ADVANCES IN FOOD ANALYSIS

INTRODUCTION TO 2D-LC BASIC PRINCIPLES

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LC *vs* MULTIDIMENSIONAL LC

- 1D HPLC is widely applied to the analysis of real world samples in several fields.

- Such a method often does not provide sufficient resolving power for the separation of target components in real samples.

- A possible solution can be the use of multidimensional systems (MD), where the dimensions are based on different separation mechanisms.

Comprehensive LC (LC×LC)

- Comprehensive two-dimensional liquid chromatography represents a very powerful technique for the analysis of complex mixtures.

It offers:

- Enhanced resolving power.
- Enhanced identification power due to the formation of 2D chemical class patterns.

- In the last three decades LC × LC methods have been developed and applied to the separation of different classes of components using many combinations of HPLC modes, based on the nature of the components to be analysed and on the selectivity of individual modes: normal phase (NP), reversed phase (RP), size exclusion (SEC), ion exchange (IEX), affinity chromatography (AC), hydrophilic interaction liquid chromatography (HILIC).

Comprehensive LC (LC×LC)

Basic principles:

- a typical comprehensive separation is achieved, generally, on two distinct columns connected in series with a special transfer system (modulator) located between them

- the type of interface used is linked to the specific methodology

- the function of the interface is to cut and then release continuous fractions of the primary column effluent onto a fast separation column

- the bands injected onto the secondary column must undergo elution before the following re-injection

ORIGIN OF MULTIDIMENSIONAL CHROMATOGRAPHY

Selective 2D Liquid Chromatography (1978) Erni & Frei

Journal of Chromatography, 149 (1978) 561–569

CHROM. 10,733

TWO-DIMENSIONAL COLUMN LIQUID CHROMATOGRAPHIC TECHNIQUE FOR RESOLUTION OF COMPLEX MIXTURES

F. ERNI and R. W. FREI*

Analytical Research and Development, Pharmaceutical Department, Sandoz Ltd., CH-4002 Basle (Switzerland)

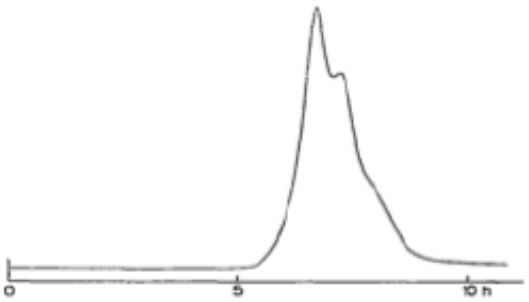


Fig. 5. GPC separation of a senna glycoside extract (trace from recorder 1 in Fig. 4). Mobile phase, buffer, pH 6 (Titrisol; Merck, Darmstadt, G.F.R.); flow-rate, 1.2 ml/h; detection, UV (254 nm). Chromatographic equipment as described in Fig. 4.

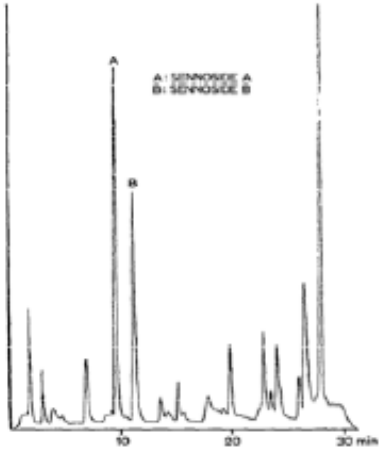


Fig. 6. RPC separation of the same senna glycoside extract as in Fig. 4. Mobile phase, seven steps of acetonitrile–0.01 *N* sodium hydrogen carbonate in water; flow-rate, 2 ml/min; detection, UV (254 nm). The gradient steps were as follows:

ORIGIN OF MULTIDIMENSIONAL CHROMATOGRAPHY

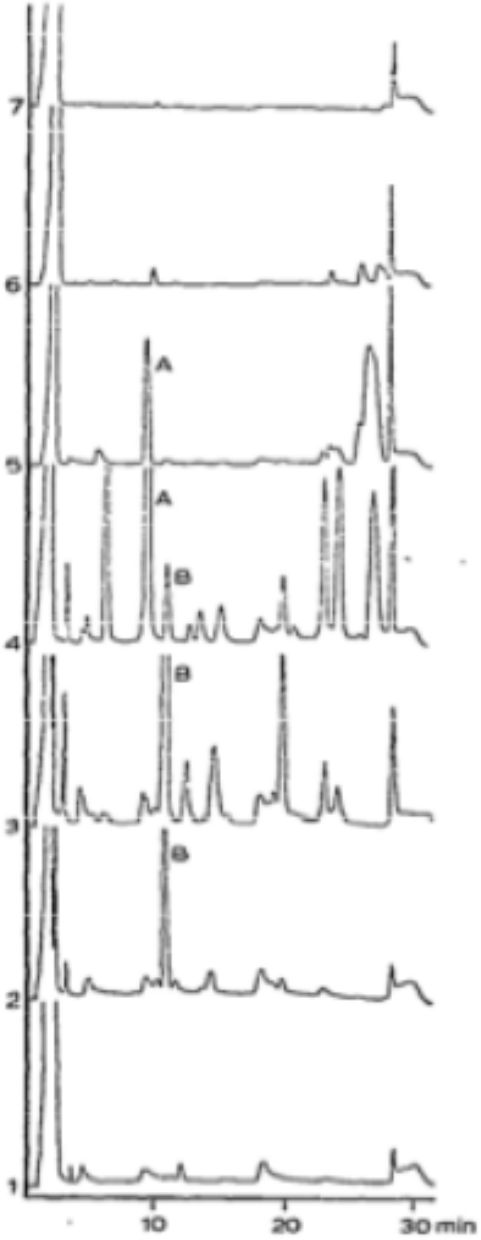


Fig. 7. RPC runs of seven fractions from the GPC run of the same senna glycoside extract as in Fig. 6. Peaks: A, sennoside A; B, sennoside B.

Selective 2D Liquid Chromatography

(1978) Erni & Frei

ORIGIN OF MULTIDIMENSIONAL CHROMATOGRAPHY

First Comprehensive 2D Liquid Chromatography (1990) Bushey & Jorgenson

Automated Instrumentation for Comprehensive Two-Dimensional High-Performance Liquid Chromatography of Proteins

Michelle M. Bushey and James W. Jorgenson*

Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina 27599-3290

ANALYTICAL CHEMISTRY, VOL. 62, NO. 2, JANUARY 15, 1990

Pp 161-167

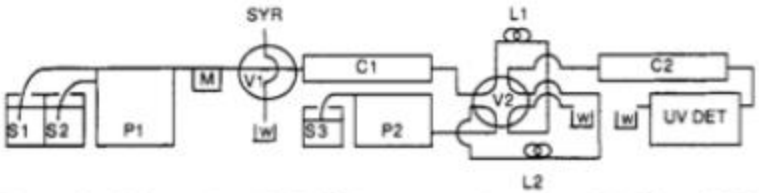


Figure 2. Schematic of 2-D LC instrumental setup. S1, S2, and S3 are buffers A, B, and C; P1, Brownlee microgradient syringe pump; M, 52-μL mixer; V1, Rheodyne 0.5-μL injection valve; SYR, injection syringe; C1, cation exchange column; V2, eight-port computer-controlled valve; L1 and L2, 30-μL loops; P2, Waters Associates Model 6000A piston pump; C2, size exclusion column; UV DET, UV detector operated at 215 nm; W, waste.

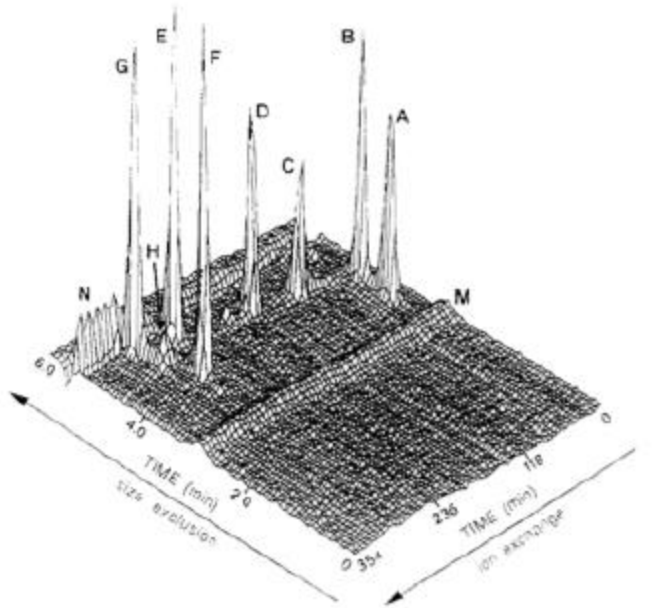
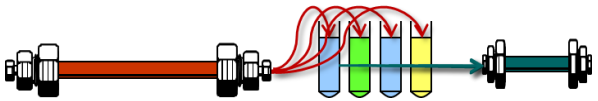


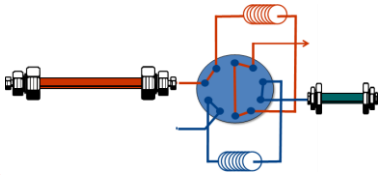
Figure 3. 2-D chromatogram of protein sample: peak A, glucose oxidase; B, ovalbumin; C, β-lactoglobulin A; D, trypsinogen; E, α-chymotrypsinogen A; F, conalbumin; G, ribonuclease A; H, hemoglobin; M, exclusion volume "pressure" ridge; N, inclusion volume "salt" ridge. Ovalbumin and α-chymotrypsinogen A at 0.2%, other proteins at 0.3% (w/v). C1 conditions: 5 μL/min, 0% to 100% buffer B from 20 to 260 min; buffer A, 0.2 M NaH_2PO_4, pH 5; buffer B, 0.2 M NaH_2PO_4/0.25 M Na_2SO_4, pH 5. Valve actuated every 6 min; detection at 215 nm, data collection rate 0.5 point/s; plot shows every other point collected for injection 1 through 60. Each line perpendicular to the IEC time axis represents one injection on the SEC column.

MULTIDIMENSIONAL LC (MD-LC)

✓ **Off-line MD-LC**



✓ **On-line MD-LC**



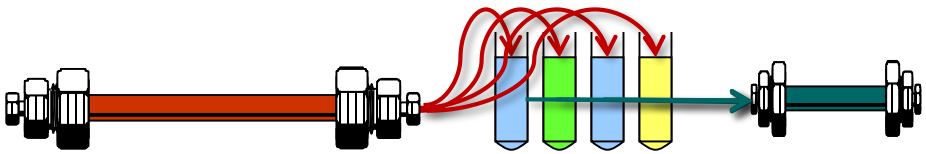
Heart-cutting LC

Comprehensive LC

- ***Continuos mode***
- ***Stop-flow mode***

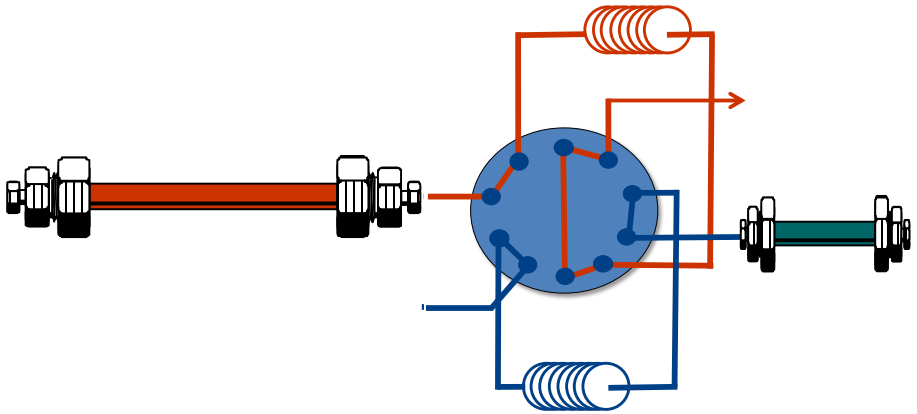
Choice of hyphenation mode

i. Off-line LC×LC



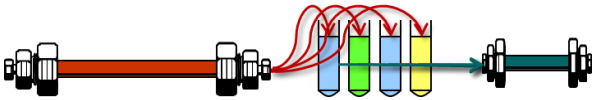
- Longer ^{2}D analyses offer much higher resolving power
- More flexible
- Simple instrumentation
- Option of altering ^{1}D fractions
- **Very long analysis times (10-50 h)**

ii. On-line LC×LC



- **Conventional analysis times (1-2 h)**
- Fast ^{2}D separations essential
- More advanced instrumentation required
- More complex

Off-line 2D-LC:



fractions isolated in the first chromatographic step are collected, separated from the solvent by evaporation, redissolved and then re-analyzed in the second step.

Advantages:

- very simple;
- great variety of different separation modes can be coupled;
- no problems related with immiscible solvents;
- the sample concentration injected in both dimensions can be easily regulated.

Disadvantages:

- time-consuming;
- difficult to automate;
- possible sample contamination and artefact formation;
- losses or degradation during solvent evaporation can occur;
- low analytical reproducibility.

On-line 2D-LC:

two columns are connected by means of a specific interface which transfers the ^{1}D eluate onto the ^{2}D column.

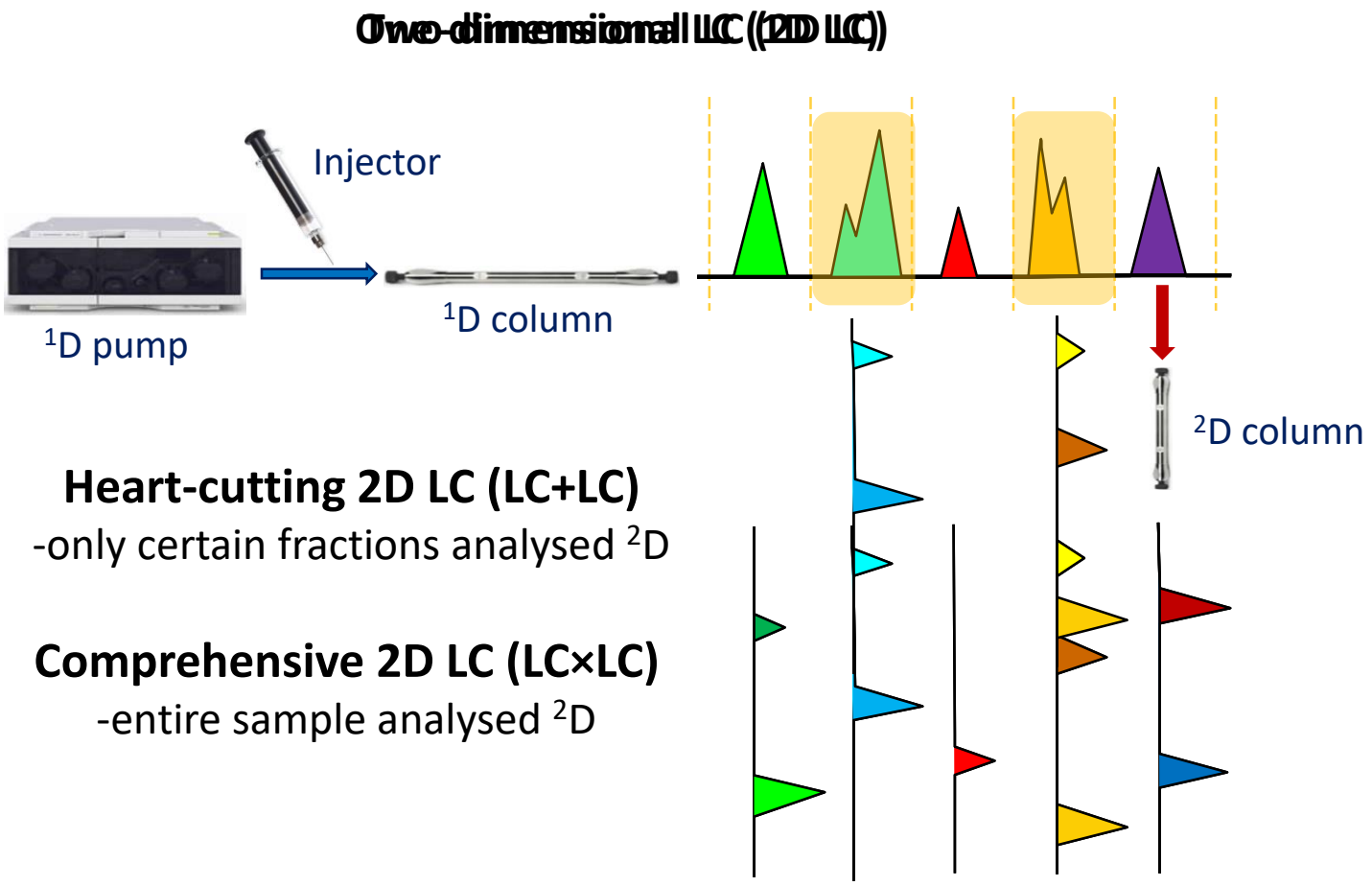
Advantages:

- ease of automation;
- greater reproducibility;
- greater amount of information with a single analyses;
- shorter treatment of the sample;
- higher resolving power;
- great potential for the identification of “unknowns”: formation of chemical class patterns on the 2D space plane.

Disadvantages:

- difficult to operate;
- need for specific interfaces;
- need for specific software;
- problems with immiscible
- solvents;
- coupling of different separation modes more complicated.

