HPLC Basics / Instrumentation

Justin Nadar

Application Specialist, HPLC/UHPLC

ThermoFisher SCIENTIFIC

UHPLC+ focused

Understanding the Principles of HPLC

Overview

- Fundamentals of Chromatography – HPLC
- Instrumentation – HPLC
- Applications

INTRODUCTION

History

Mikhail Tswett, Russian Botanist, 1872-1919

In 1906 Tswett used chromatography to separate plant pigments.

He called the new technique chromatography

Chromatography means color writing

Chroma means “color” and graphein means to “write”

He used liquid –adsorption column chromatography with calcium carbonate as adsorbent and petrol ether / ethanol mixtures as eluent to separate chlorophylls & carotenoids

Chromatography

Chromatography is an analytical technique commonly used for separating a mixture of chemical substances into its individual components so that the individual components can be thoroughly analyzed

Chromatography

UHPLC+ focused

Column Chromatography

Planar Chromatography

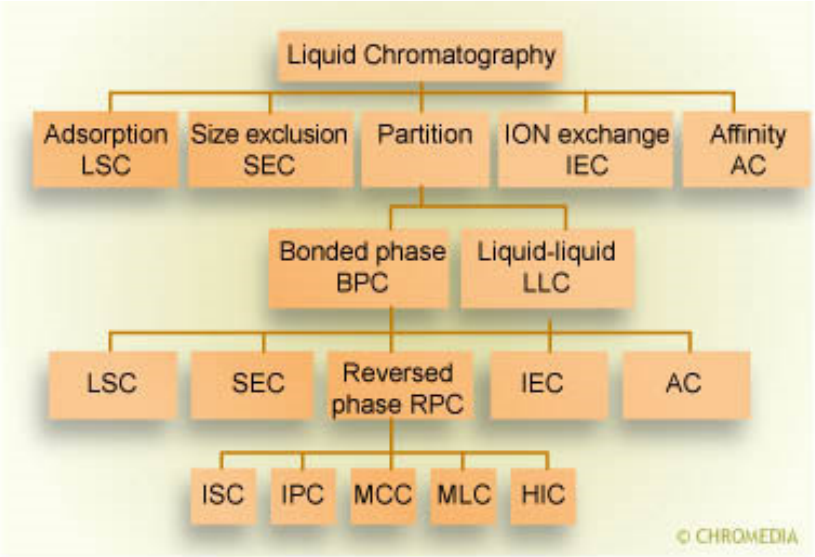
Gas Chromatography

Liquid Chromatography

Super Critical Fluid Chromatography

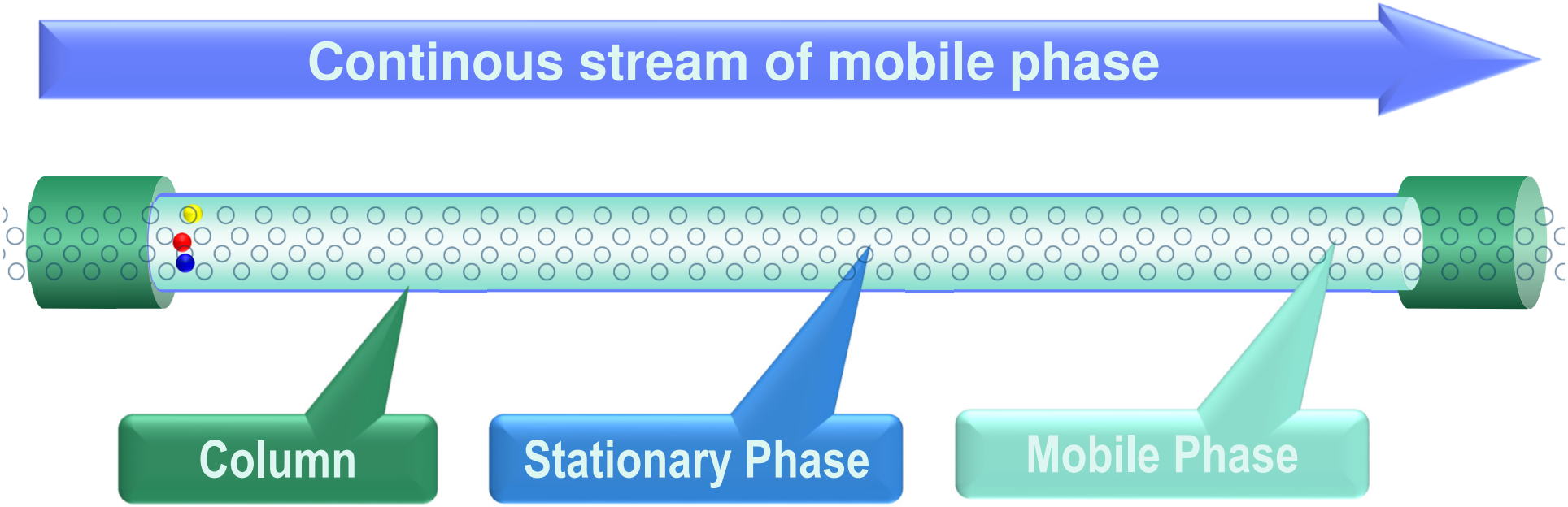
Simulating Moving Bed Chromatography

Liquid Chromatography



LSC = Liquid-solid (Or Normal-phase) Chromatography
SEC = Size-exclusion Chromatography
IEC = Ion-exchange Chromatography
AC = Affinity Chromatography
BPC = Bonded-phase Chromatography
LLC = Liquid-liquid Chromatography
RPC = Reversed-phase Chromatography
ISC = Ion-suppression Chromatography
IPC = Ion-pair Chromatography
MCC = Metal-complexation Chromatography
MLC = Micellar-liquid Chromatography
HIC = Hydrophobic-Interaction Chromatography

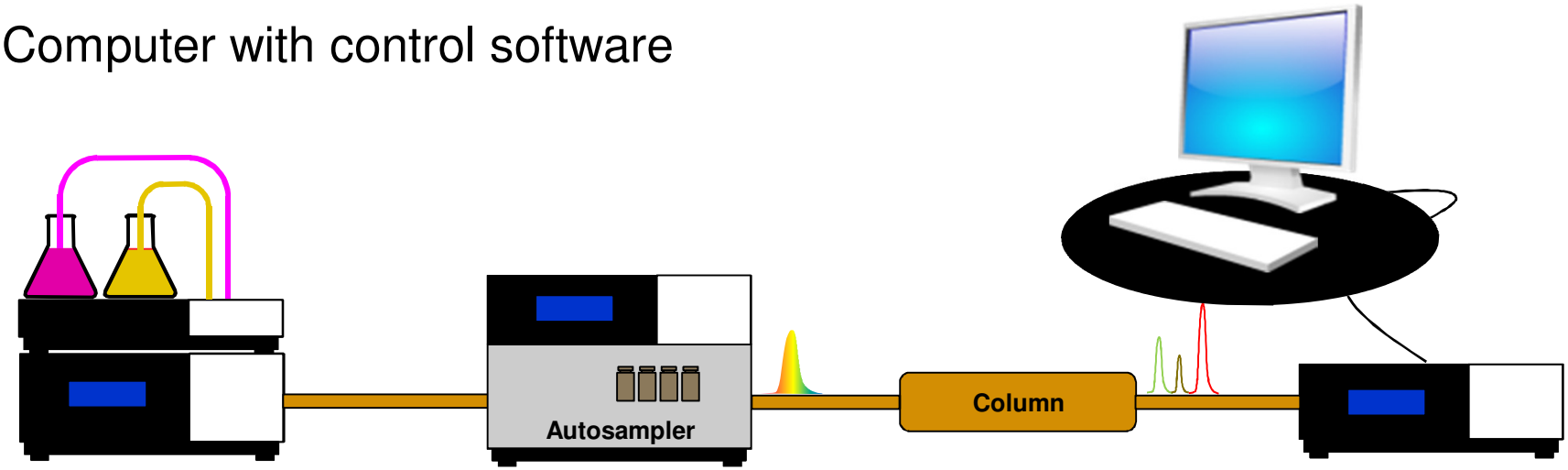
Introduction – The Chromatographic Process



- The separation is based on differential partitioning between the mobile and stationary phases
- Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus affect the separation

Introduction – HPLC-System: General Design

- Pump with Degasser
- Autosampler
- Column (installed in a Column Compartment)
- Detector
- Computer with control software



Detectors

UHPLC+ focused

Detector	Response – Solute	Selectivity	Sensitivity	Linear Range	Destructive	Flow Sensitive
RI/RID	Universal – Bulk	No	microgram	10^4	No	Yes
UV-PDA	Selective - Solute	Chromophore	nanogram	10^5	No	No
FL/FD	Selective - Solute	Fluorophore	picogram	10^3	No	No
ELS/ELD/ ELSD	Selective - Bulk	Volatization	picogram	Non-Linear	Yes	Yes
MS	Selective- Solute	Ionization	femtogram	10^4 - IS*	Yes	Yes
MS/MS	Selective - Solute	Ionization	femtogram attogram	10^5 - IS*	Yes	Yes
ECD	Selective - Solute	Redox	picogram	10^6	Yes	Yes
CAD	Selective - Bulk	Aerosols	nanogram	10^4	yes	Yes

ThermoFisher SCIENTIFIC

Chromatography

Characteristics that are to be fulfilled by a detector to be used in HPLC determination are:

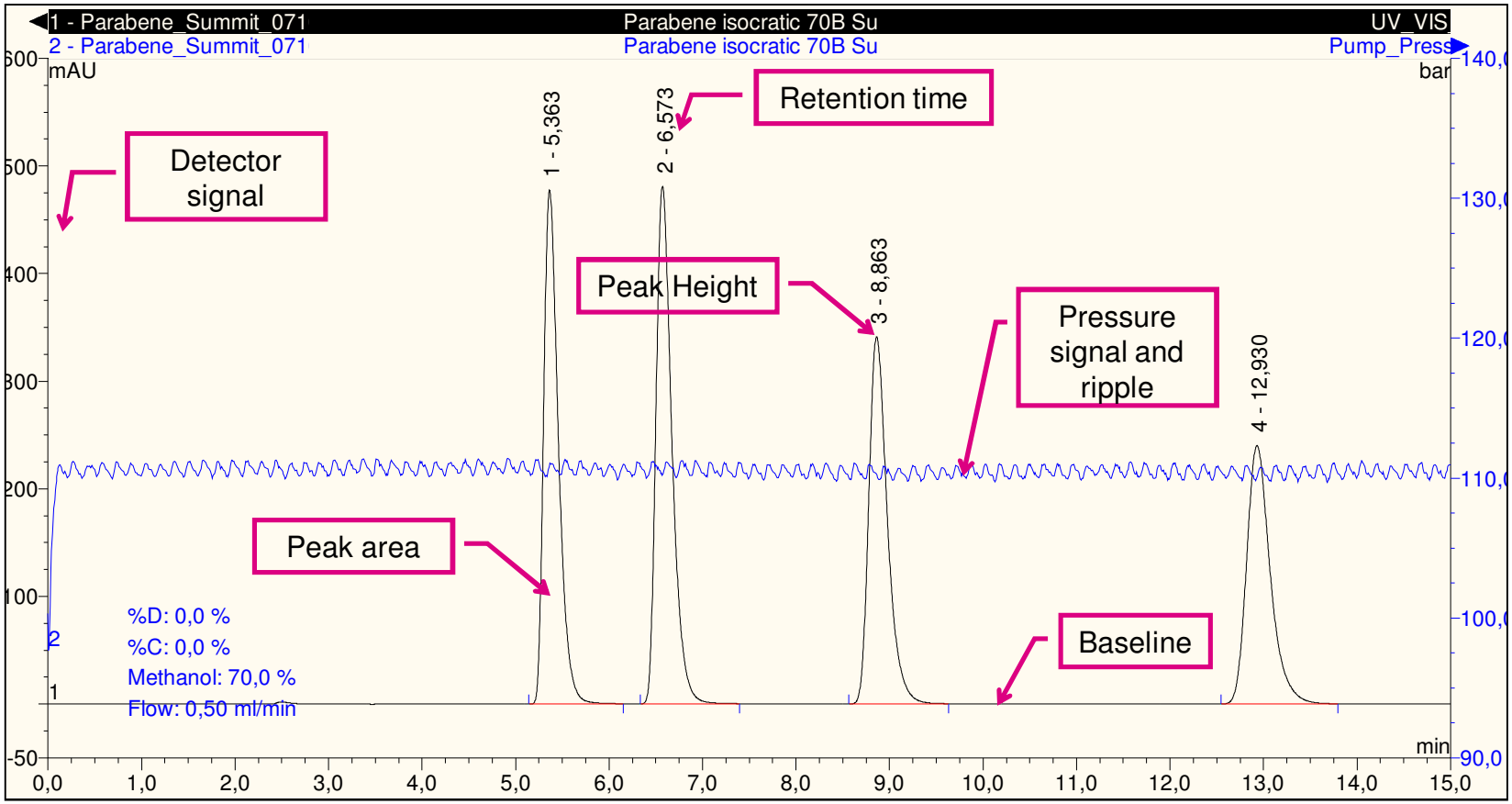
- High sensitivity, facilitating trace analysis
- Negligible baseline noise to facilitate lower detection
- Low drift and noise level
- Wide linear dynamic range (this simplifies quantitation)
- Low dead volume (minimal peak broadening)
- Cell design that eliminates remixing of the separated bands
- Insensitivity to changes in type of solvent, flow rate and temperature
- Operational simplicity and reliability
- Tunability so that detection can be optimized for different compounds

