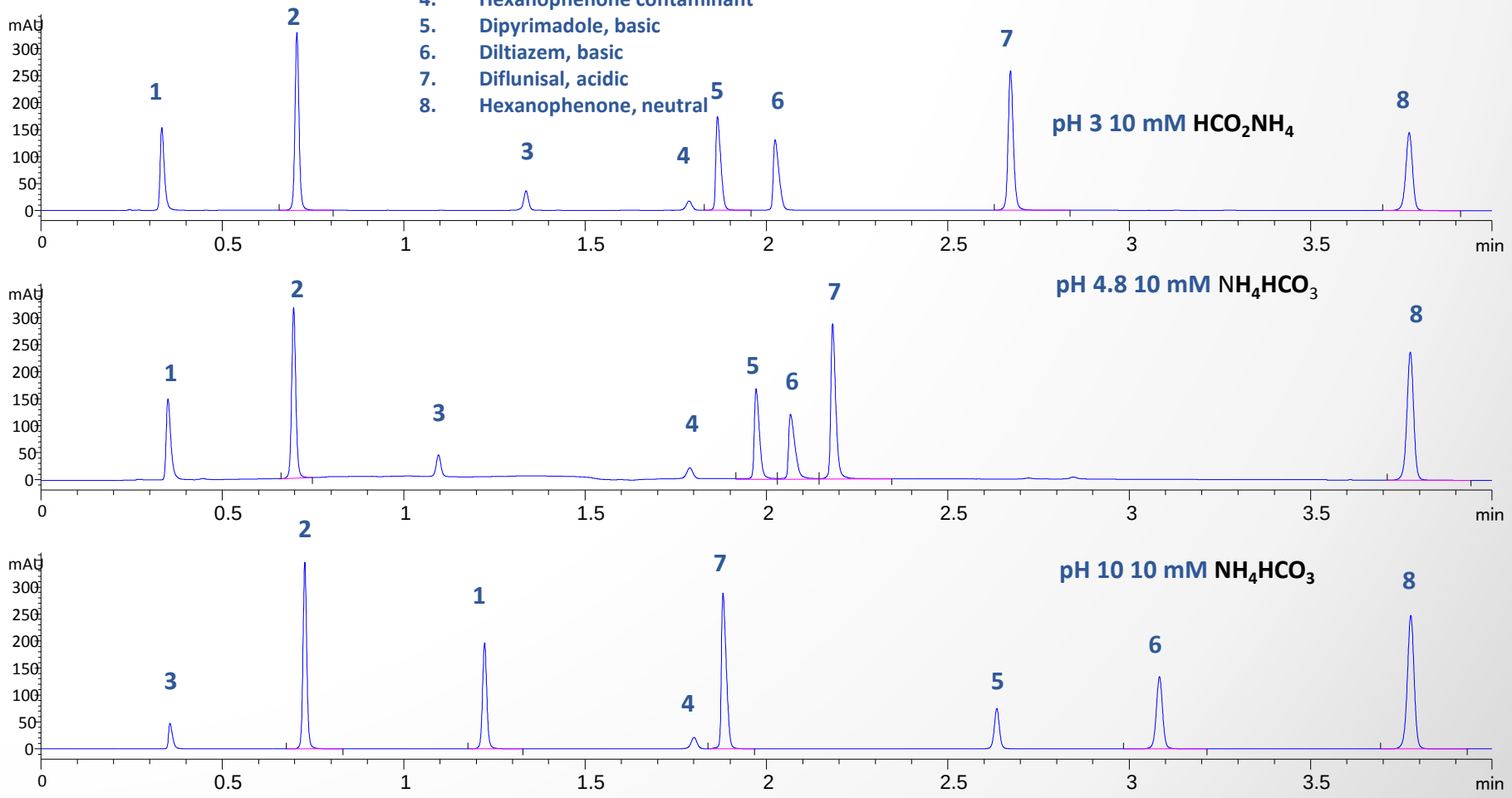
2D LC Column Selection

Orthogonality in Dependence of Mobile Phase pH*

1. Procainamide, pKa 9.2
2. Caffeine, pKa 14
3. Acetyl Salicylic Acid, pka 3.5
4. Hexanophenone contaminant
5. Dipyrimadole, basic
6. Diltiazem, basic
7. Diflunisal, acidic
8. Hexanophenone, neutral