COMPREHENSIVE LC×LC *vs*. 2D LC



Heart-cutting 2D LC (LC+LC)

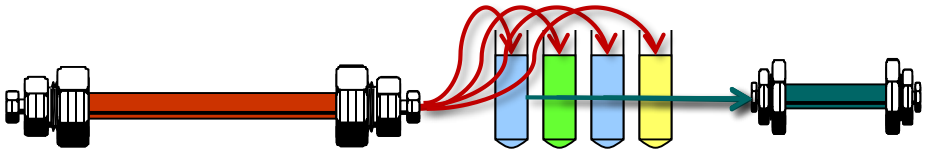
-only certain fractions analysed ^{2}D

Comprehensive 2D LC (LC×LC)

-entire sample analysed ^{2}D

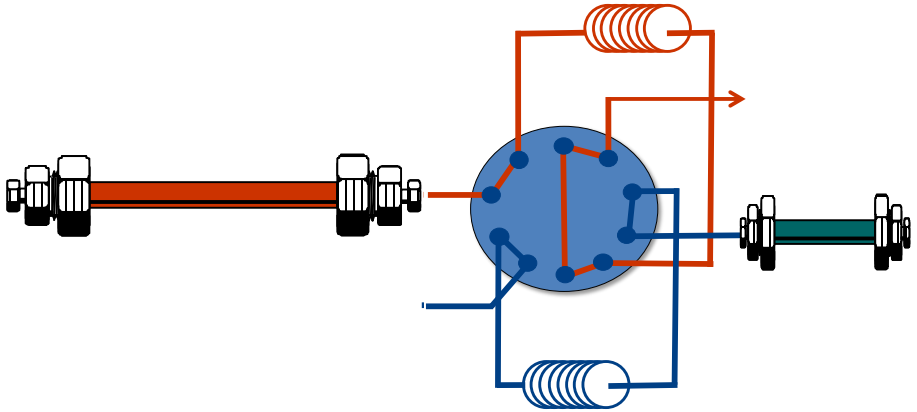
Practical aspects: Hyphenation modes

i. Off-line LC×LC



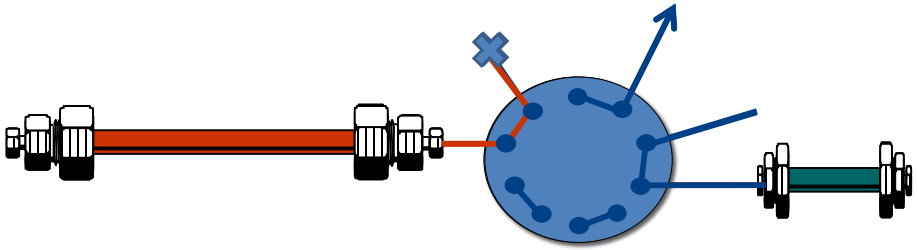
Two dimensions operated independently

ii. On-line LC×LC



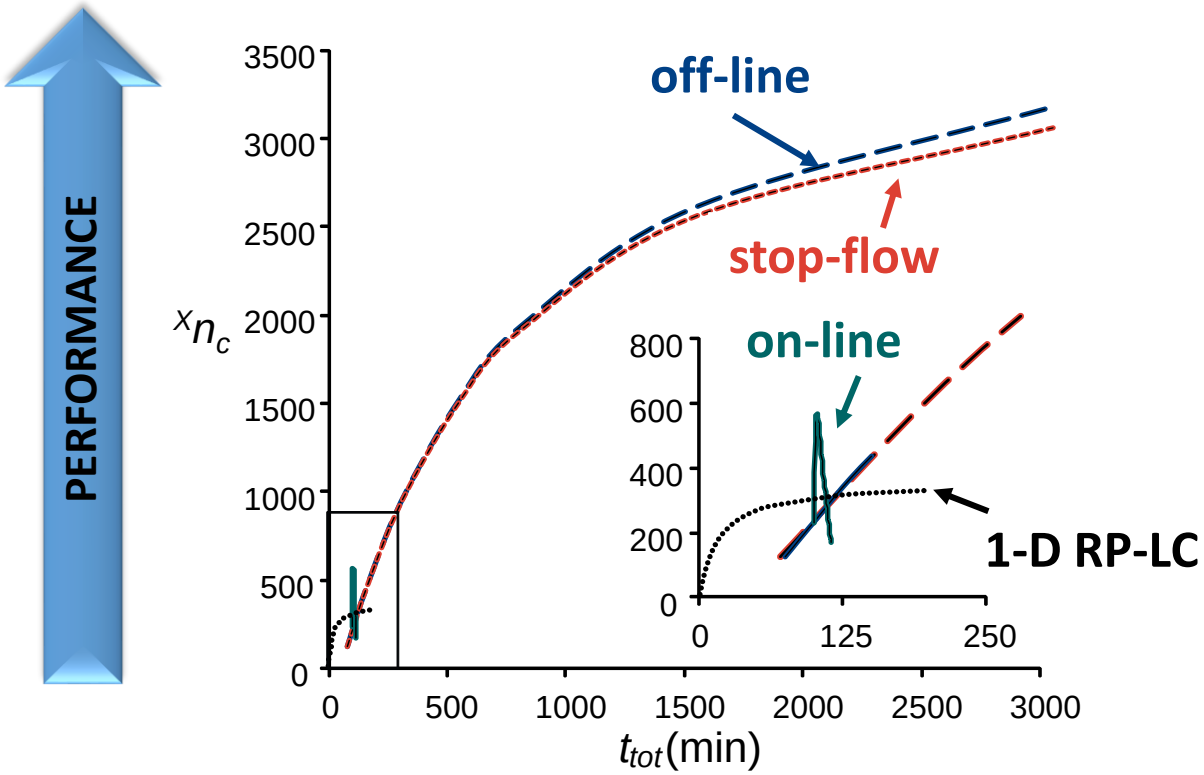
Second dimension separation completed during fraction collection

iii. Stop-flow LC×LC



First dimension flow stopped during second dimension separation

COMPARISON COMPREHENSIVE 2D-LC: OFF-LINE, ON-LINE AND STOP-FLOW



Instrumentation

To build an LC × LC system, special attention has to be devoted to the choice of:

- First dimension (^{1}D)
- Modulator
- Second dimension (^{2}D)
- Detectors
- Data elaboration

First dimension (^{1}D)

- Most of the frequently used LC × LC systems employ a microbore/narrowbore column in the ^{1}D, operated at low flow rate, under isocratic or gradient conditions.

This enables the transfer of fractions of small volume *via* the multiport valve equipped with two identical sample loops, into the ^{2}D column.

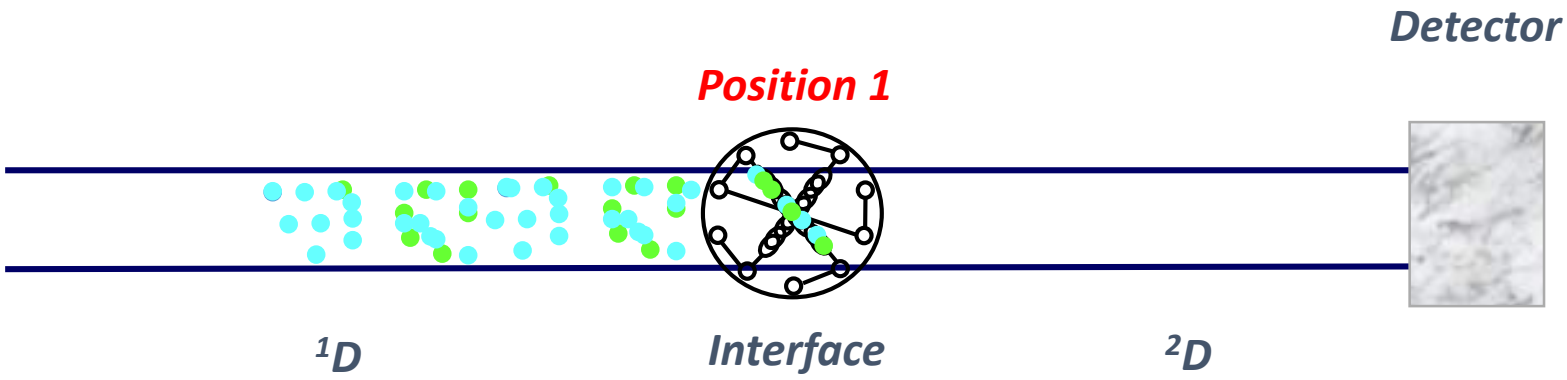
The loop volume usually corresponds to the mobile phase quantity per modulation time eluting from the ^{1}D.

MODULATORS IN 2D-LC

MODULATORS IN 2D-LC

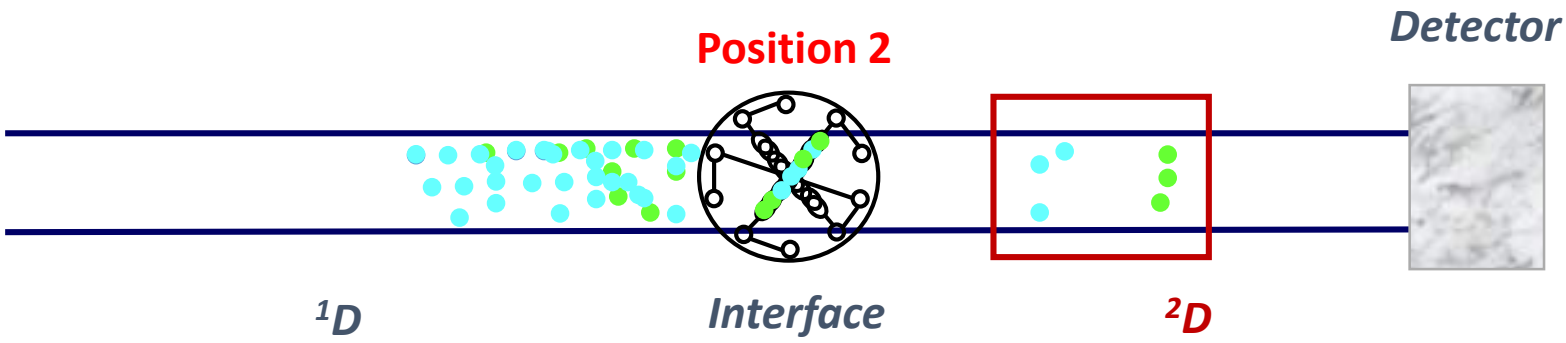
- Critical part to get a proper LC × LC separation.
- Part of instrument where the physical interaction between dimensions takes place.
- The modulator should collect ^{1}D effluent continuously and make its transfer to the ^{2}D.

