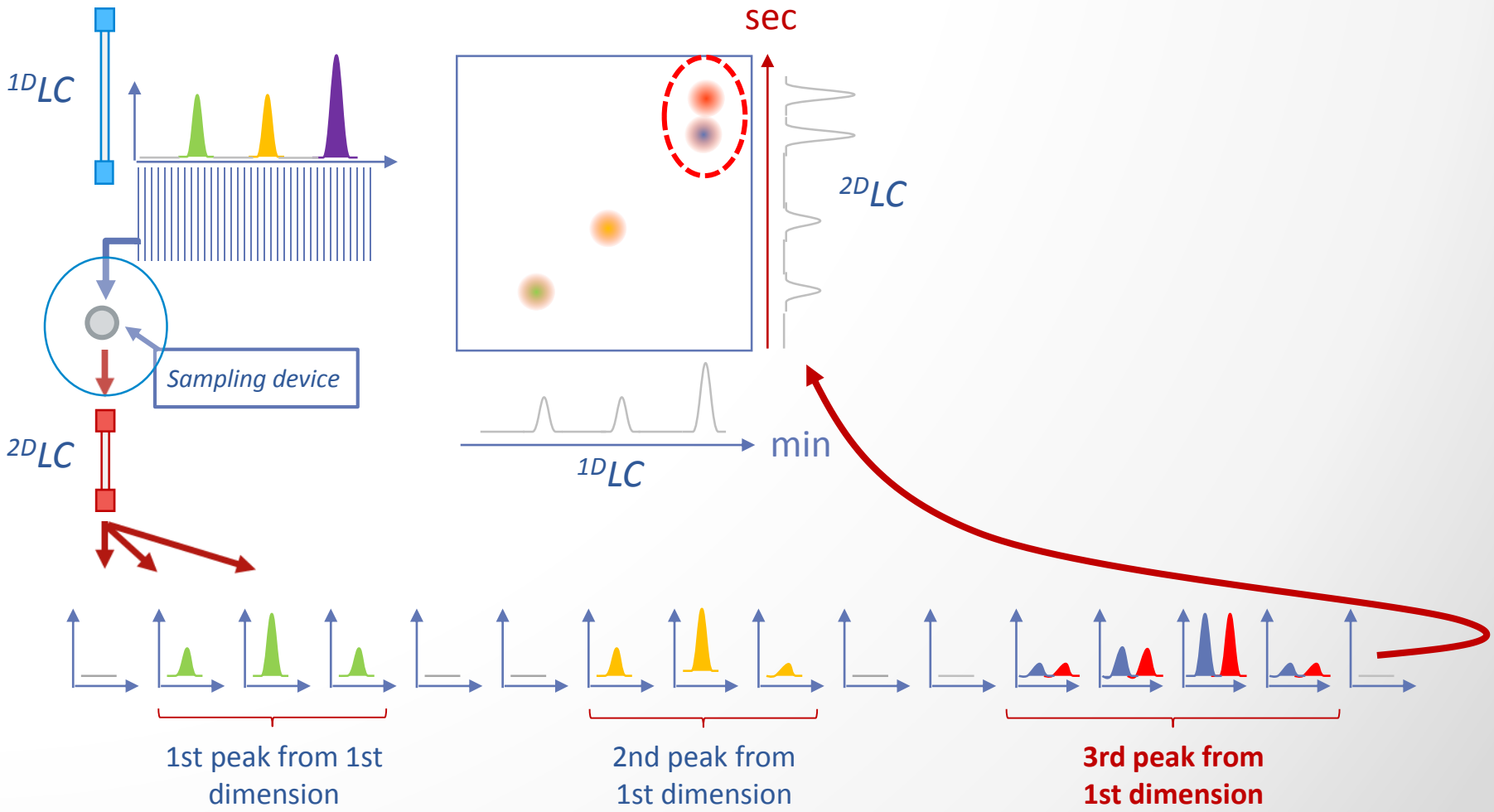
Multidimensional HPLC Tutorial Part - 2

Practical Implementation of 2D-LC, Column Selection, Data Handling of 2D-LC, Method Development for 2D-LC