Column: Agilent Poroshell HPH

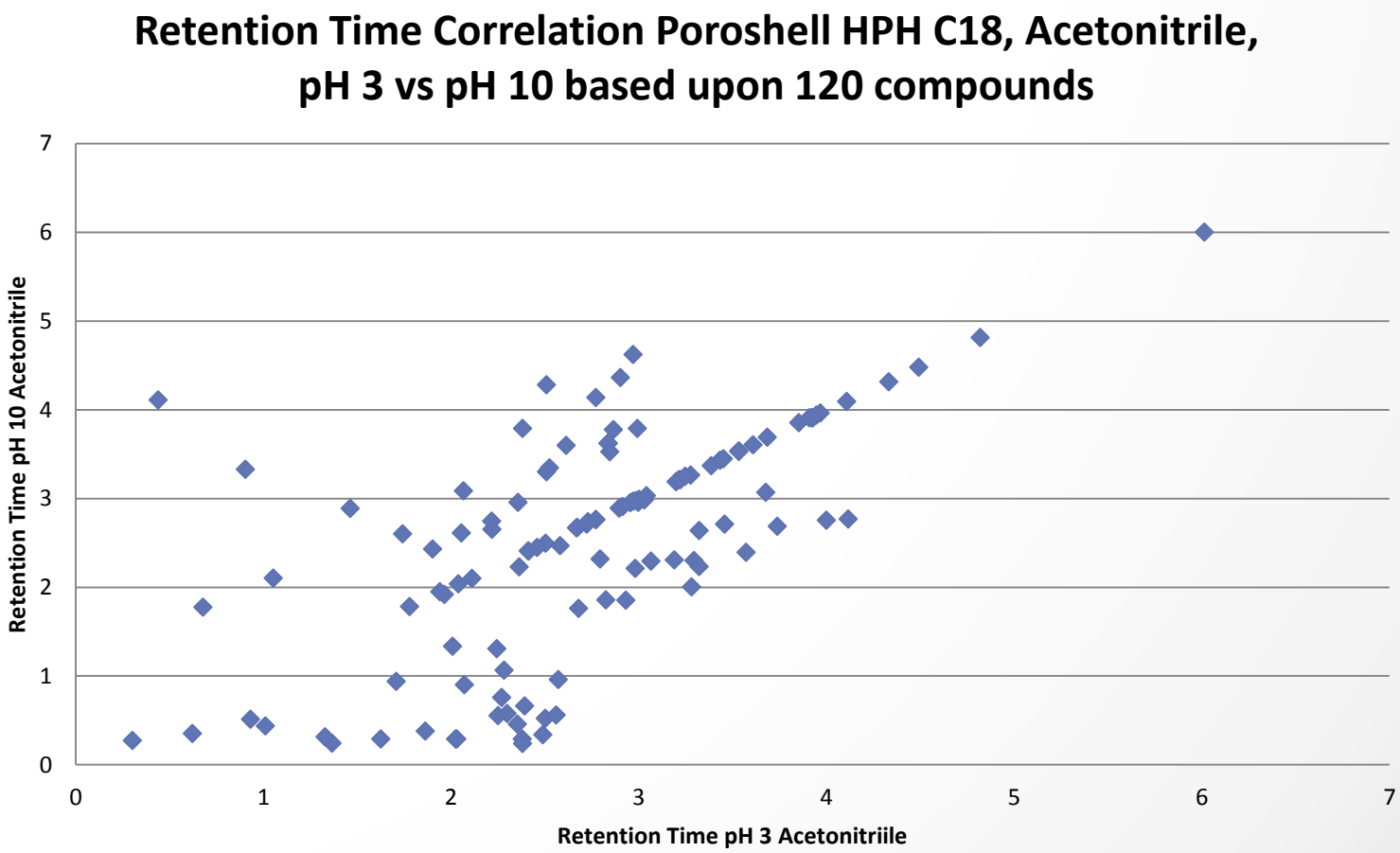
*Slide courtesy Jason Link, Agilent Technologies



Ionizable compounds – acids and bases change retention and selectivity significantly upon changes in eluent pH

2D LC Column Selection

Orthogonality in Dependence of Mobile Phase pH*



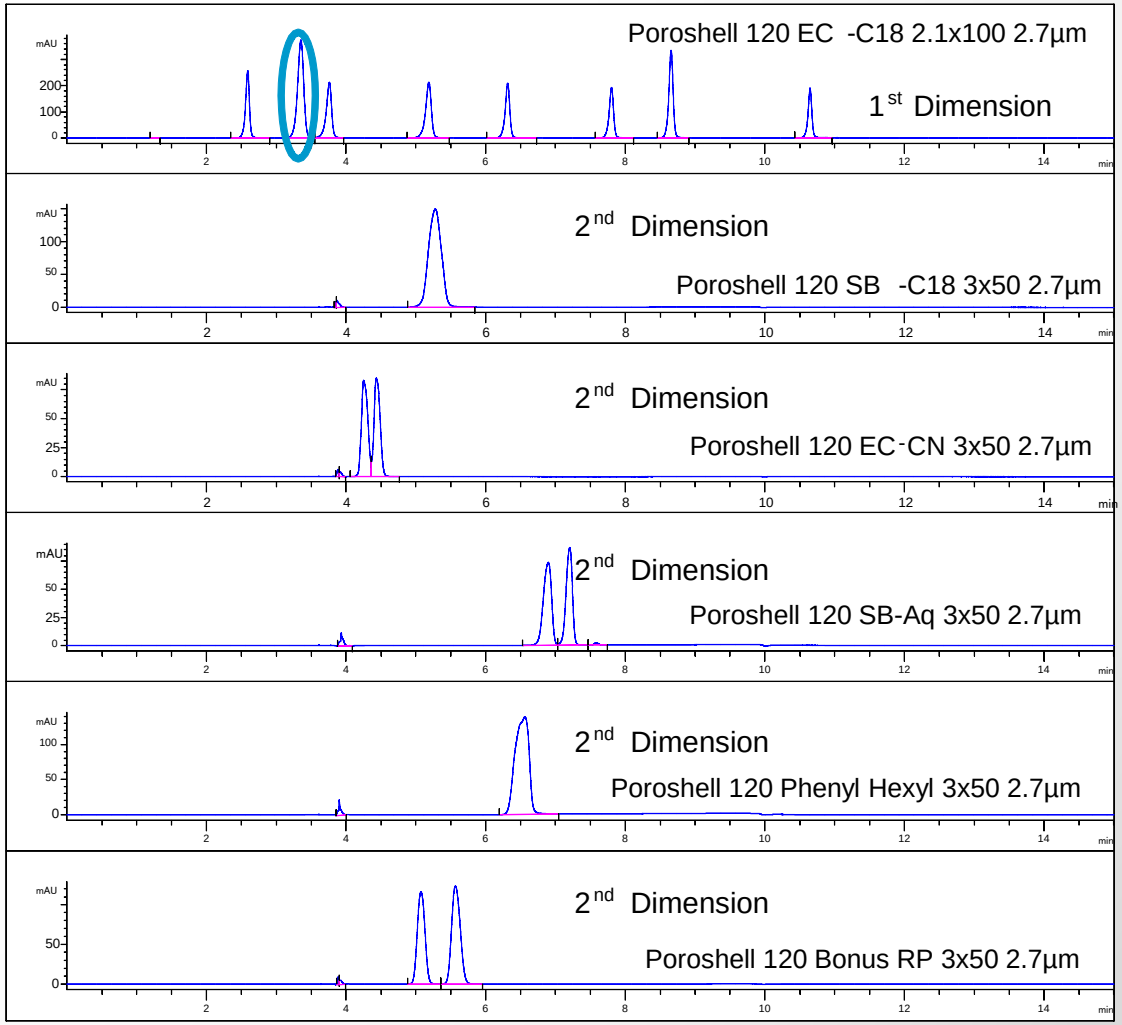
5 % to 95% over 4 minutes. Hold 1 minutes Chromatograms at pH 3 (ammonium formate), and pH 10 (ammonium bicarbonate) are shown using mass spec compatible buffers. The flow rate 0.42 ml/min. 254 nm Agilent 1260 2.1x 50 mm column Poroshell HPH C-18