2D-LC: HOW A MODULATOR WORKS



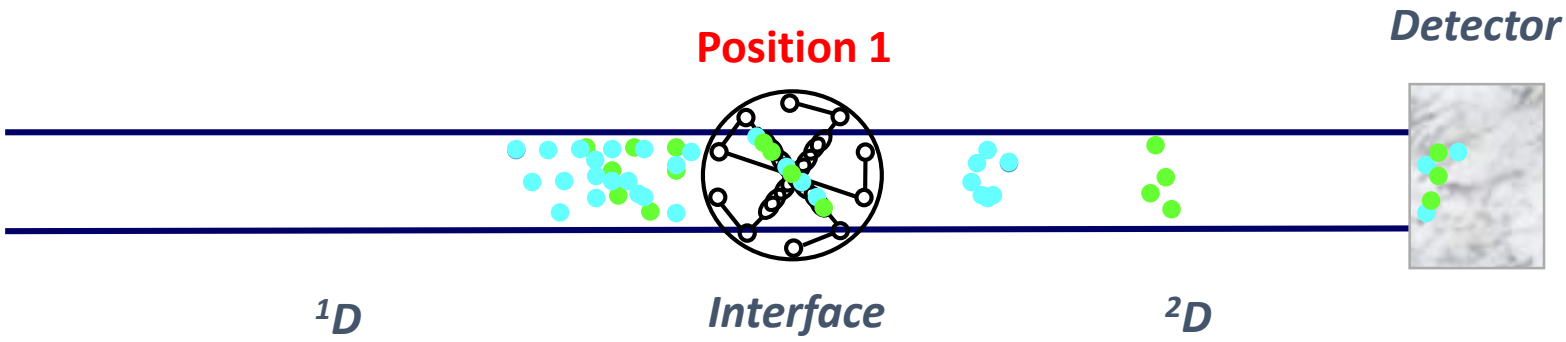
CHROMATOGRAM

2D-LC: HOW A MODULATOR WORKS



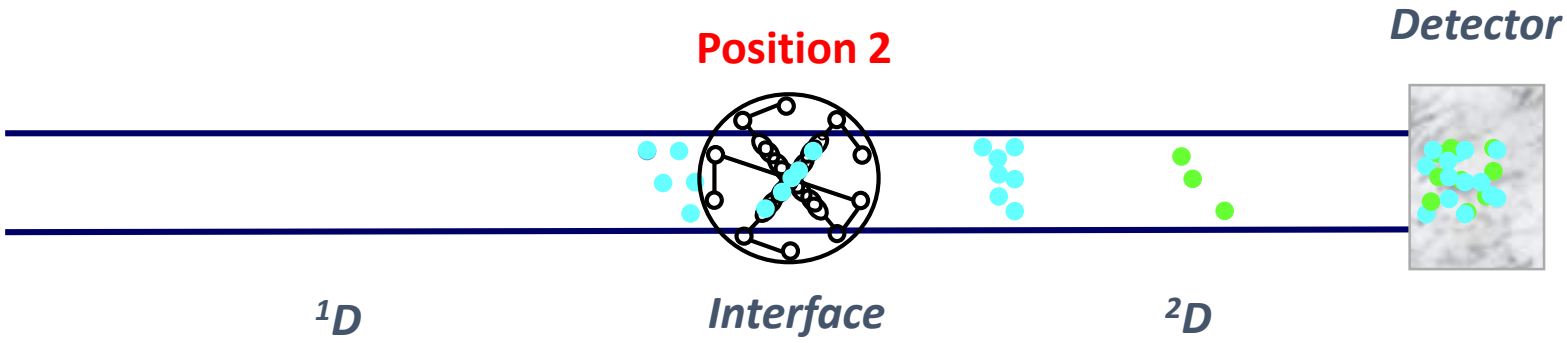
CHROMATOGRAM

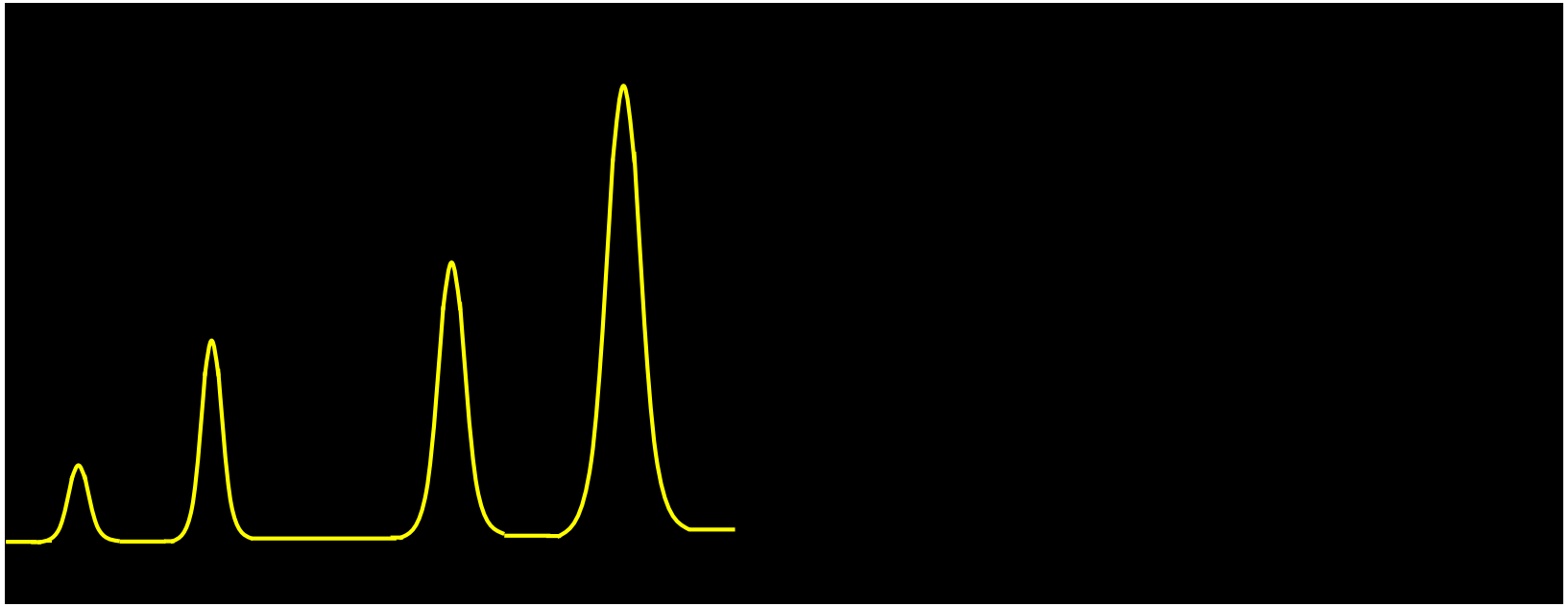
2D-LC: HOW A MODULATOR WORKS



CHROMATOGRAM

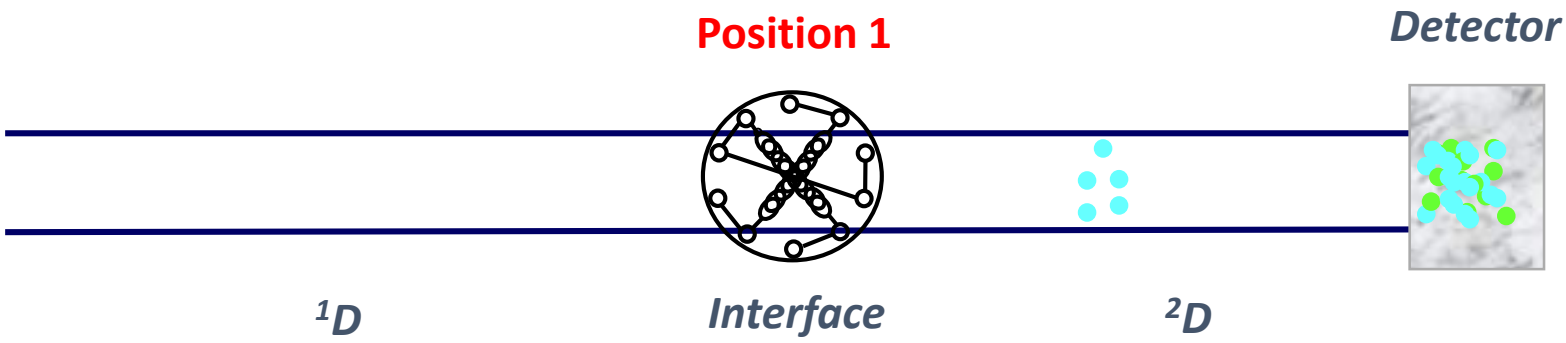
2D-LC: HOW A MODULATOR WORKS

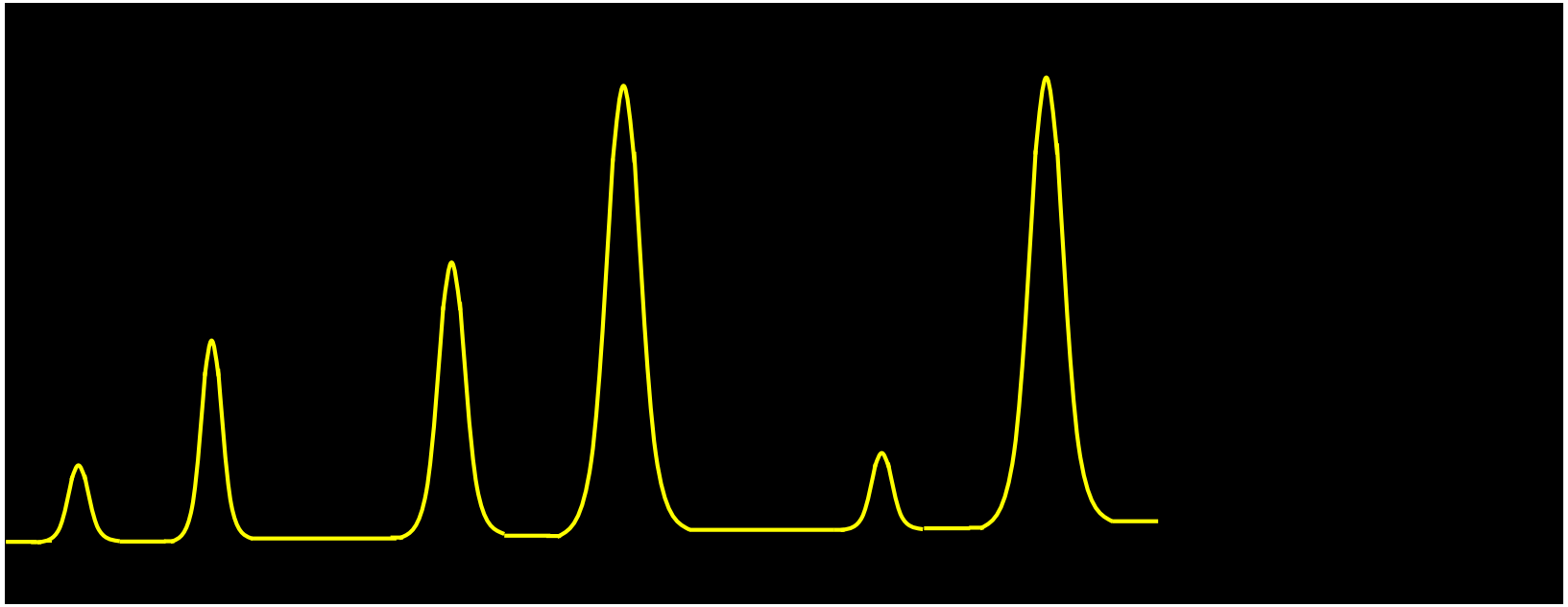




CHROMATOGRAM

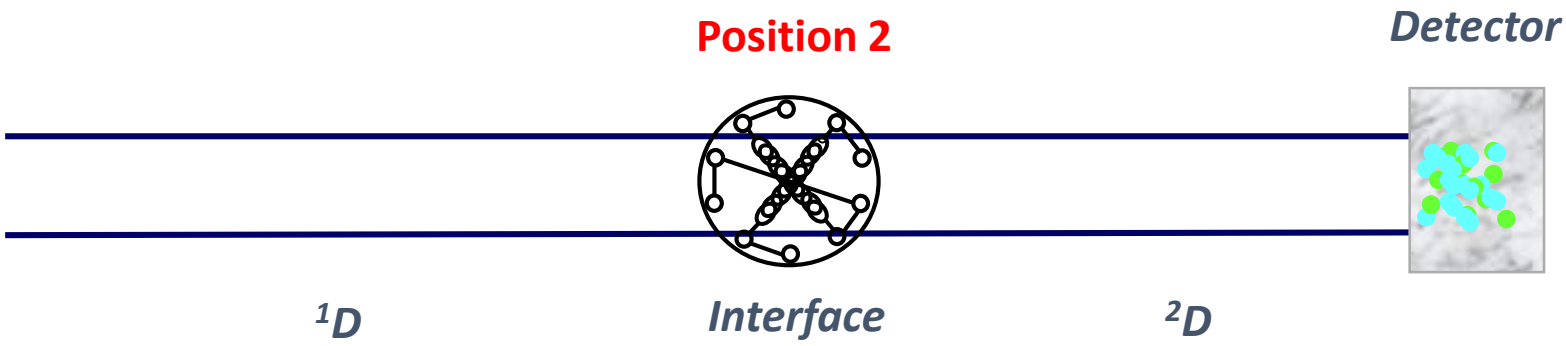
2D-LC: HOW A MODULATOR WORKS

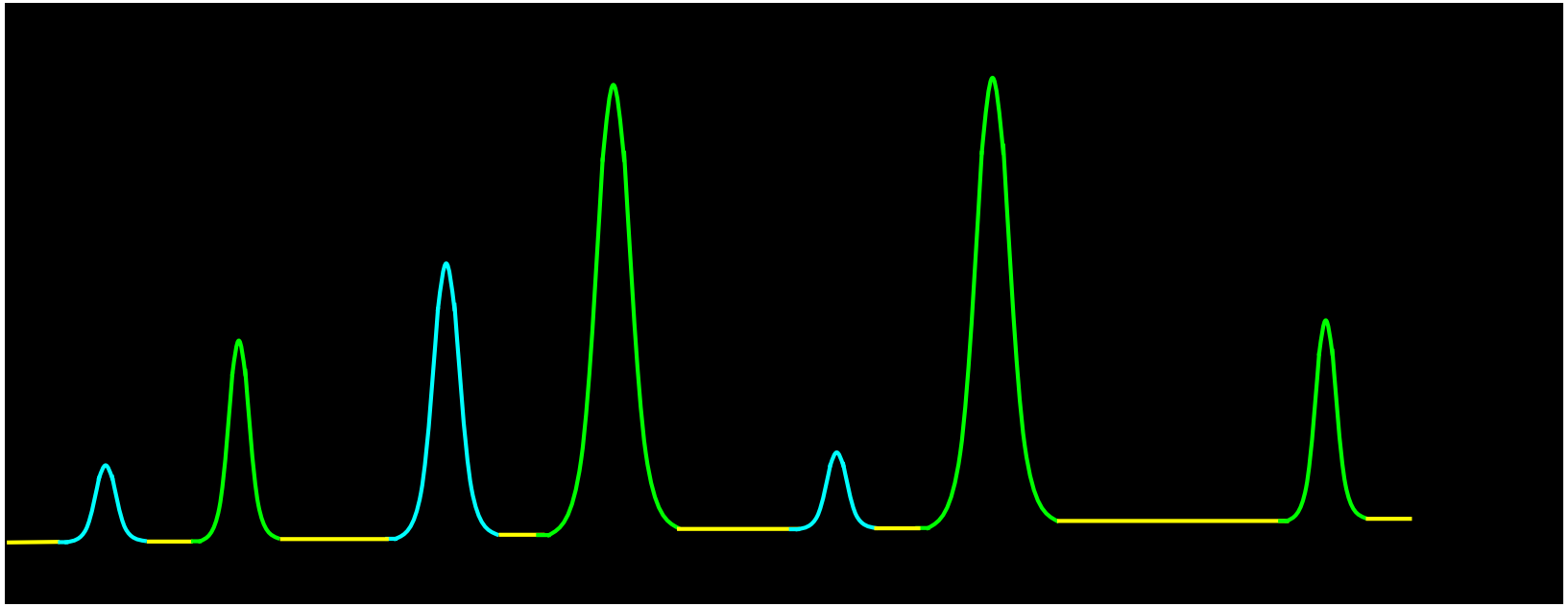




CHROMATOGRAM

2D-LC: HOW A MODULATOR WORKS





CHROMATOGRAM

MODULATOR

- Critical part to get a proper LC × LC separation.
- Part of instrument where the physical interaction between dimensions takes place.
- The modulator should collect ^{1}D effluent continuously and make its transfer to the ^{2}D.

TYPE OF MODULATORS

LC × LC

Non-focusing interfaces.

» Switching valves generally equipped with sampling loops.

Focusing interfaces.

» Switching valves: trapping columns, active modulation.

» Vacuum-assisted evaporative interface.

» Thermal modulation.

2D-LC

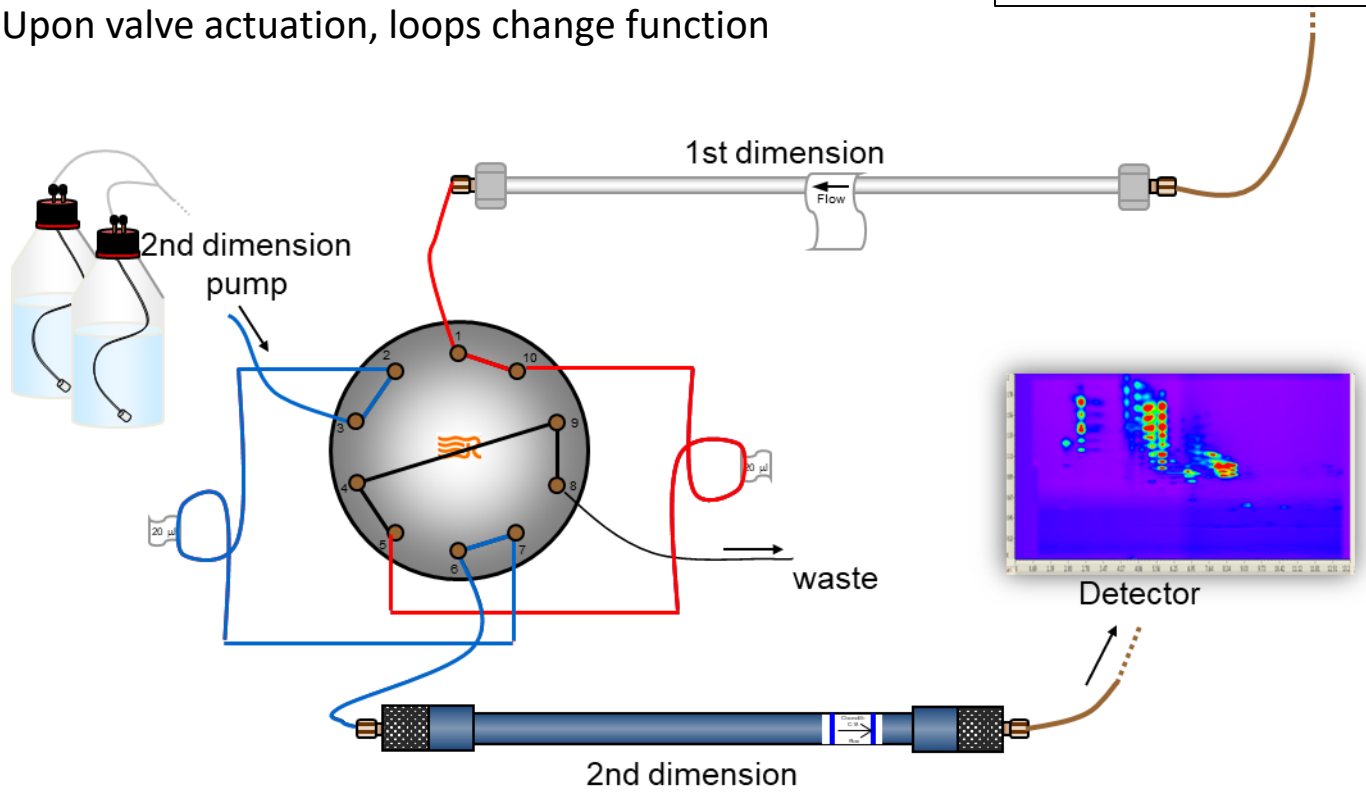
Other modulators

MODULATOR

NON-FOCUSING INTERFACES (LC × LC)

- By far, the most-used approach up to date.
- Set-up controlled by one or more switching valves equipped with two sampling loops of identical internal volume.
- Sampling loops function: collect / inject
- Upon valve actuation, loops change function

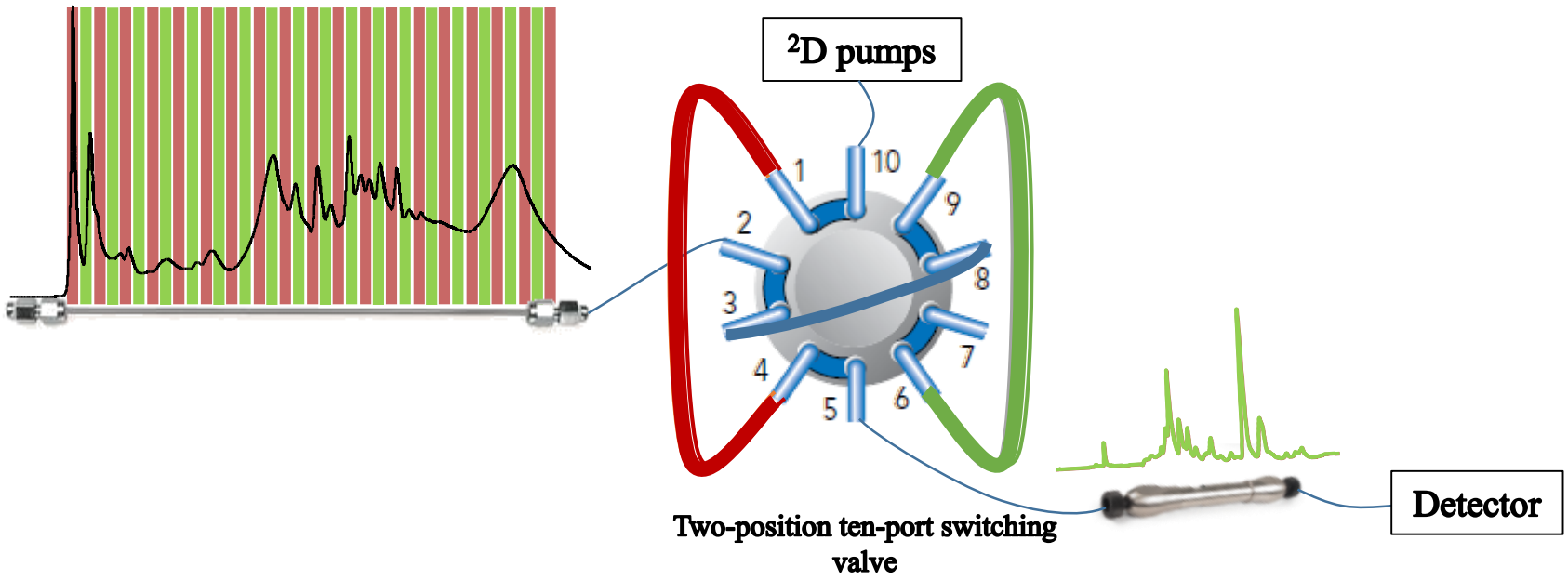
Installation arrangement:



MODULATOR

NON-FOCUSING INTERFACES (LC × LC)

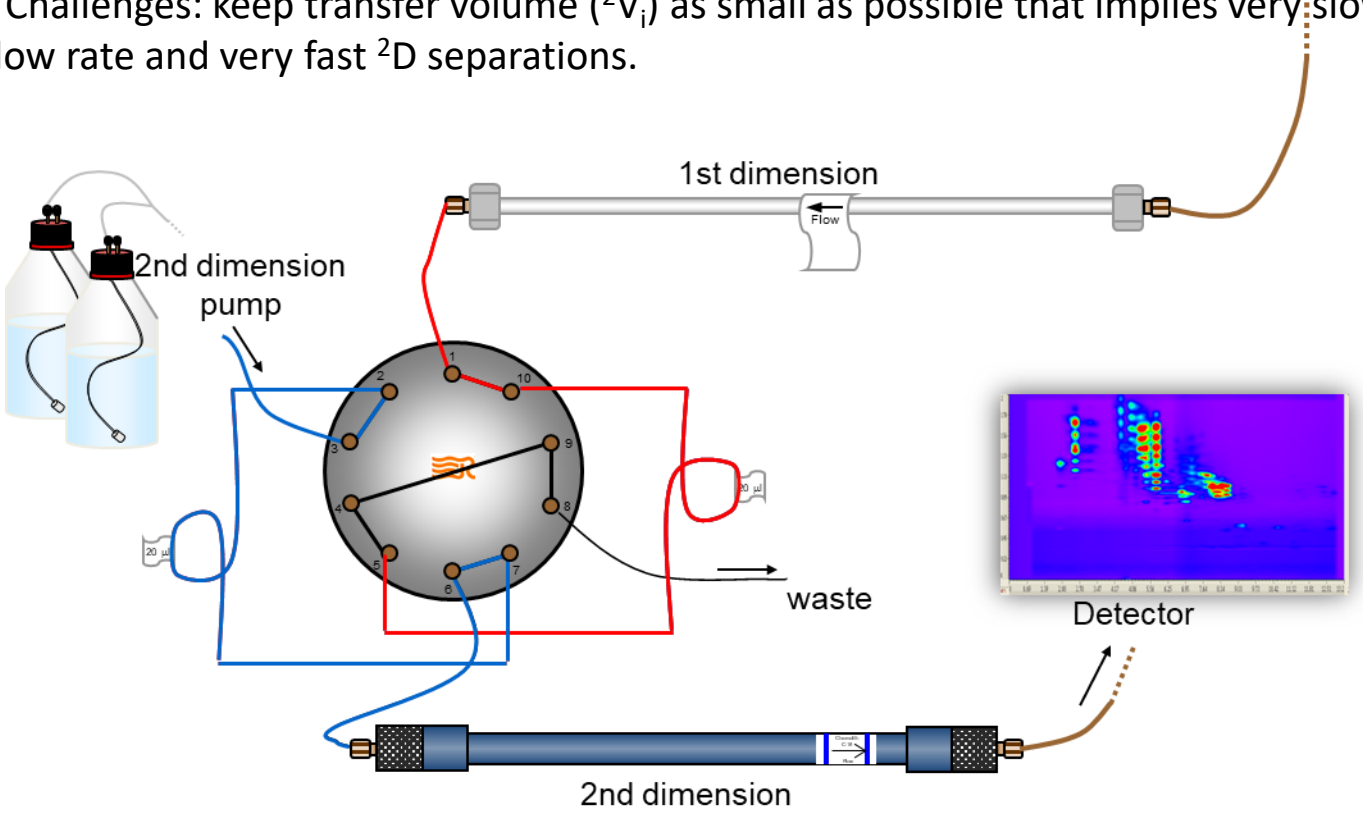
TYPICAL WORKING SEQUENCE



MODULATOR

NON-FOCUSING INTERFACES (LC × LC)

- Loop volume directly related to ^{1}D flow rate and modulation time. → $V_L = {}^1F \times t_s$
- Loop volume = injection volume in each ^{2}D analysis.
- Therefore, each ^{2}D analysis (separation + column reconditioning) should last at most the modulation time.
- Challenges: keep transfer volume (^2V_i) as small as possible that implies very slow ^{1}D flow rate and very fast ^{2}D separations.