Practical Implementation for 2D HPLC

Sampling Device for LCxLC

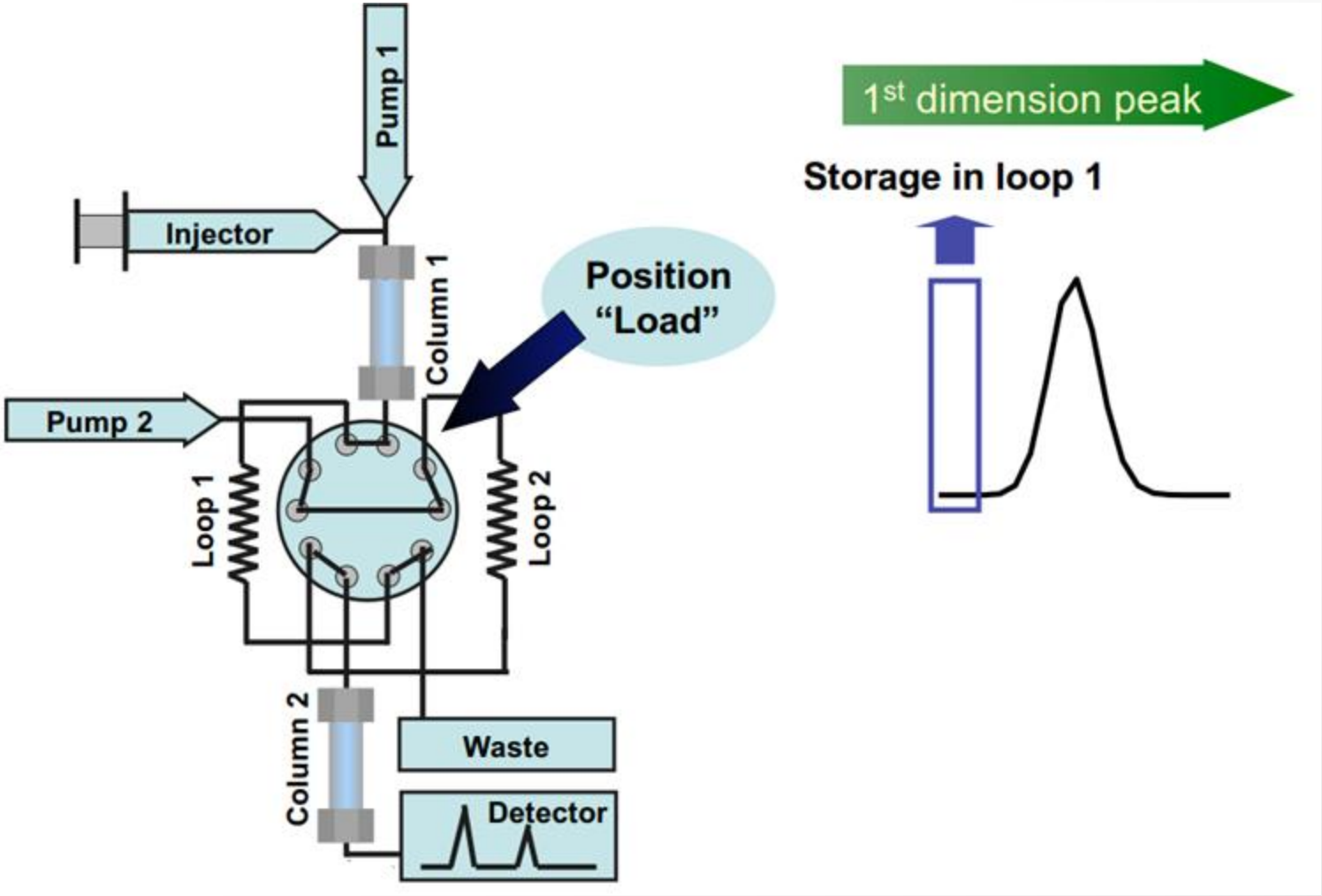


Slide courtesy of Agilent Technologies

Sampling Device for LCxLC

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

Microscale Separations and Bioanalysis

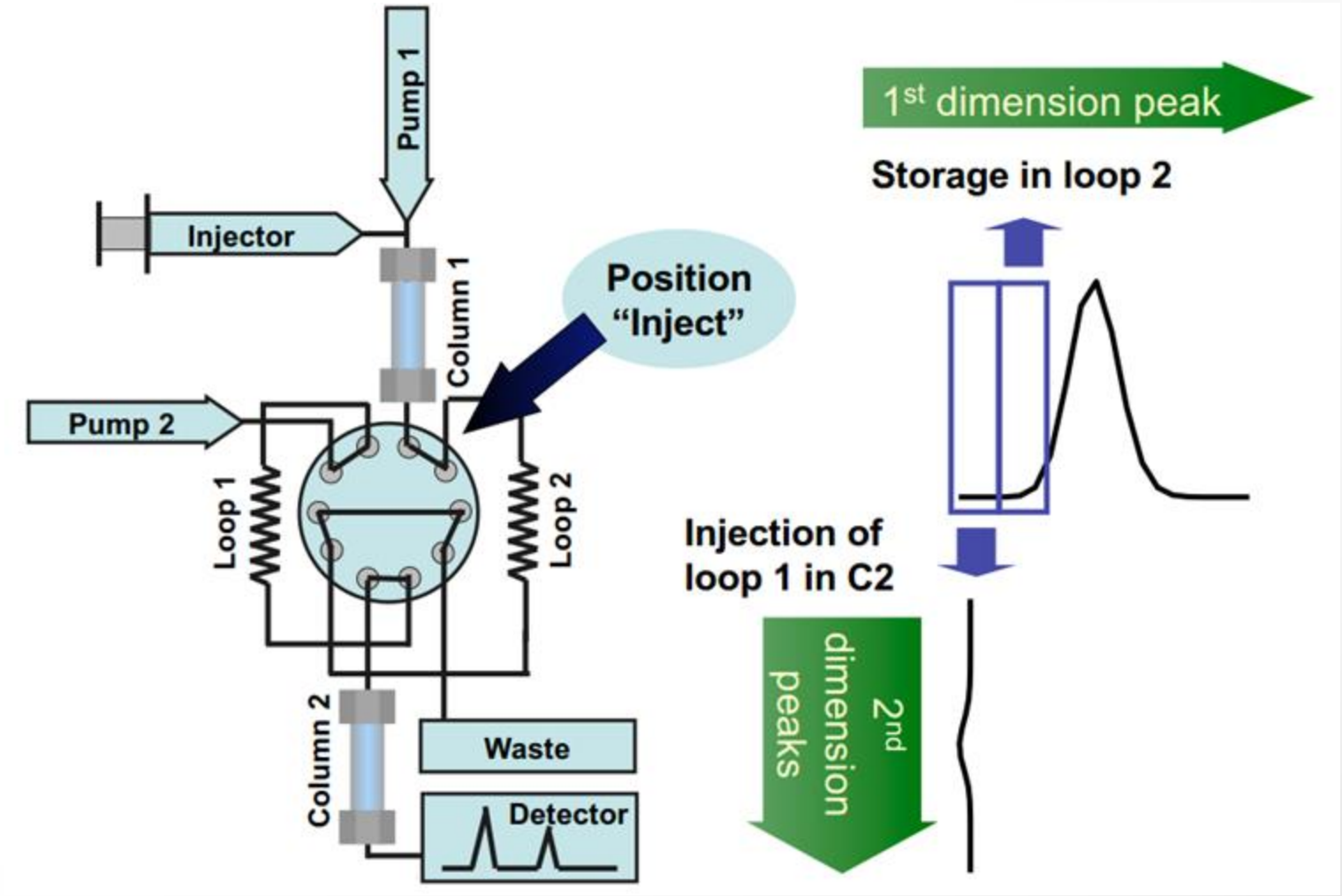


Slide courtesy of Prof. P. Schoenmakers

Sampling Device for LCxLC

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

Microscale Separations and Bioanalysis

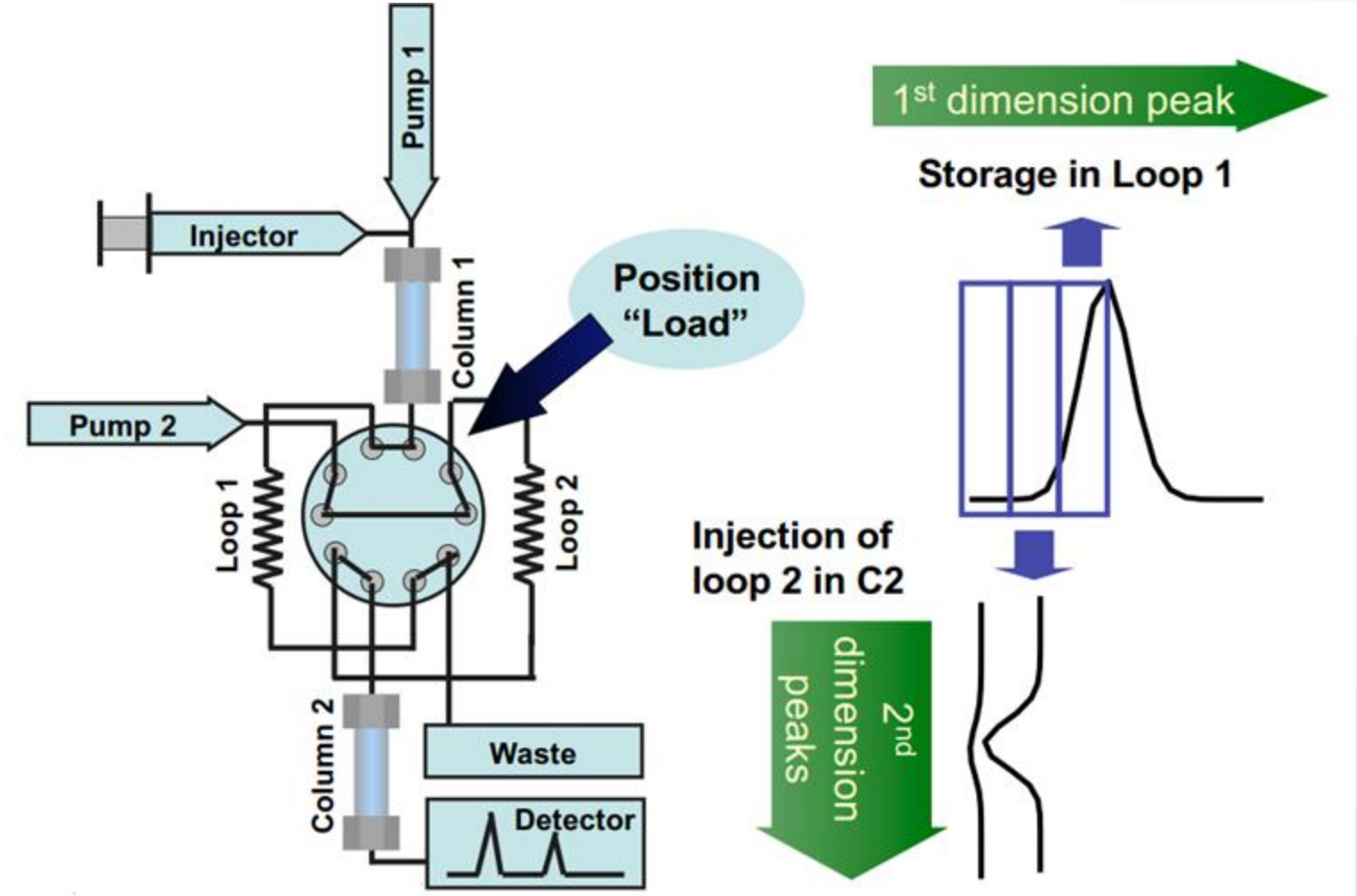


Slide courtesy of Prof. P. Schoenmakers

Sampling Device for LCxLC

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

Microscale Separations and Bioanalysis

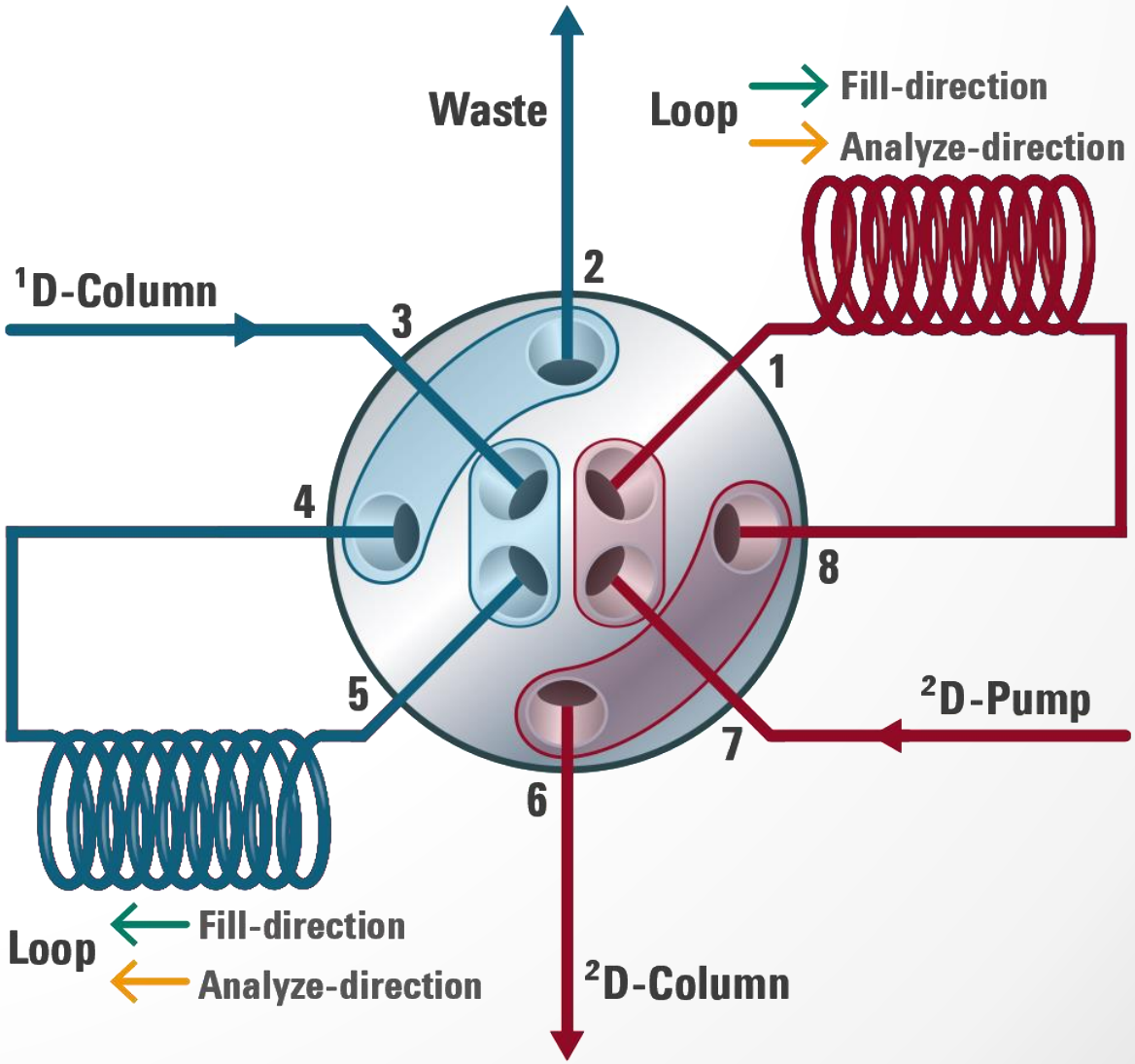


Slide courtesy of Prof. P. Schoenmakers

Sampling Device for LCxLC

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

Microscale Separations and Bioanalysis

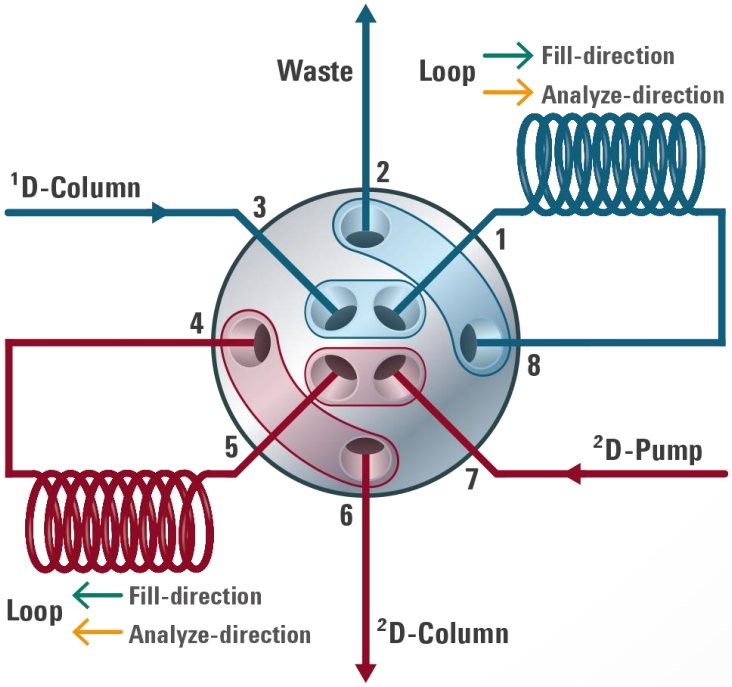


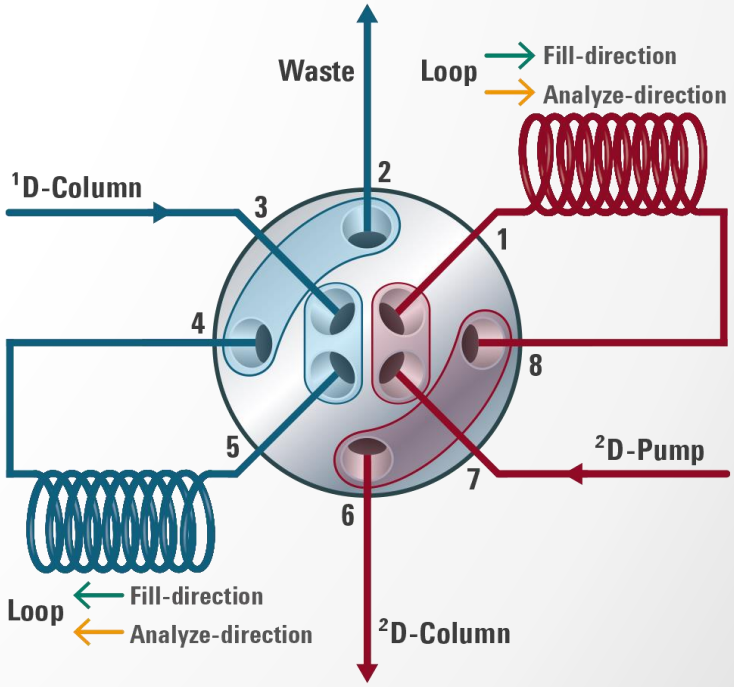
Slide courtesy of Agilent Technologies

Sampling Device for LCxLC

2x 4 port, 2 position valve (Agilent Technologies Duo Valve)

Microscale Separations and Bioanalysis





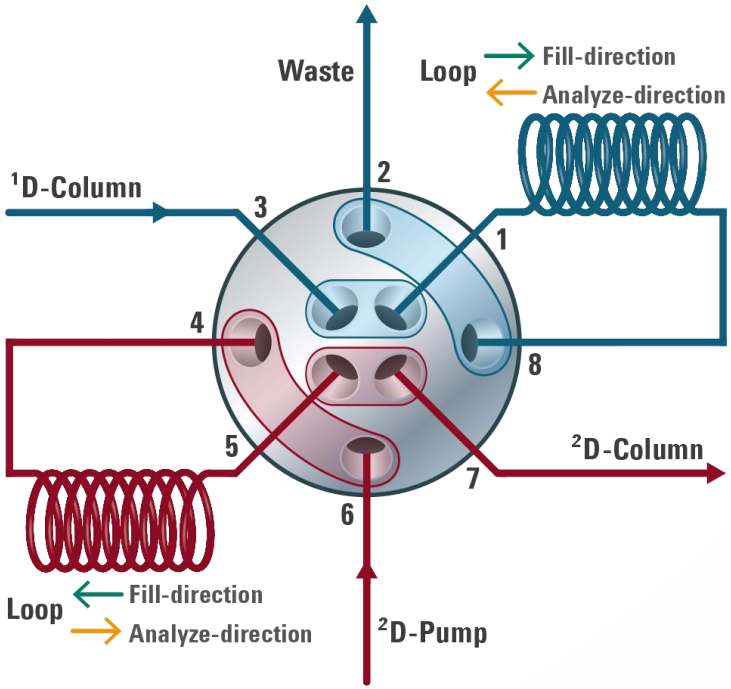
First-In-First-Out (FIFO)
co-current mode

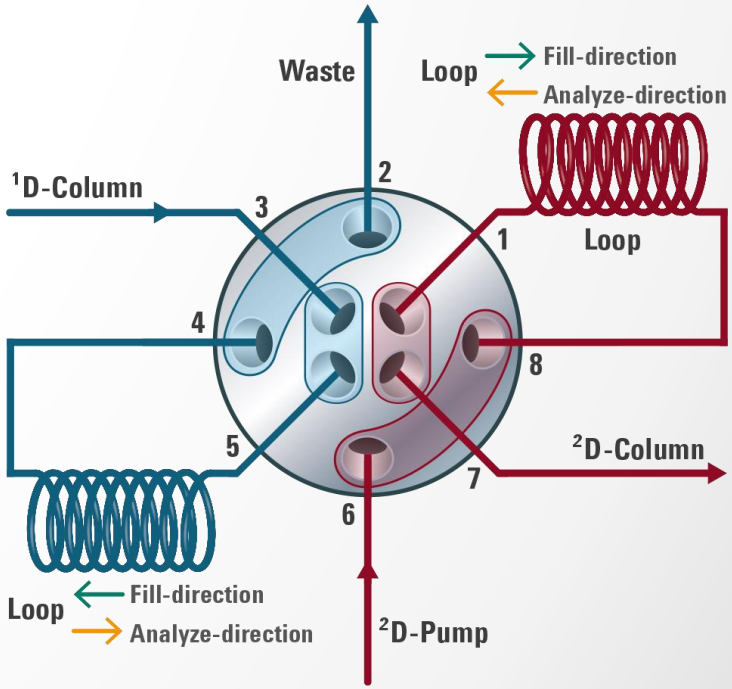
Slide courtesy of Agilent Technologies

Sampling Device for LCxLC

2x 4 port, 2 position valve (Agilent Technologies Duo Valve)

Microscale Separations and Bioanalysis





First-In-Last-Out (FILO)

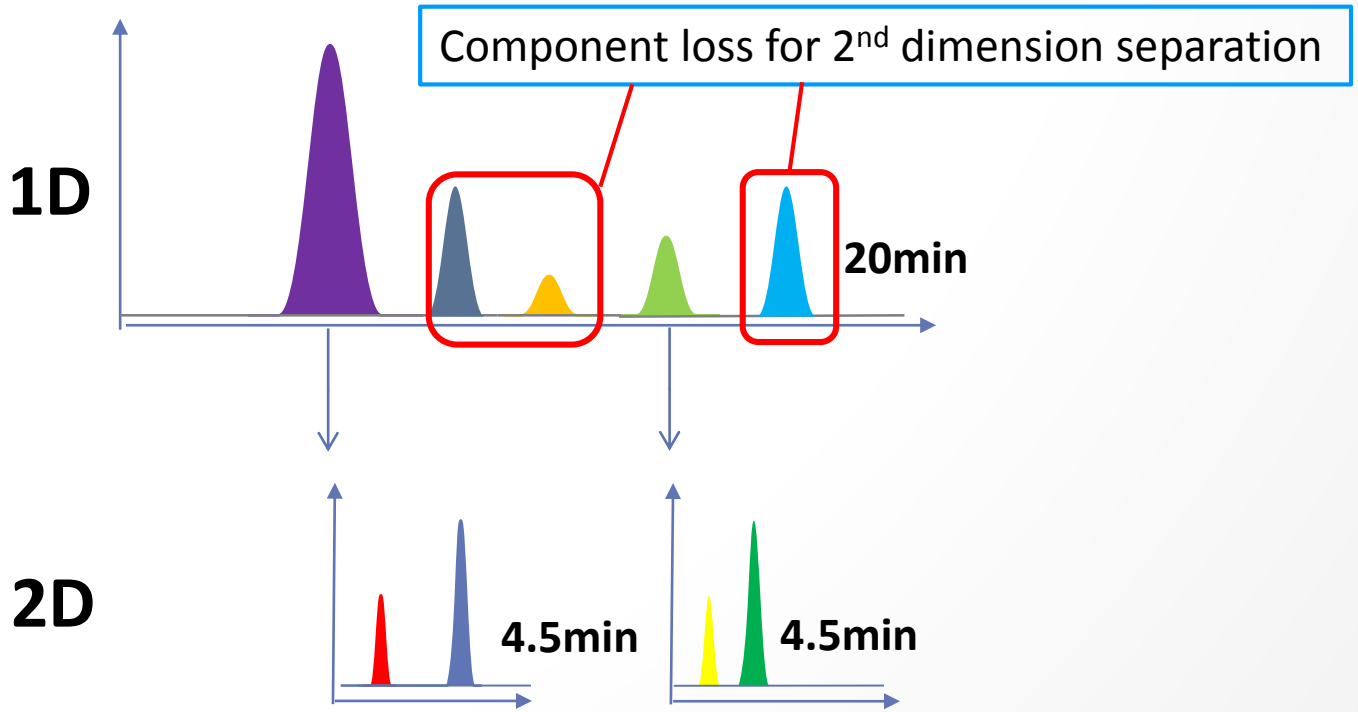
Counter-Current Mode: connections on port 6 and 7 reversed!