Chromatography

UHPLC+ focused

Assay
Related substances
Dissolution
Content Uniformity
Genotoxic impurities
Determination of Molecular weight
Reverse engineering
Characterisation
Carry-over studies
Bioequivalence studies (ADME/PDPK)
Reaction monitoring
Chemical kinetics
Toxicological studies
Clinical and pharmacological studies

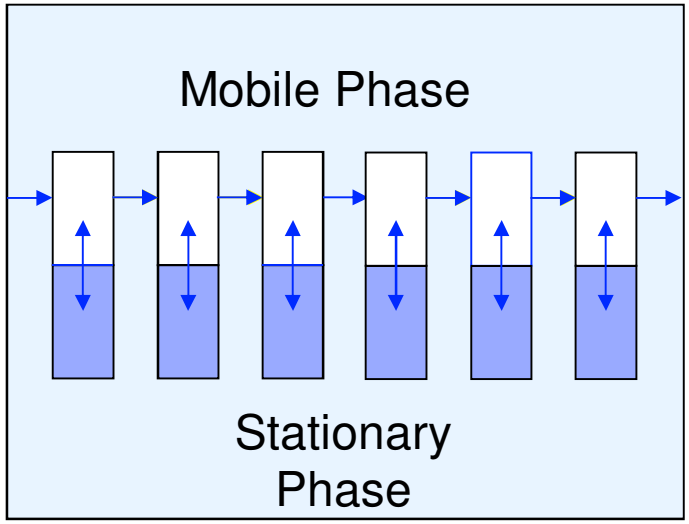
Introduction – Characteristics of a Chromatogram



THEORY OF CHROMATOGRAPHY

Plate Theory and Column Efficiency
Band Broadening (van Deemter plot)
Resolution
Influence of Smaller Particle Diameter

Theory of Chromatography – Plate Theory



- Plate model assumes that the chromatographic column contains a large number of separate layers, called theoretical plates.
- **These plates do not really exist**; they are a figment of the imagination to help us describe the processes in the column.
- Within the plates, the mobile and stationary phases, are described as being in equilibrium.
- The partition coefficient K is based on this equilibrium and is defined as:
  $K = c_{stat} / c_{mob}$
- As K increases it takes longer for solute to elute and the retention time increases.

Theory of Chromatography – Plate Number and Column Efficiency

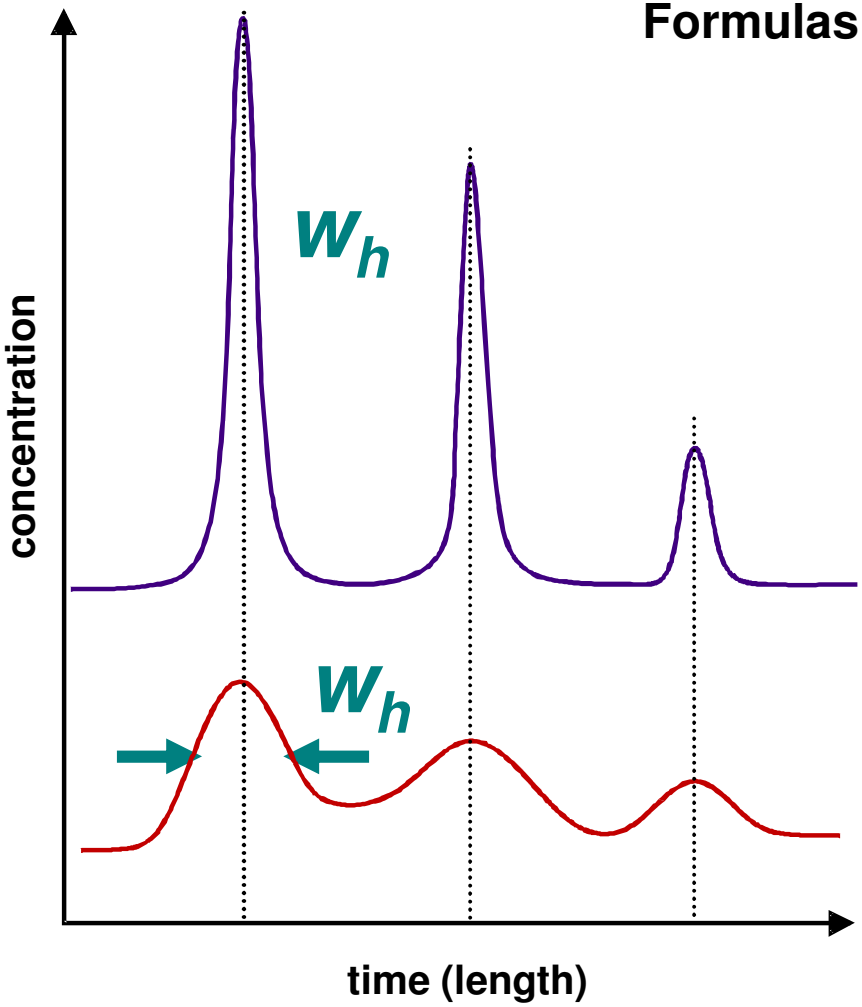
UHPLC+ focused

- The plate theory serves as a way of measuring column quality
  - Either by stating the number of theoretical plates (N) in a column (the more plates the better)

    or

  - by stating the plate height (HETP, H); the Height Equivalent to a Theoretical Plate (the smaller the better)
- Columns with high plate numbers are more efficient than columns with lower plate numbers.
- A column with a high number of plates will have a narrower peak at a given retention time than a column with a lower number of plates.

Theory of Chromatography – Influence of Efficiency on the Separation Quality



Formulas are only valid for isocratic methods!

$$N = 5.54 \cdot \left(\frac{t_R}{w_h}\right)^2 = \frac{L}{H}$$

$$H = \frac{L}{8 \ln 2} \cdot \left(\frac{w_h}{t_R}\right)^2$$

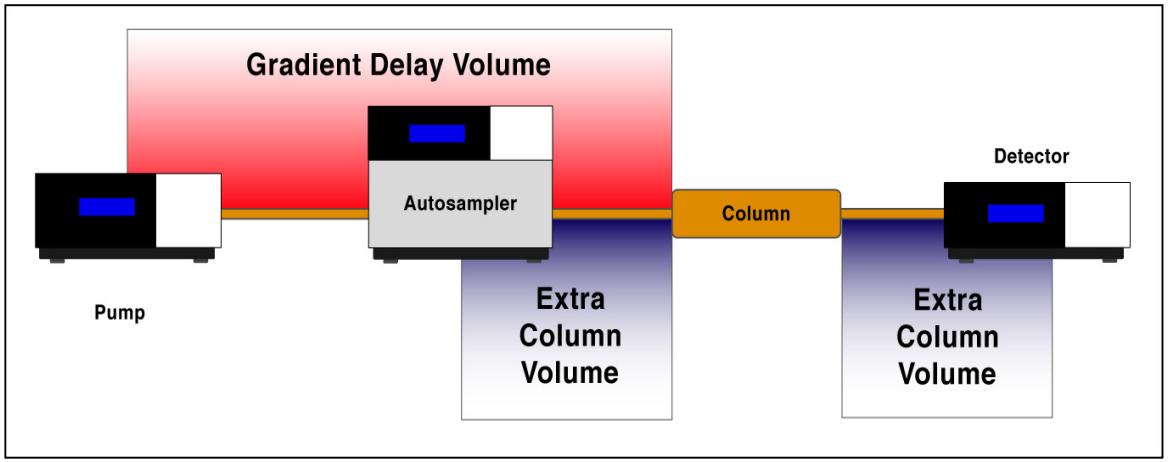
H - plate height; N - plate number
L - column length
t_R - retention time
w_h - peak width at half height

Small H or high N means sharp peaks!

N is related to band broadening in the chromatographic system.

Theory of Chromatography – Causes of Band Broadening

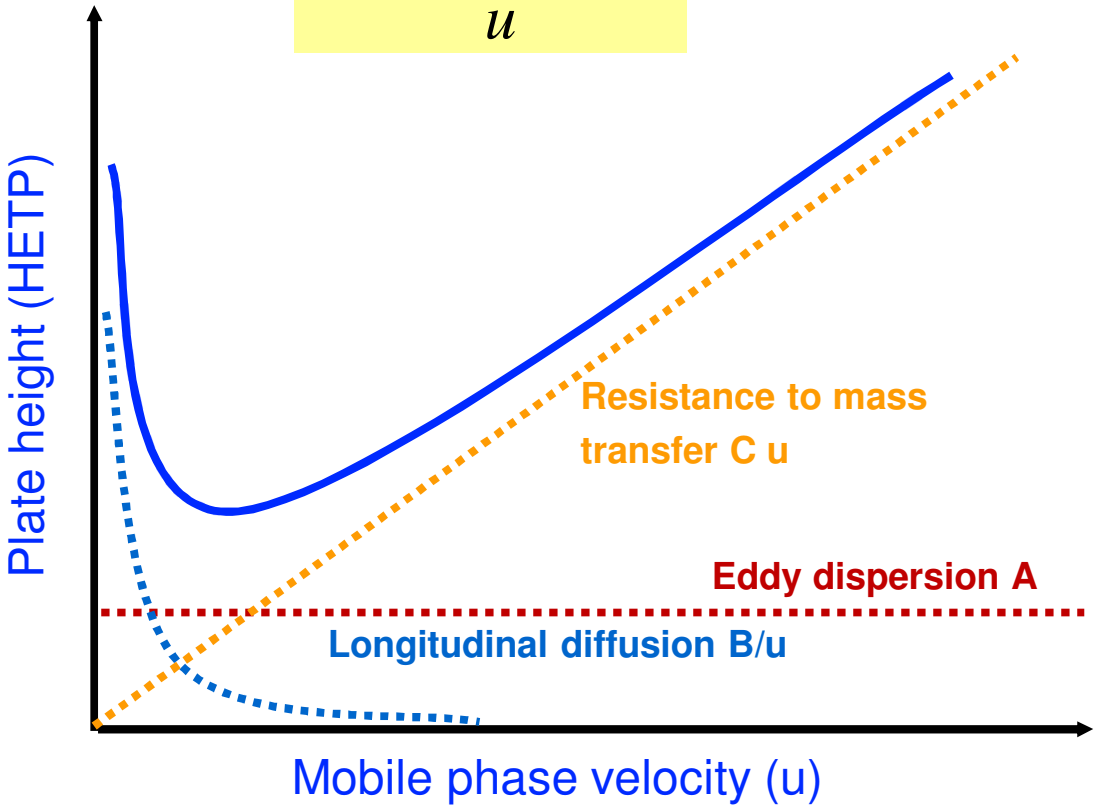
- Extra column effects
  - Tubing and connections
  - Detector flow cell
  - Eluent pre-conditioner
  - In-line filter
- In-column effects
  - Unwanted processes
    - Damaged packing, void volumes
    - Immobilized impurities on stationary phase or column inlet frit
    - Thermal mismatch
- Natural processes
  - Eddy dispersion
  - Longitudinal diffusion
  - Resistance to mass transfer

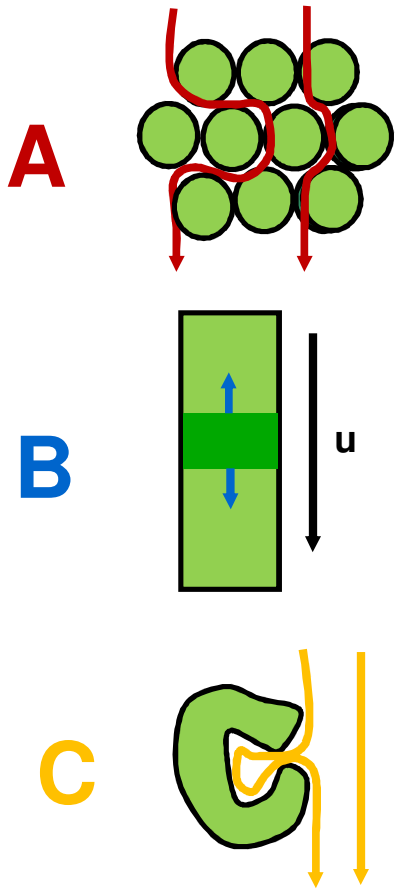


Van Deemter Theory explains natural processes.