MODULATOR

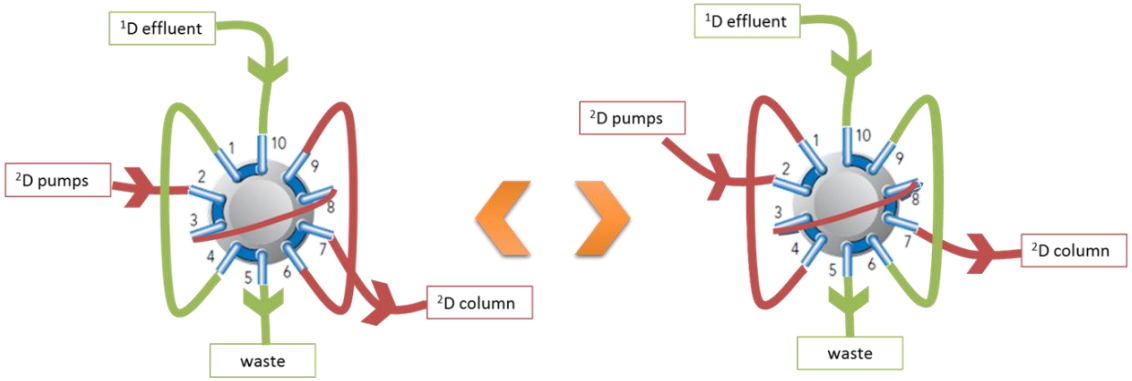
NON-FOCUSING INTERFACES (LC × LC)

SWITCHING VALVES SET-UPS

"Traditional": 10-port 2-position

Need of bridge modifies path in one of the two positions (small but observable retention shifts)

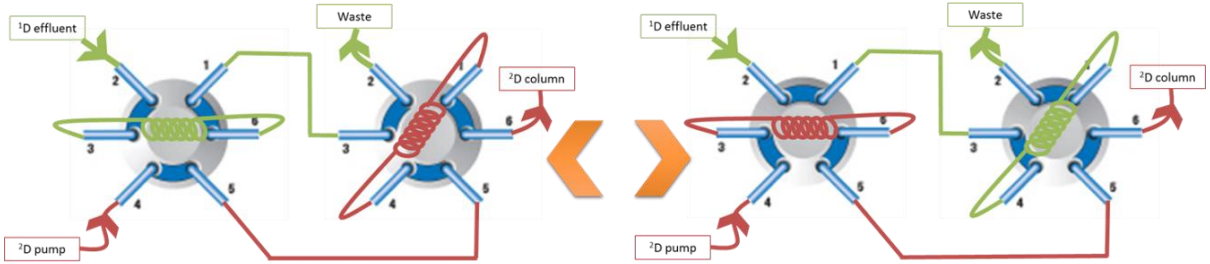
A



2 × 6-port 2-position

B

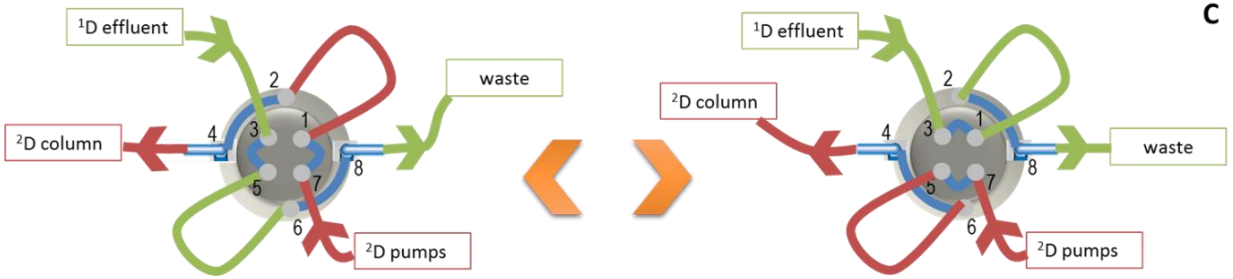
SHIMADZU
Excellence in Science



8-port 2-position

C

Agilent

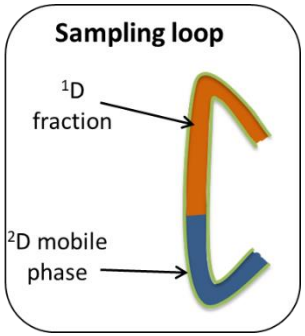


MODULATOR

NON-FOCUSING INTERFACES (LC × LC)

SAMPLING LOOP VOLUME

$$V_L = {}^1F \times t_s$$



- Loop volume directly related to ^{1}D flow rate and modulation time.
- Loop volume could be modified but both should be identical.
- Higher internal volumes than strictly needed could contribute to dilution of ^{1}D solvent in ^{2}D mobile phase, reducing solvent strength.
- Thus, change in sampling loop volume offers modification of fraction solvent.

	^{2}D - C_{18} 50 × 4.6 mm, 2.7 µm			^{2}D - C_{18} 30 × 4.6 mm, 2.7 µm		
Sampling loop volume						
	20 µL	**30 µL**	**50 µL**	**20 µL**	**30 µL**	**50 µL**
^{2}Vinj (V dilution)	20 µL (0.5 µL)	30 µL (10.5 µL)	50 µL (30.5 µL)	20 µL (5.0 µL)	30 µL (15.0 µL)	50 µL (35.0 µL)
% ^{2}D column void volume	4%	6%	10%	7%	10%	17%
A_O	68%	76%	79%	82%	82%	84%
Normalized sensitivity	0.85	1.00	1.37	1.08	1.32	1.61
$^{2D}n_c$ corr.	1176	1493	1780	1399	1424	1495

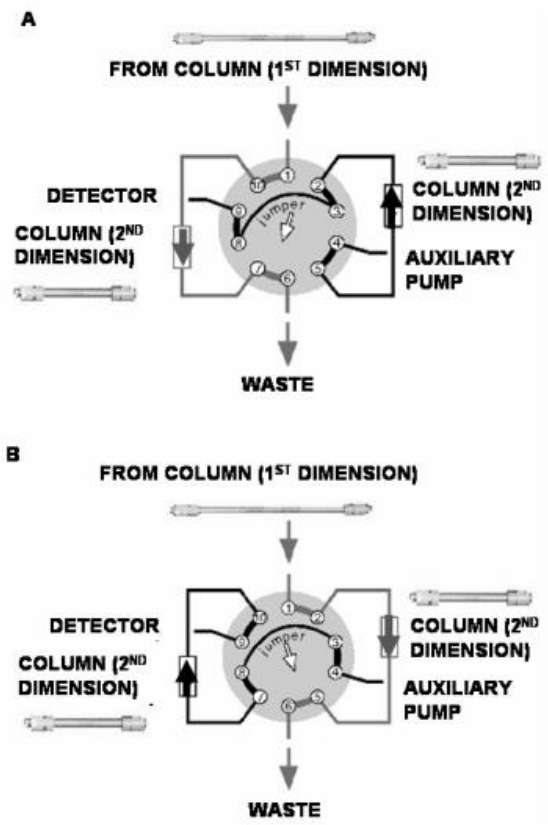
Montero et al., Analytica Chimica Acta 985 (2017) 202-212

MODULATOR

NON-FOCUSING INTERFACES (LC × LC)

RELATED LESS-USED ARRANGEMENTS

Two parallel ^{2}D columns

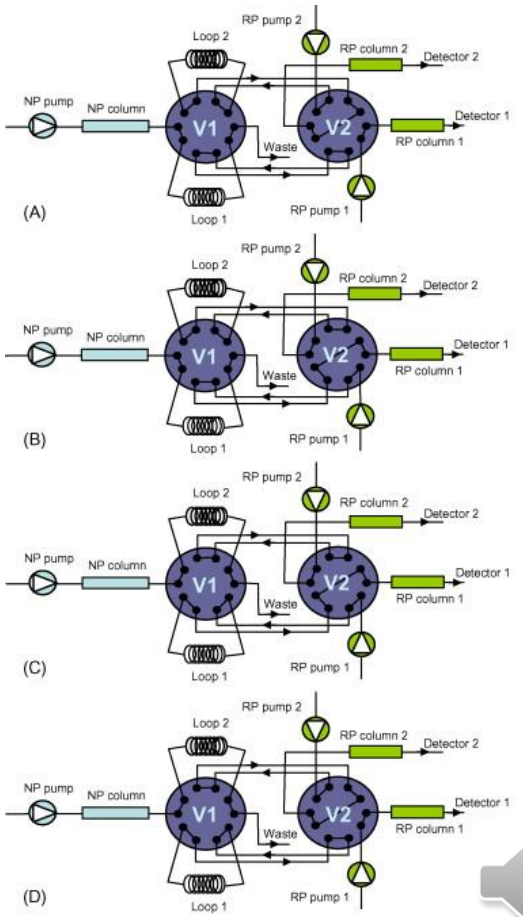


Cacciola et al., J. Sep. Sci. 2006, 29, 2500 – 2513

- Low reproducibility of parallel columns
- Severe undersampling (without loops)
- Very sophisticated and complicated set-up (with loops)

Although successful approaches, no widespread use

Two parallel ^{2}D columns combined with loops

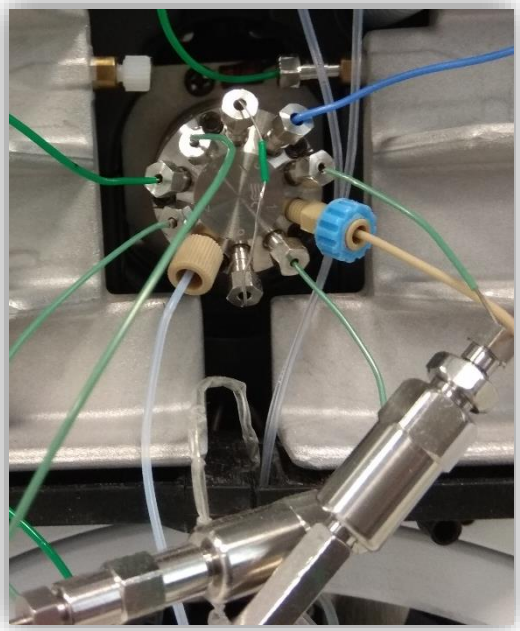


François et al., J. Chromatogr. A. 2008, 1178, 33-42

MODULATOR

FOCUSING INTERFACES (LC × LC)

- Tool to solve the problems related to solvent strength mismatch between dimensions.
- HILIC × RP or NP × RP are examples of very orthogonal couplings in which the solvent transfer presents poor compatibility.
- Generally, the stronger solvent in ^{1}D is the weaker in ^{2}D and vice versa.
- ^{2}D seriously hampered: broad and/or distorted peaks.

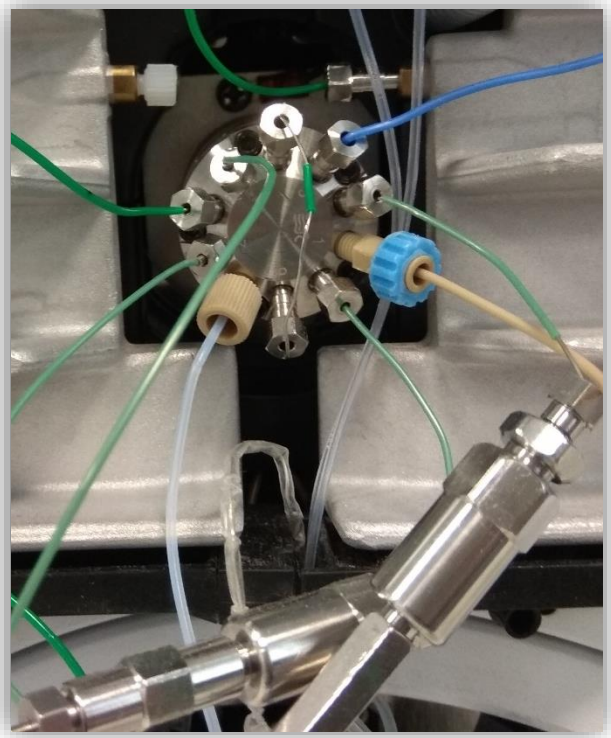


MODULATOR

FOCUSING INTERFACES (LC × LC)

I) TRAPPING COLUMNS

- Similar valve configuration than loops interface.
- Trapping columns of suitable stationary phase material instead of loops.



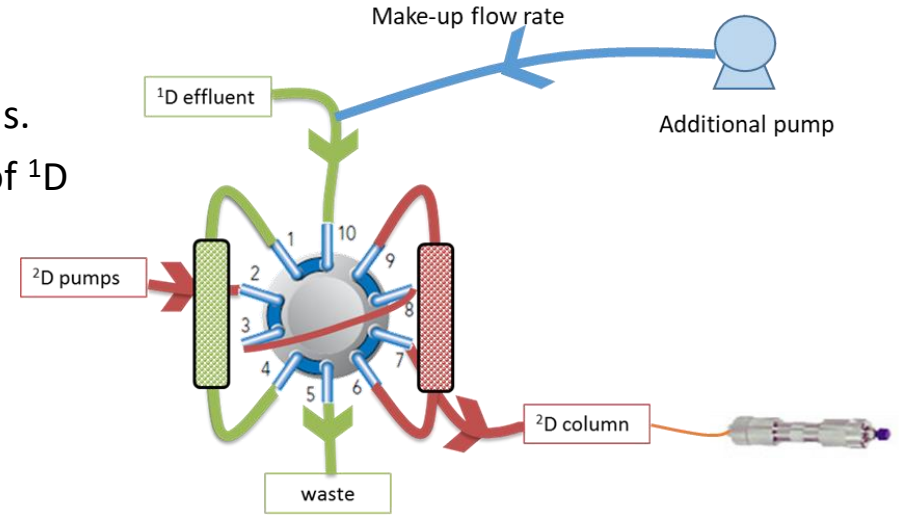
- Aim: to foster a concentration step in the trapping column
- Retention no too strong to allow subsequent desorption of analytes.
- Higher flow rates (thus, bigger fractions) possible in ^{1}D since retention of analytes may produce an elimination of ^{1}D solvent
- Forward or back-flush elution modes.

MODULATOR

FOCUSING INTERFACES (LC × LC)

II) ACTIVE MODULATION

- Similar valve configuration than trapping columns.
- Additional make-up flow introduced at the exit of ^{1}D before entering the valve.

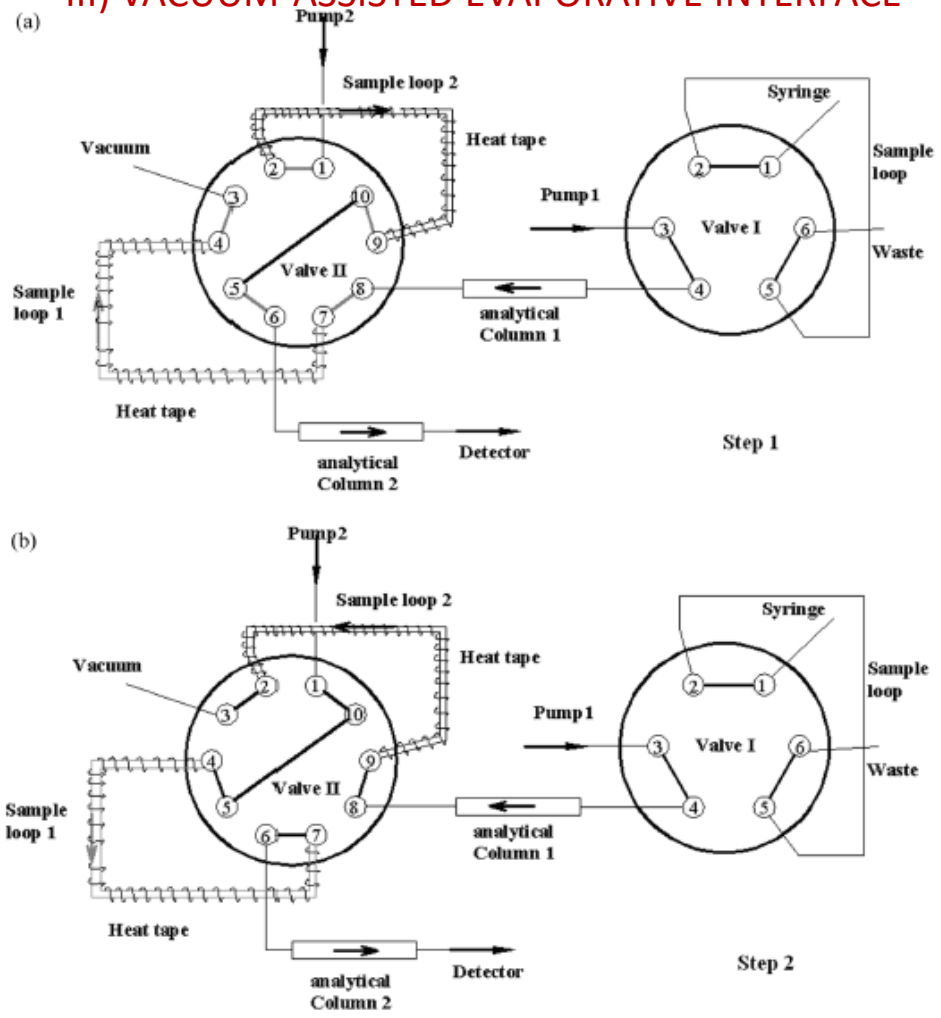


- Aim: to decrease solvent strength to make it compatible with ^{2}D starting mobile phase.
- On-column focusing on the trapping column as analytes are dissolved in a more appropriate solvent.
- Injection in ^{2}D in fully compatible solvent.
- Narrower peaks and higher efficiency in ^{2}D.
- Potentially, more sample can be loaded in ^{1}D column resulting in higher sensitivity.

MODULATOR

FOCUSING INTERFACES (LC × LC)

III) VACUUM-ASSISTED EVAPORATIVE INTERFACE



- Modification of a regular loop-based interface for NP × RP.
- Application of vacuum at 25ºC.
- ^{1}D solvent evaporated and analytes deposited in the inner wall of the loop.
- ^{2}D mobile phase should be able to redissolve analytes.

PROS

» No dilution, reduced band broadening

» No dependence between fraction volume and ^{2}D injection volume

CONS

» Reduced analyte recovery

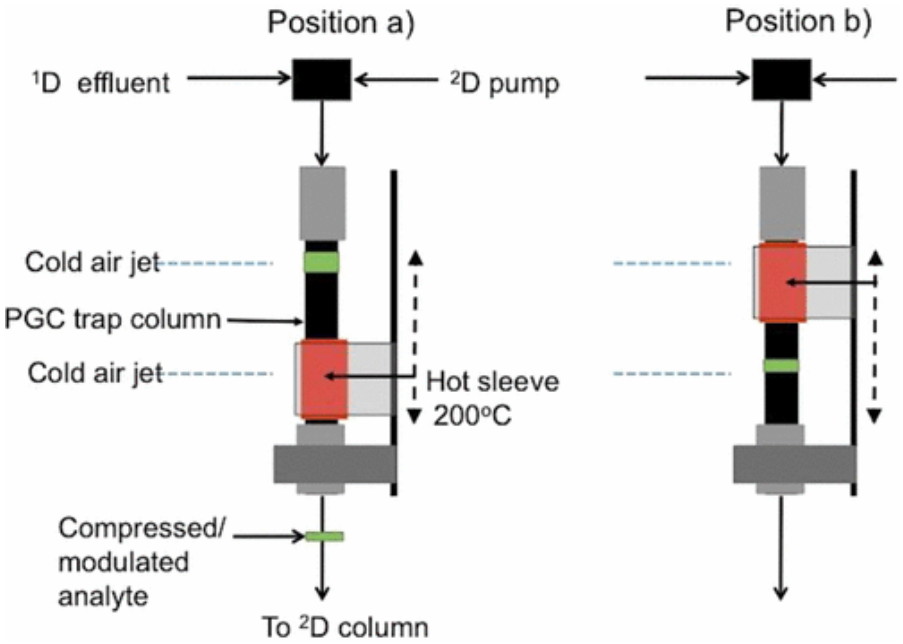
» High risk of sample loss

Tian et al., J. Sep. Sci. 2008, 31, 1677 – 1685

MODULATOR

FOCUSING INTERFACES (LC × LC)

IV) THERMAL MODULATION



- The only design presented so far involving a *valveless* modulator.
- Inspired in GC × GC modulators.
- Heating and cooling cycles to capture analytes in the trap.