Slide courtesy of Agilent Technologies

Sampling Device for LC-LC (Heart-Cut)

Long Analysis Time of 2nd Dimension Separation

Microscale Separations and Bioanalysis



Heart-cutting Data Viewer

Slide courtesy of Agilent Technologies

Sampling Device for LC-LC (Heart-Cut)

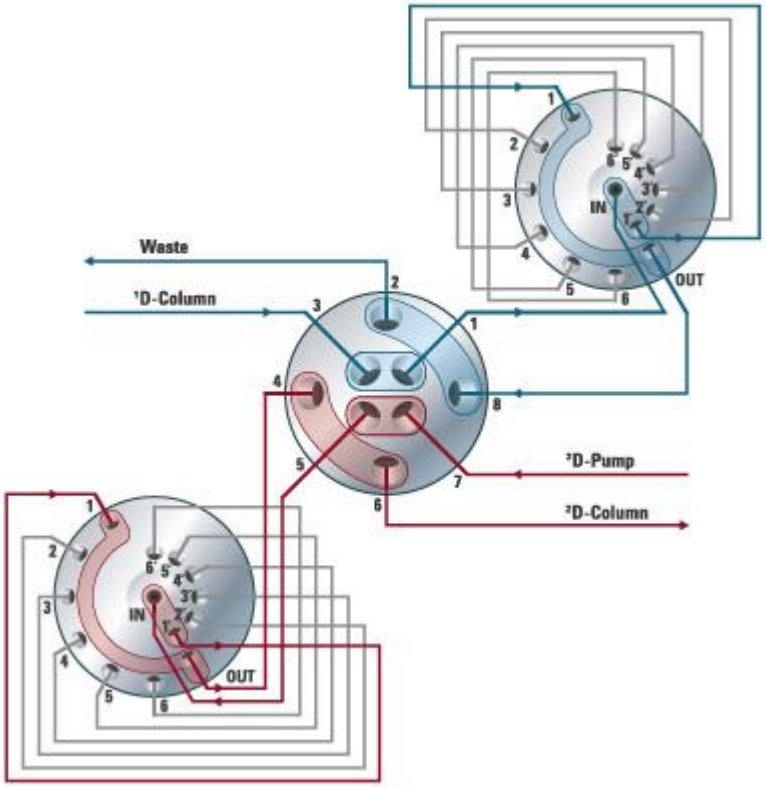
Agilent Multiple Heart-Cutting 2D-LC

Microscale Separations and Bioanalysis

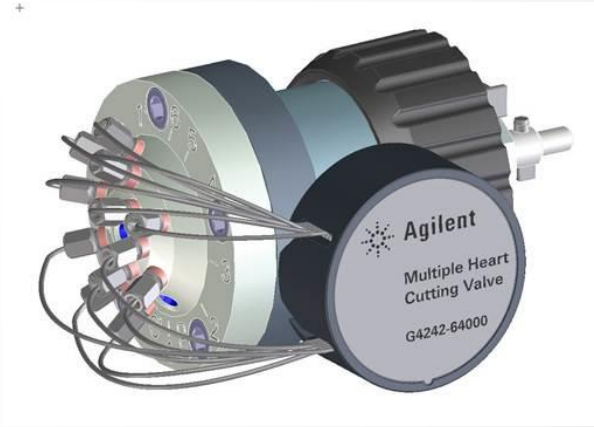
ROZING.COM Consulting

Smart Valve-Loop Setup with 12 loops

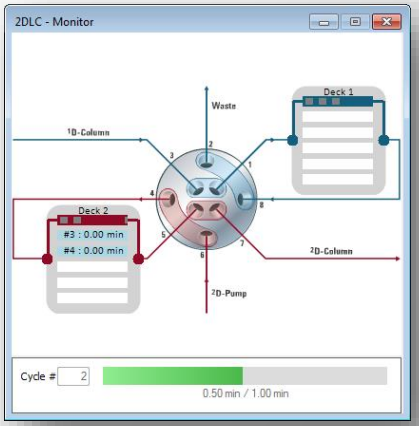
→ **2D-LC valve + two 6/14 valves**



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



Online status monitoring

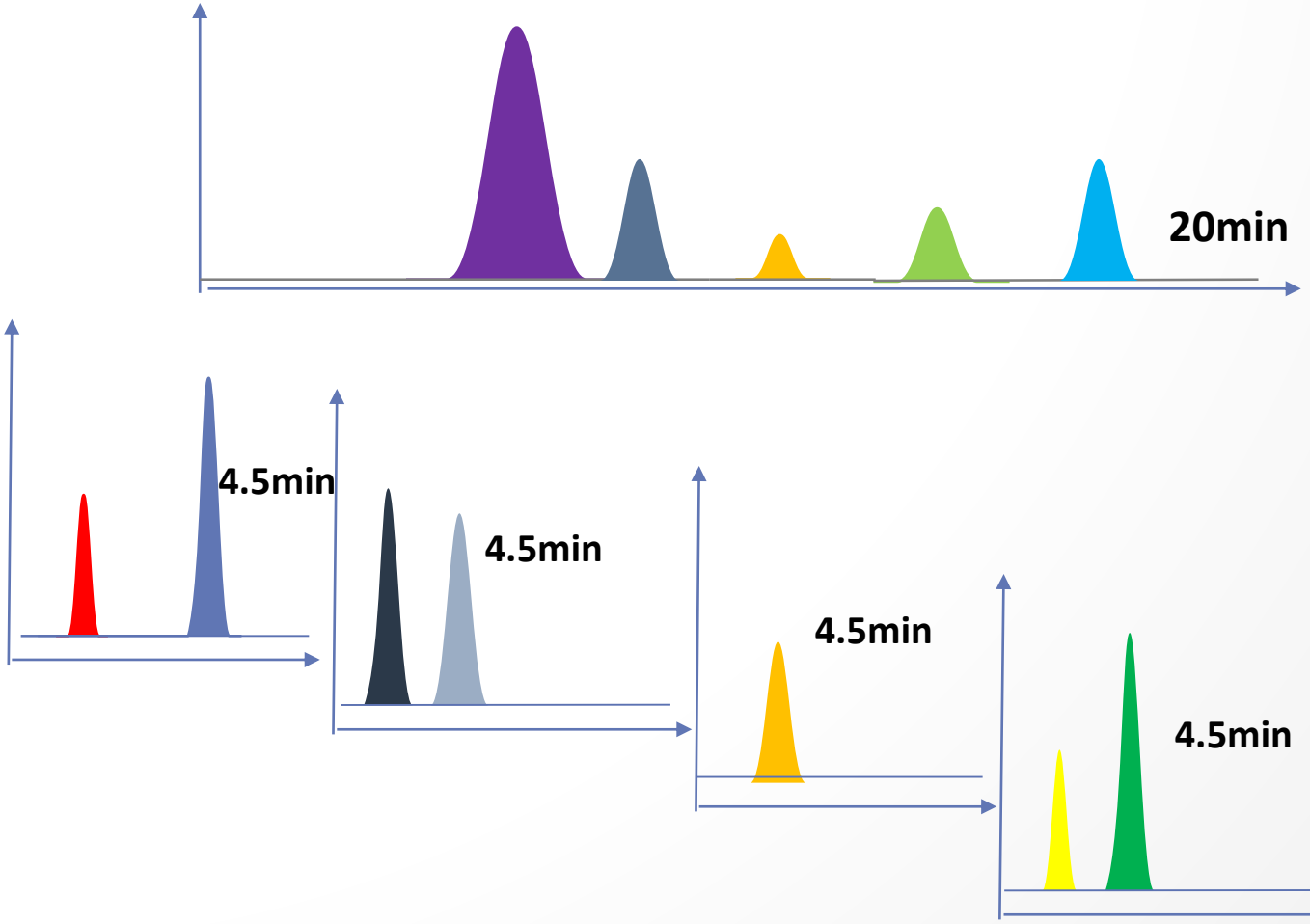


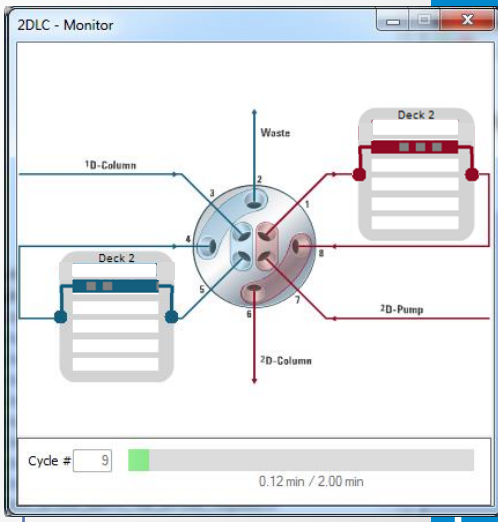
Slide courtesy of Agilent Technologies

Sampling Device for LC-LC (Heart-Cut)

Agilent Multiple Heart-Cutting 2D-LC

Microscale Separations and Bioanalysis





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

Slide courtesy of Agilent Technologies

2nd Dimension Separation

Cycle Time

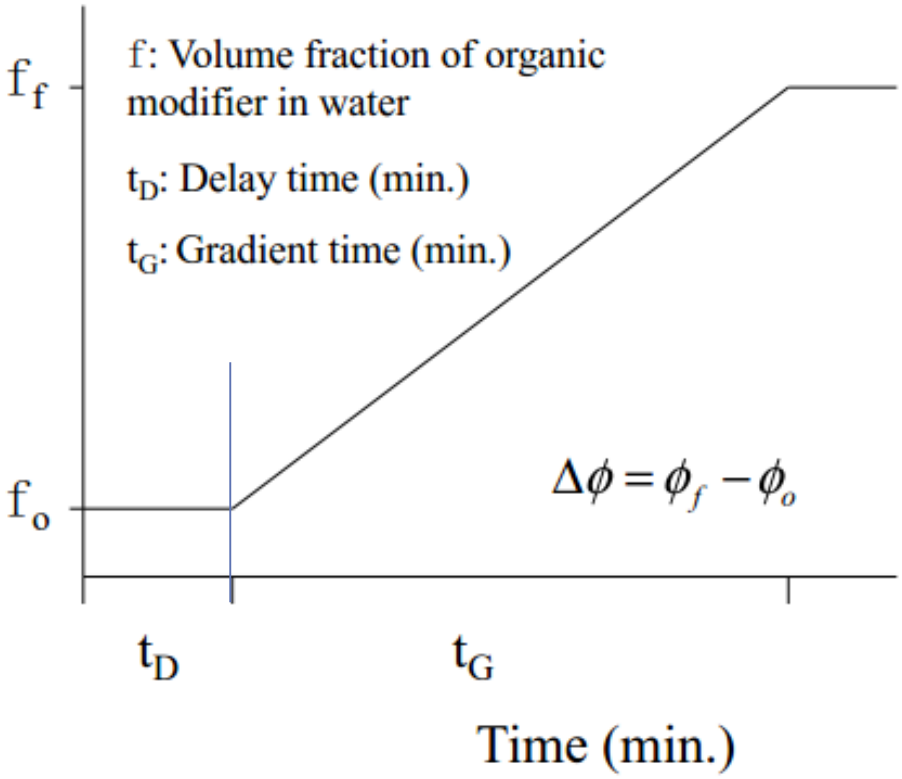
Microscale Separations and Bioanalysis

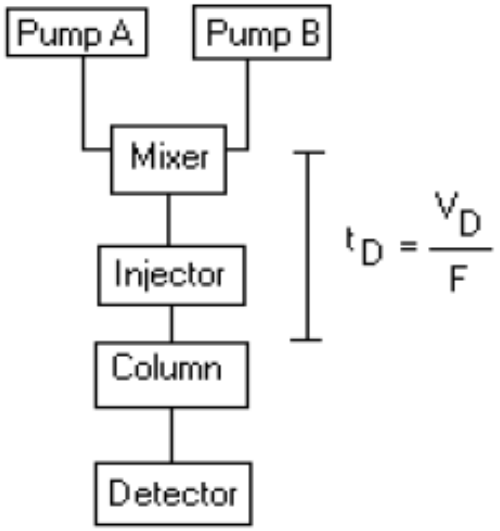
- We want to make the sampling time short
- In LC x LC $^1t_{sample} = {}^2t_{cycle}$
- Prefer $^1t_{sample} < {}^2t_{cycle}$ (under fill the sample loop!)
- $^2t_{cycle} = {}^2t_{gradient} + {}^2t_{re\text{-}equilibration}$
- So we don't want to make $^1t_{sample}$ too short since 2D separation peak capacity decreases if $^2t_{gradient}$ decreases
- ➔Fast, very high efficiency separation in the 2nd dimension

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.)