Theory of Chromatography – Band Broadening (Van Deemter Plot)

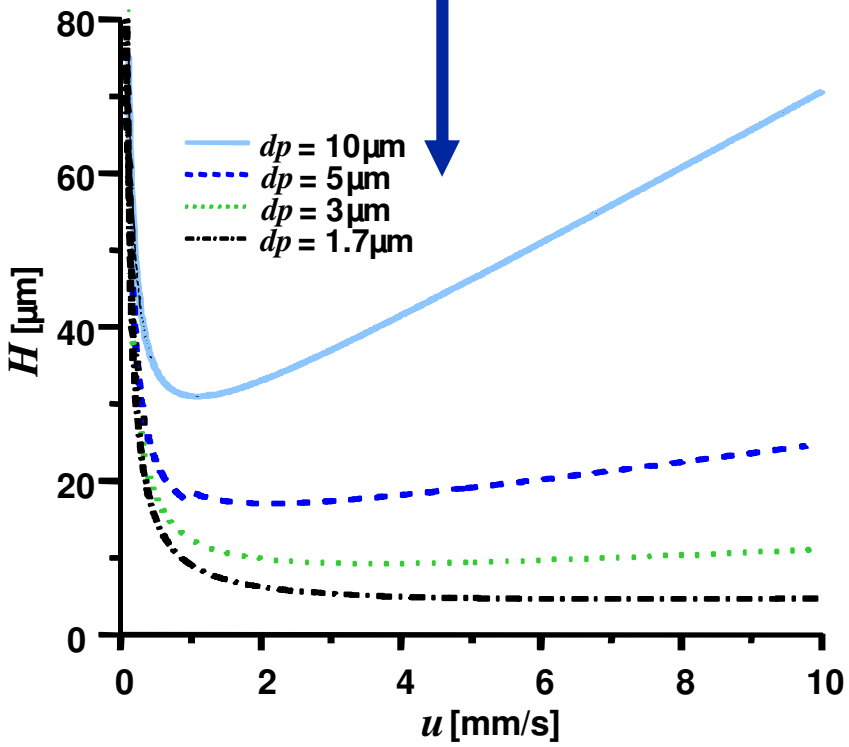
$$H = A + \frac{B}{u} + C \cdot u$$





Theory of Chromatography – Parameters which Influence Band Broadening

- Particle diameter
- Column packing quality
- Linear velocity of mobile phase
- Sample characteristic
- Eluent viscosity
- Retention factor

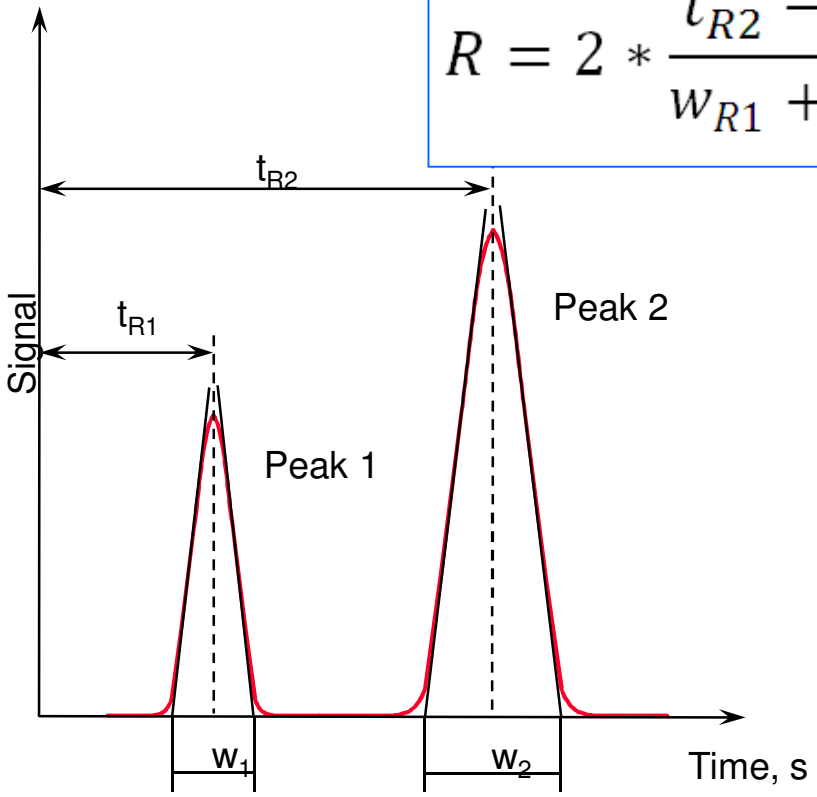


Van Deemter describes only **efficiency** optimization but we are actually looking into **resolution** optimization

Theory of Chromatography

Resolution R of two peaks: Goal of every chromatographic method!

$$R = 2 * \frac{t_{R2} - t_{R1}}{w_{R1} + w_{R2}}$$



- Distance between the peak centers of two peaks divided by the average base width of the peaks.
- From theory R > 1.50 indicates baseline separation.
- In real life R ≥ 2 is usually the goal (requested in regulated environment).
- Much more resolution than 2 does not improve separation quality but increases analysis time.

Theory of Chromatography

UHPLC+ focused

Thermodynamic and Kinetic Factors that determine Resolution

$$R = 1/4\sqrt{N} \cdot \frac{(\alpha - 1)}{\alpha} \cdot \frac{k}{k+1}$$

Term for Efficiency

Term for Selectivity

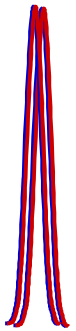
Term for Retention Characteristics

The fundamental parameters

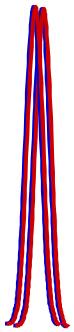
- Efficiency, *N*
- Selectivity, α
- Retention characteristics, *k*

Theory of Chromatography – Effects on Resolution

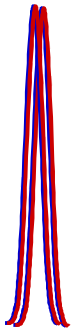
N



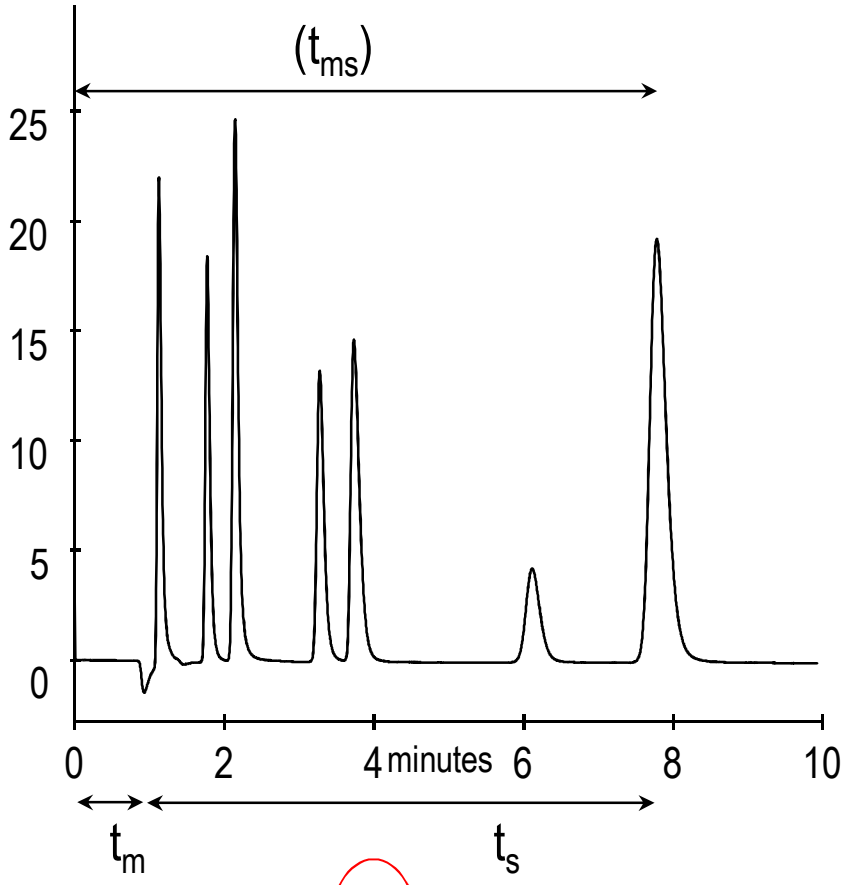
α



k



Theory of Chromatography – Selectivity



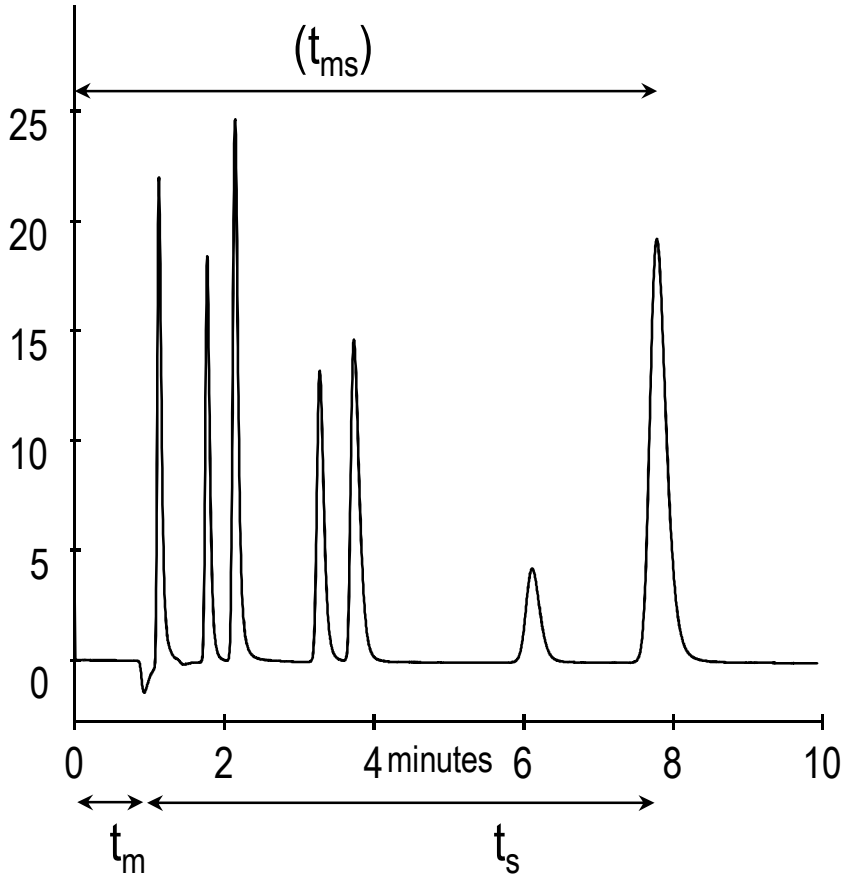
$$R = \frac{1}{4}\sqrt{N}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k}{k+1}\right)$$

$$\alpha = \frac{t_{s2}}{t_{s1}} = \frac{t_{ms2} - t_m}{t_{ms1} - t_m}$$

Selectivity can be changed by

- Changing the stationary phase
- Changing the mobile phase or ionic strength
- Changing the temperature (column oven)
- Changing the charge on the solute (pH)

Theory of Chromatography – Retention Characteristics

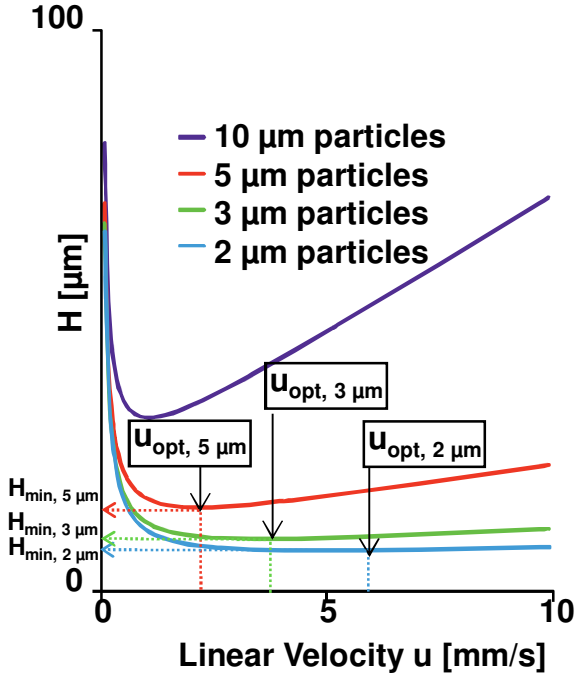


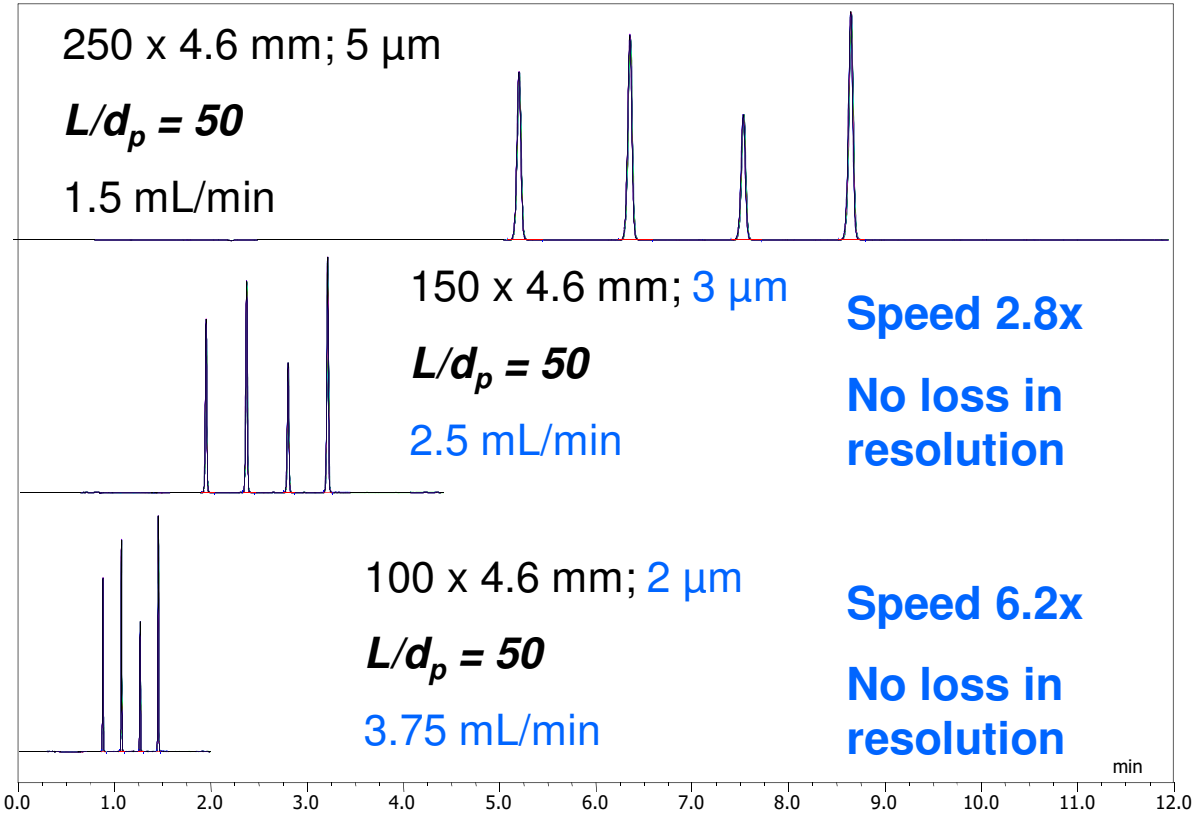
$$R = \frac{1}{4}\sqrt{N}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k}{k+1}\right)$$