PROS

» Fast transfer, no pressure fluctuations (better signal-to-noise ratios)

» Narrower bands transferred

CONS

» Very sophisticated

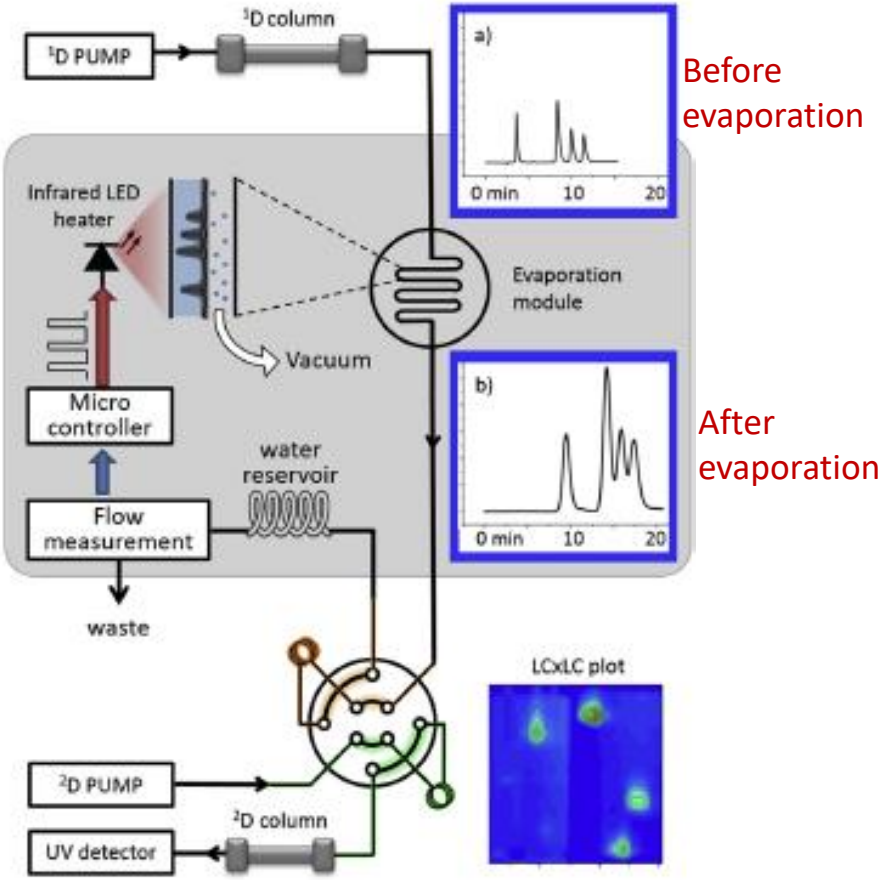
» Still to be further developed

Creese et al., Anal. Chem., 2017, 89, 1123–1130

MODULATOR

FOCUSING INTERFACES (LC × LC)

V) EVAPORATIVE MEMBRANE MODULATION



- Evaporative device placed on-line at the exit of ^{1}D.
- Solvent evaporation to a fixed factor using a porous hydrophobic membrane.
- Fraction volume to ^{2}D greatly reduced improving peak shapes

PROS

» Better sensitivity

» Peak shape improvement

CONS

» Reduction in ^{1}D peak capacity

Fornells et al., Anal. Chim. Acta, 2018, 1000, 303–309

MODULATOR

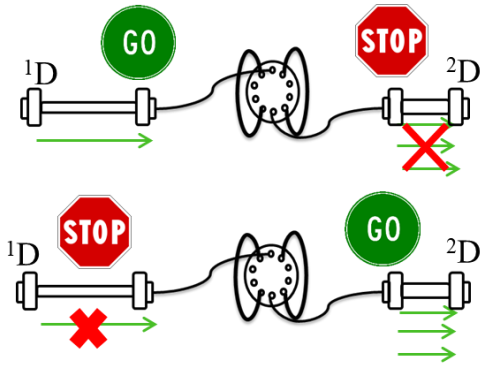
INTERFACES (LC × LC)

Interface	Description	Pros	Cons
Sampling loops	One 8-, 10-, 12-port 2-position switching valve or 4-port duo valve or two 6-port 2-position switching valves equipped with two sampling loops with identical volume	› Versatility › Simple configuration › High reproducibility	› No focusing effect › Short ^{2}D analysis time required
Two parallel ^{2}D columns	Use of two ^{2}D columns directly connected to the valve	› Longer ^{2}D analysis, higher ^2n_c	› Severe ^{1}D undersampling due to long modulation times › Lack of reproducibility or drift appearance because of non-identical ^{2}D columns
Trapping columns	One of the possible configurations for switching valves using two trapping columns with similar ^{2}D column selectivity.	› Focusing effect › Reduction of ^{2}D band broadening	› Potential loss of some components due to un-efficient trapping › Short ^{2}D analysis time required
Active modulation (LC/a × m/LC)	Use of trapping columns with the incorporation of a make-up flow at the exit of the ^{1}D effluent, before entering the trap	› Reduction of the ^{1}D effluent solvent strength › Effective trap retention › Strong focusing effect › Increase in selectivity	› Need of an additional pump › Short ^{2}D analysis time required
Vacuum-assisted evaporation	Conventional loop interface with the incorporation of heat and vacuum connected to the valve.	› Complete removal of ^{1}D solvent, making compatible the coupling of almost all separation modes	› High risk of sampling loss
Thermal modulation	Modulator formed by a trapping column with the same stationary phase than the ^{2}D column, a LTM and a longitudinally modulated cryogenic system.	› Reduction of ^{2}D band broadening	› ^{2}D analysis time limited by the modulator cycles › Very sophisticated instrumentation

MODULATOR

OTHER MODULATORS (2D-LC)

STOP-FLOW LC × LC

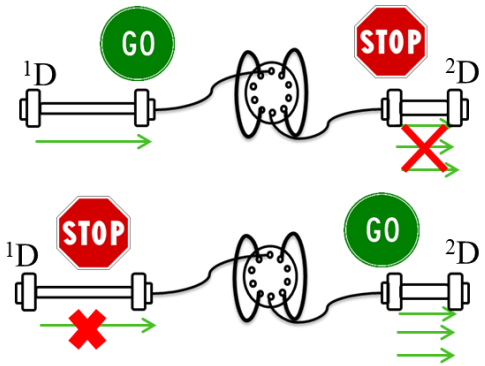


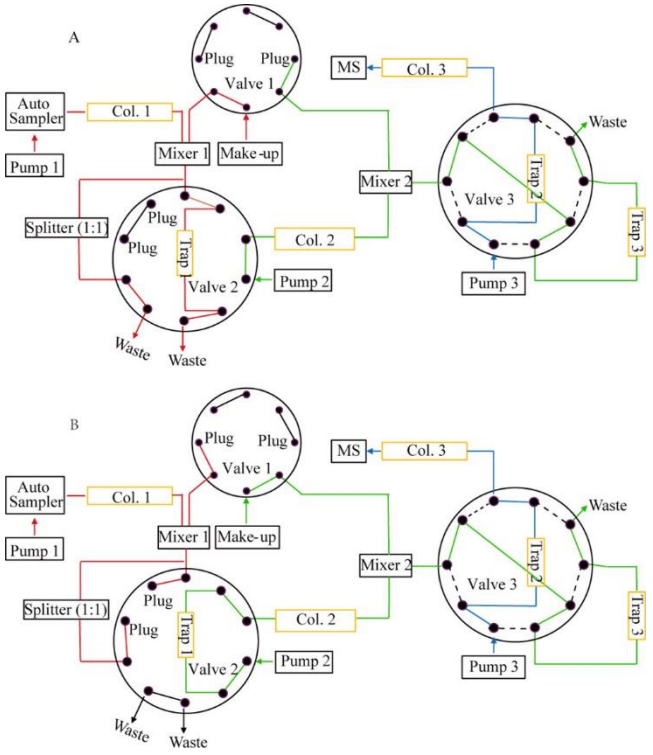
- ^{1}D flow stopped to allow longer ^{2}D separations.
- ^{2}D peak capacity increased (also total $^{2D}n_c$)
- Loss of first dimension separation – dispersion
- Total analysis time significantly longer (several hours)

MODULATOR

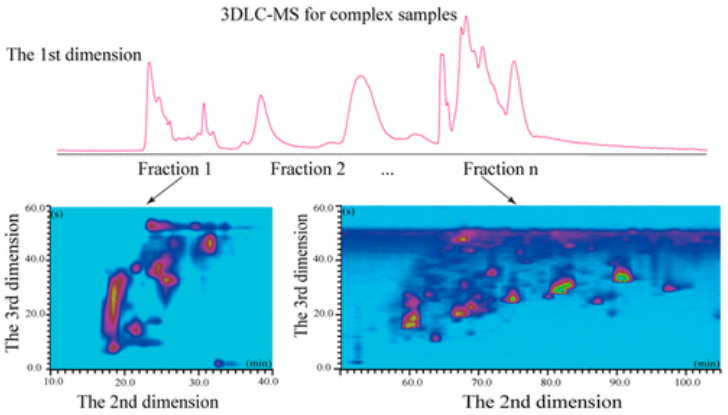
OTHER MODULATORS (2D-LC)

STOP-FLOW LC × LC





Metabolites in a soybean extract



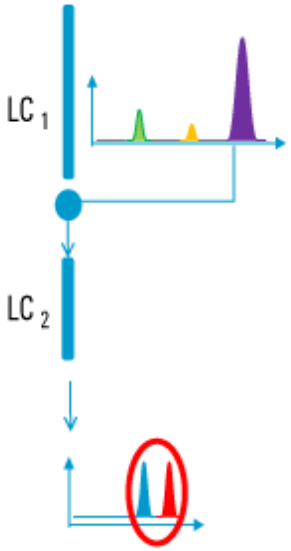
Wang et al., Anal Chem., 2017, 89, 1443-1438

- Sample fractionated in ^{1}D and transferred to LC × LC system through a stop-flow interface
- Each fraction analyzed by LC × LC
- Alternative selectivity in each dimension

MODULATOR

OTHER MODULATORS (2D-LC)

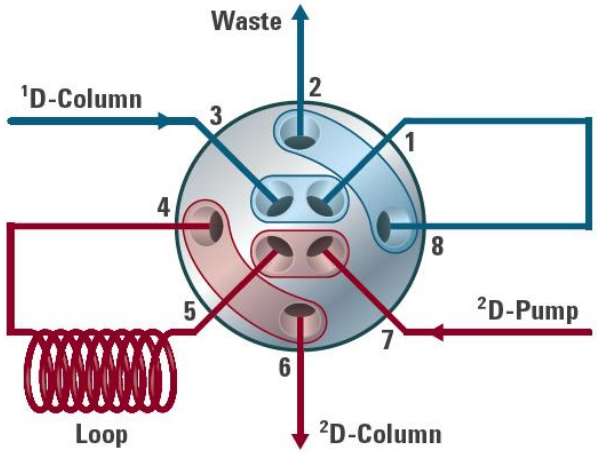
HEART-CUTTING (LC-LC)



» Just selected fractions submitted to second separation.

» Targeted approach.

» Long ^{2}D analysis possible, which might be advantageous with respect to LC × LC



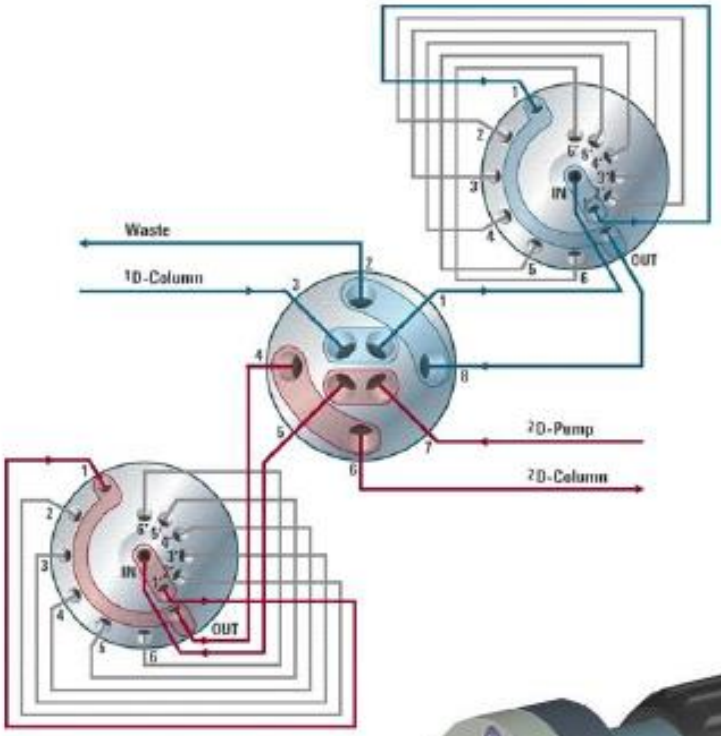
SEQUENCE:

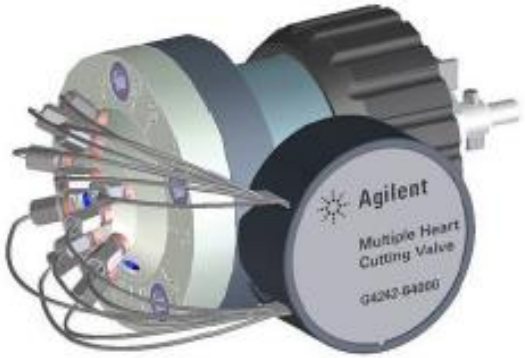
1) Loop filled with fraction
2) Valve actuation
3) Analysis in ^{2}D

MODULATOR

OTHER MODULATORS (2D-LC)

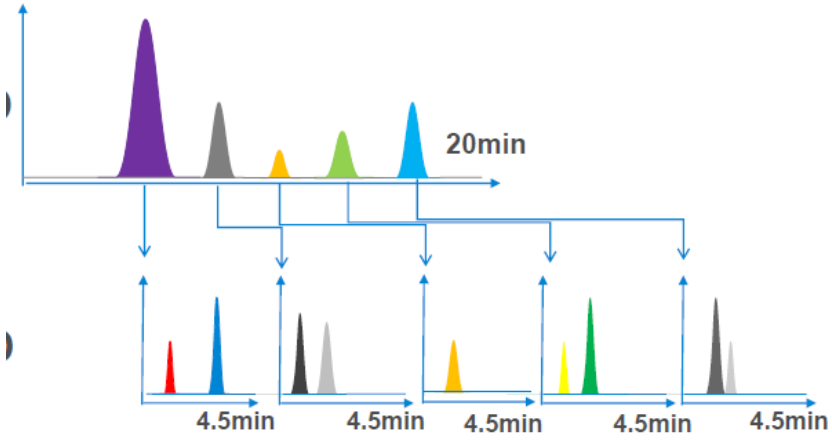
MULTIPLE HEART-CUTTING





» Possibility of storing different cuts for further analysis.

» No need to stop ^{1}D separation.



Concepts and Comparison in Multidimensional Separations (1987)

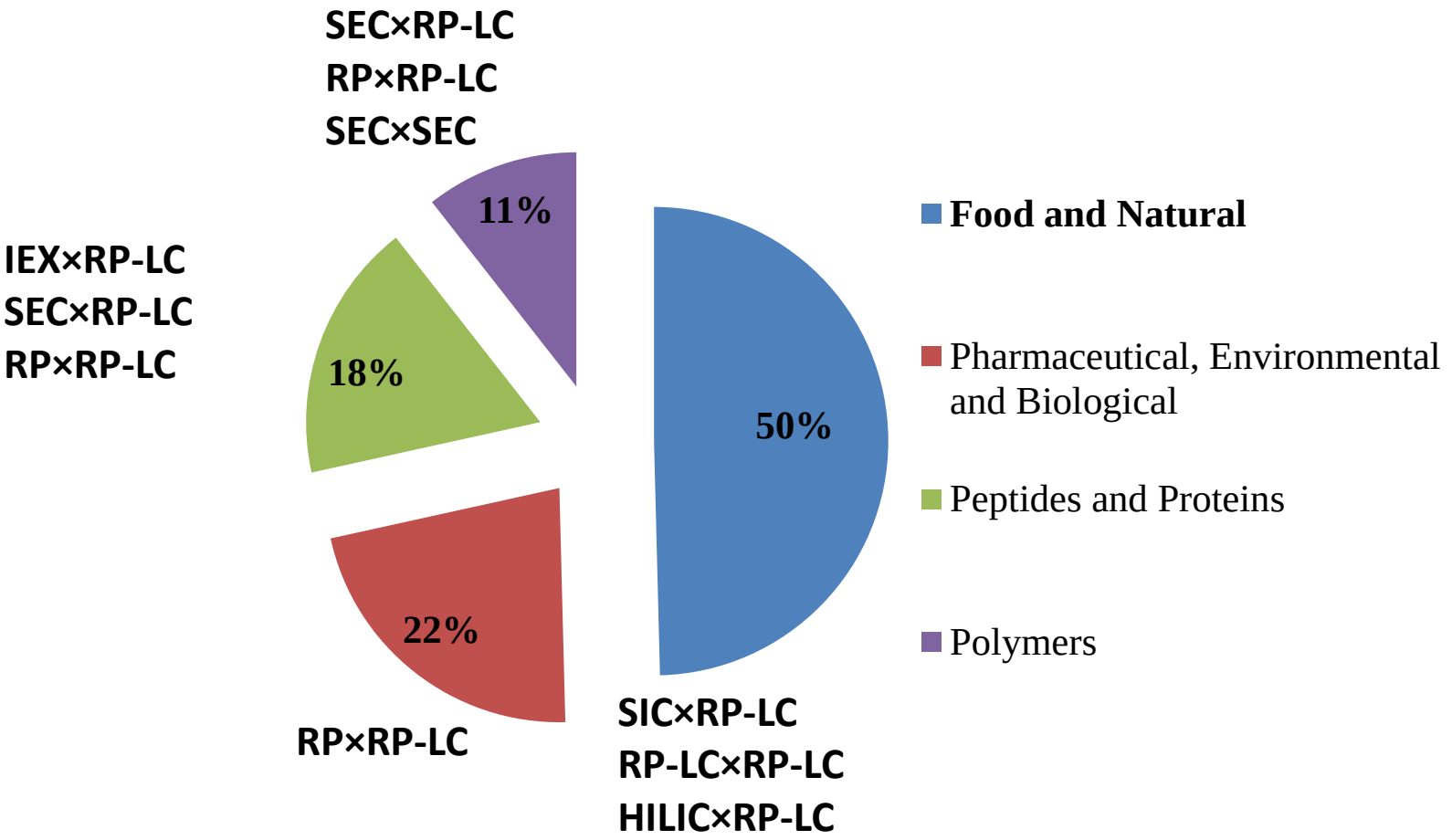
J. C. Giddings
Department of Chemistry, University of Utah, Salt Lake City, UT 84112, USA

We use here the term *multidimensional separation* in a relatively broad context, which requires first that components be subjected to two or more largely independent separative displacements. However, a second criterion is imposed: the separation must be structured such that whenever two components are adequately resolved in any one displacement step, they will generally remain resolved throughout the process.