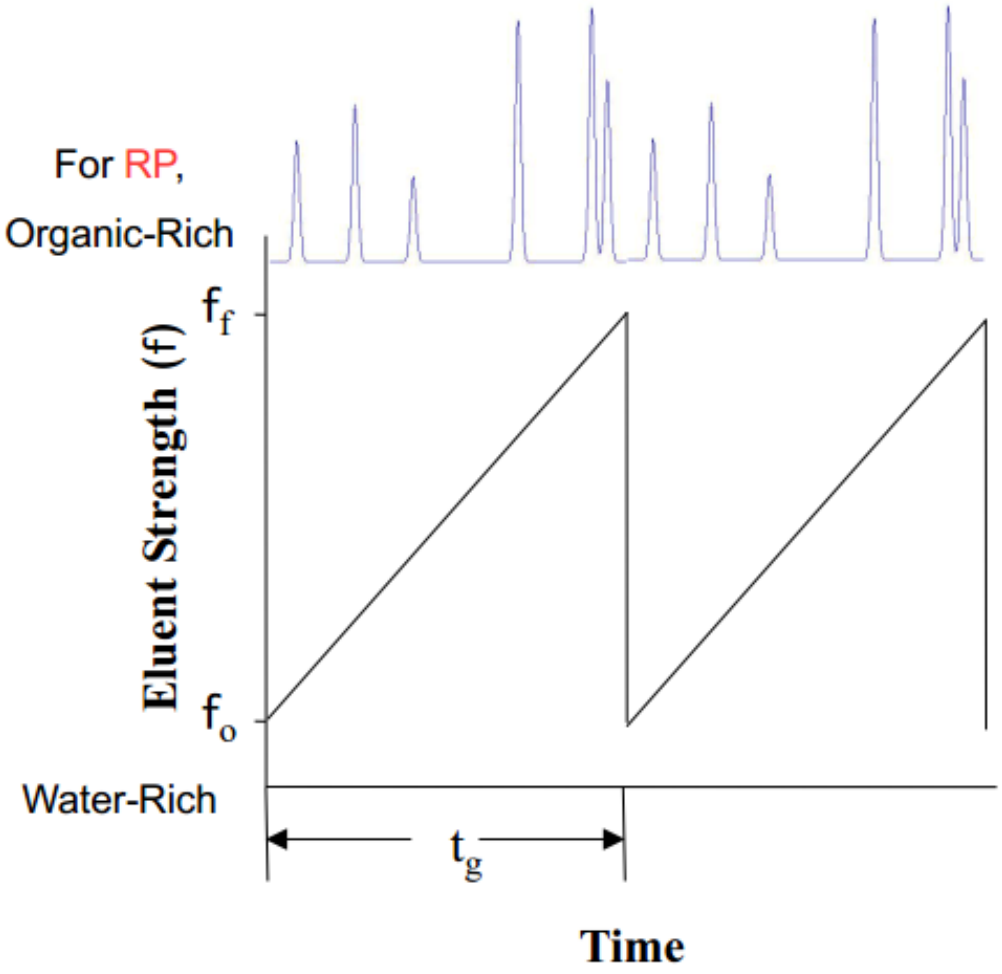
The delay volume is a unique and important property of the instrument and a BIG PROBLEM for the second dimension of 2DLC

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

2nd Dimension Separation

Ideal Situation

Microscale Separations and Bioanalysis

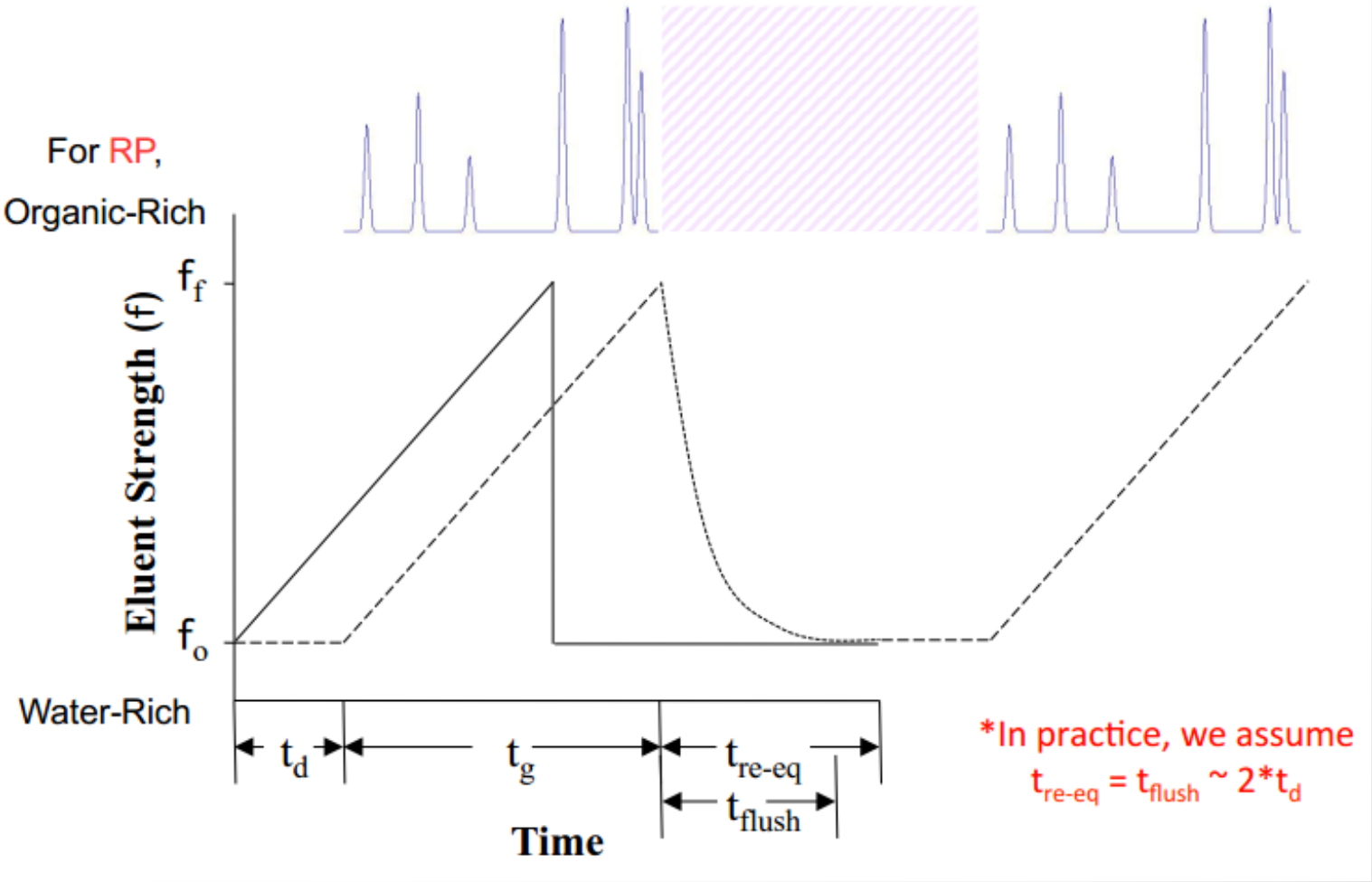


Slide courtesy of Prof. P. Carr & Dr. D. Stoll

2nd Dimension Separation

Real Situation

Microscale Separations and Bioanalysis



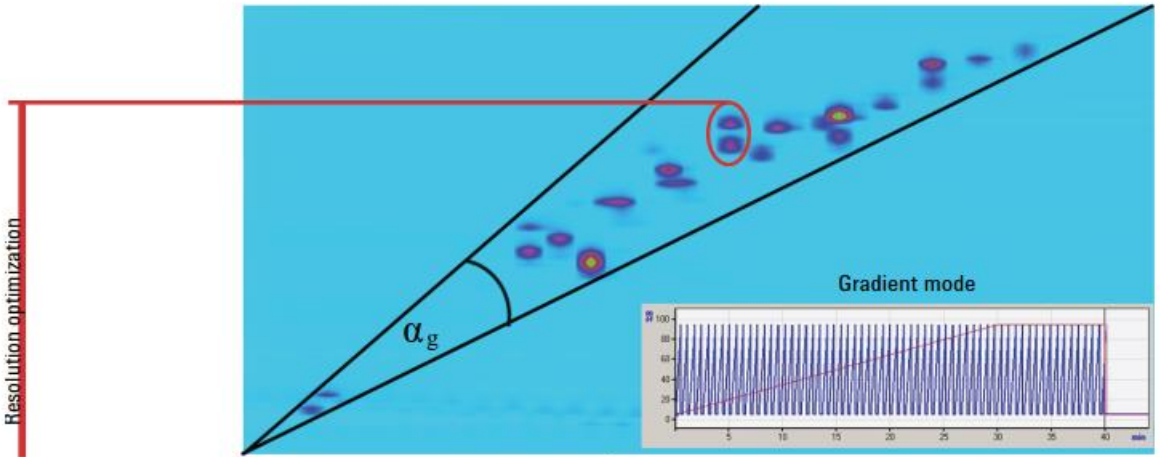
$$t_{cycle} = t_d + t_{gradient} + t_{re-equilibrate} = t_d + t_{gradient} + 2.V_{2Dcolumn}.F$$

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

2nd Dimension Separation

Optimize Gradient Separation

Microscale Separations and Bioanalysis



First dimension gradient

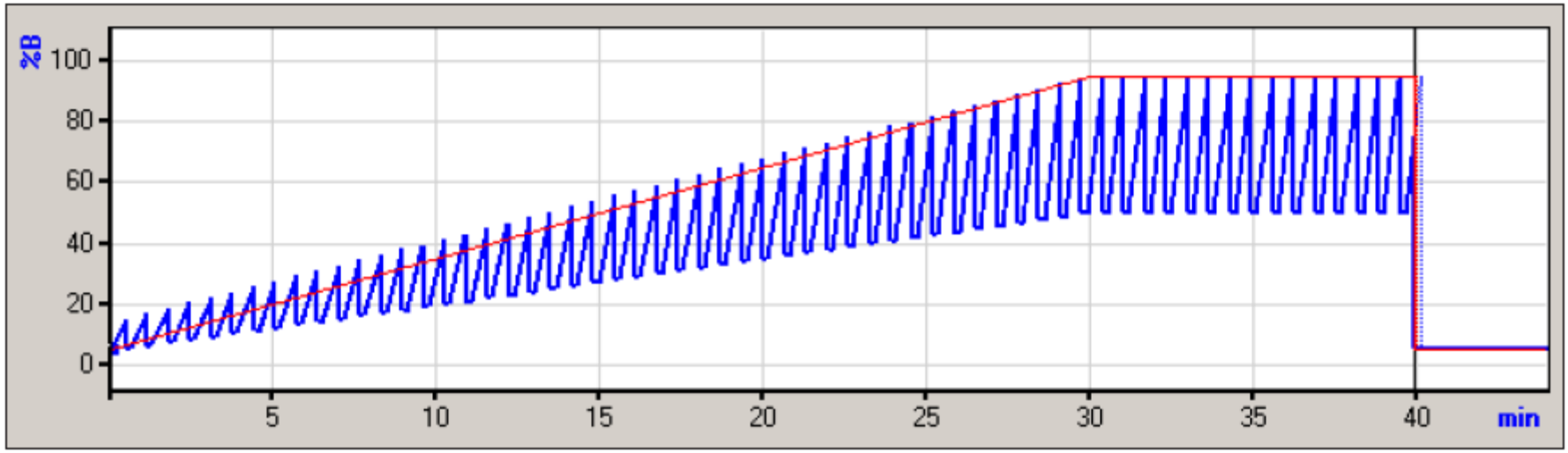
Second dimension gradient

ROZING.COM Consulting

2nd Dimension Separation

Optimize Gradient Separation

Microscale Separations and Bioanalysis



Adapt start composition of 2nd dimension gradient separation!!

Slide courtesy of Agilent Technologies

Column Selection for 2D LC

2D-LC Column Stationary Phase Selection

Considerations

Microscale Separations and Bioanalysis

ROZING.COM Consulting

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 Stationary Phase Selection

Is RPxRP a good choice?

Microscale Separations and Bioanalysis

- 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
- 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 Stationary Phase Selection

Mode Combinations

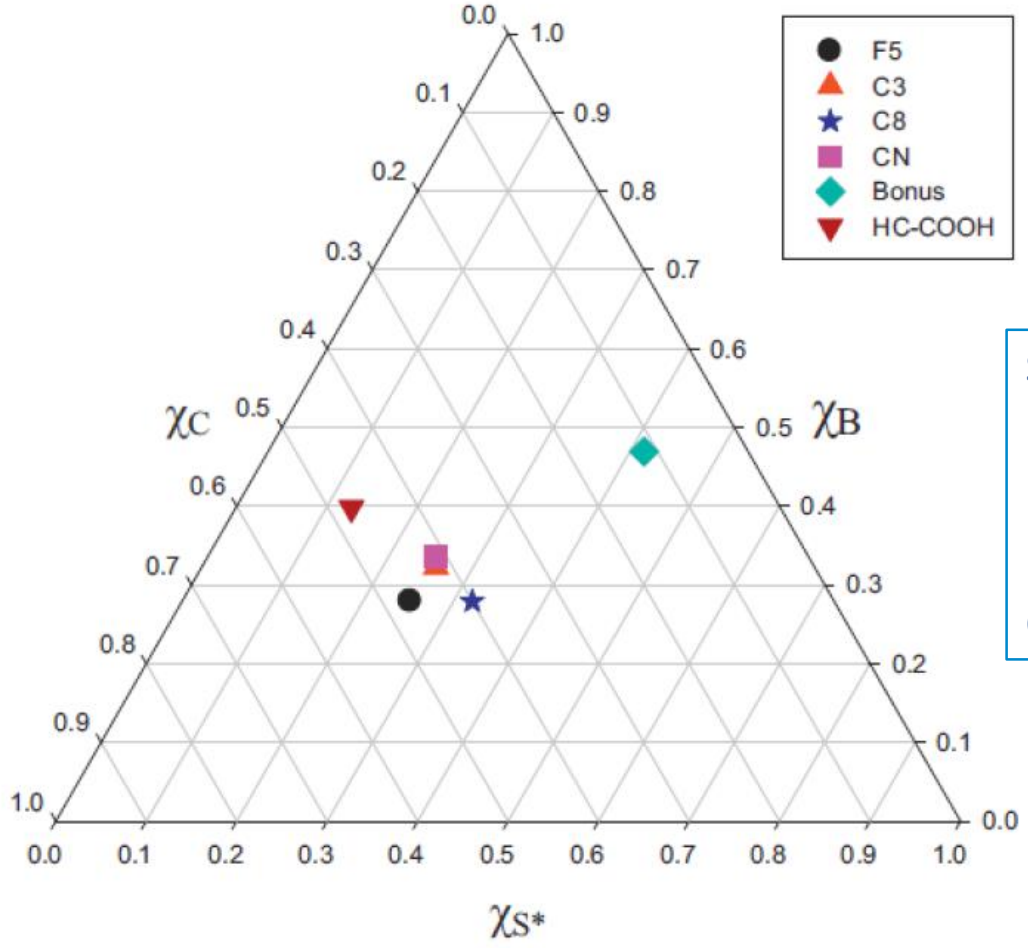
Microscale Separations and Bioanalysis

Modes	Orthogonality	Peak Capacity	Applications	Comments
RP x RP	**	******	Metabolomics, Food, Consumer Goods, Environment	Broadest Application Range, Wide Stationary Phase Range, UHPLC possible
IEC x RP	*****	*****	Proteomics, Peptide Analysis	
SEC x RP	**	***	Polymers, Proteomics	
HILIC x RP	****	****	Polymers, Pharmaceuticals, Natural Products	Mind Solvent Compatibility!
AC x RP	****	***	Bio- activity, bio-marker discovery	
SEC x NP	***	***	Polymer Characterization	
SEC x IEC	***	**	Proteomics	