$$k = t_s/t_m = (t_{ms}-t_m)/t_m$$

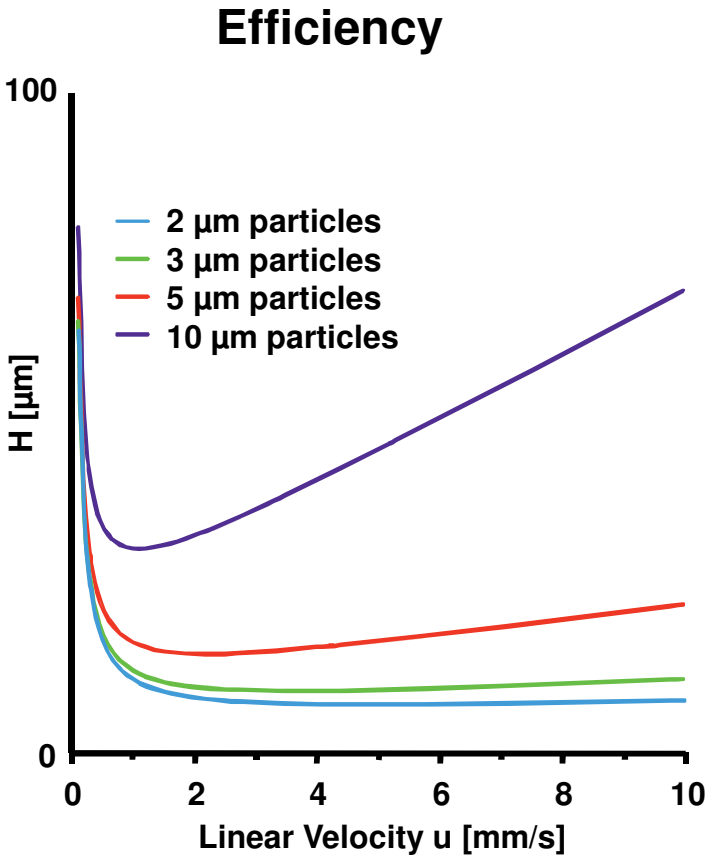
- Small k values
  - the components elute close to void volume
  - are not well resolved
- Large k values
  - good separations
- Very large k values
  - longer retention times
  - peak broadening which can compromise sensitivity
- Typical working range
  - $2 < k < 10$

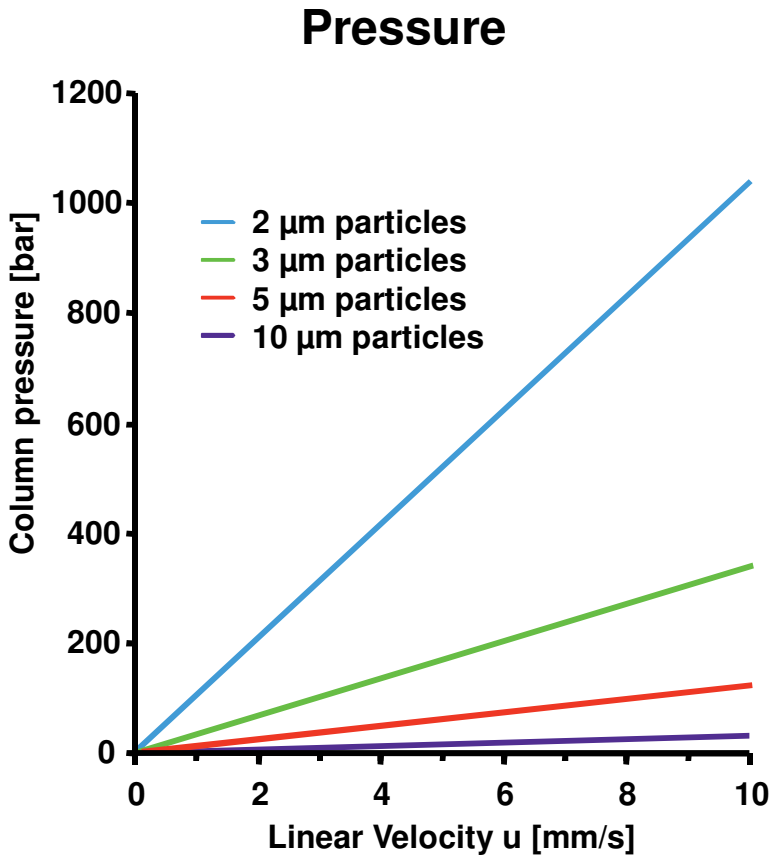
Theory of Chromatography – The Influence of the Particle Diameter on Speed





Theory of Chromatography – Influence of the Particle Diameter on Pressure





For separation speed – We Need High Pressure and Fast Flows

Introduction – Size of Silica Particles

- The smaller the particles, the better the separation performance
- But: Smaller particles generate higher back pressure

	Typical Particle Size	**Typical Back Pressure**	**Typical Column Diameter**
Preparative HPLC	100 – 10 µm	10 - 100 bar	21 mm
Conventional HPLC	5 – 3 µm	100 – 300 bar	4.6 mm
UHPLC	≤ 3 µm	≥ 600 bar	2.1 mm

- HPLC: High Performance Liquid Chromatography
- UHPLC: Ultra High Performance Liquid Chromatography

Theory of Chromatography – Summary

- Plate theory and column Efficiency
  - The higher the plate number N the better the efficiency of the column
- Band broadening (van Deemter plot)
  - Natural processes result in band broadening
- Resolution is a function of column efficiency, selectivity and retention characteristics.
- Influence of smaller particle diameter:
  - Higher resolution
  - More speed possible
  - Higher back pressure generated

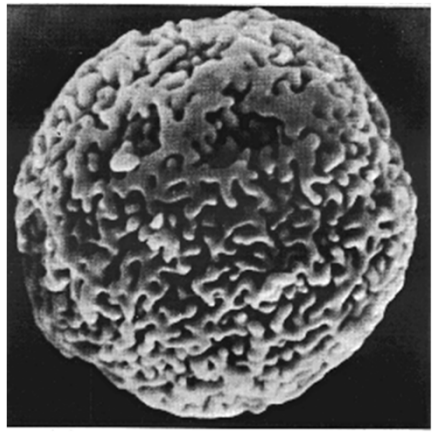
Normal Phase Chromatography
Reversed Phase Chromatography
Isocratic elution
Gradient Elution
Mobile Phase Additives

COLUMN CHEMISTRY

Column Chemistry – The Column

UHPLC+ focused

- Solid Support - Backbone for bonded phases
  - Usually porous silica or polymeric particles



- Bonded Phases
  - Functional groups chemically bound to the solid support.

- Frits or Sieves as Stationary Phase Retainers
  - Protects the packing from sample matrix particles
  - Mesh size shrinks with particle size ⇨ ≤ 3 µm require thorough eluent filtration!

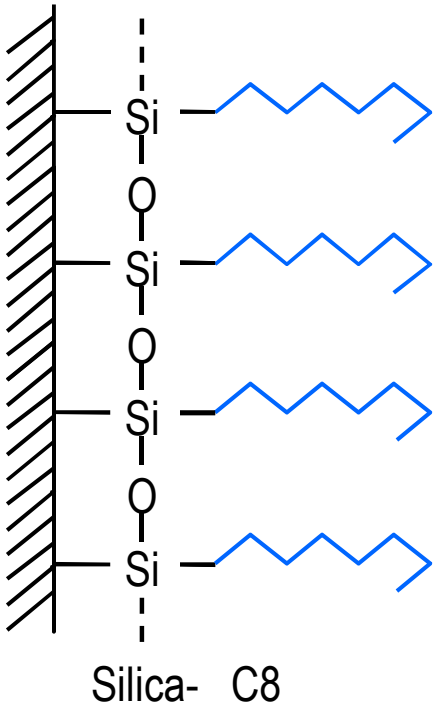
Column Chemistry – HPLC-Modes

- **Normal Phase** (NP): The stationary phase is polar and the mobile phase is non-polar.
  - ➢Niche mode in modern (prep) LC (e.g. separation of enantiomers)
- **Reversed Phase** (RP): The stationary phase is made of chemically modified silica (normally with non-polar surface) and the mobile phase is polar.
  - ➢Most common HPLC mode
  - ➢Good for separation of broad polarity range
- Early HPLC experiments worked with pure silica and polar solvents.

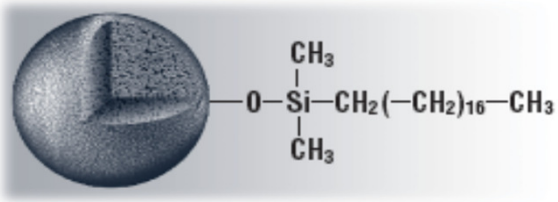
General rule: ”Similia similibus attrahuntur“ (“like dissolves like”).

Column Chemistry – Reversed Phase Chromatography

Example: C8



Silica- C8



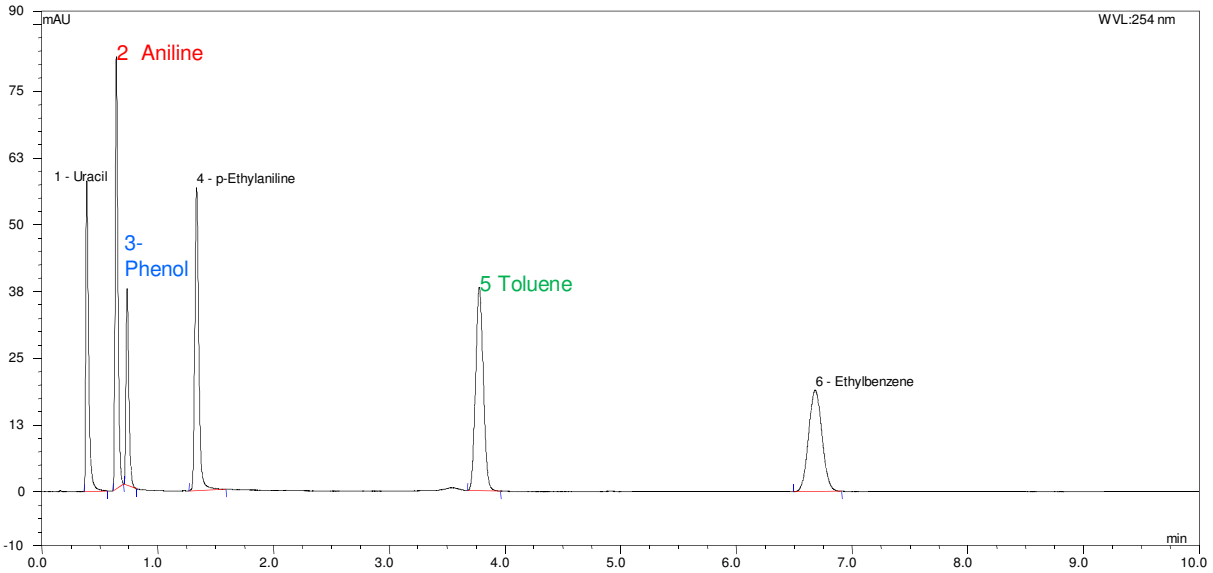
- Common **RP stationary phases**:
  Normal silica treated with **R**Me_2SiCl
  R=$(CH_2)_{17}CH_3$: C18
  R=$(CH_2)_7CH_3$: C8

- Common **RP mobile phases**:
  Water < Methanol < Acetonitrile
  (elutropic series for RP: decreasing retention)

Column Chemistry – Mobile Phase Additives in Reversed Phase

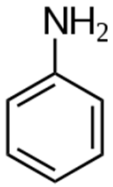
- Inorganic salts:
  - Altered surface tension affects retention characteristics by altering the analyte/surface interaction
  - Example: Ammonium sulfate for peptide separation
- Ion pairing reagents
  - Neutralize charged analytes for better retention
  - Example: Trifluoroacetic acid (TFA), Triethylamine (TEA)
- Buffering agents
  - Neutralize charged analytes for better retention and increase reproducibility of retention time
  - Example: Sodium acetate buffer, sodium phosphate buffer