This second requirement rules out purely tandem arrangements of two or more columns in which the stream emerging from one column is fed directly to another. In such systems the resolution gained in one column can be partially or entirely nullified by a different order of migration rates in a subsequent column.

/OL. 10, MAY 1987 Journal of High Resolution Chromatography & Chromatography Communications pp 319-323

Distribution of LC × LC papers as a function of the application field (2019)



Second dimension (^{2}D) – some requirements

- The second dimension separation of the transferred fraction **must** be completed before the injection of the successive fraction eluting from the first column and should be fast enough to permit that (ideally) 3-4 transfers of the same ^{1}D peak.

- This is important because undersampling of ^{1}D peaks can cause serious loss of information in the two dimensional separation.

- These aspects emphasizes the need for very fast ^{2}D separations

Second dimension (^{2}D) – gradient mode

1. ^{2}D gradient

- A repetitive gradient is necessary when the differences in polarity and hydrophobicity of the components present in the sample are very large. In this context, isocratic conditions for the separation of these components in a very short time are difficult or impossible to optimize.

2. ^{2}D separation requirements in gradient mode

- Perform successive cycles with a very brief equilibration time.
- Work at high flow rates without loss of resolution, thus reducing the analysis time.

Second dimension (^{2}D) – Fast separation

Fast separations in ^{2}D can be achieved with:

✓ **Fast short columns**

- Monolithic
- Shell packed
- sub-2 μm

They enable to work at high flow rates without loss of resolution

✓ **An array of second dimension columns used in parallel**

This approach is critical due to the fact that different columns are rarely identical

✓ **High temperature HPLC**

The decreased viscosity of the eluent at high temperature allows a much higher linear velocity through the column with faster gradient development without significant loss in efficiency

FAST ^{2}D separations

Disadvantages

- **Column packed with microparticles**
  - **Elevated pressures**
  - **Long reconditioning times**

Advantage → **UHPLC**

Effective Height Equivalent to a Theoretical Plate (H) and the Van Deemter Equation

- The number of theoretical plates (N) is an index used to indicate the level of peak separation. The larger the N value, the sharper the peaks, which is thought to enable higher separation. However, the N value also increases in proportion to the column length (L). Therefore, it does not indicate the separation efficiency (performance) of packing materials.
- Consequently, it has become popular to express the separation efficiency of a packing material independently of column length, in terms of the height equivalent to a theoretical plate (H), which is calculated as the column length (L) divided by the number of theoretical plates (N).
- In other words, it is the column length per theoretical plate.

Effective Height Equivalent to a Theoretical Plate (H) and the Van Deemter Equation

$$H = \frac{L}{N} \quad \cdots\cdots \text{Equation 1}$$

- Equation 1 indicates that a smaller H value, a smaller L value or larger N value, means a more efficient packing material.

Packing material particle diameter

Linear velocity

$$H = A \cdot dp + \frac{B}{v} + C \cdot dp^2 \cdot v \quad \cdots\cdots \text{Equation 2}$$

- The van Deemter equation (Equation 2) expresses the relationship between the height equivalent to a theoretical plate (H) and column packing particle diameter and mobile phase linear velocity.

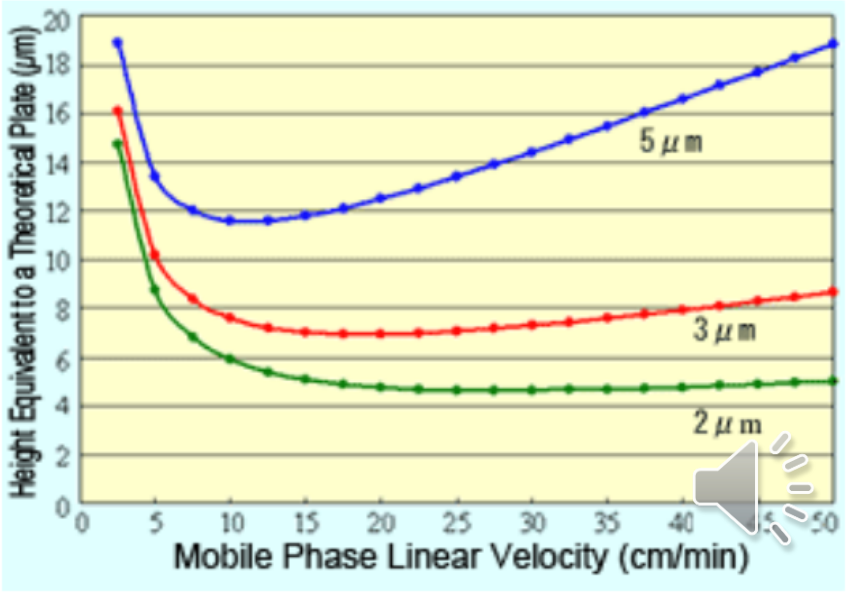
Effective Height Equivalent to a Theoretical Plate (H) and the Van Deemter Equation

Reducing the Packing Material Particle Size in Terms of the Van Deemter Curve

- Plotting the van Deemter equation results in curves like those shown in ther figure below. It shows how reducing particle size of the packing material reduces the height equivalent to a theoretical plate (H), which means a more efficient packing material. Also, it shows that when the packing material particle size is reduced, the linear velocity with the minimum H value is higher and the optimal linear velocity increases.

- This means that **reducing the packing material particle size allows using a shorter column and faster mobile phase flowrates without sacrificing separation.**

- **<u>In other words, it enables performing analyses faster.</u>**



Effective Height Equivalent to a Theoretical Plate (H) and the Van Deemter Equation

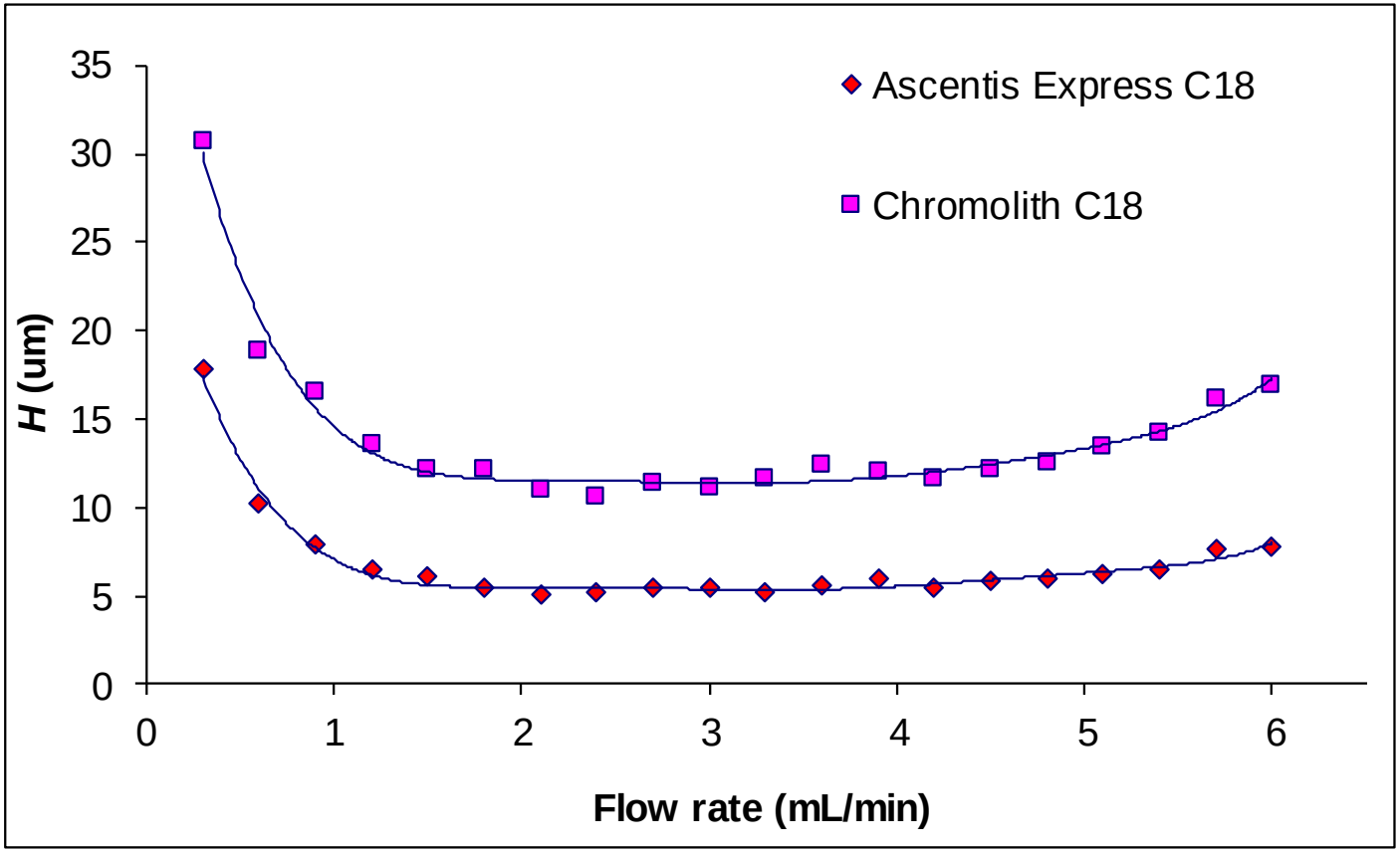
Advantages and Disadvantages of Microparticle Packing Materials

- Theoretically, the van Deemter equation also means that reducing the particle size of the packing material enhances the column efficiency and optimal mobile phase linear velocity, allowing faster analysis. However, even if microparticle packing materials provide such advantages, they also have the disadvantage of increasing pressure losses in the column.

$$\Delta P = \frac{\rho \cdot L \cdot v}{dp^2} \qquad \cdots\cdots \text{Equation 3}$$

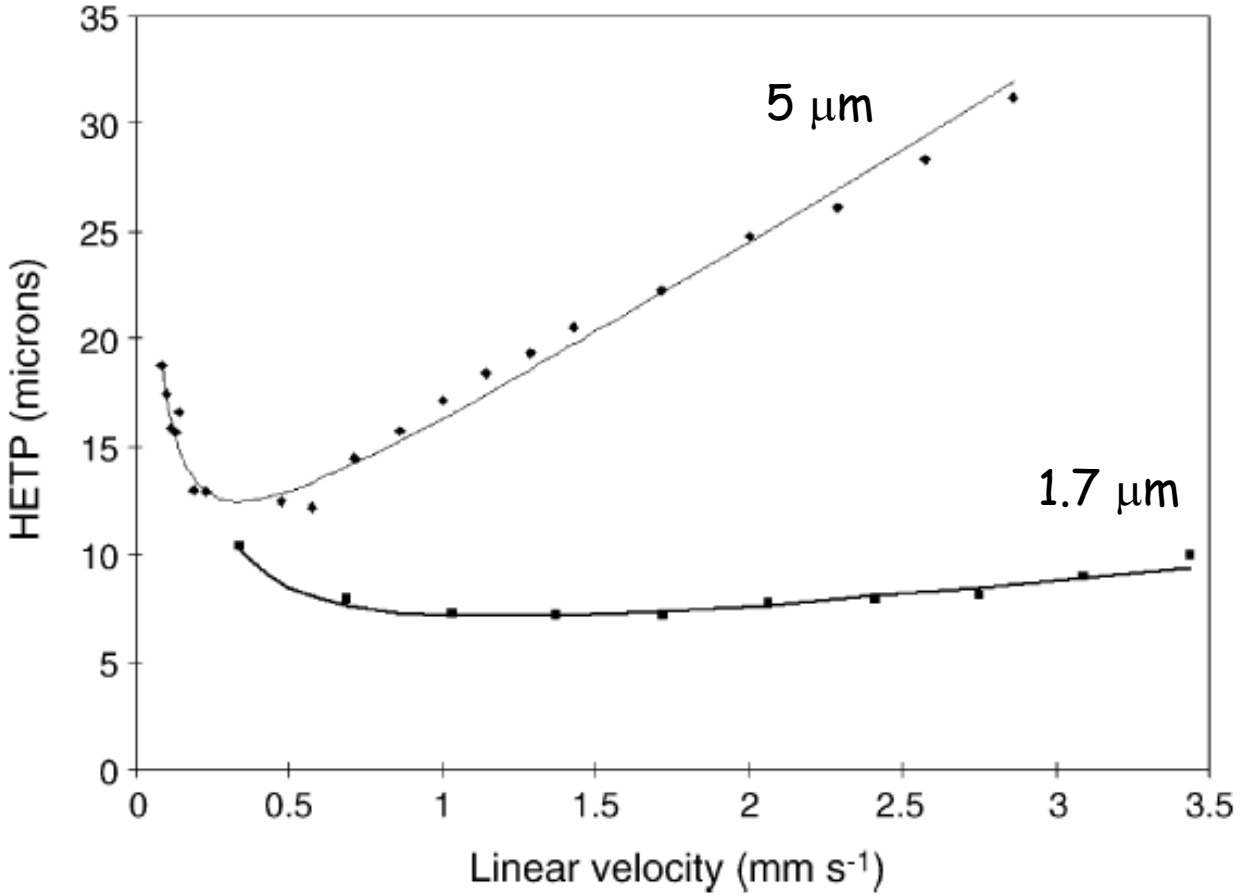
- Pressure losses in the column are described by equation 3 and are proportional to factors such as the mobile phase viscosity coefficient (ρ) for viscosity, column length (L), and the mobile phase linear velocity (v), and is inversely proportional to the square of the particle diameter (dp).

Shell-packed vs. Monolithic column in the ^{2}D of an LC×LC system



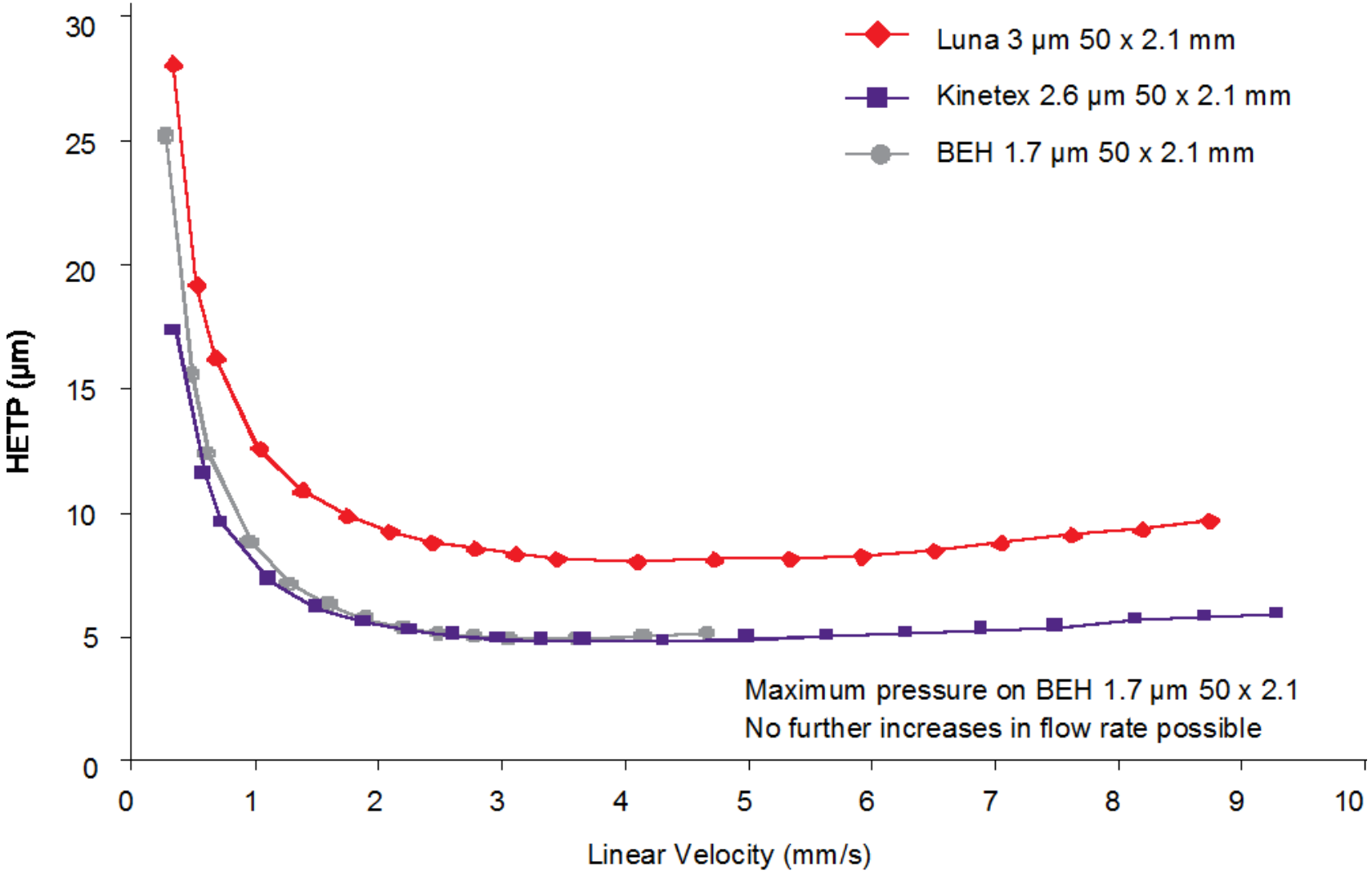
(Dugo et al. J. Sep. Sci. 2008, 31, 3297)