*Slide courtesy Jason Link, Agilent Technologies

2D LC Column Selection

Orthogonality of RP phases

- Example mixture of several sulfa drugs
- Multiple chemistries utilized in the 2nd dimension
- Bonus RP demonstrated the best heart cut separation of the selected peak

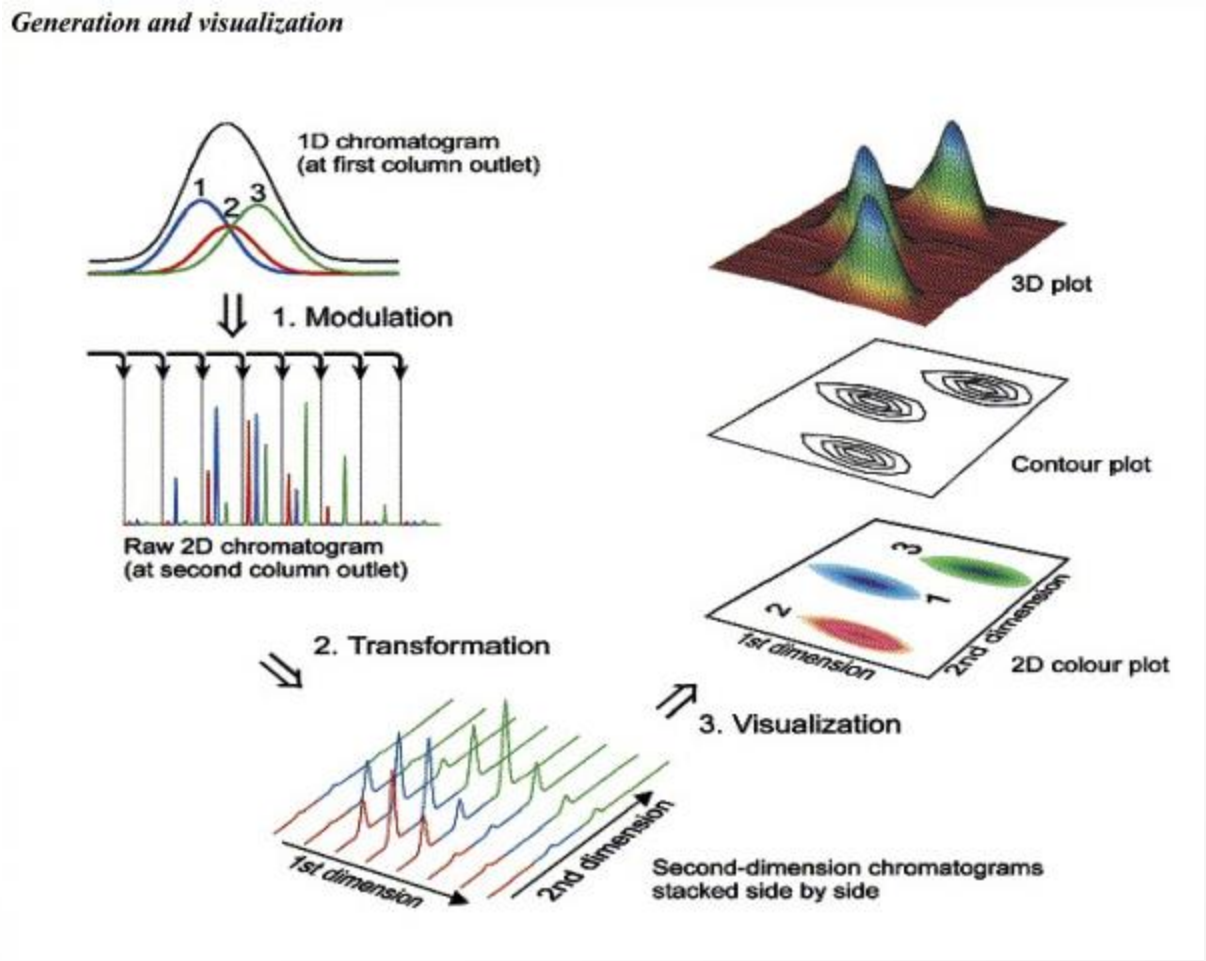


*Slide courtesy Jason Link, Agilent Technologies

Data Handling in Comprehensive 2D LC

Comprehensive 2D-LC

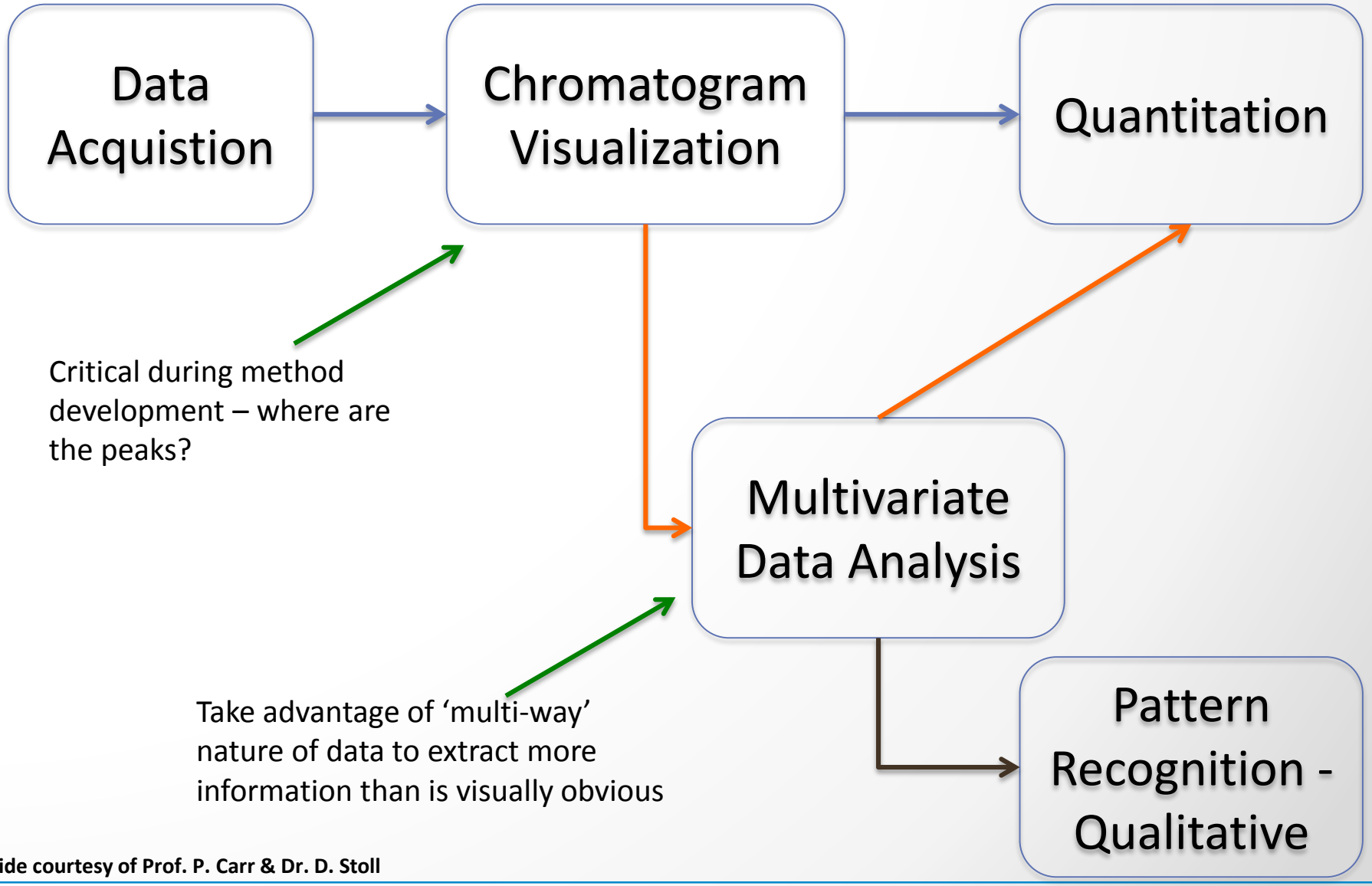
Data Handling



U.T.A. Brinkman et al., Trends in Analytical Chemistry, 2006, 25, 438–454.

Comprehensive 2D-LC

Data Handling



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

Comprehensive 2D-LC

Data Handling Features

- Detector signal background removal
- Peak detection
- Chromatogram visualization
- Chromatogram comparison; statistical analysis

Slide adapted from Prof. P. Carr & Dr. D. Stoll

Comprehensive 2D-LC

Data Handling Software

Commercial

ChromSquare - http://www.chromaleont.it/chromsquare.html

Kroungold Analytical - http://www.kroungold.com/LCLC.html

LC Image - http://www.gcimage.com/lcxlc/index.html (with Agilent Technologies)

Approaches described in the literature

S. Peters, G. Vivó-Truyols, P.J. Marriott, P.J. Schoenmakers, Development of an algorithm for peak detection in comprehensive two-dimensional chromatography, *Journal of Chromatography A*. 1156 (**2007**) 14–24.

Comprehensive 2D LC Method Development

Comprehensive 2D LC Method Development

Primary Considerations

- Comprehensive or (multiple) heartcutting
  - If n_c <20 consider heart cutting
  - If n_c >>20 do comprehensive 2D LC
- Select the stat. phase of the 1st and 2nd dimension