Column Chemistry
– Separation of Acidic, Basic and Neutral Compounds

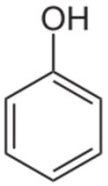


C18, 2x100 mm, 1.8 µm
H_2O/MeOH (50/50), 450 µL/min

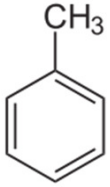
Basic compound:
Aniline pK_b=2.7



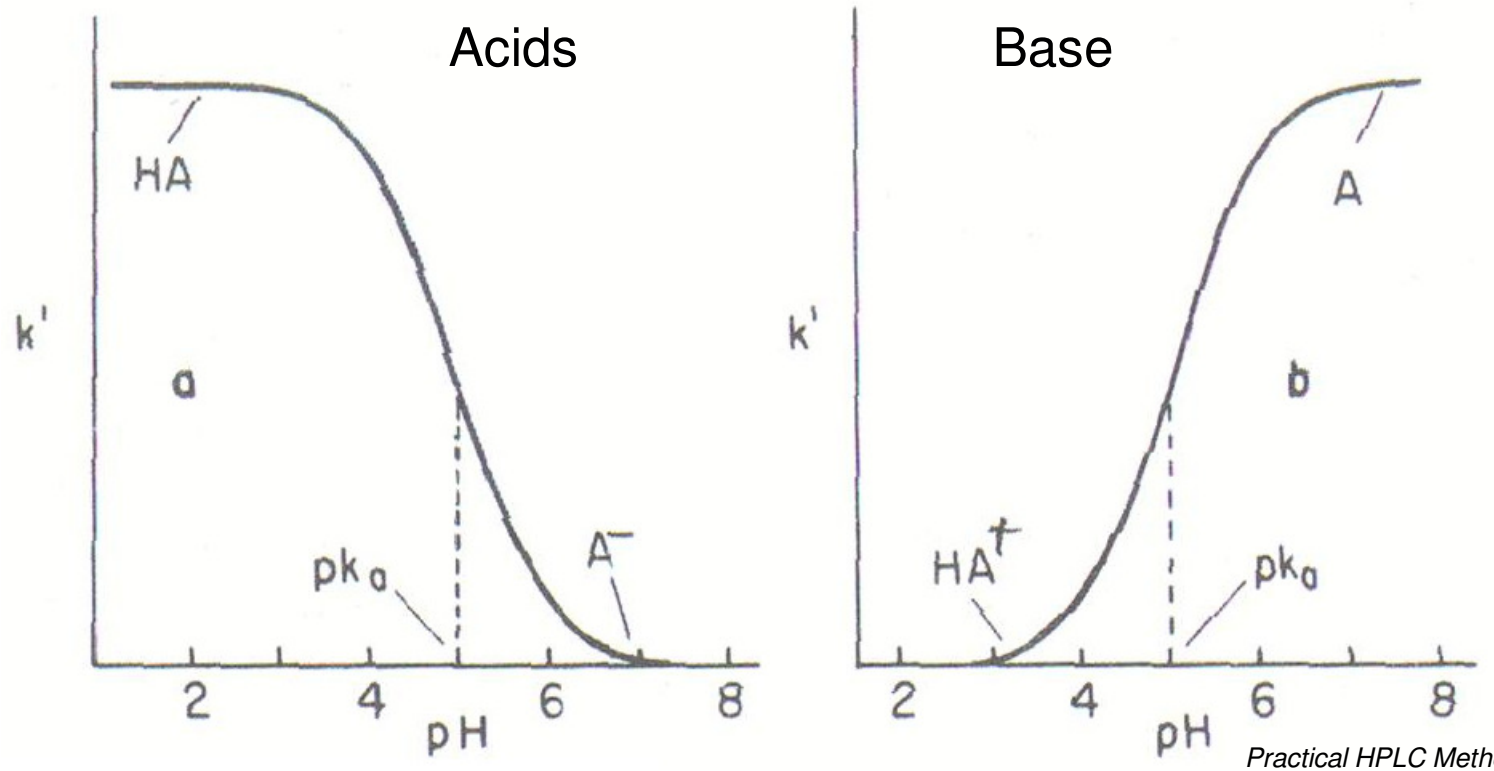
Acidic compound:
Phenol pK_a=9.9



Neutral compound:
Toluene



Column Chemistry – Acids and Bases – pH Effects



Practical HPLC Method Development
Snyder, Glajch, Kirkland 1988

The stronger an analyte is charged, the less its retention on a RP-Phase

Eluent
Capillaries
Column
Pump
Autosampler
Oven
Detector

SYSTEM CONTRIBUTION

System Contribution –
How do the Modules Contribute to the Chromatogram?

Pump — Retention time; Pressure ripple; Detector baseline noise

Autosampler — Peak area

Oven — Retention time; Sensitivity

Detector — Baseline noise; Sensitivity

System Contribution – Summary

- **Eluents** have to be prepared and handled with reasonable care to avoid particle contamination.
- Dead-volume free connections and minimized extra-column volume by use of appropriate **tubing and fitting** systems.
- The **pump** has to mix the eluents precisely and accurately and provide a constant flow rate and back pressure.
- The **autosampler** has to work accurately for successful method transfer and preciscely for good result reproducibility.
- The temperature effect compound retention and peak shape and has to be controlled reliably by the column oven.
- Reasonable **detector** settings are essential for good peak mapping.

Dionex' UHPLC-Compatible Detectors

VWD

100Hz VWD-3100

200Hz VWD-3400RS

MWD

100Hz MWD-3000SD

200Hz MWD-3000RS

DAD

100Hz DAD-3000SD

200Hz DAD-3000RS

FLD

100Hz FLD-3100

200Hz FLD-3400RS

ECD

200Hz ECD-3000RS

Corona Ultra (CAD)

200Hz Corona Ultra RS (CAD)

The most complete UHPLC detector range

Note: 200 Hz only available with Chromeleon 7

ThermoFisher SCIENTIFIC

Vanquish HPLC / UHPLC System

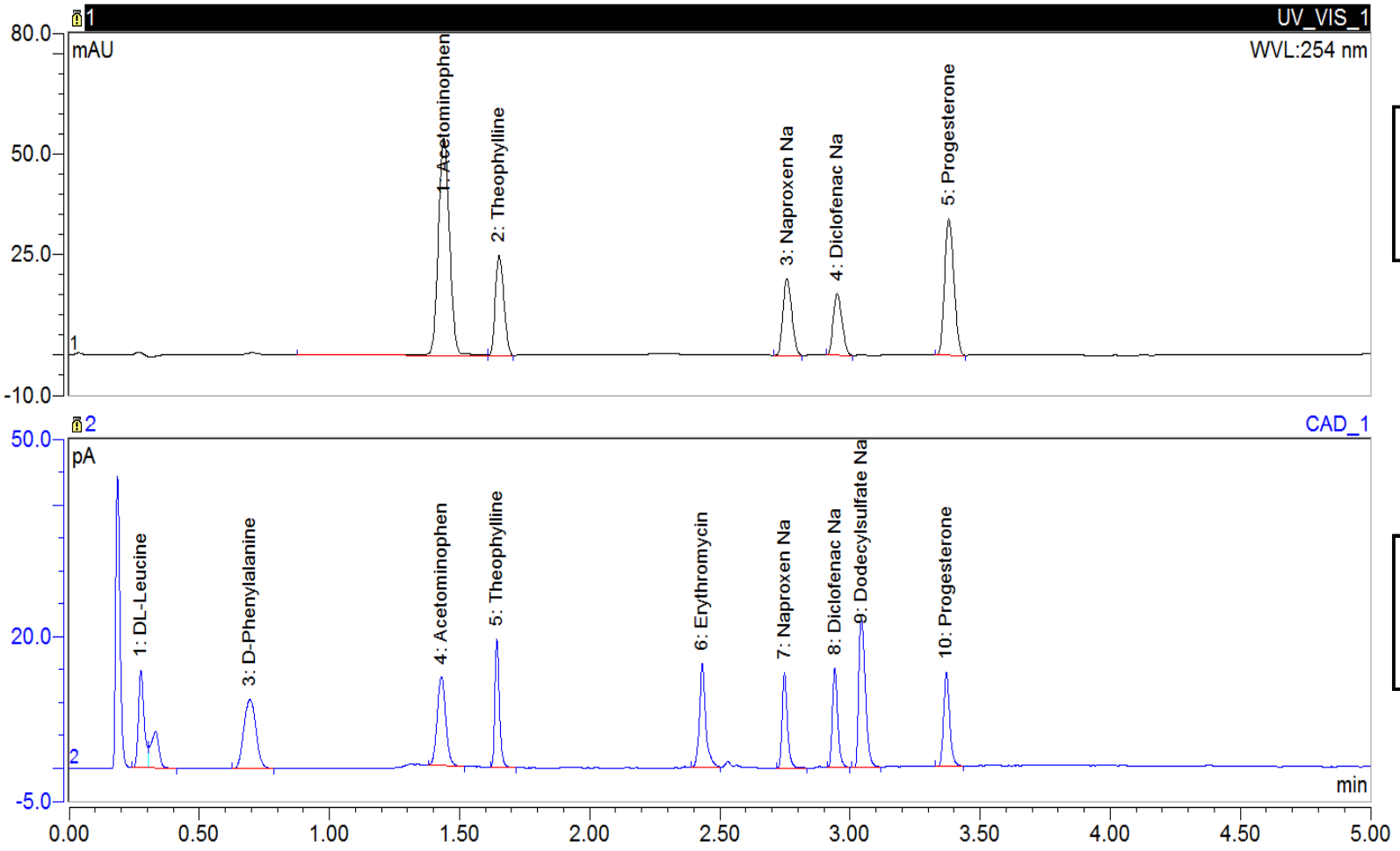
- Integrated modularity
- Biocompatible, iron-free flow path (default)
- Viper-based, tool-free fluidic connections
- Support of latest innovation in column technology
- Enhanced LC/MS support and integration
- Portable system control with tablet PC (planned)
- Revolutionary module drawer system for outstanding service accessibility
- Removable doors for easy access
- Reduced system height
- Variable system GDV in standard configuration as low as 40 µL

Slide 42

M16 Slide 7 bis 12 kommen auch in der Customer-Präsentation vor - da sind sie auch verfeinert worden