2D LC Column Stationary Phase Selection

Selectivity matters; RP phases

Microscale Separations and Bioanalysis



F5: Discovery HS-F5 (Pentafluorophenylpropyl)
C3: Zorbax 300SB-C3;
C8 Zorbax 300SB-C8
CN: Zorbax SB-CN
Bonus: Zorbax Bonus-RP (embedded amide C14-alkyl)
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

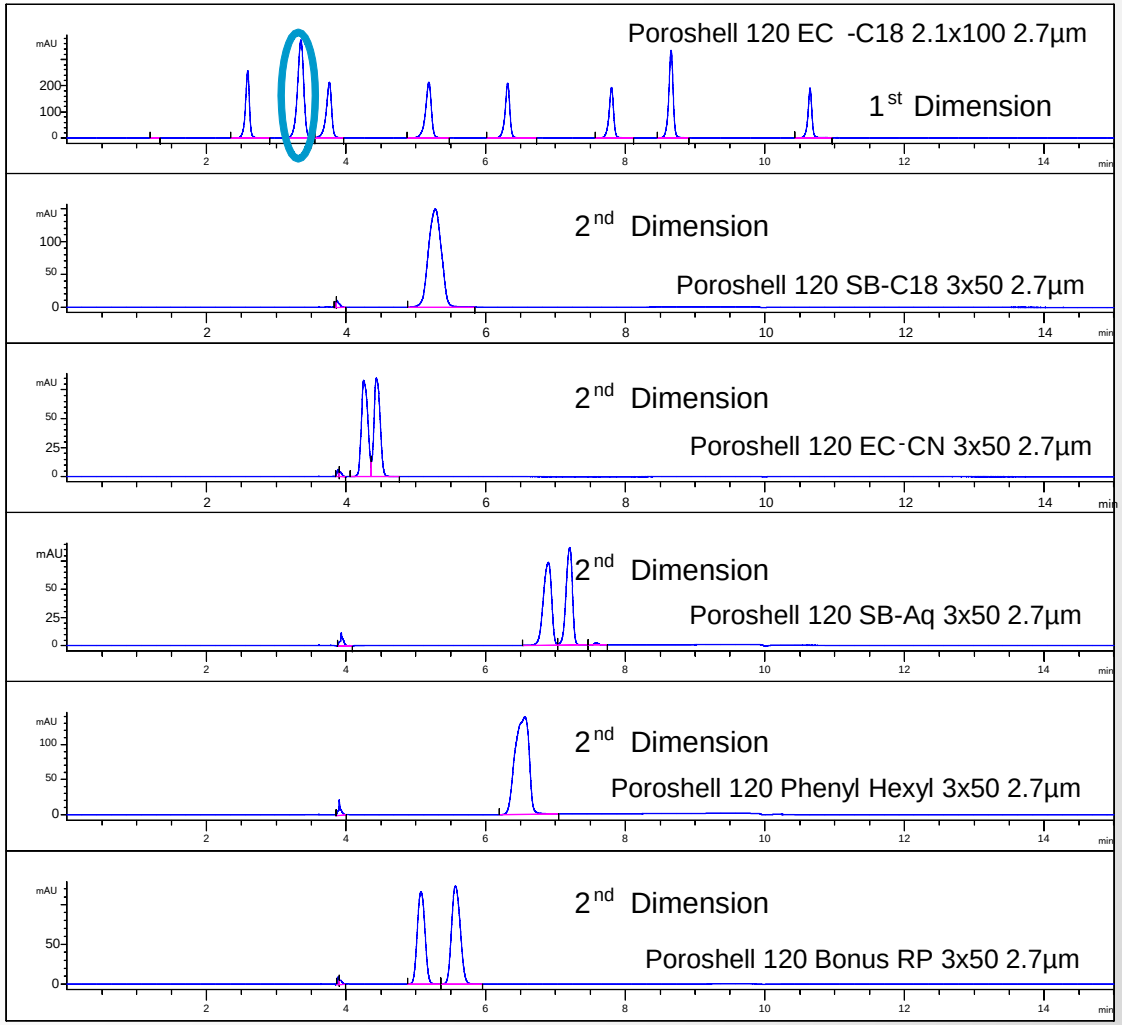
Carr, P.W. et al., J. of Chrom. A, (2011) 1218, 6675–6687

2D LC Column Selection

Orthogonality of RP phases

Microscale Separations and Bioanalysis

- **Example mixture of several sulfa drugs**
- **Multiple chemistries utilized in the 2nd dimension**

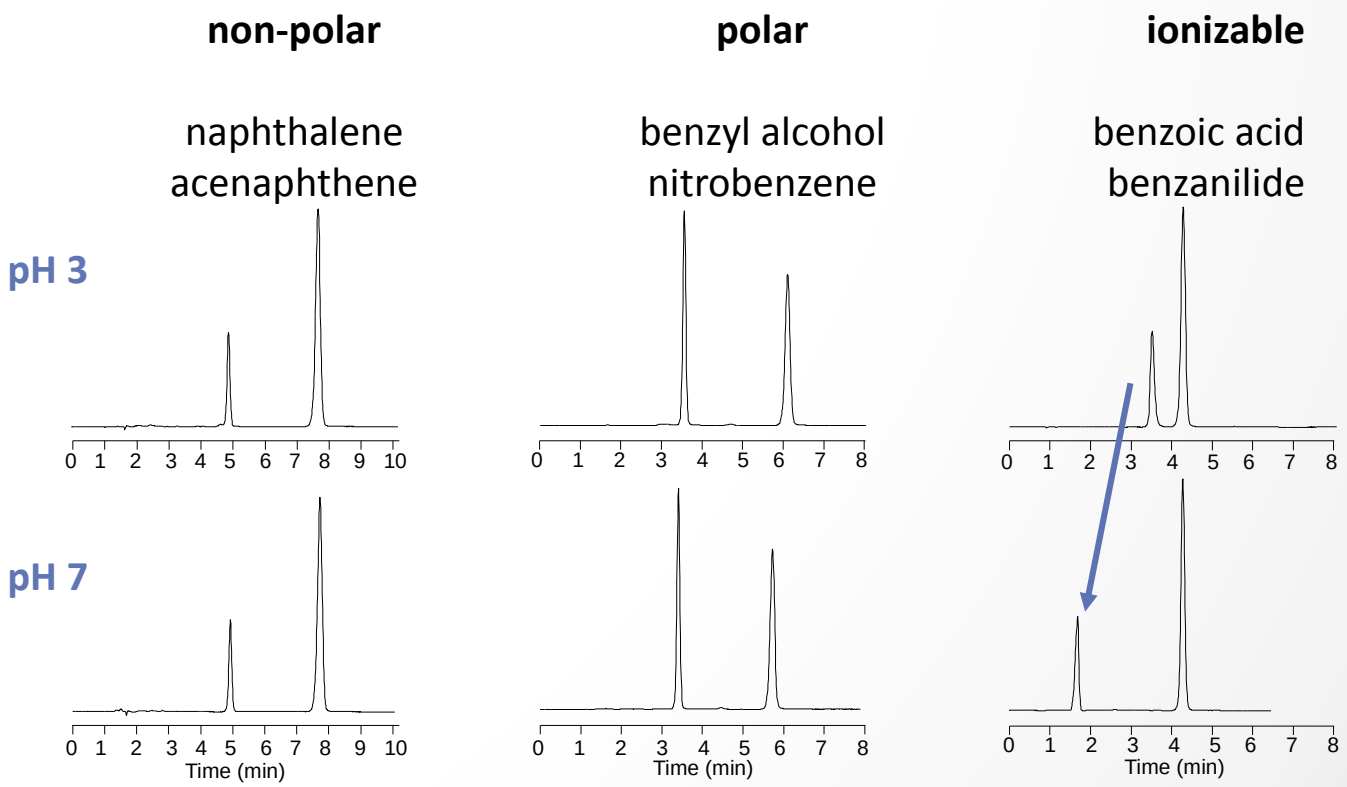


*Slide courtesy Jason Link, Agilent Technologies

2D LC Column Stationary Phase Selection

Selectivity Matters; Mobile Phase pH*

Microscale Separations and Bioanalysis



*Slide courtesy Jason Link, Agilent Technologies

2D LC Column Stationary Phase Selection

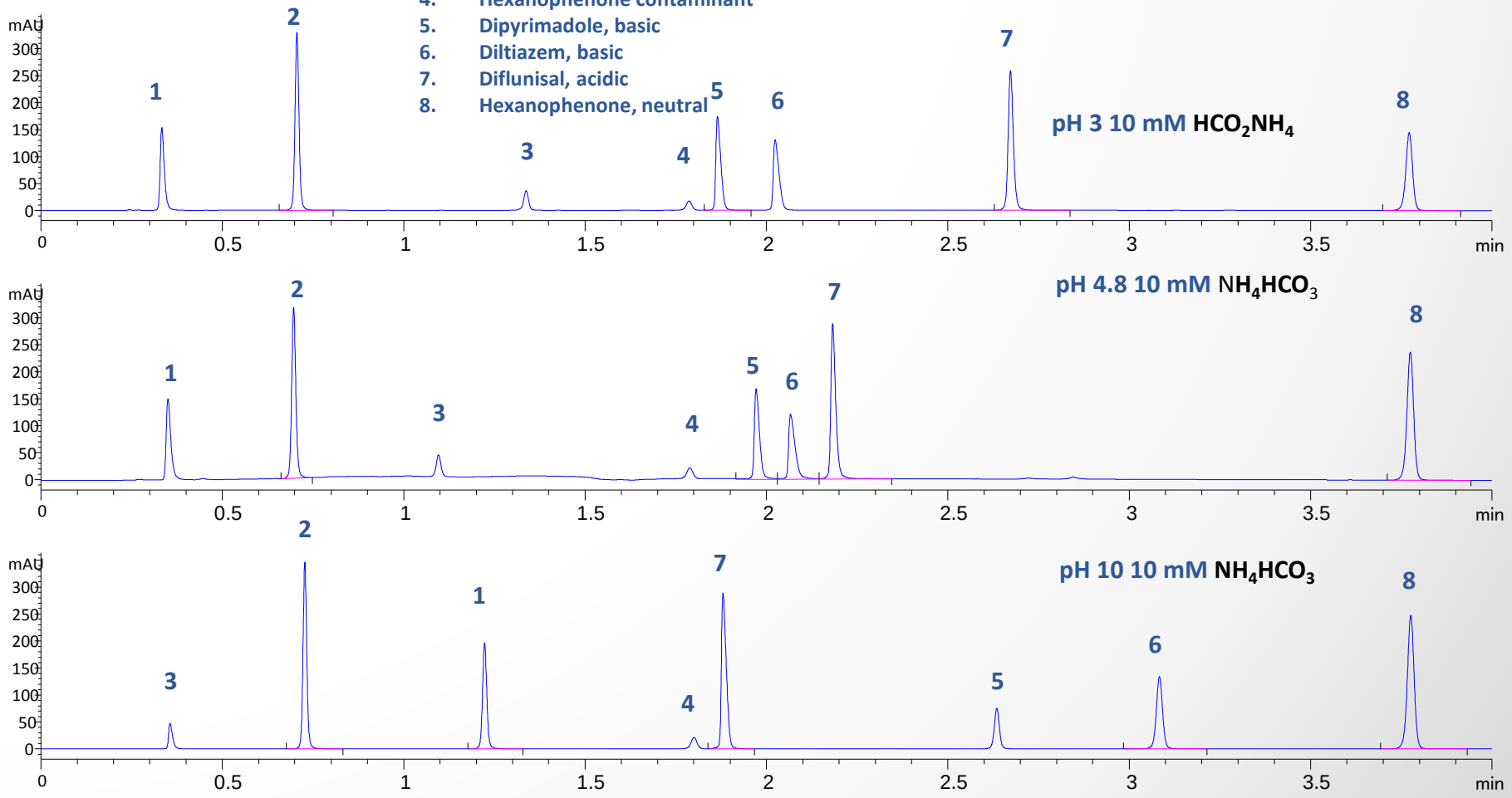
Orthogonality in Dependence of Mobile Phase pH*

Microscale Separations and Bioanalysis

1. Procainamide, pKa 9.2
2. Caffeine, neutral
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

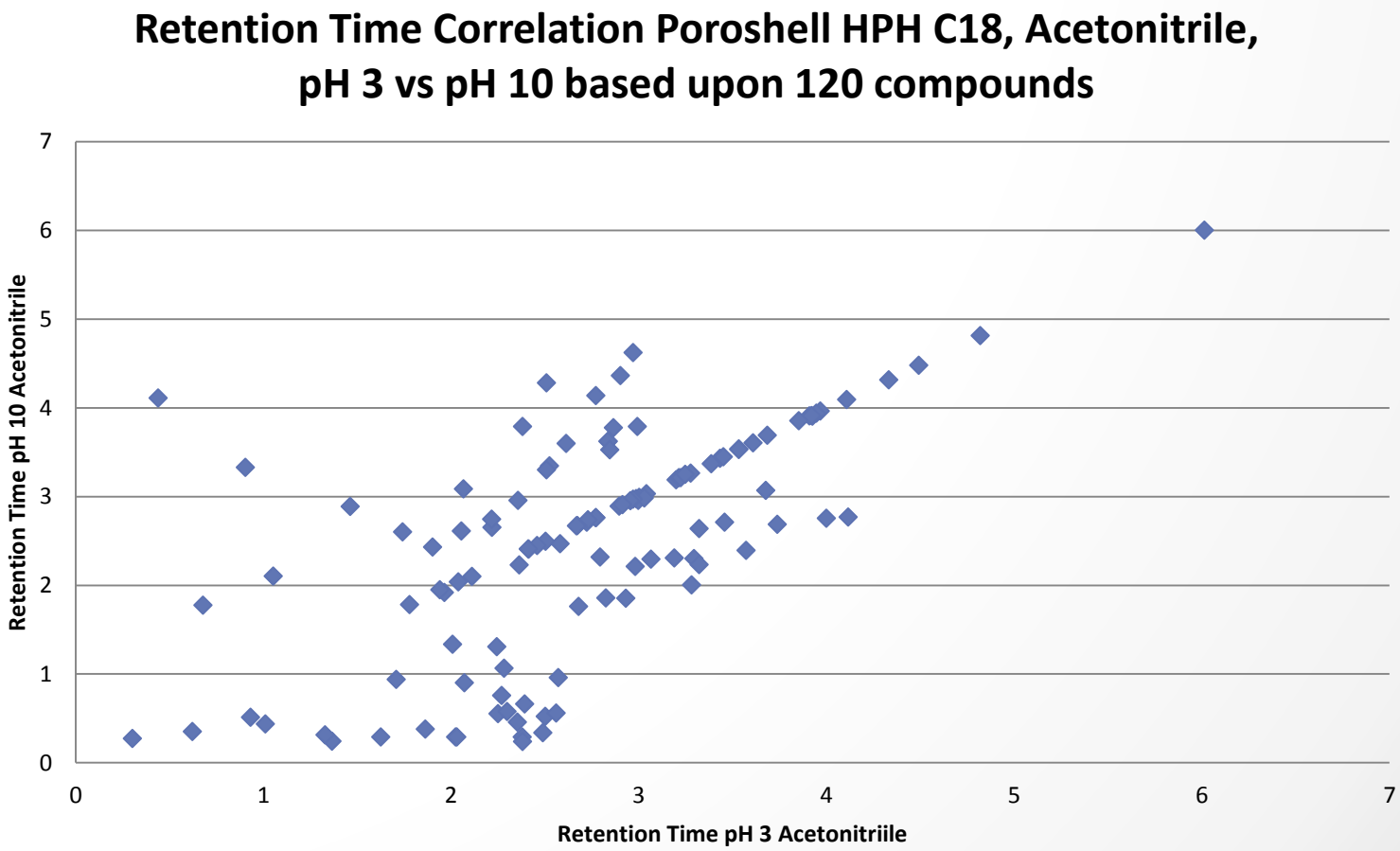


Ionizable compounds – acids and bases change retention and selectivity significantly upon changes in eluent pH