  - Consider structural properties of the solutes, hydrophobicity, polarity, H-bonding, ionization, size to make an informed choice
  - Use more retentive separation column for the 2nd dimension
- Isocratic or gradient separation
  - Consider the range of polarity, pK, hydrophobicity

Adapted from slide of Prof. P. Carr & Dr. D. Stoll

Comprehensive 2D LC Method Development

Further Considerations

- Specify the 2nd dimension separation
  - Particle size required for N_r
  - Column diameter and length; use 2.1 mm in modern UHPLC equipment and 4.6 mm in conventional HPLC equipment
  - Instrument; magnitude of the delay volume, allowable gradient speed, re-equilibration time
  - Calculate cycle time of 2nd dimension separation
- Fix the loop size ($V_{loop} = t_{cycle} \times {}^{1}F$)
- Determine flow rate of 1st dimension
- Determine modulation time (under filling of the loop is recommended strongly)
- Select the appropriate column diameter for 1D separation
- Set the injection volume for the 1D separation

Adapted from slide of Prof. P. Carr & Dr. D. Stoll

Comprehensive 2D LC Method Development

Example: System Evaluation*

Columns

- First dimension: Agilent ZORBAX RRHD Eclipse Plus C18, 150 × 2.1 mm, 1.8 µm
- Second dimension: Agilent ZORBAX RRHD Eclipse Plus Phenyl Hexyl, 50 × 3.0 mm, 1.8 µm

Separation 1st Dimension

- Solvent A: Water + 0.1% formic acid. Solvent B: Acetonitrile + 0.1% formic acid; Flow rate: 0.1 mL/min, Gradient: 5% B at 0 min 95% B at 30 min 95% B at 40 min; Stop time: 40 min. Post time: 15 min
- Sample: 20 component RP standard, 5 µL

Separation 2nd Dimension

- Solvent A: Water + 0.1% formic acid. Solvent B: Methanol + 0.1% formic acid. Flow rate: 3 mL/min; Gradient: 5% B at 0 min15% B at 0.5 min 5% B at 0.51 min 5% B at 0.65 min