MNeubaue, 6/30/2014

Universal UHPLC Detection with Corona Ultra

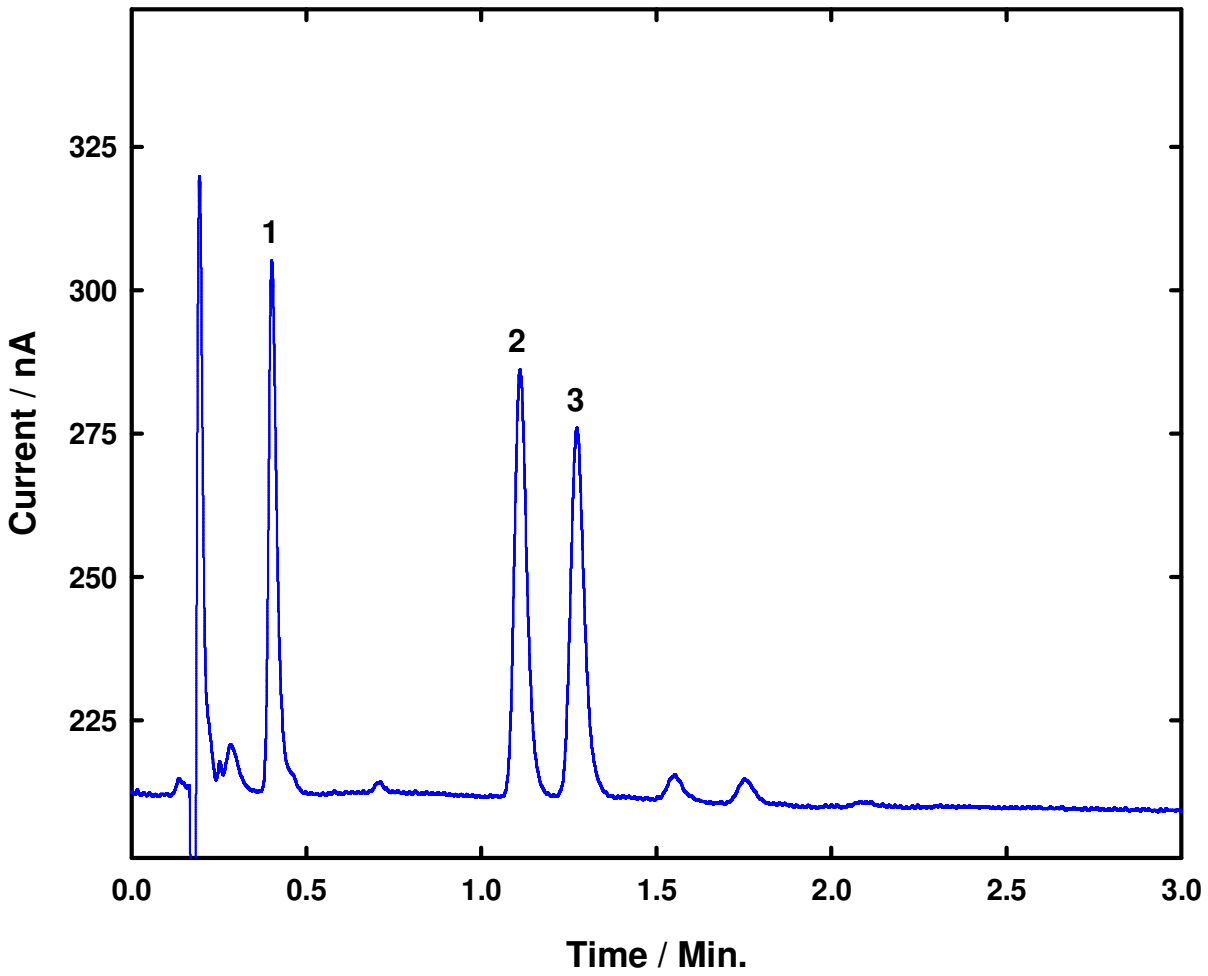


UV
5 Peaks

Corona
9 Peaks

Separation of 9 pharmaceutical compounds in less than 5 minutes

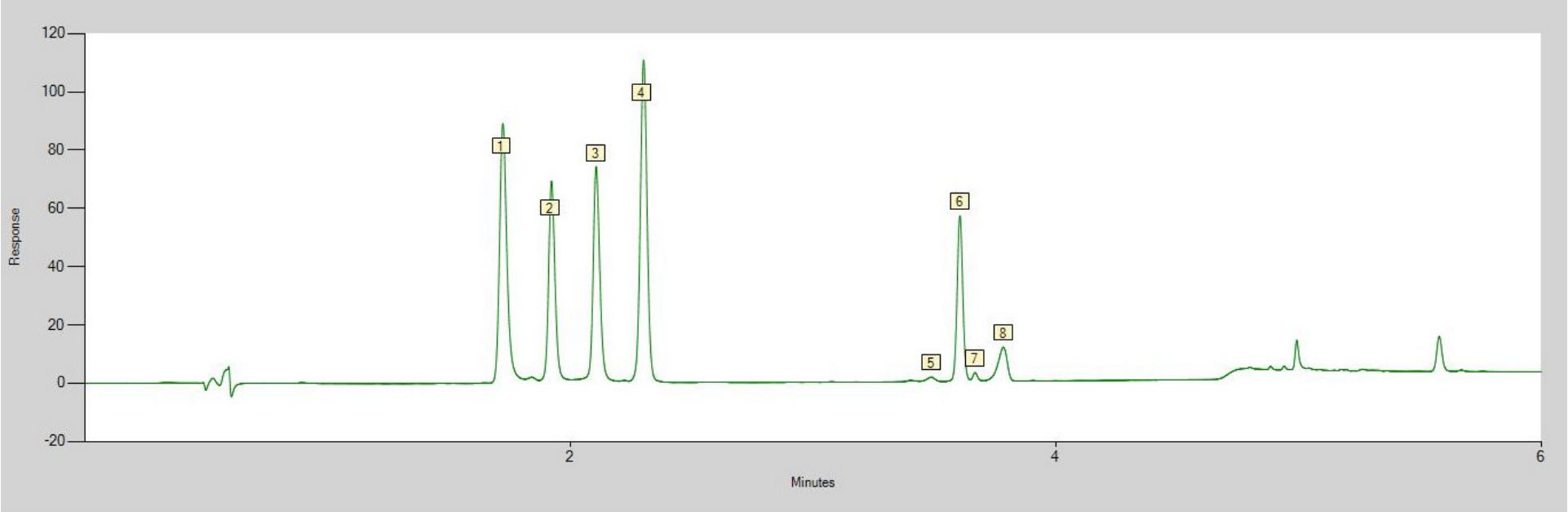
Vitamin D - Coulochem III and ultra Analytical cell



1 – Internal Std
2 – D_3
3 – D_2

500 ng on column
Run Time = 3 min

AN109: Rapid HPLC Separation of Multiclass Antibiotics in Food and Water

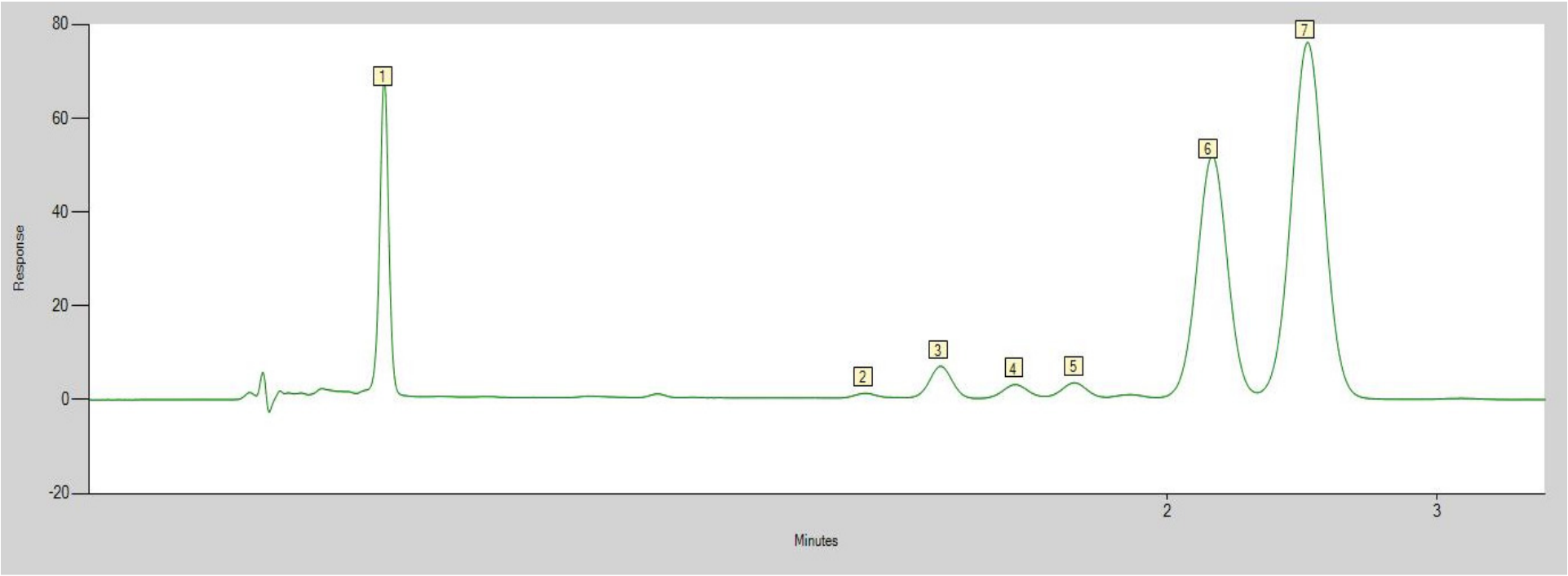


No	Peak_Name	*Compound_Class	Retention_Time	Peak_Area	Peak_Area_pc	Peak_Height	Peak_Height_pc	Peak_EP_Plates
1	Sulfathiazole	Antibiotic	1.722	3.077	23.18	88.557	21.39	17098
2	Oxytetracycline	Antibiotic	1.923	2.080	15.67	68.469	16.54	27416
3	Tetracycline	Antibiotic	2.107	2.356	17.75	73.512	17.76	30275
4	Nitrofurantoin	Antibiotic	2.302	3.558	26.81	110.404	26.67	33740
5	Tylosin_related product a	Antibiotic	3.488	0.055	0.42	1.480	0.36	55533
6	Chloramphenicol	Antibiotic	3.606	1.550	11.68	56.859	13.74	115229
7	Tylosin_related product b	Antibiotic	3.668	0.073	0.55	3.055	0.74	169032
8	Tylosin_related product c	Antibiotic	3.785	0.524	3.95	11.637	2.81	47987

F&B

Column: Dionex Acclaim RSLC PA2, 2.2 µm, 150 x 2.1 mm

Separation of Fat-Soluble Vitamins on Acclaim RSLC PolarAdvantage II (PA2)

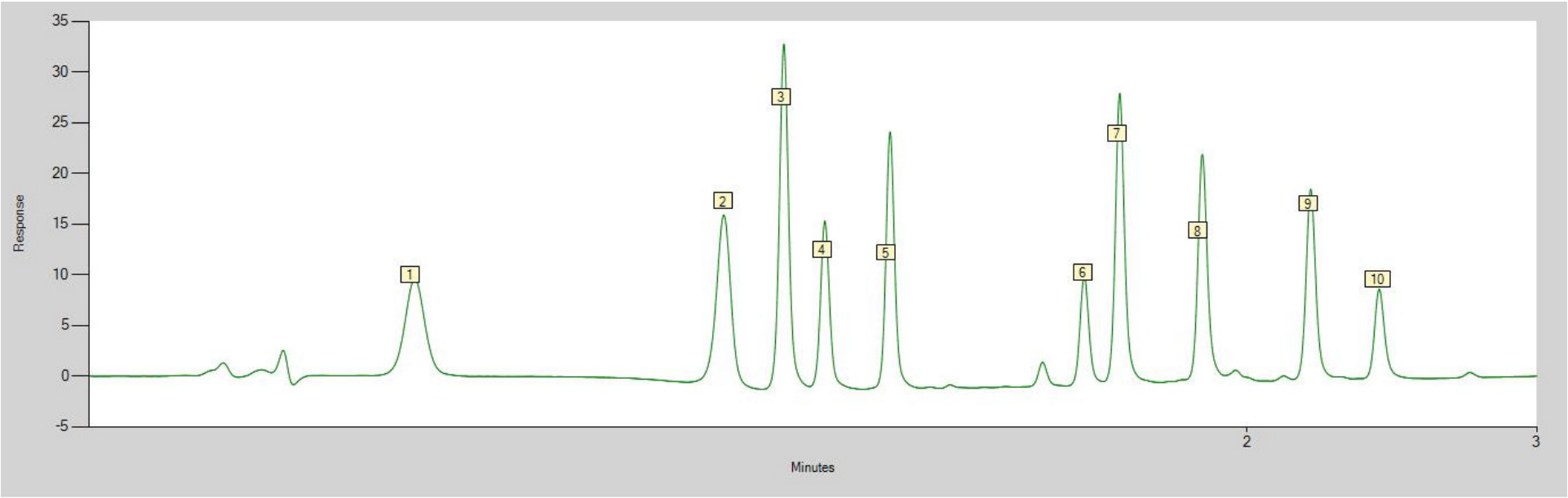


F&B

No	Peak_Name	*Compound_Class	Retention_Time	Peak_Area	Peak_Area_pc	Peak_Height	Peak_Height_pc	Peak_EP_Plates
1	Vitamin A acetate	Vitamins	0.547	1.323	12.26	67.735	32.42	5409
2	Impurity in vitamin E	Impurity	1.440	0.039	0.36	0.917	0.44	6726
3	Vitamin E acetate	Vitamins	1.579	0.324	3.00	6.762	3.24	6817
4	Degradation product of vitamin D	Impurity, Degradant	1.719	0.154	1.42	2.858	1.37	6501
5	Degradation product of vitamin D	Impurity, Degradant	1.827	0.162	1.50	3.124	1.49	7648
6	Vitamin D2	Vitamins	2.084	3.389	31.39	51.651	24.72	6463
7	Vitamin D3	Vitamins	2.260	5.406	50.07	75.914	36.33	6497