2D LC Column Stationary Phase Selection

Orthogonality in Dependence of Mobile Phase pH*

Microscale Separations and Bioanalysis



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

2nd Dimension Separation

Sample Zone Focusing

Microscale Separations and Bioanalysis

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:
$^{2}V_m$ = 55 µL, and $^{2}Vinj./^{2}V_m$ ~ 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

Microscale Separations and Bioanalysis

Approach	Disadvantage	Advantage
1) Inject less ^{1}D effluent into ^{2}D column	A. Decreases detection sensitivity B. Requires either low ^{1}D flow or flow splitting (1^{st} dimension detector!) C. Sample time is reduced	Relatively easy to implement
2) Use larger ^{2}D column volume	A. Decreases detection sensitivity B. 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. Requires additional hardware B. More variables to consider in method development	Very effective when it works

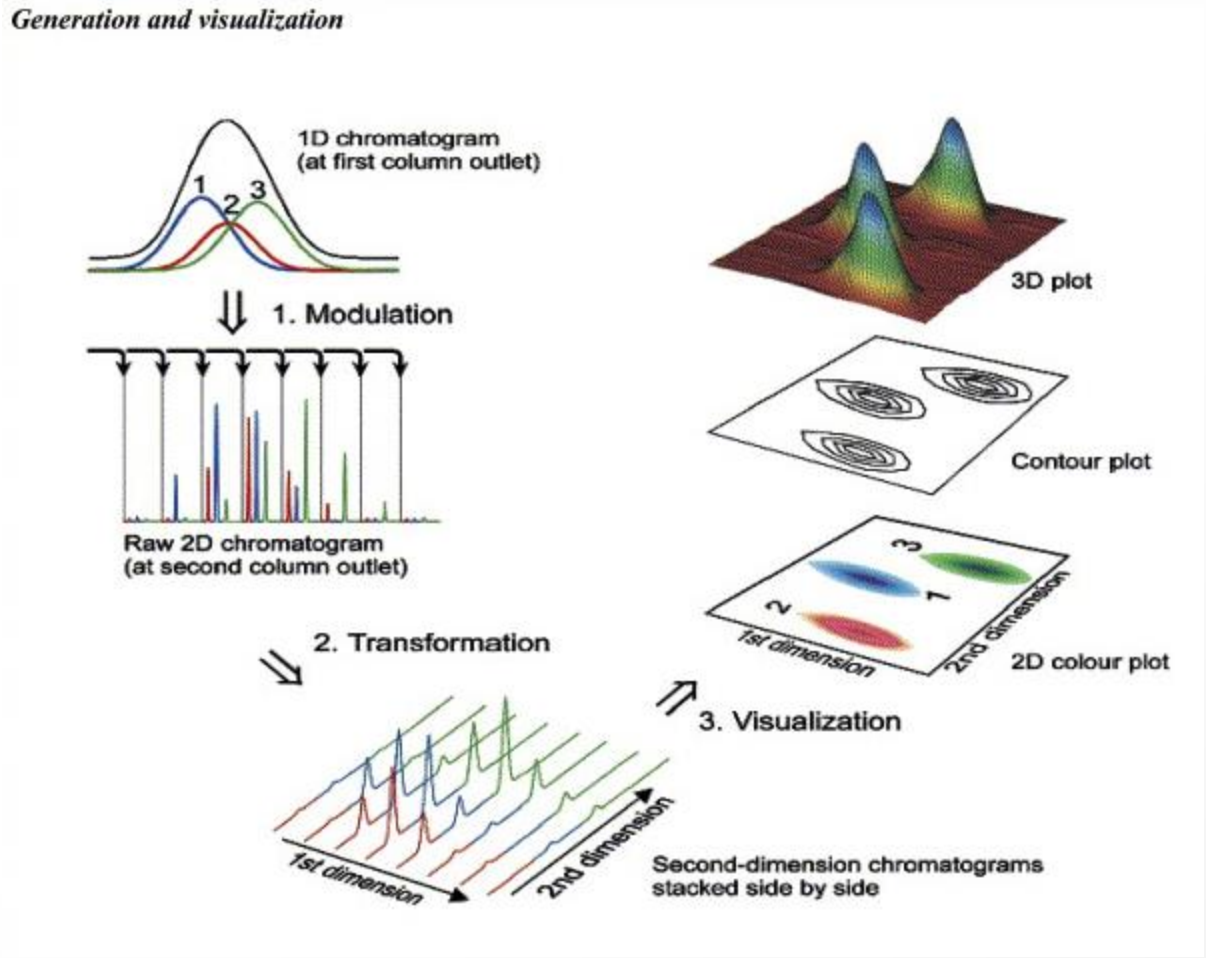
Slide courtesy of Prof. P. Carr & Dr. D. Stoll

Data Handling in Comprehensive 2D LC

Comprehensive 2D-LC

Data Handling

Microscale Separations and Bioanalysis

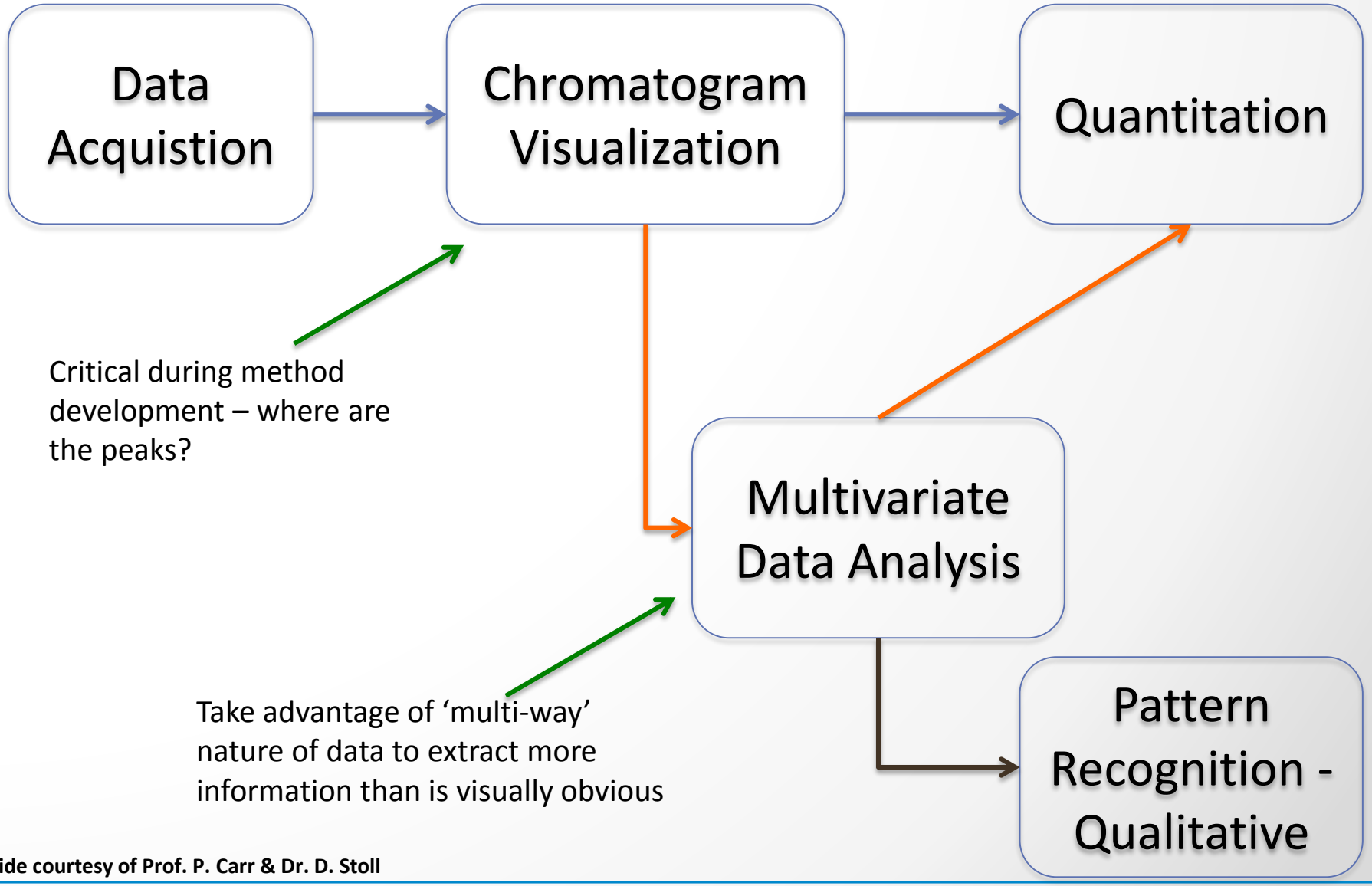


U.T.A. Brinkman et al., Trends in Analytical Chemistry, 2006, 25, 438–454.

Comprehensive 2D-LC

Software for Data Handling

Microscale Separations and Bioanalysis



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

Comprehensive 2D-LC

Data Handling Features

Microscale Separations and Bioanalysis

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

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

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

- (multiple) heart-cutting?
  - Group separation in the 1st dimension
  - 2nd dimension separation takes too long
  - 1st dimension detection required (peak trigger)
- Comprehensive?
  - If n_c >>>20
  - Wide concentration range of the solutes in the sample
  - High # of unknown components in the sample
- Select the stat. phase of the 1st dimension
  - Consider structural properties of the solutes, hydrophobicity, polarity, H-bonding, ionization, size to make an informed choice
  - Always use more retentive separation column for the 2nd dimension
- Isocratic or gradient elution

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

Comprehensive 2D LC Method Development

Further Considerations

Microscale Separations and Bioanalysis

- Specify the 2nd dimension separation
  - More retentive separation column ➔ solute focusing
  - Highest plate number ➔particle size
  - Column diameter and length; use (short) 2.1 mm in modern and (short) 4.6 mm in conventional HPLC equipment
  - Instrument properties; delay volume, allowable gradient speed, re-equilibration time
  - Calculate cycle time of 2nd dimension separation
- Fix the loop size ($V_{loop} = t_{cycle} \times {}^{1}F$) (avoid volume overload 2D column)
- Set flow rate of 1st dimension separation
- Determine modulation time (under filling of the loop is recommended)
- Select the appropriate column diameter for 1D separation
- Set the injection volume for the 1D separation (dilution in the 1st dimension!!)

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

Comprehensive 2D LC Method Development

Example: System Evaluation*

Microscale Separations and Bioanalysis

ROZING.COM Consulting

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

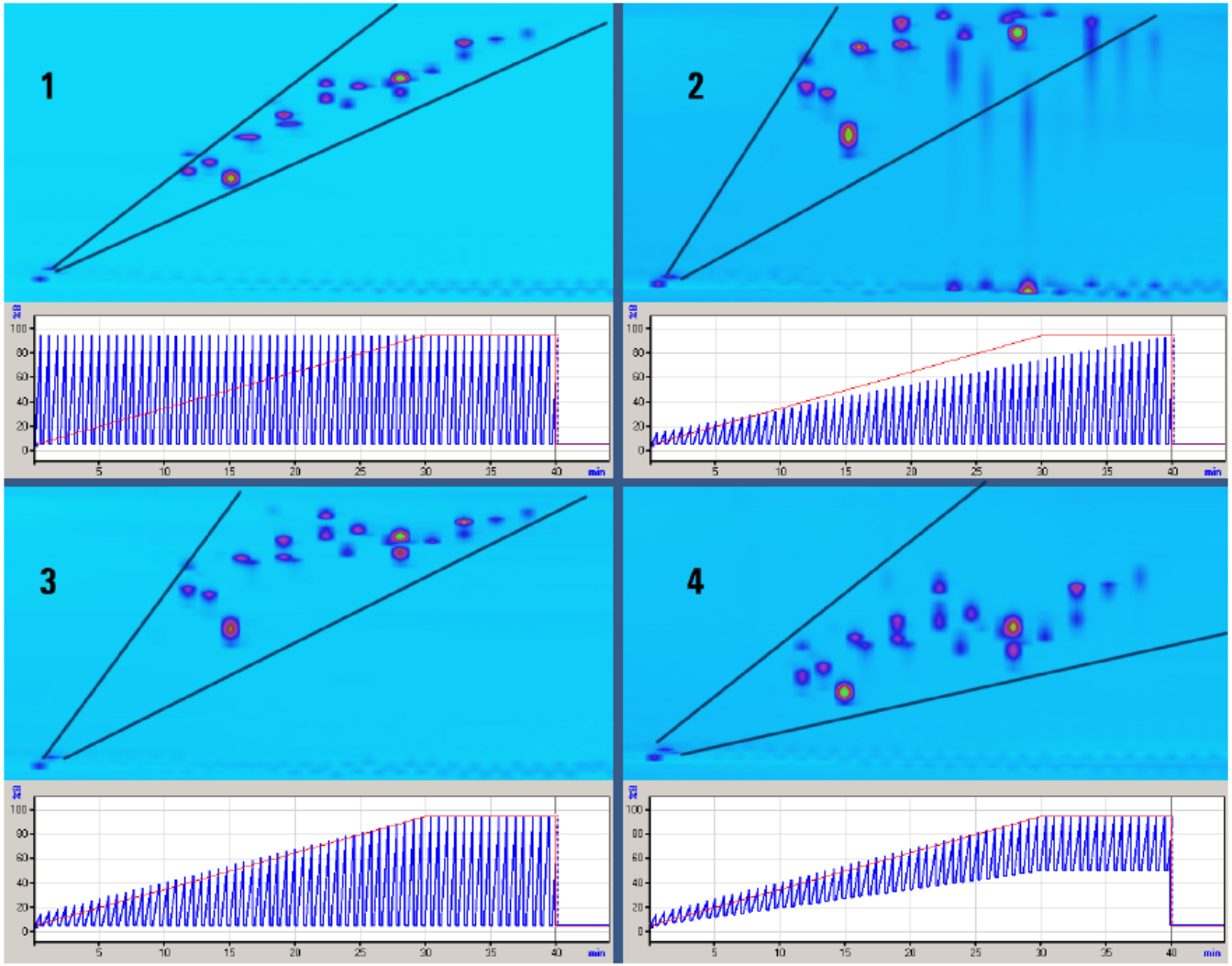
- Agilent OpenL AB CDS ChemStation, Edition, version C.01.03 with
- 2D-LC add-on Software for 2D-LC data analysis from GC Image LLC, Lincoln, NE, USA