Column Thermostate

- Agilent 8/4 Port/2 Position Valve (“Duo”)
- Two loops 80 µL, First-in-last-out configuration
- Switching time 0.65 min ➔ injection volume 2nd dimension separation 65 µL
- Temperature 1st dimension 25°C; temperature 2nd dimension 60°C

Gradient Optimization

- See next slide

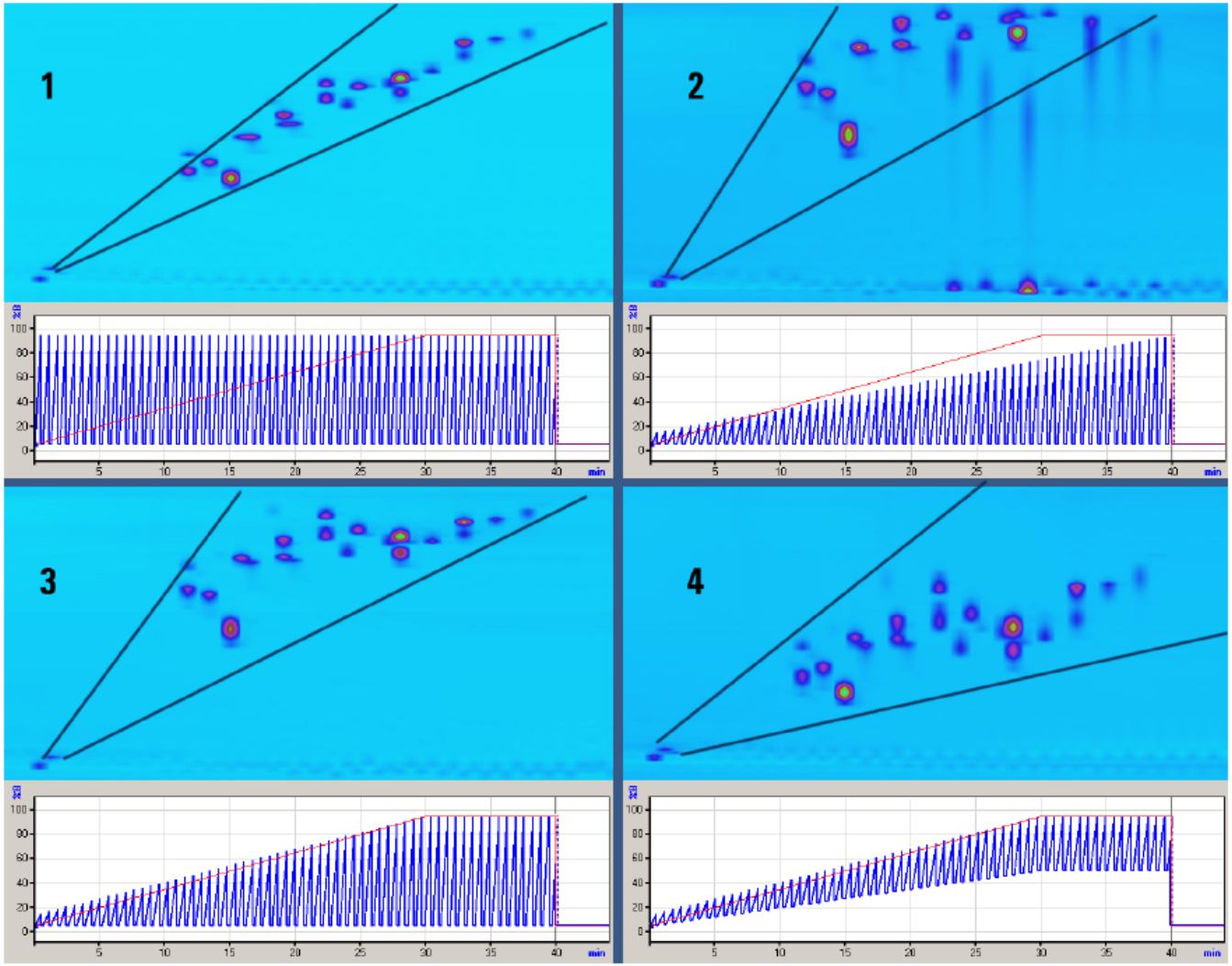
Software

- Agilent OpenL AB CDS ChemStation, Edition, version C.01.03 with
- 2D-LC add-on Software for 2D-LC data analysis from GC Image LLC, Lincoln, NE, USA

Taken from Agilent AppNote 5991-0138EN

Comprehensive 2D LC Method Development

2nd Dimension Gradient Optimization*



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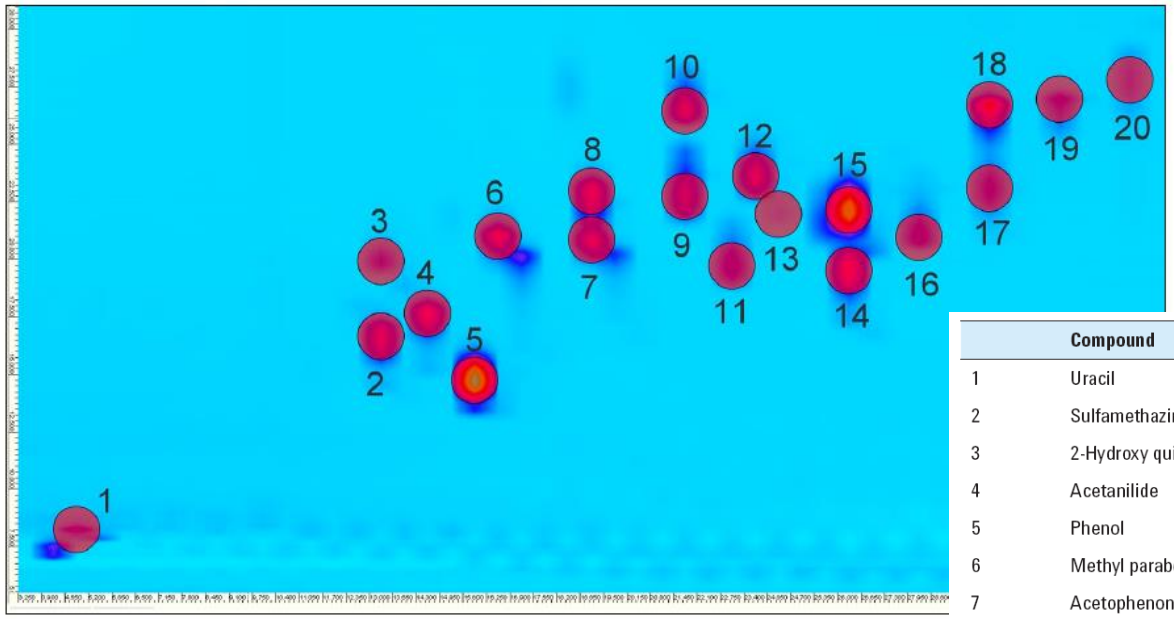
Comprehensive 2D LC Method Development