Column: Acclaim RSLC PA2, 2.2 µm, 100 x 2.1 mm

AN245: Fast HPLC Analysis of Dyes in Foods and Beverages



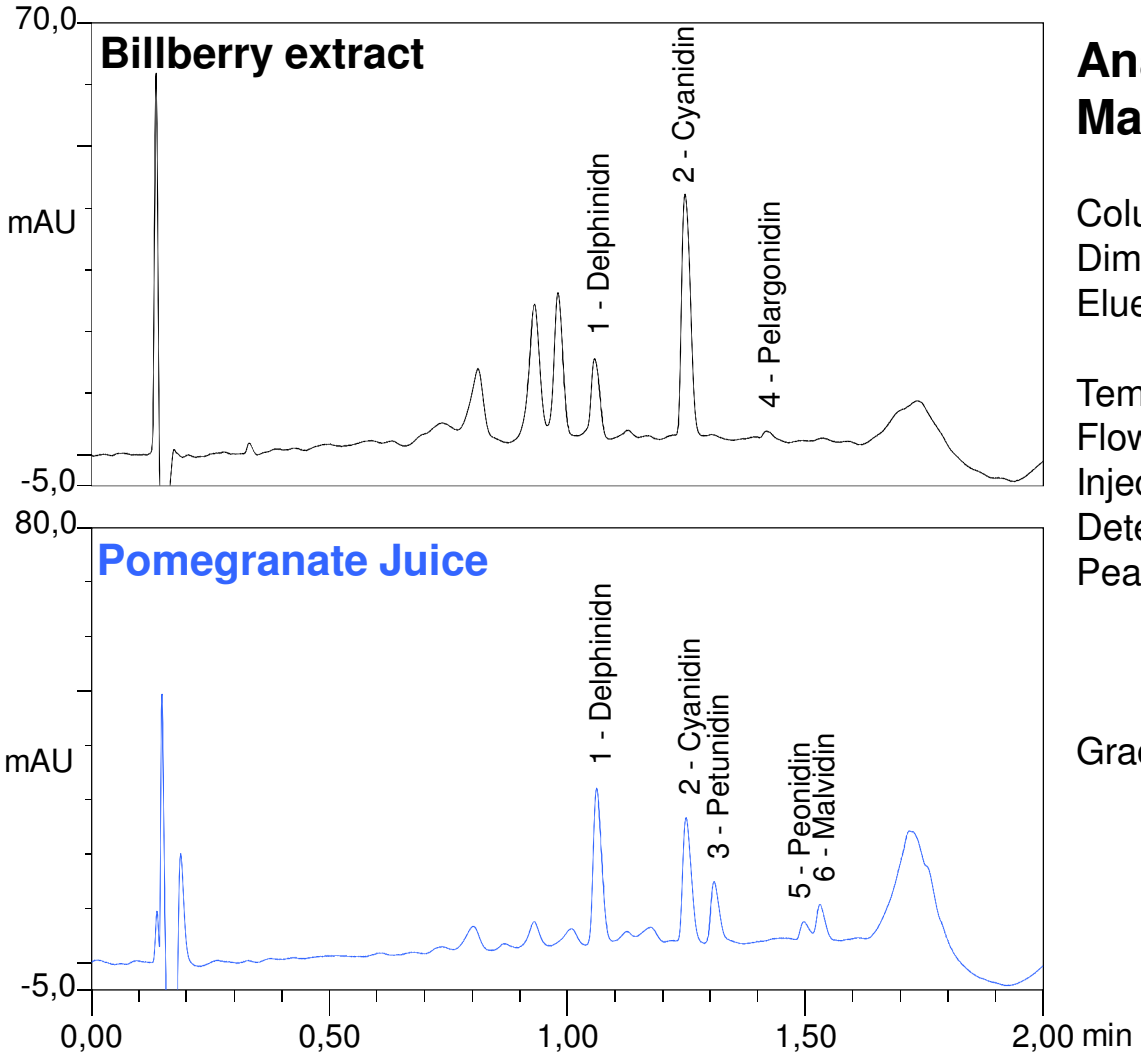
F&B

No	Peak_Name	*Compound_Class	Retention_Time	Peak_Area	Peak_Area_pc	Peak_Height	Peak_Height_pc	Peak_EP_Plates
1	Tartrazine	Dyes	0.56	0.395	9.92	9.570	5.02	1270
2	Amaranth	Dyes	1.10	0.505	12.67	16.869	8.85	9361
3	Indigo Carmine	Dyes	1.20	0.636	15.98	33.884	17.78	28161
4	New Coccine	Dyes	1.27	0.300	7.53	16.372	8.59	33538
5	Sunset Yellow FCF	Dyes	1.38	0.452	11.36	25.326	13.29	43203
6	Fast Green FCF	Dyes	1.72	0.189	4.74	10.583	5.55	61352
7	Eosin Y	Dyes	1.78	0.530	13.31	28.409	14.91	64486
8	Erythrosine	Dyes	1.92	0.417	10.46	22.034	11.56	69506
9	Phloxine B	Dyes	2.11	0.374	9.38	18.724	9.82	80499
10	Bengal Rose B	Dyes	2.23	0.185	4.66	8.828	4.63	80364

Column: Dionex Acclaim RSLC PA2, 2.2 µm, 50 x 2.1 mm

Anthocyanidins analysis with

UHPLC+ focused



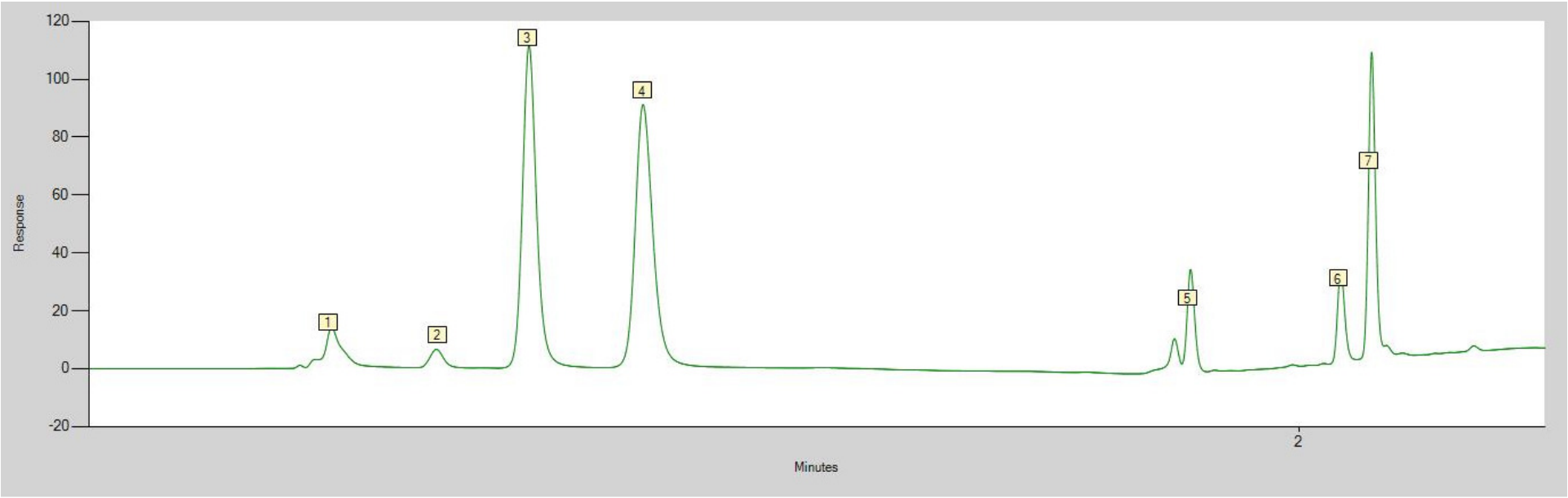
Analysis Time: 2 min
Max Pressure: 600 bar

Column:	AcQuity BEH, C18,1.7 µm
Dimensions:	50 x 2.1 mm ID
Eluent:	A: 0.3% H_3PO_4
	B: ACN
Temperature:	40 °C
Flow rate:	1.0 mL/min
Injection Vol:	1 µL sample in 80% EtOH
Detection:	UV 520 nm
Peaks:	1. Delphinidin 2. Cyanidin
	3. Petunidin 4. Pelargonidin
	5. Peonidin 6. Malvidin

Gradient Table:	0.00 min	10.0% B
	2.00 min	20.0% B

ThermoFisher SCIENTIFIC

Ultrafast Analysis of Water Soluble Vitamins by Parallel UHPLC

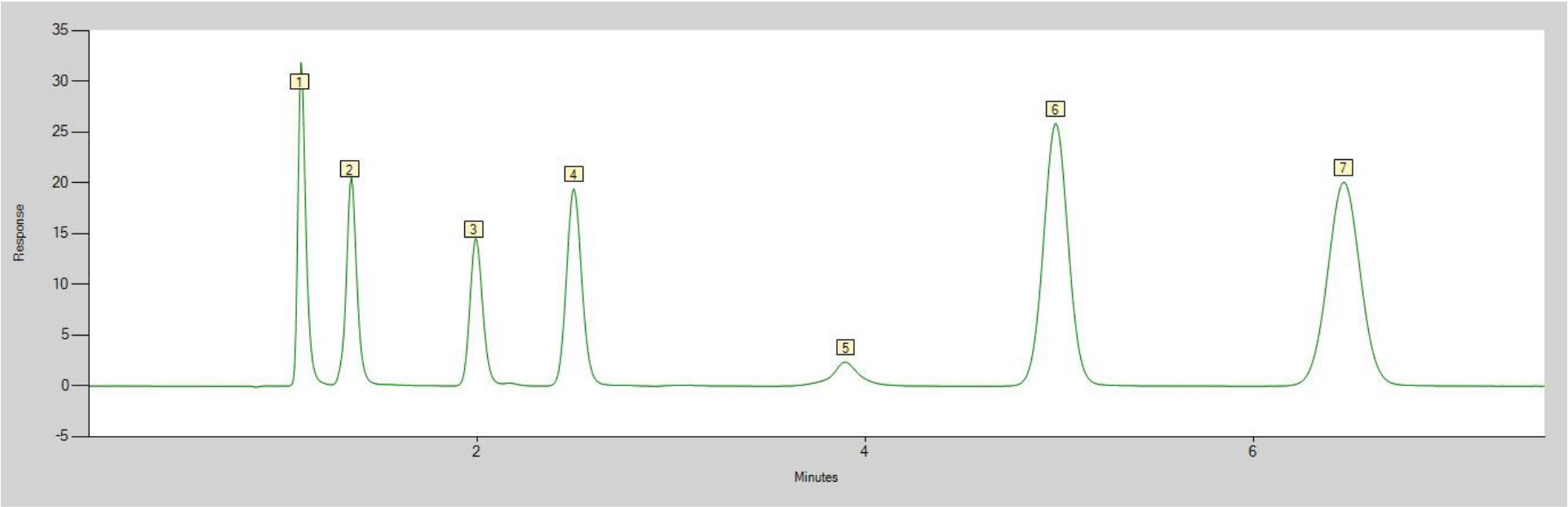


No	Peak_Name	*Compound_Class	Retention_Time	Peak_Area	Peak_Area_pc	Peak_Height	Peak_Height_pc	Peak_EP_Plates
1	Thiamine	Vitamin	0.402	0.481	5.24	13.885	3.54	1429
2	Ascorbic Acid	Vitamin	0.575	0.166	1.81	6.340	1.61	3134
3	Niacinamide	Vitamin	0.728	3.033	33.00	111.623	28.42	5090
4	Pyridoxine	Vitamin	0.916	3.150	34.28	90.932	23.15	4999
5	Pantothenic Acid	Vitamin	1.822	0.438	4.77	32.667	8.32	111606
6	Folic Acid	Vitamin	2.070	0.444	4.83	31.850	8.11	140396
7	Riboflavin	Vitamin	2.120	1.476	16.06	105.444	26.85	163715

F&B

Column: Dionex Acclaim RSLC PA2, 2.2 µm, 100 x 2.1 mm

AN193: Determination of Additives in Carbonated Beverages using Mixed-mode WAX-1 columns

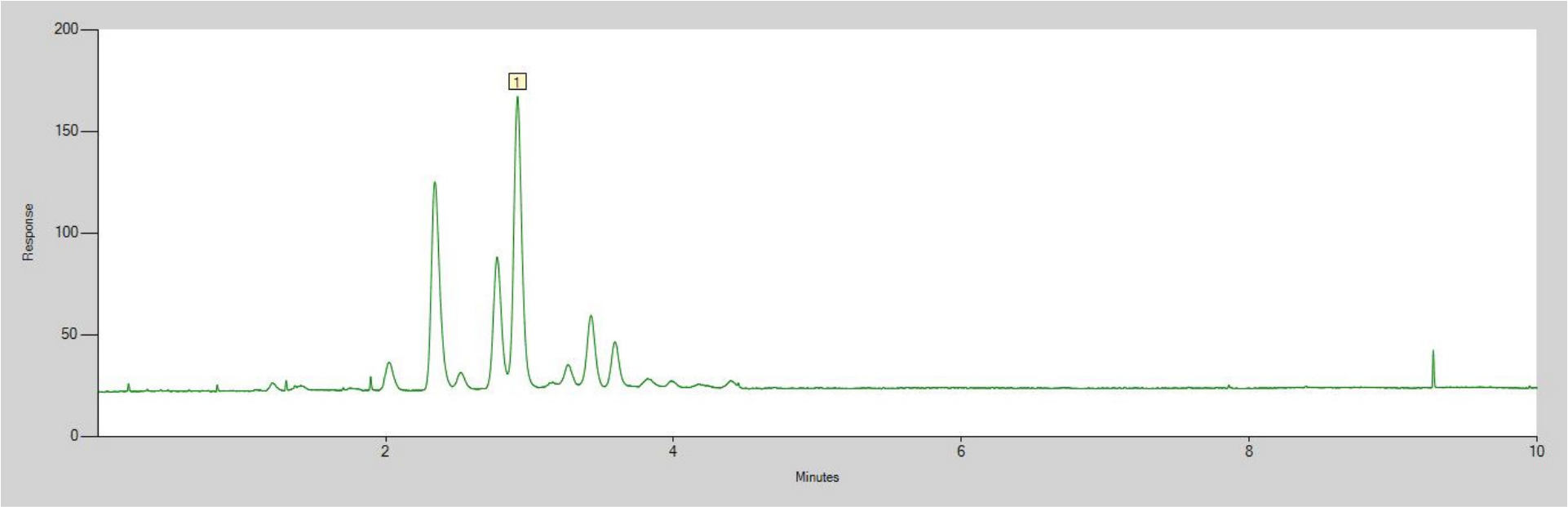


No	Peak_Name	*Compound_Class	Retention_Time	Peak_Area	Peak_Area_pc	Peak_Height	Peak_Height_pc	Peak_EP_Plates
1	Caffeine	Alkaloid	1.093	1.531	10.95	31.863	23.67	3666
2	Aspartame	Sweetener	1.353	1.337	9.56	20.585	15.29	3294
3	Sorbate	Preservative	1.993	1.095	7.83	14.425	10.72	4429
4	Benzoate	Preservative	2.500	1.807	12.92	19.428	14.43	4774
5	Citrate	Food Acid	3.900	0.402	2.87	2.365	1.76	4740
6	Acesulfame	Sweetener	4.980	3.844	27.49	25.846	19.20	7250
7	Saccharin	Sweetener	6.467	3.970	28.38	20.101	14.93	6930