Sub-2 mm vs. particle packed column in the ^{2}D of an LC×LC system



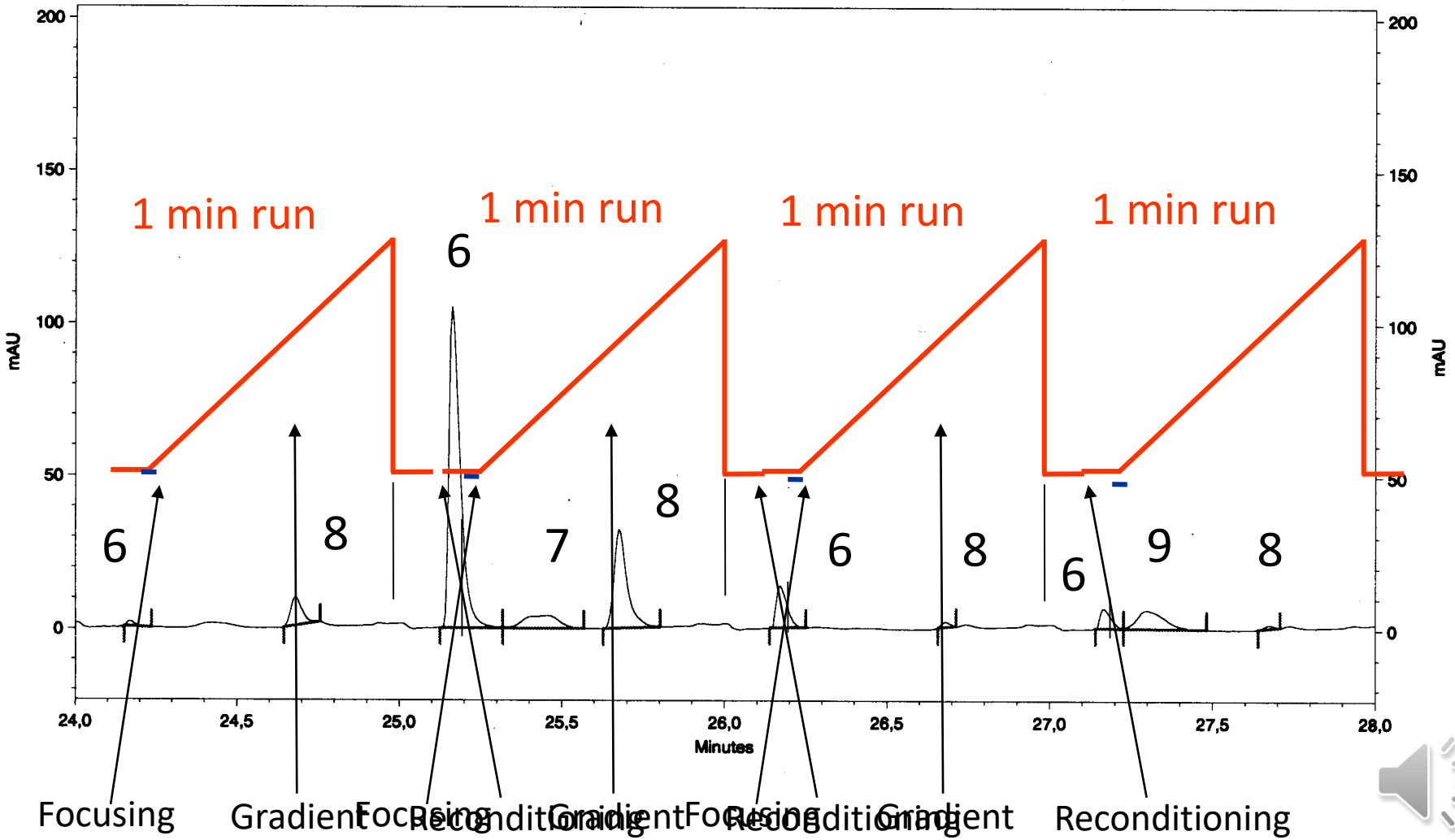
(Wren & Tchelitcheff, J. Chromatogr. A 2006, 1189, 140)

Comparison of different sub-3 µm stationary phases



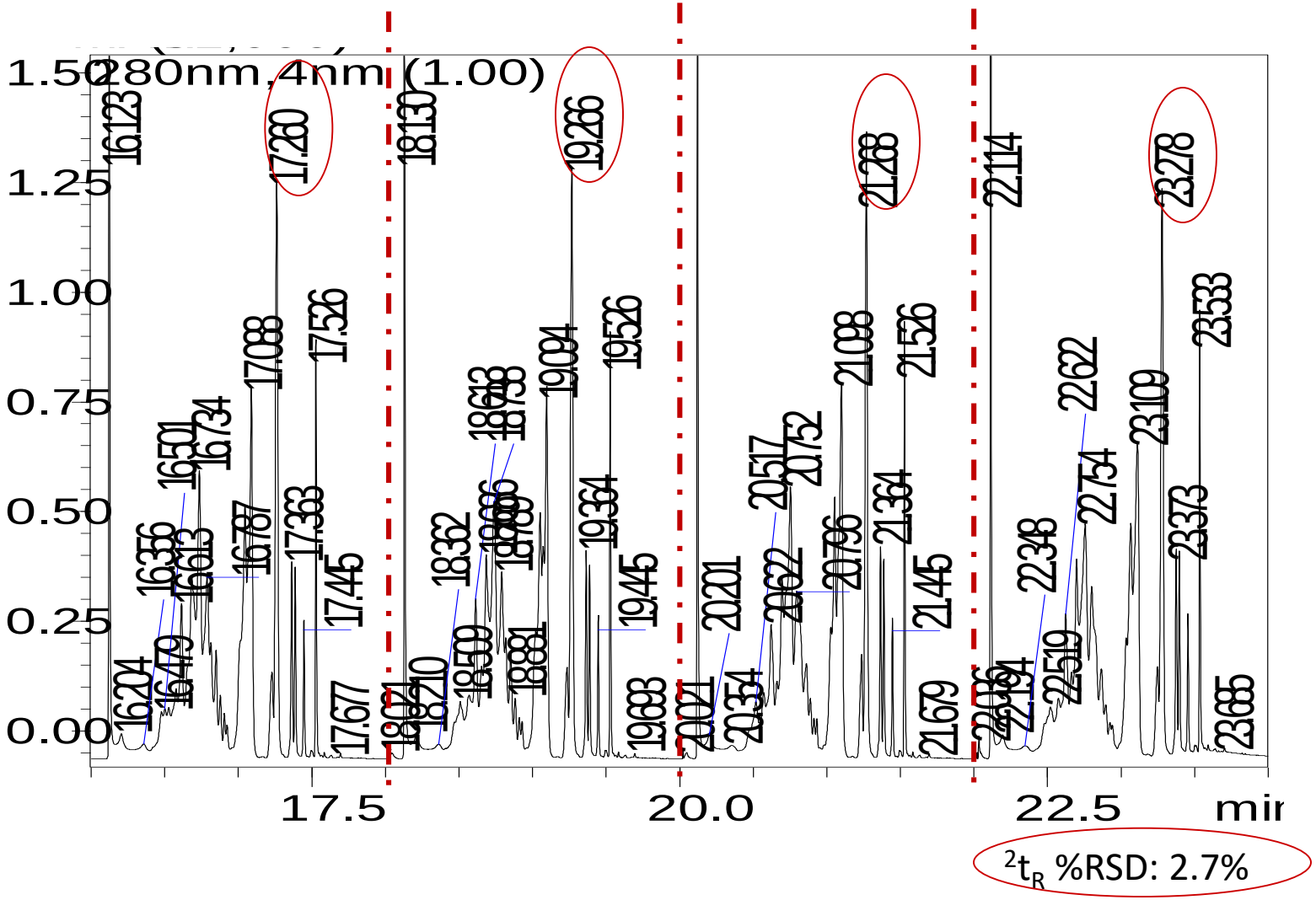
Van Deemter Plot – Agilent 1200

Fast ^{2}D separation:
1-min gradient analysis on a monolithic column in the ^{2}D of an LC×LC system *(18 sec reconditioning time)*



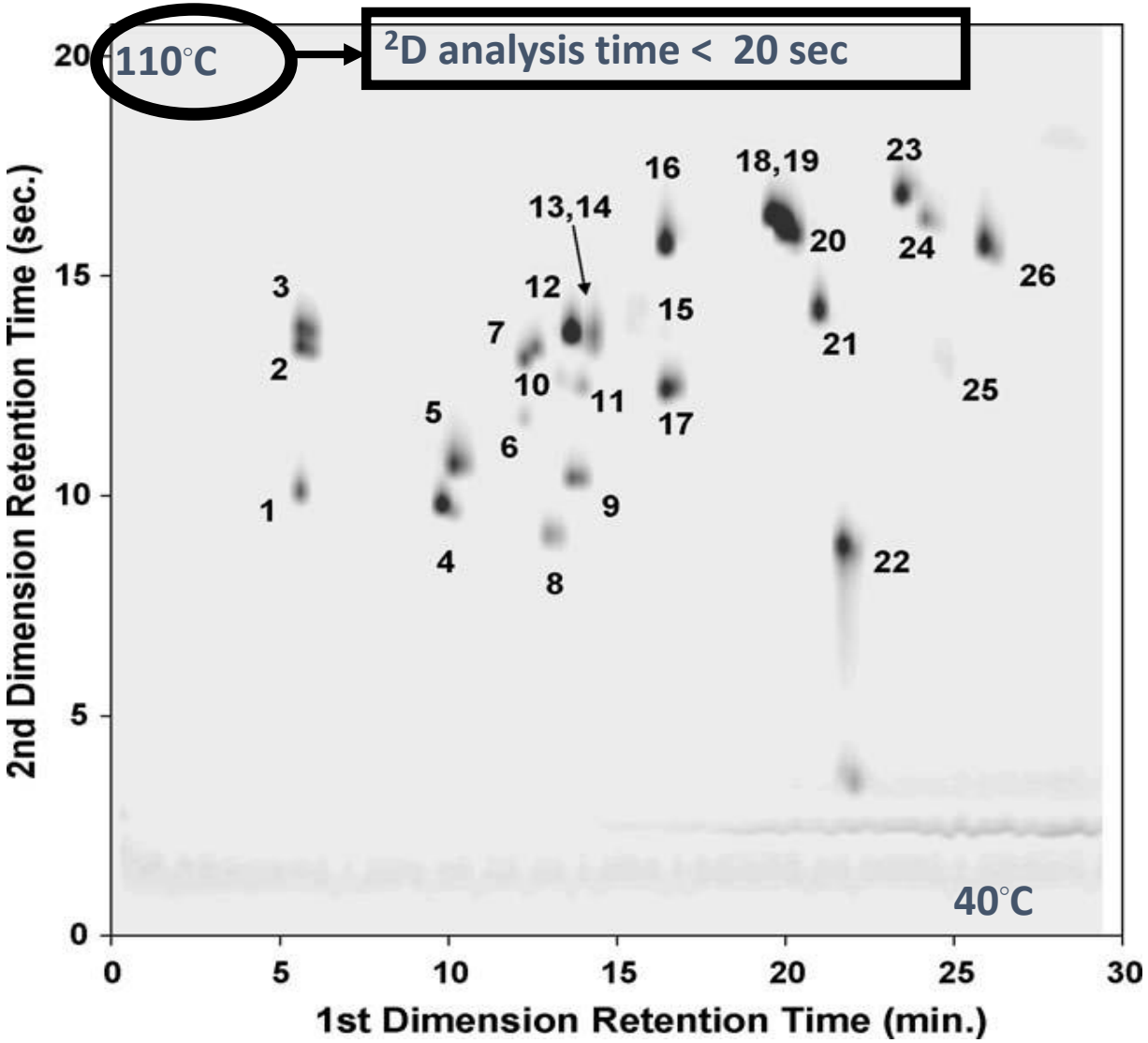
2-min gradient analysis on a shell packed column in the ^{2}D of an LC×LC system

(6 sec reconditioning time)



Use of high temperature ultra-fast gradient elution in the ^{2}D of an LC × LC system

(Stoll *et al.* J. Chromatogr. A 1122, 2006, 123-137)



^{1}D column: Discovery HS-F5 50x2.1 mm i.d.; 5 μm dp. Solvent A: 20 mM sodium phosphate, 20mM sodium perchlorate, pH 5.7 Solvent B: acetonitrile; gradient elution. Temperature 40 ◦C; Flow rate, 0.10 mL/min; Inj. Vol. 10 μL.

^{2}D column: ZirChrom-CARB 50x2.1 mm i.d.; 5 mm dp. Solvent A: 20mM perchloric acid in water Solvent B: acetonitrile gradient elution. Temperature 110 ◦C; Flow rate, 3 mL/min; Inj. Vol. 34 μL.

UV: 220 nm

Sample: indolic metabolite standard mixture

Method development in LC×LC

Method development in LC×LC:

Column selectivity, orthogonality, peak capacity

Selectivity of the columns used in the two dimensions must be different. It has a direct effect on system **orthogonality** and on **peak capacity**.

The best results are achieved in so-called “orthogonal” systems with **non-correlated** retention times in both dimensions.

Two-dimensional systems with fully non-correlated selectivities are rarely found in practice.

Method development in LC×LC

- LC techniques are characterised by a wider variety of separation mechanisms with truly different selectivities.

The number of theoretically achievable orthogonal combinations is high.

Compatibility between the two dimensions need to be considered.

- However, combination of certain LC modes can present difficulties if not impossibilities:

- *mobile phases immiscibilities*
- *precipitation of buffer salts*
- *^{1}D mobile phase–^{2}D stationary phase incompatibility*

- RP×RP; IEX×RP; SEC×RP; SEC×NP; HILIC×RP are examples of compatible hyphenation.

- **It is more difficult to combine NP and RP, due to mobile phase immiscibility.**

Method development in LC×LC
Coupling with less problems of solvent incompatibility

RP×RP

Mainly applied to natural and environmental compounds but recently also to peptides separation.

HILIC×RP and RP×HILIC

Mainly applied to natural antioxidants and lipids separation.

IEX×RP, IEX×SEC

Mainly applied to biological (peptides and proteins) and organic compounds.

SEC×LC (NP or RP) or LC×SEC

Mainly applied to synthetic and natural polymers and oligomers but also to proteins and peptides.

Detectors

- Most of the traditional HPLC detectors can be applied to LC × LC analyses.

- Usually only one detector is installed after the second dimension column, but the use of multiple detectors is possible.

- Monitoring of the first dimension separation can be performed only during method development. If micro HPLC is used, adequate detector equipped with microcell needs to be used.

- Operating the second dimension in fast mode, fast detectors with high data acquisition rate need to be used.

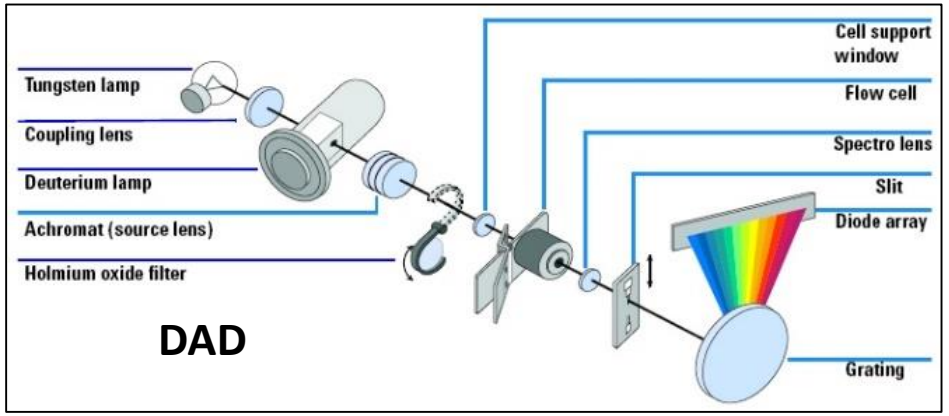
Detectors

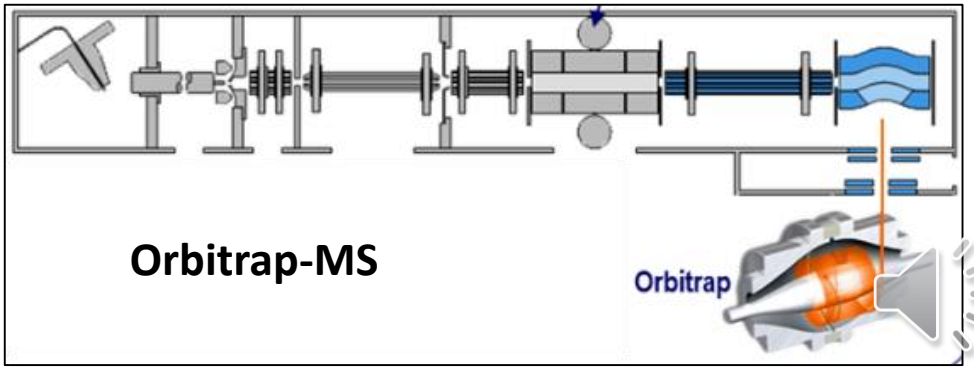
DETECTION IN LC×LC

- One or two detectors is possible.
- Detector after ^{1}D is frequently not used in comprehensive 2D-LC
- Detection not different from any other HPLC instrument.
- Need to reduce the ^{2}D flow rate in some cases.
- Special importance of acquisition rate.

Detectors reported

- DAD
- UV-Vis
- Evaporative light-scattering
- MS

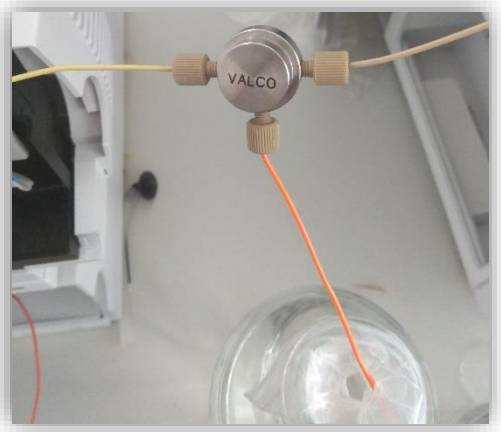




Detectors

DETECTION IN LC×LC

CONSIDERATIONS



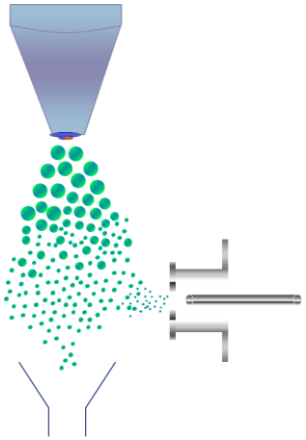
» DAD

- Operated at maximum sampling rate (that can reach 400 Hz).
- The entire flow from ^{2}D column goes to detection cell.