***Taken from Agilent AppNote 5991-0138EN**

Comprehensive 2D LC Method Development

2nd Dimension Gradient Optimization*

Microscale Separations and Bioanalysis

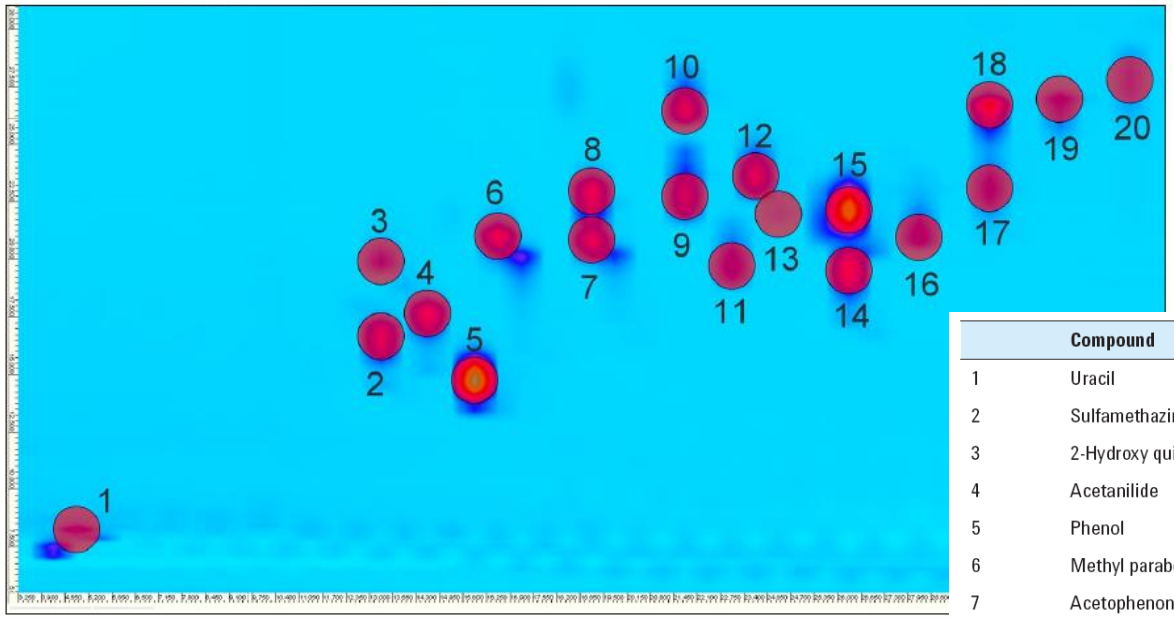


Taken from Agilent AppNote 5991-0138EN

Comprehensive 2D LC Method Development

Results*

Microscale Separations and Bioanalysis



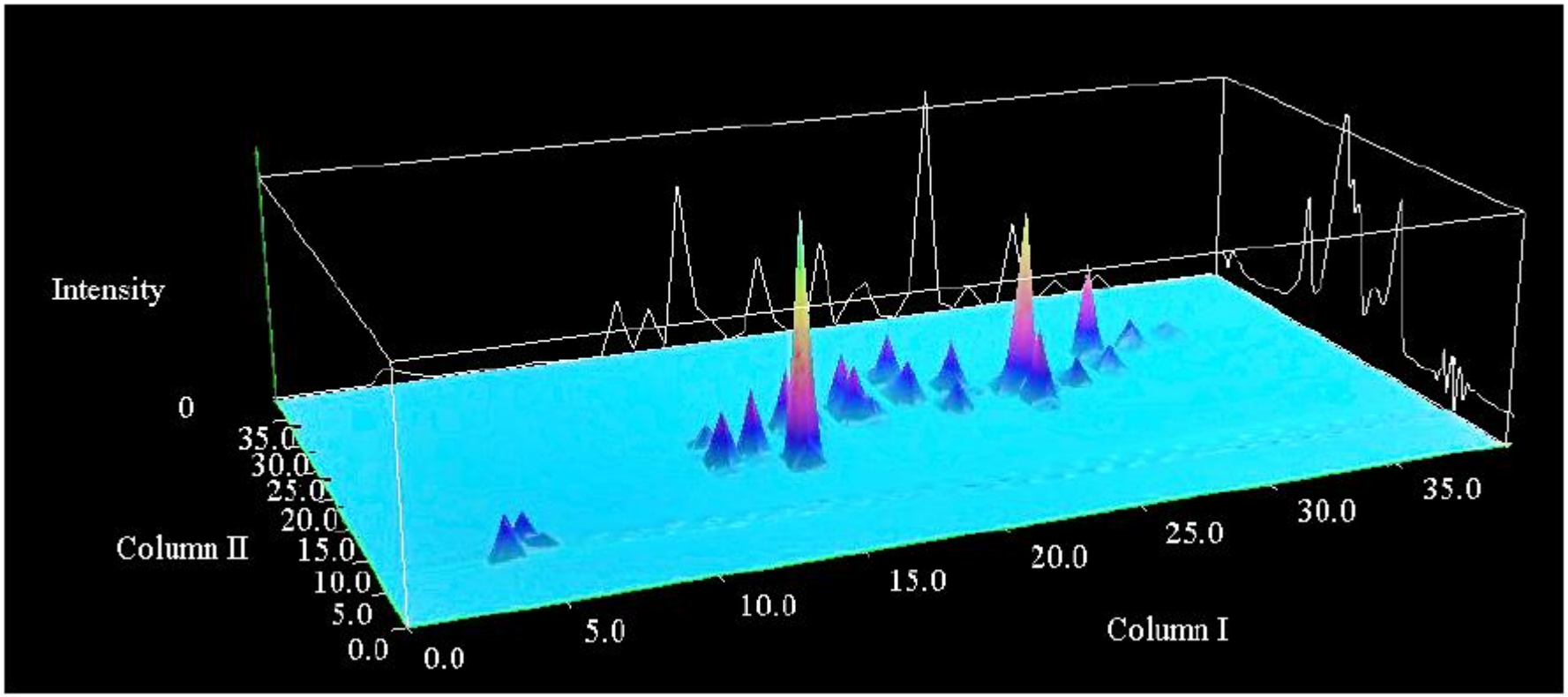
	Compound	RT I (min)	RT II (sec)
1	Uracil	4.55	8.32
2	Sulfamethazine	13.00	16.71
3	2-Hydroxy quinoline	13.00	20.06
4	Acetanilide	14.30	17.61
5	Phenol	15.60	14.56
6	Methyl paraben	16.25	20.95
7	Acetophenone	18.85	20.58
8	Ethyl paraben	18.85	22.88
9	Propyl paraben	21.45	22.65
10	N,N-Diethyl-m-toluamide	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*

Microscale Separations and Bioanalysis



Taken from Agilent AppNote 5991-0138EN

Comprehensive 2D LC Method Development

Example: Extra Virgin Olive Oil

Microscale Separations and Bioanalysis

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

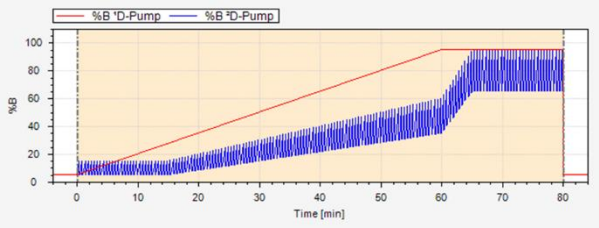
Separation 2nd Dimension

- 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

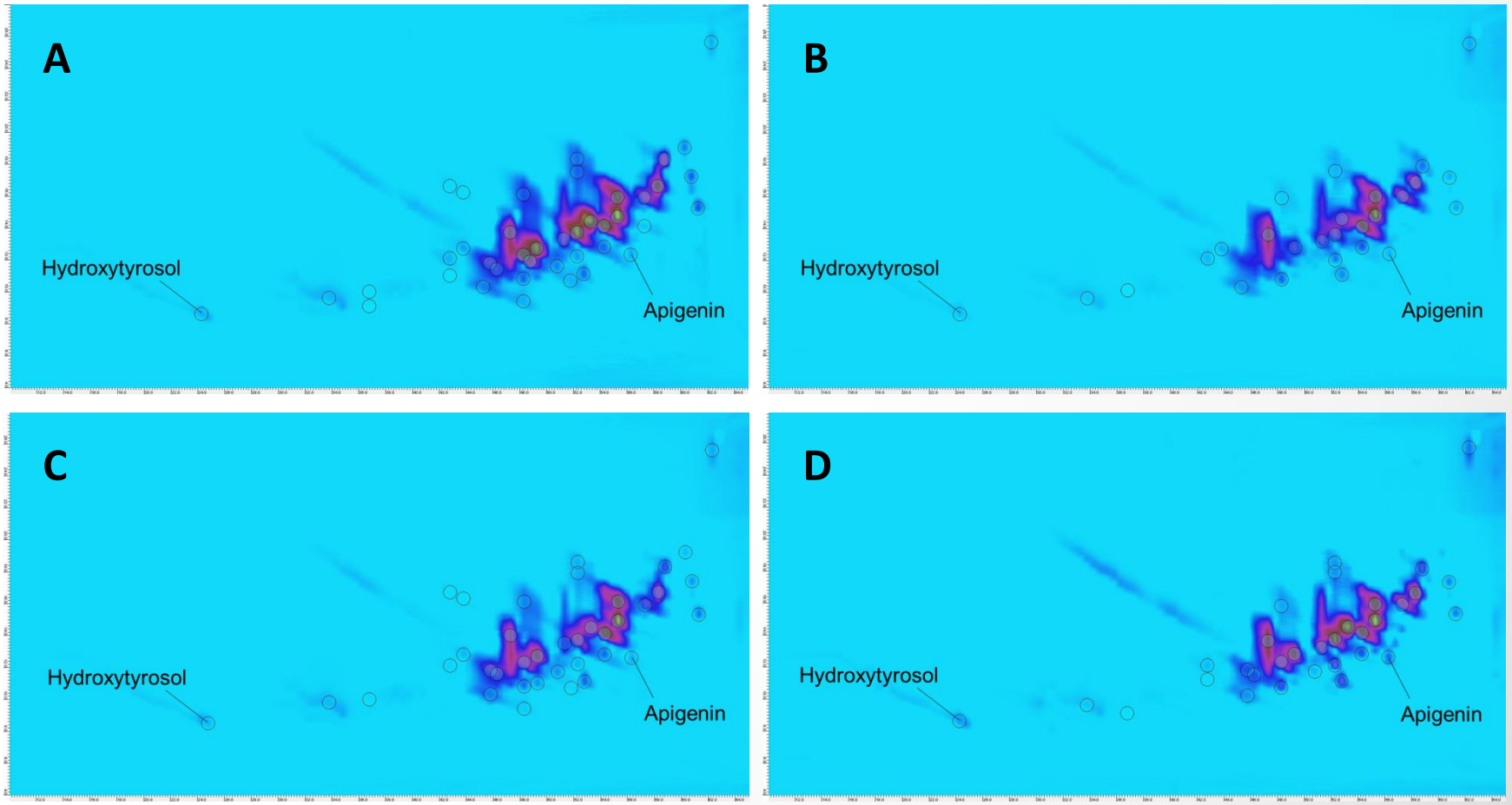
- Agilent OpenL AB CDS ChemStation, Edition, version C.01.03 with
- 2D-LC add-on Software for 2D-LC data analysis from GC Image LLC, Lincoln, NE, USA

Taken from Agilent AppNote 5991-4515EN

Comprehensive 2D LC Method Development

Hydrophilic phenols from extra virgin olive oils

Microscale Separations and Bioanalysis



Taken from Agilent AppNote 5991-4515EN

Comprehensive 2D LC Method Development

Hydrophilic phenols from extra virgin olive oil

Microscale Separations and Bioanalysis

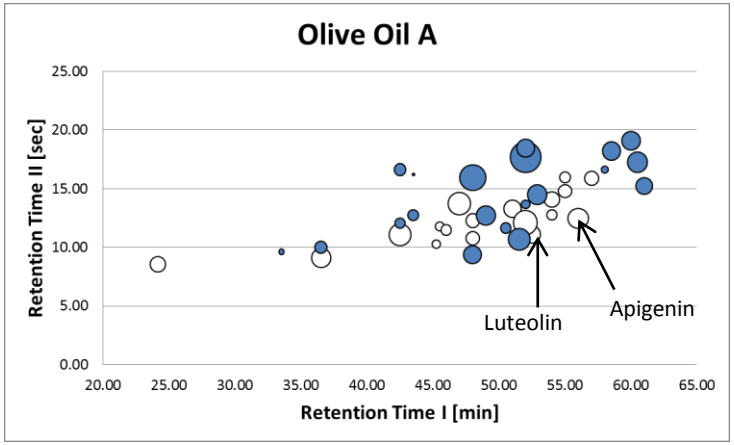
Compound name	Formula	Theoretical *m/z*	Mean Retention time I [min]	Mean Retention time II [sec]
Oleuropein aglycon	$C_{19}H_{22}O_8$	377.1242	48 - 55	13.6 - 16.0
Ligstroside aglycon	$C_{19}H_{22}O_7$	361.1293	52 - 61	14.5 - 17.7
Decarboxymethyl oleuropein aglycon	$C_{17}H_{20}O_6$	319.1187	47 - 52	12.1 - 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