F&B

Column: Acclaim Mixed-Mode WAX-1, 5 µm, 150 x 4.6 mm

High-Resolution Triglyceride Profile of Cooking Oils

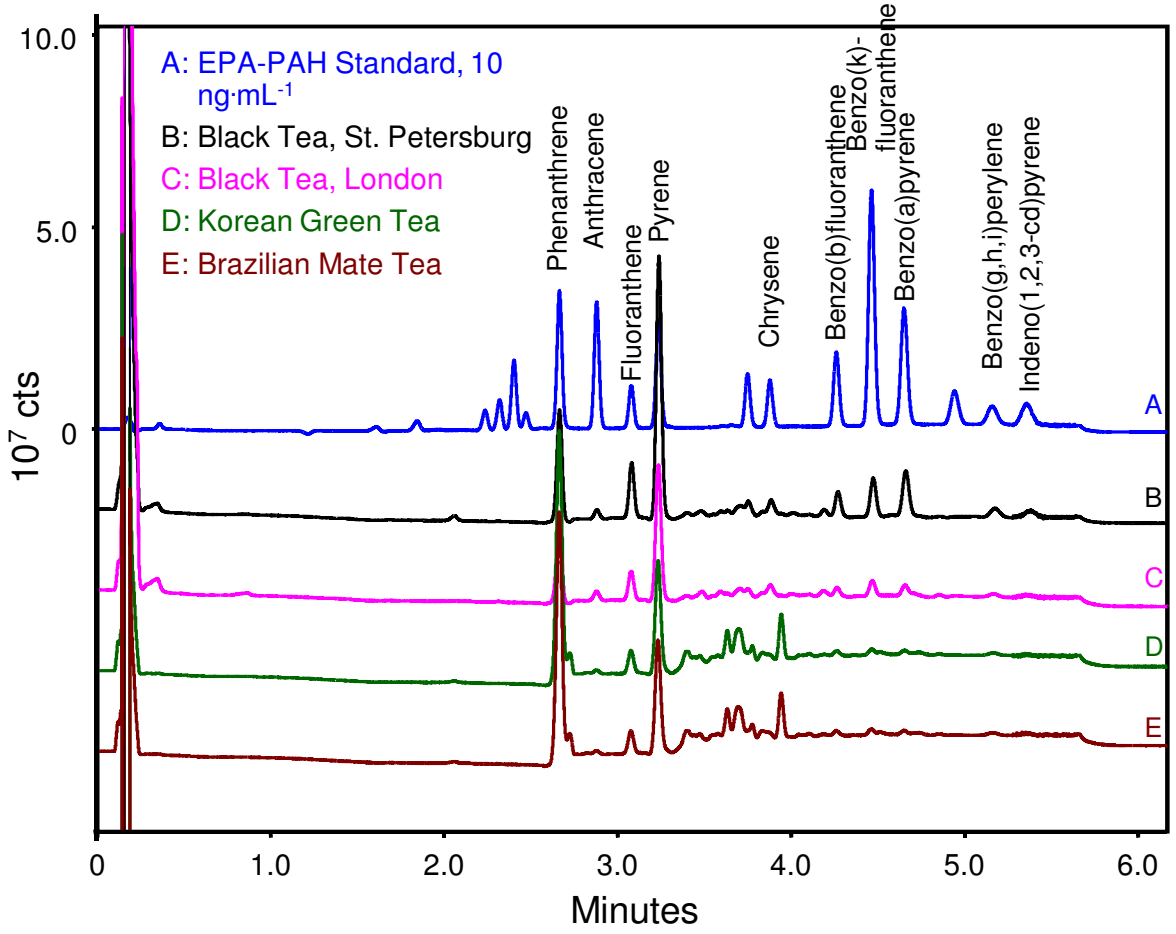


No.	Peak_Name	*Compound_Class	Retention_Time	Peak_Area
1	Triglycerides Profile	Triglycerides	2.916	30.212

F&B

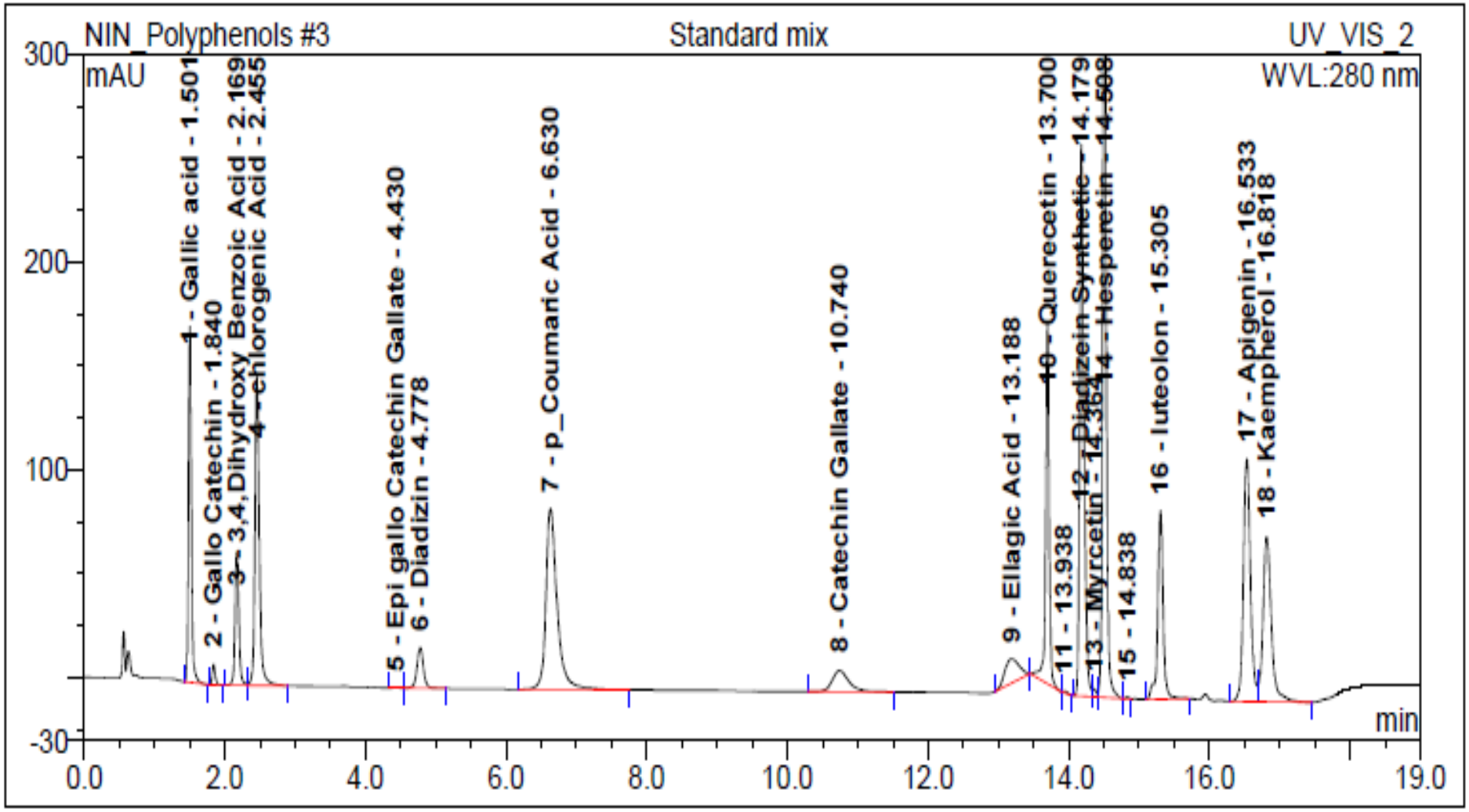
Column: Acclaim RSLC 120 C18, 2.2 µm, 100 x 2.1 mm

EPA-PAHs in Tea Samples with Standard System

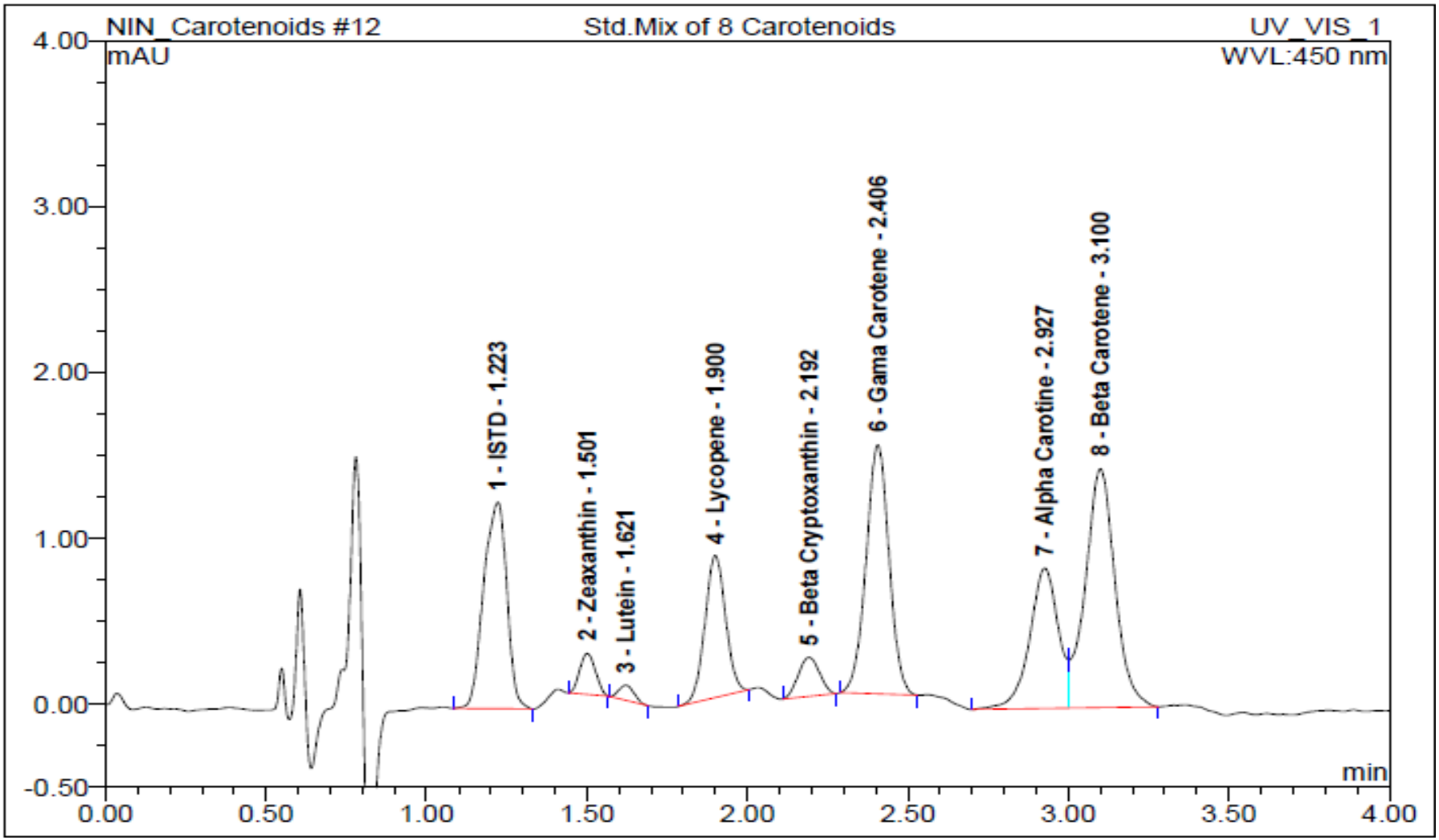


Column: MN Nucleodur C18 PAH, 3 µm (100 x 3.0 mm)
Eluents: A:Water
B:Acetonitrile
Gradient: 0 – 0.5 min: 45% B
45 – 90% B in 3.262 min
90 – 95% B in 0.513 min
95% B for 1.013 min
Flow Rate: 2.0 mL/min @ 537 bar
Temperature: 30 °C
Inj. Vol.: 12.5 µL
Detection: FLU
Sample: see fig.

Polyphenols Analysis with RSLC System



Carotenoides Analysis with RSLC System



AppsLibrary Conclusion

- Web Based Application Search Engine
- Offers easy search option for Dionex IC/LC applications
- Provides unique structured searching tools for
  - Analyte name or class
  - Market and instrument type
  - Column information such as particle size and packing material
- Modern application view screen
  - See all details in a single glance
- Unique download of all application settings for immediate use

ThermoFisher SCIENTIFIC

UHPLC+ focused

Thank You!

The world leader in serving science

QUIZ

- **NAME THE LAW THAT FORMS THE PRINCIPLE OF UV DETECTORS.**
- **NAME THE LAW THAT FORMS THE PRINCIPLE OF REFRACTIVE INDEX DETECTOR.**
- **NAME THE EQUATION THAT DEFINES THE PEAK BROADENING WITHIN A CHROMATOGRAPHIC COLUMN.**
- **IN REVERSE PHASE CHROMATOGRAPHY, THE STATIONARY PHASE IS _____. (POLAR/NON-POLAR)**
- **IN NORMAL PHASE CHROMATOGRAPHY, THE MOBILE PHASE IS _____ (POLAR/NON-POLAR)**
- **WHAT DOES THE 'N' STAND FOR IN RESOLUTION EQUATION?**
- **WHAT DOES THE 'α' STAND FOR IN RESOLUTION EQUATION?**
- **WHAT DOES THE 'k' STAND FOR IN RESOLUTION EQUATION?**
- **IN FLUORESCENCE DETECTION, THERE ARE TWO WAVELENGTHS, WHAT ARE THEY?**
- **FOR _____ DETECTION, THE COMPOUND SHOULD HAVE REDOX PROPERTIES.**