Resuls*

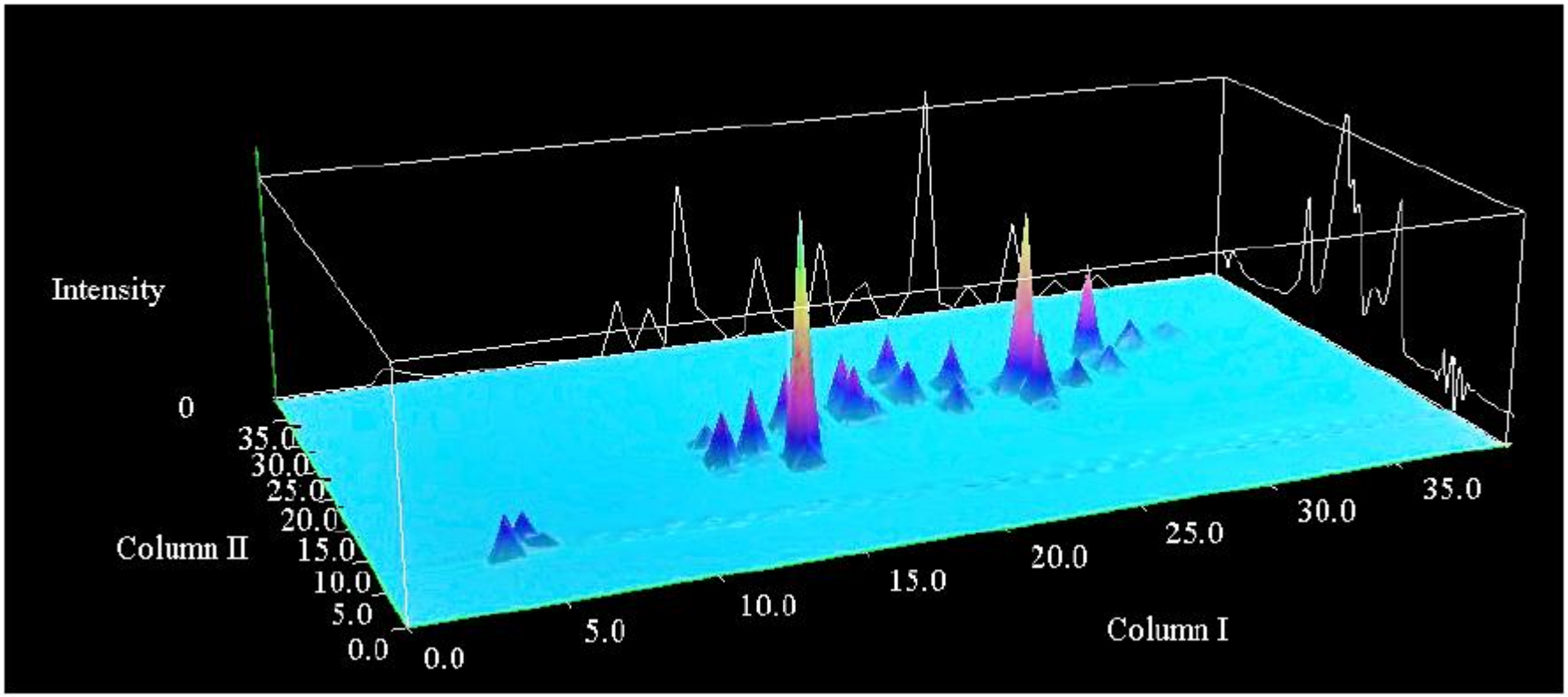


	Compound	RT I (min)	RT II (sec)
1	Uracil	4.55	8.32
2	Sulfamethazine	13.00	16.71
3	2-Hydroxy quinoline	13.00	20.06
4	Acetanilide	14.30	17.61
5	Phenol	15.60	14.56
6	Methyl paraben	16.25	20.95
7	Acetophenone	18.85	20.58
8	Ethyl paraben	18.85	22.88
9	Propyl paraben	21.45	22.65
10	N,N-Diethyl-m-toluamide	21.45	26.38
11	Propiophenone	22.75	19.81
12	Butyl paraben	23.40	23.71
13	Butyrophenone	24.05	21.65
14	Toluene	26.00	19.57
15	Benzophenone	26.00	22.21
16	Valerophenone	27.95	21.28
17	Hexanophenone	29.90	22.95
18	Heptyl paraben	29.90	26.62
19	Heptanophenone	31.85	26.89
20	Octanophenone	33.80	27.94

Taken from Agilent AppNote 5991-0138EN

Comprehensive 2D LC Method Development

Result*



Taken from Agilent AppNote 5991-0138EN

Comprehensive 2D LC Method Development

Example: Extra Virgin Olive Oil

Columns

- First dimension: Agilent ZORBAX RRHD Eclipse Plus, Phenyl-Hexyl, 2.1 x 150 mm, 1.8 µm
- Second dimension: Agilent ZORBAX RRHD Eclipse Plus, C18, 3.0 x 50 mm, 1.8 µm