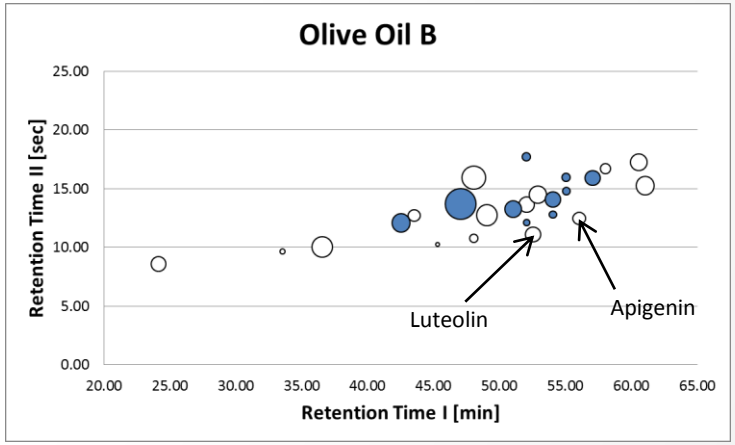
Taken from Agilent AppNote 5991-4515EN

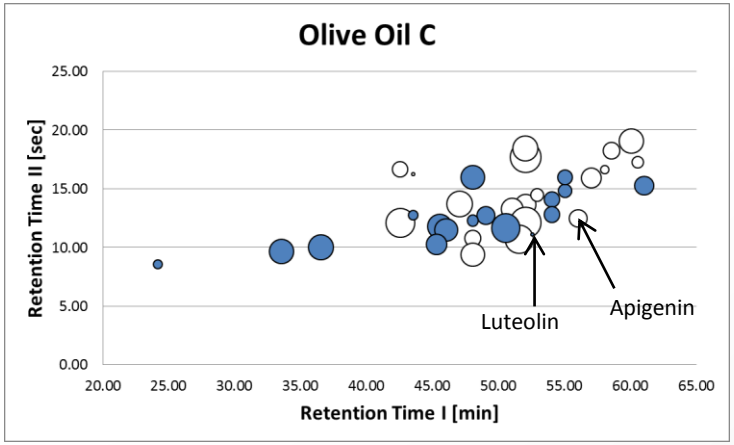
Comprehensive 2D LC Method Development

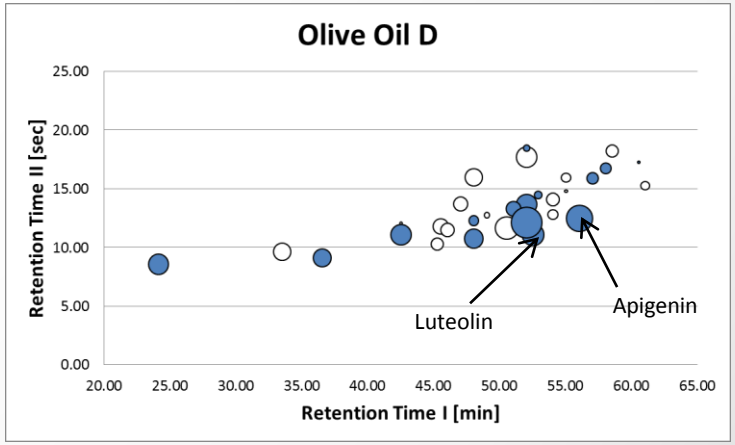
Differences of hydrophilic phenols from extra virgin olive oil

Microscale Separations and Bioanalysis









Taken from Agilent AppNote 5991-4515EN

Multiple Heartcutting 2D LC Method Development

Example: Lipidomics

Microscale Separations and Bioanalysis

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First dimension HILIC:

- Column Agilent RX-SIL, 1.0 × 150 mm, 3.5 µm (custom made)
- Solvent A 0.1 % formic acid in acetonitrile;
- Solvent B 20 mM ammonium formate in acetonitrile/methanol/water, 50/20/30, v/v, Flow rate 40 µL/min
- Gradient 0–2 minutes: 30–60 %B, 40 µL/min
- 2–4 minutes: 60–70 %B, 40 µL/min,
  4–5 minutes: 70–100 %B, 40 µL/min,
  5–12 minutes: 100 %B, 40 µL/min,
  12–54 minutes: 100 %B, 20 µL/min,
  54–65 minutes: 30 %B, 40 µL/min
- Temperature 40 °C

Second dimension RPLC:

- Column Agilent ZORBAX Eclipse Plus C18 RRHD, 2.1 × 100 mm, 1.8 µm
- Solvent A 20 mM ammonium formate/0.1 % formic acid in methanol/water, 10/90, v/v
- Solvent B 20 mM ammonium formate/0.1 % formic acid in acetonitrile/methanol/isopropanol, 20/30/50, v/v, Flow rate 0.6 mL/min
- Idle flow rate 0.2 mL/min
- Gradient 0–6.5 minutes: 40–100 %B
  6.5–8.5 minutes: 100 %B
  8.5–10 minutes: 40 %B
- Temperature 60 °C

Loop filling:

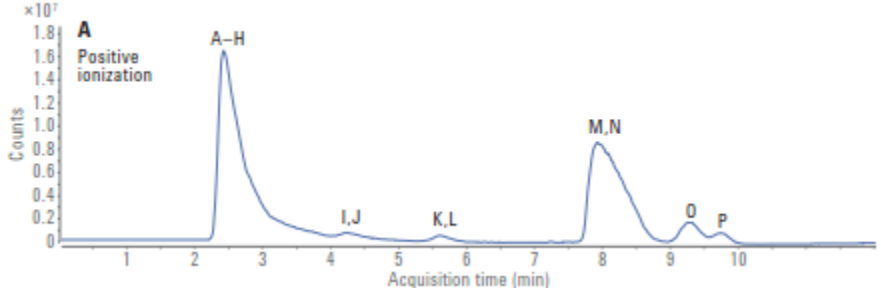
- Time segments Fraction 1: 2.00–3.80 minutes (Loop 80 µL)
- Fraction 2: 3.90–4.80 minutes
- Fraction 3: 5.25–6.15 minutes
- Fraction 4: 7.80–8.75 minutes
- Fraction 5: 9.05–10.00 minutes

Injection:

- Sample; plasma or long sputum reconstituted in MTBE/isopropanol 50/50
- Volume 1 µL

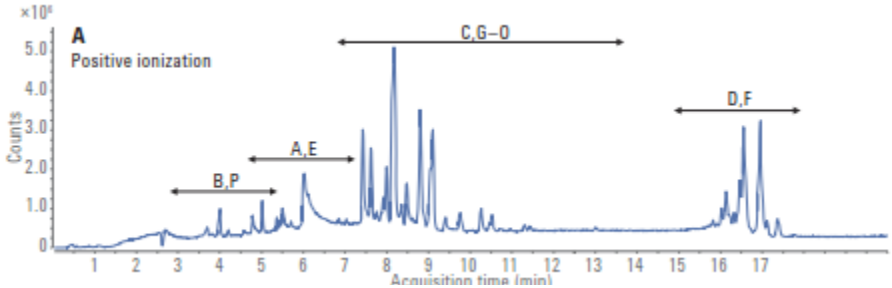
Multiple Heartcutting 2D LC Method Development

Example: Lipidomics



Separation on the 1st dimension column

Lipid class (abbreviation)	Code	MHC fraction
Free fatty acids (FFA)	A	1
Monoacylglycerols (MG)	B	1
Diacylglycerols (DG)	C	1
Triacylglycerols (TG)	D	1
Cholesterol (Chol)	E	1
Cholesterol esters (CE)	F	1
Ceramides (CER)	G	1
Glycoceramides (Hex-CER)	H	1
Glyconceramides (Hex_n-CER)	I	2
Phosphatidylinositols (PI)	J	2
Phosphatidylethanolamines (PE)	K	3
Glycosphingolipids (GSL)	L	3
Phosphatidylserines (PS)	M	4
Phosphatidylcholines (PC)	N	4
Sphingomyelins (SM)	O	5
Lysophospholipids (LysoPL)	P	5

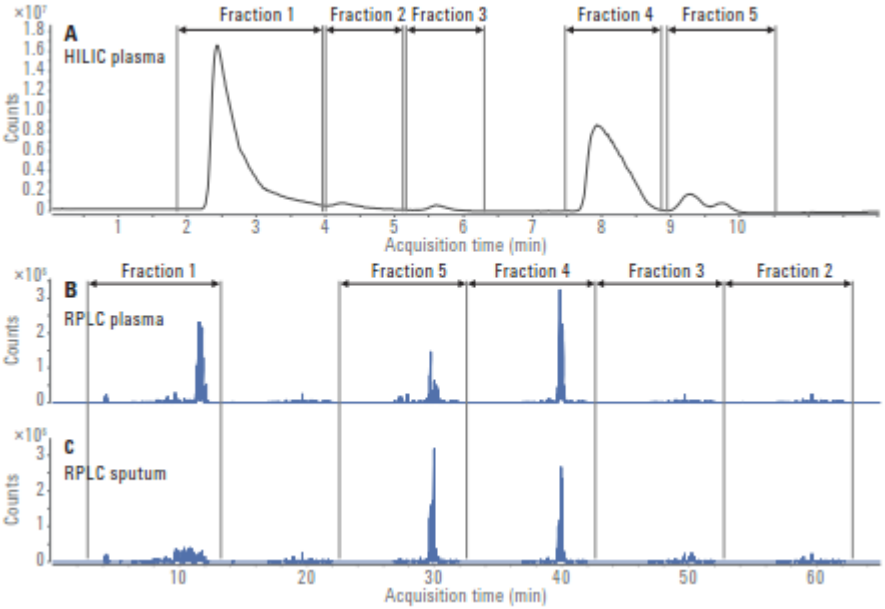


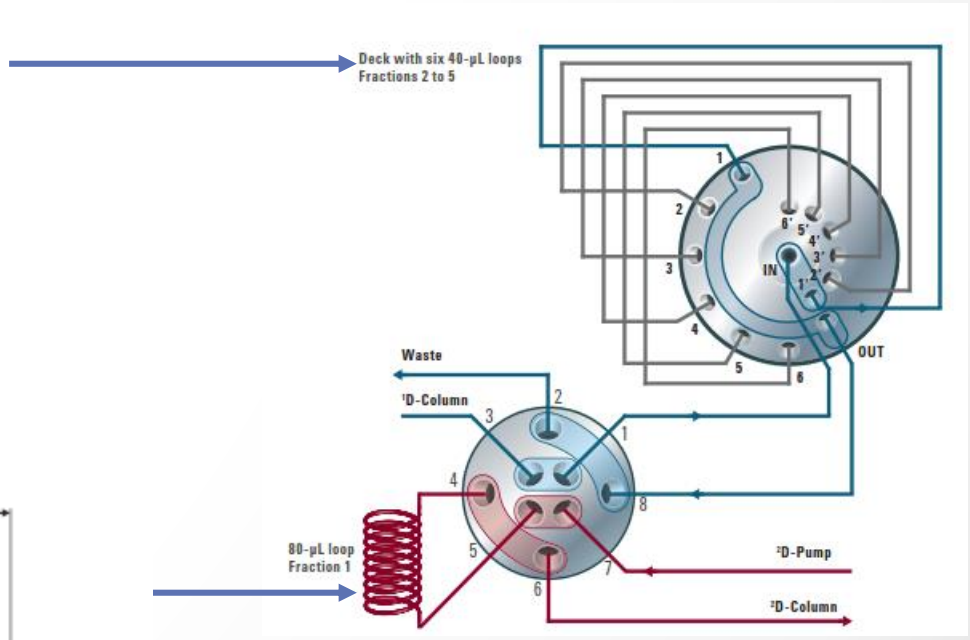
Separation on the 2nd dimension column

Multiple Heartcutting 2D LC Method Development

Example: Lipidomics

Microscale Separations and Bioanalysis





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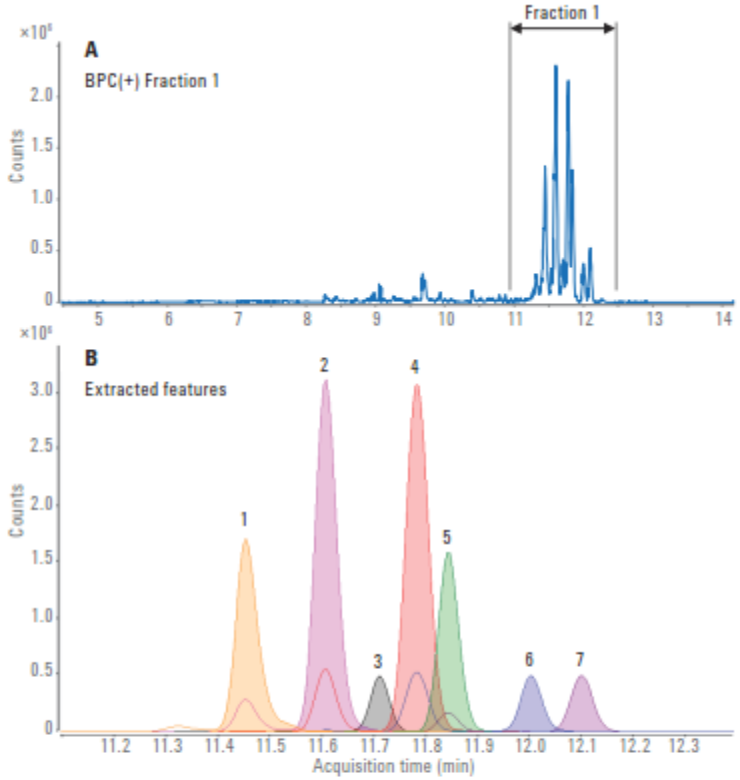
Multiple Heartcutting 2D LC Method Development

Example: Lipidomics

Microscale Separations and Bioanalysis



Feature	Formula	t_R (min)	*m/z*	Identity
1	$C_{55}H_{101}O_6N$	11.45	871.7629	TG(52:4)NH_3
2	$C_{55}H_{103}O_6N$	11.61	873.7786	TG(52:3)NH_3
3	$C_{47}H_{79}NO_2$	11.71	689.6111	CE(20:4)NH_3
4	$C_{55}H_{105}O_6N$	11.78	875.7942	TG(52:2)NH_3
5	$C_{45}H_{79}NO_2$	11.84	665.6111	CE(18:2)NH_3
6	$C_{55}H_{107}O_6N$	12	877.8099	TG(52:1)NH_3
7	$C_{45}H_{81}NO_2$	12.1	667.6267	CE(18:1)NH_3

Fraction 1, positive ionization, triglycerides and cholesterol esters in plasma. TG and CE represent the most apolar lipids. Both groups are detected as ammonia adducts in the positive ESI mode.

Further Reading

- User Guide Infinity 2D-LC Solution

  Agilent Technologies p.n. G2198-90001

Acknowledgements

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Thank You for Your Attention

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