» MS

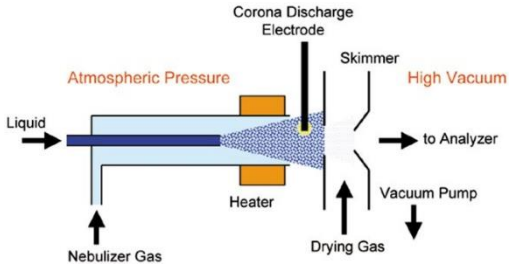
- Need to reduce flow rate entering the MS depending on interface used.

ESI



Max. Flow: < 1 mL min^{-1}

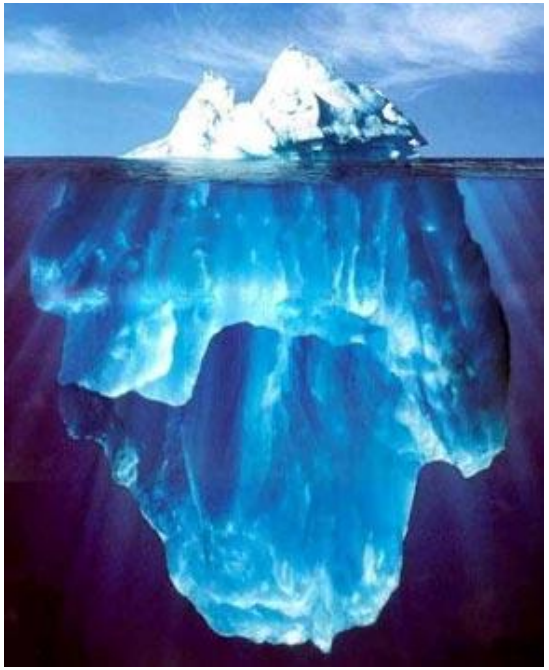
APCI



Max. Flow: < 1.5 mL min^{-1}

Combining LC × LC with Mass Spectrometry

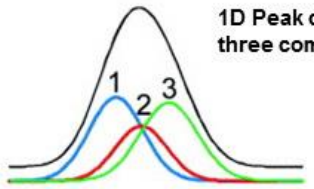
- handle complex sample
- attain more robust quantification
- reduce ion suppression
- detect even low abundant signals
- get structural information
- increasing confidence in the result



Detectors

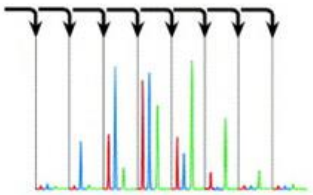
DATA TREATMENT

Direct acquisition from the detector: raw chromatogram, sum of each ^{2}D separations



1D Peak composed of three compounds

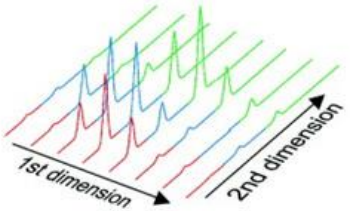
MODULATION



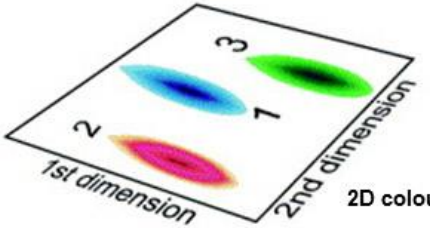
Raw 2D chromatogram

SAMPLING RATE CRITICAL!!

ELABORATION PROCESS



2D chromatograms stacked side by side



2D colour plot

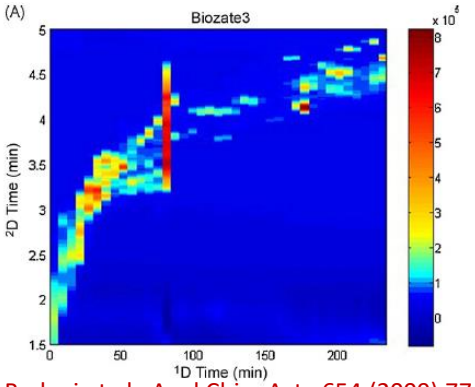
VISUALIZATION

Detectors

DATA PROCESSING

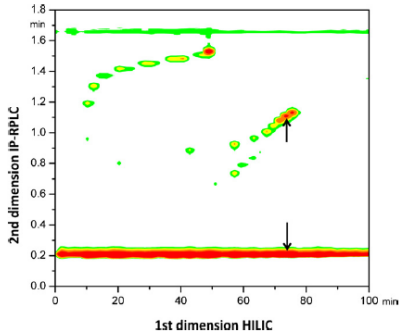
Home-made visualization software

Based on Matlab



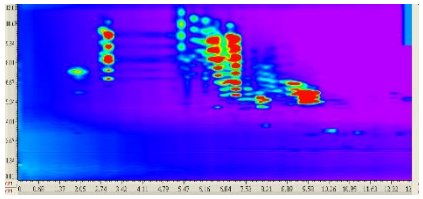
Bedani et al., Anal Chim Acta 654 (2009) 77-84

Based on Origin

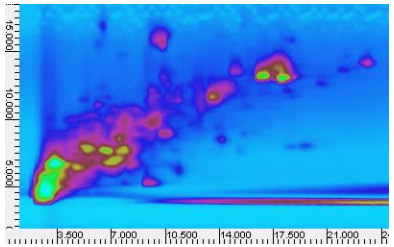


Li et al., J. Chromatogr. A 1255 (2012) 237-243

Commercial advanced dedicated software



ChromSquare



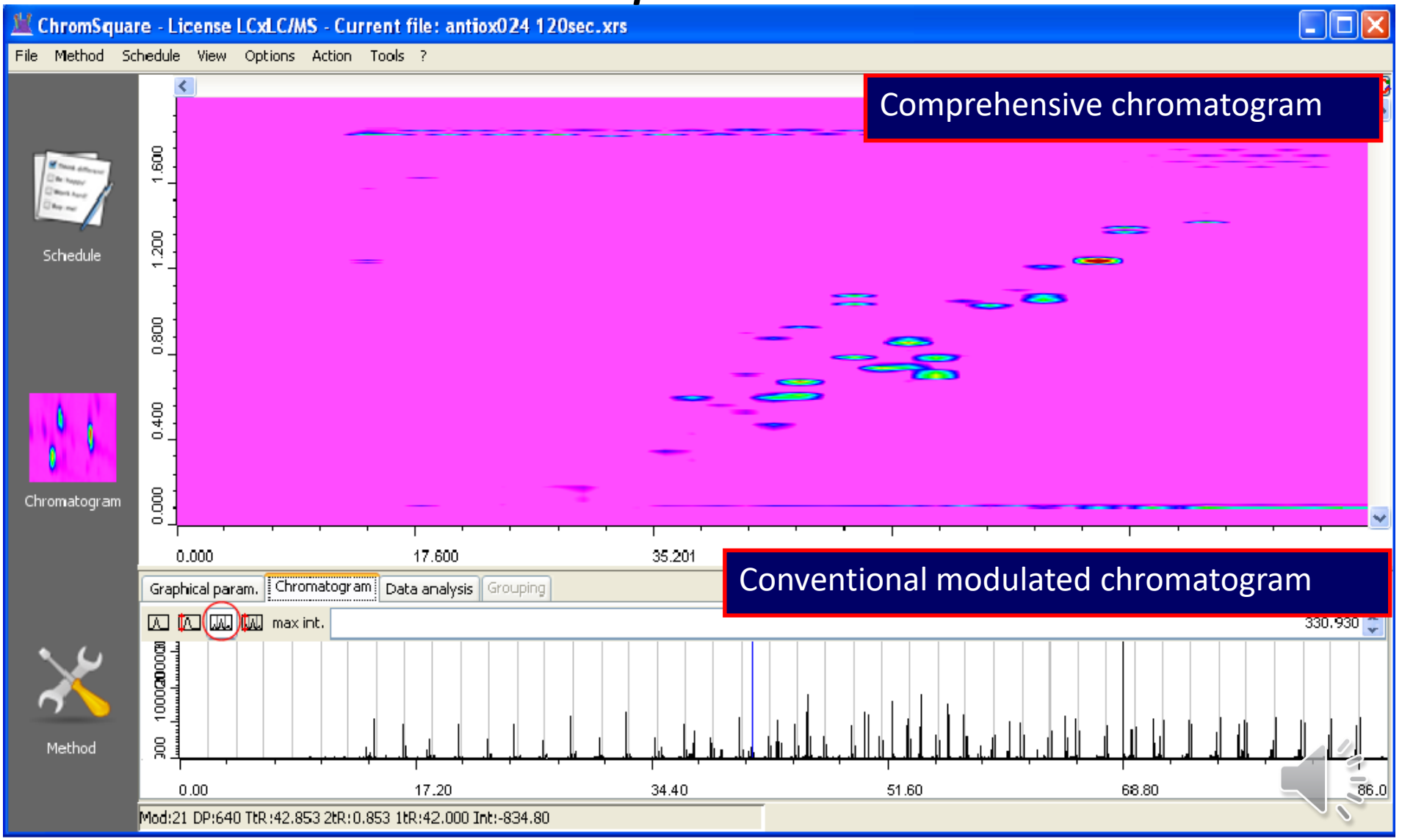
LC Image

- More or less merged with control software
- Automatic data visualization
- Quantitative functions
- Integrated info (UV-Vis and MS spectra)

Comprehensive LC software

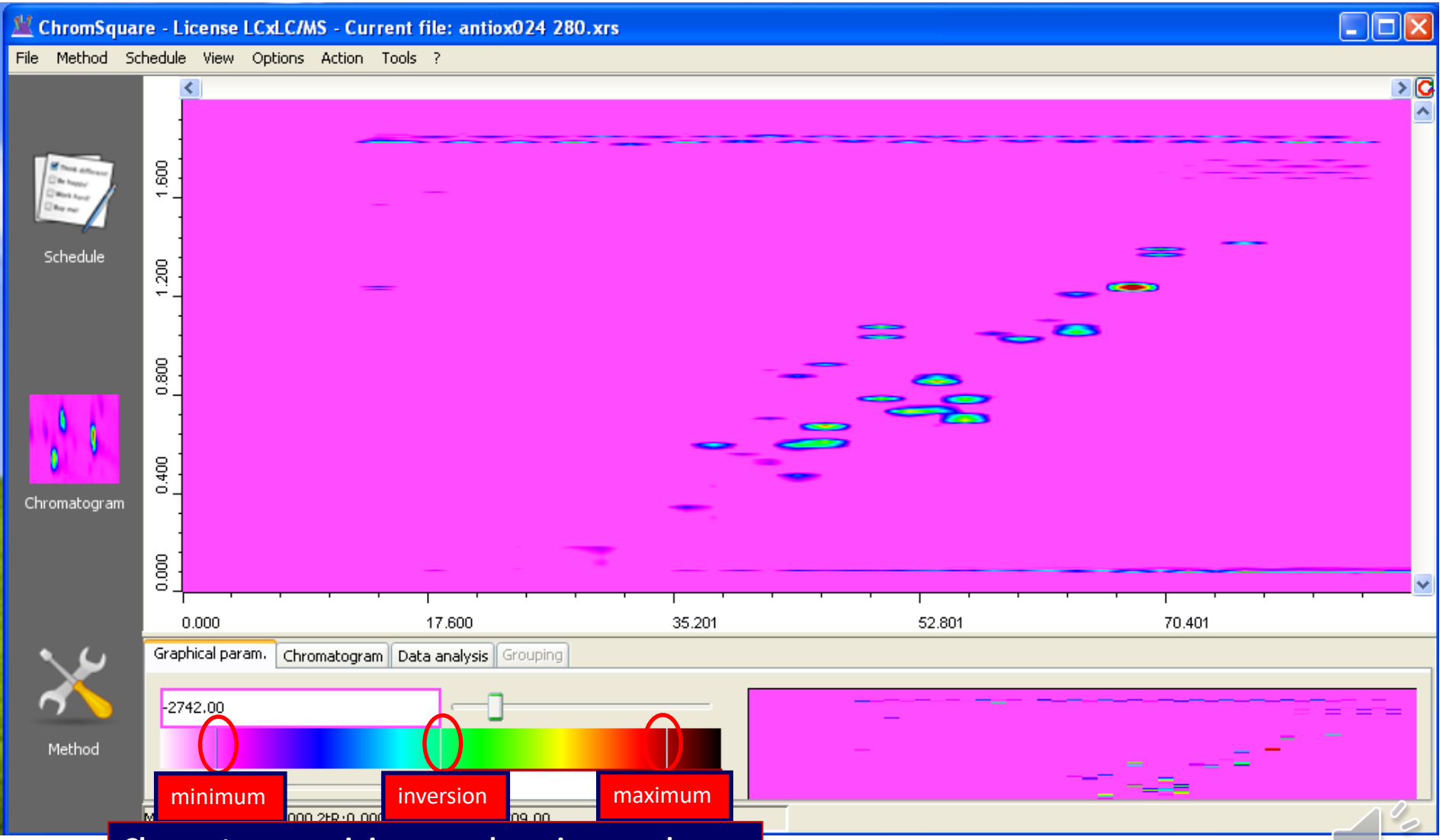
Conventional modulated and comprehensive chromatogram

View comparison



Comprehensive LC software

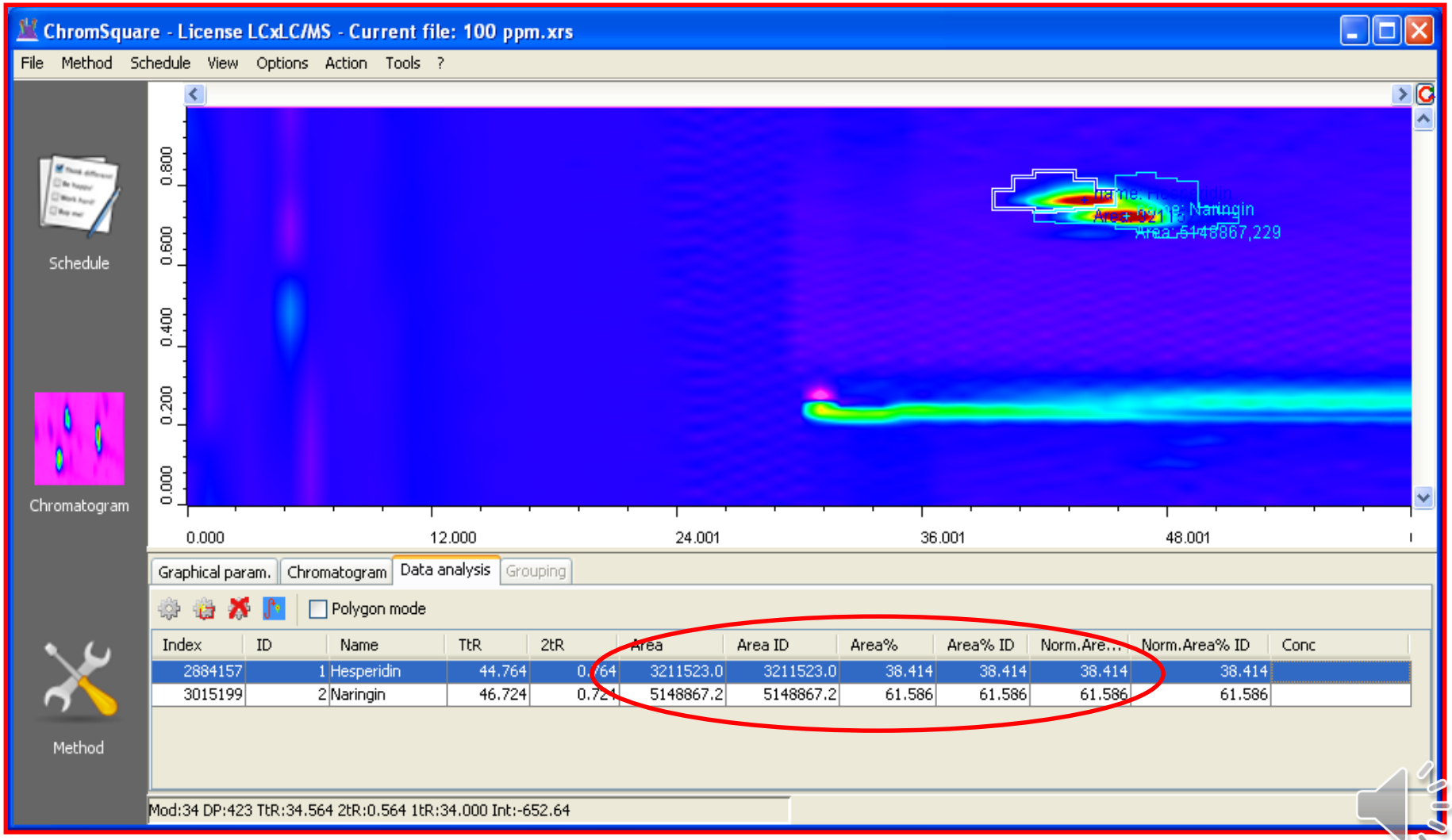
2D Topographic View



Chromatogram minimum and maximum values

Comprehensive LC software

Integrated chromatogram of recognized blobs



Conclusions

- ✓ This course lecture aimed to illustrating the advantages of the LC × LC over the classical 1D-LC approaches allowing to attain a quantity of information much higher if compared to single-column chromatography.

- ✓ Nowadays, robust and full-featured instrumentations are available from most LC manufactures.

- ✓ Miniaturization and downscaling of LC × LC instrumentations will most likely be a future “niche” and consequently a significant rise of 2D capillary-based LC systems is expected in several research fields.

Suggested reviews on Multidimensional LC

- **Shelley & Haddad**, *Comprehensive two-dimensional liquid chromatography, Anal. Bioanal. Chem. 2006, 386, 405.*

- **Stoll et al**, *Fast comprehensive two-dimensional liquid chromatography,* J. Chromatogr. A, 2007, 1168, 3.

- **Dugo et al**, *Comprehensive multidimensional liquid chromatography: theory and application*, J. Chromatogr. A, 2008, 1184, 353.

- **François et al,** *Comprehensive liquid chromatography: fundamental aspects and practical considerations-a review. Anal. Chim. Acta 2009, 641, 14*

- **Donato et al.** *Mass spectrometry detection in comprehensive liquid chromatography: Basic concepts, instrumental aspects, applications and trends, Mass Spectrom. Rev. 2012, 31, 523.*

- **Tranchida et al.** ***Potential of comprehensive chromatography in food analysis***. *TrAC, Trends Anal. Chem. 2013, 52, 186.*

- **Li et al,**. *Practical considerations in comprehensive two-dimensional liquid chromatography systems (LC×LC) with reversed-phases in both dimensions, Anal. Bioanal. Chem. 2015, 407, 153.*

- **Cacciola et al.** ***Comprehensive liquid chromatography and other liquid-based comprehensive techniques coupled to mass spectrometry in food analysis***, *Anal. Chem. 89. 414, 2017.*