Separation 1st Dimension

- Solvent A: water + 0.1% formic acid. Solvent B: methanol + 0.1% formic acid; Flow rate: 0.05 mL/min, Gradient: 5% B at 0 min 95% B at 60 min 95% B at 80 min; Stop time: 80 min. Post time: 30 min
- Sample: 20 µL

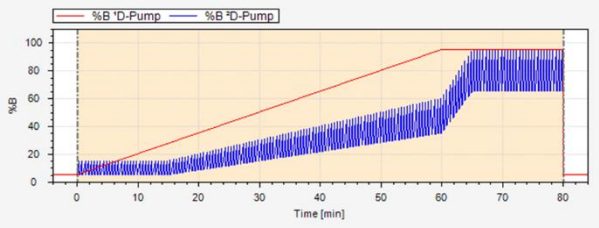
Separation 2nd Dimension

- Solvent A: Water + 0.1% formic acid. Solvent B: acetonitrile + 0.1% formic acid. Flow rate: 3 mL/min; Gradient: 5% B at 0 min15% B at 0.5 min 5% B at 0.51 min 5% B at 0.65 min

Column Thermostate

- Agilent 8/4 Port/2 Position Valve (“Duo”)
- Two loops 80 µL, First-in-last-out configuration
- Switching time 0.65 min ➔ injection volume 2nd dimension separation 65 µL
- Temperature 1st dimension 25°C; temperature 2nd dimension 60°C

Gradient Modulation



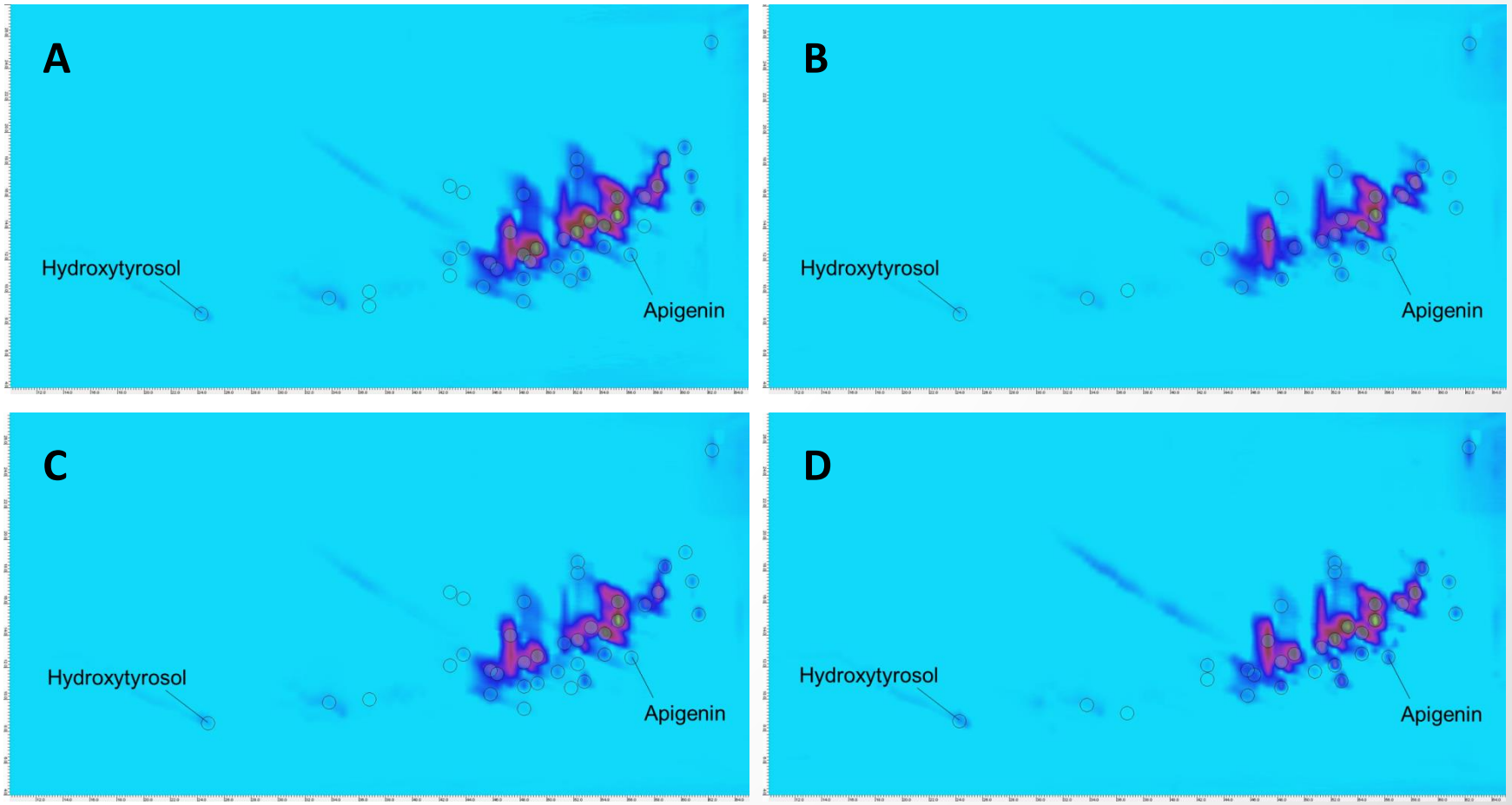
Software

- Agilent OpenL AB CDS ChemStation, Edition, version C.01.03 with
- 2D-LC add-on Software for 2D-LC data analysis from GC Image LLC, Lincoln, NE, USA

Taken from Agilent AppNote 5991-4515EN

Comprehensive 2D LC Method Development

Hydrophilic phenols from extra virgin olive oil



Taken from Agilent AppNote 5991-4515EN

Comprehensive 2D LC Method Development

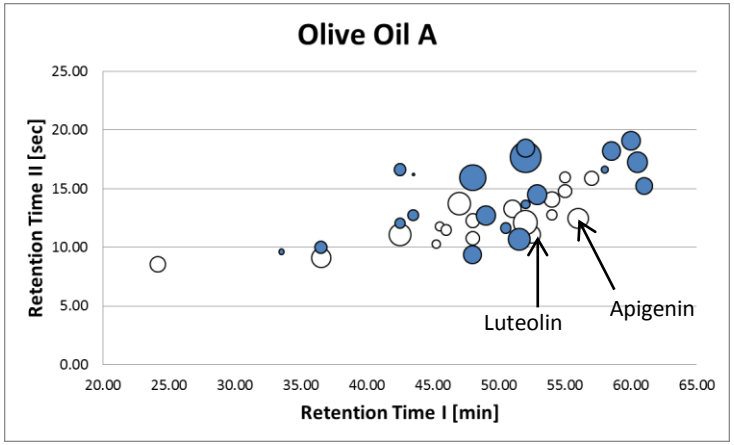
Hydrophilic phenols from extra virgin olive oil

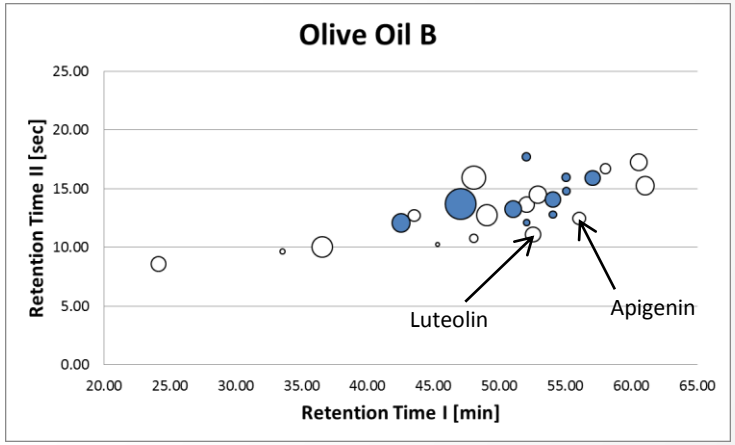
Compound name	Formula	Theoretical *m/z*	Mean Retention time I [min]	Mean Retention time II [sec]
Oleuropein aglycon	$C_{19}H_{22}O_8$	377.1242	48 - 55	13.6 - 16.0
Ligstroside aglycon	$C_{19}H_{22}O_7$	361.1293	52 - 61	14.5 - 17.7
Decarboxymethyl oleuropein aglycon	$C_{17}H_{20}O_6$	319.1187	47 - 52	12.1 - 13.7
Decarboxymethyl ligstroside aglycon	$C_{17}H_{20}O_5$	303.1238	51.03	13.29
Decarboxymethyl 10-hydroxy-oleuropein aglycon	$C_{17}H_{20}O_7$	335.1136	48.03	10.77
Elenolic acid	$C_{11}H_{14}O_6$	241.0718	33 - 45	9.6 - 10.3
Luteolin	$C_{15}H_{10}O_6$	285.0405	52.53	11.09
Apigenin	$C_{15}H_{10}O_5$	269.0455	56.03	12.47
Hydroxytyrosol	$C_8H_{10}O_3$	153.0557	24.16	8.57
Hydroxytyrosol acetate	$C_{10}H_{12}O_4$	195.0663	42.53	12.09

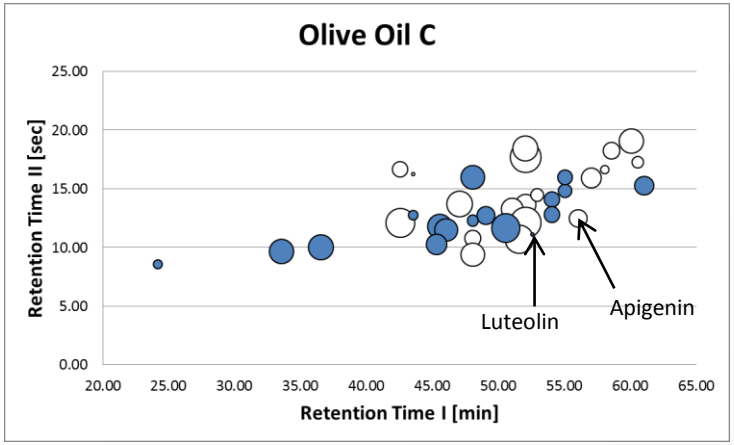
Taken from Agilent AppNote 5991-4515EN

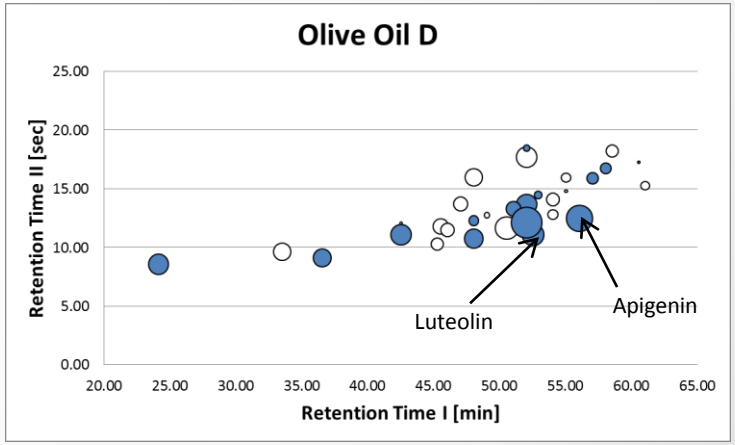
Comprehensive 2D LC Method Development

Differences of hydrophilic phenols from extra virgin olive oil









Taken from Agilent AppNote 5991-4515EN

Acknowledgements

- Dwight Stoll, Gustavus Adophus College, Saint Peter, MN USA
- Pete Carr, Univ. of Minnesota, Minneapolis, MN USA
- Jens Trafkowski, Stephan Buckenmaier, Agilent Technologies, Waldbronn, Germany
- Jason Link, Agilent Technologies, Wilmington, DE USA

Thank You for Your Attention

谢谢

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