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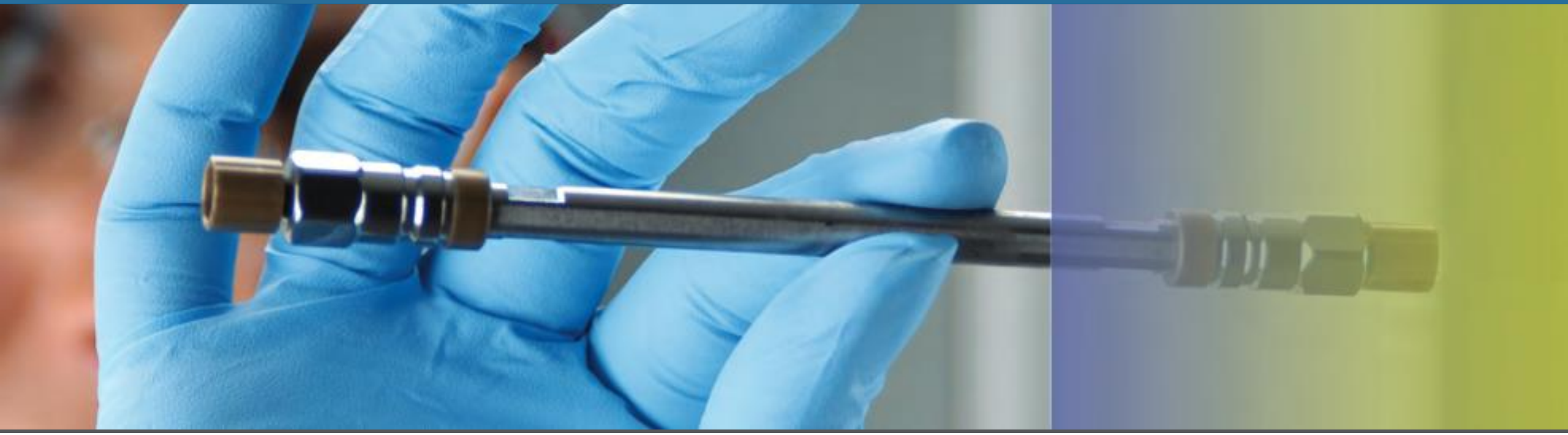
HPLC Column Selection: Solve the Separation Mystery

Overview

- Introduction
- Historical Overview in Stationary Phases
- Basics in Column Selection
  - Resolution, Efficiency and Selectivity
- Thinking Outside of the C18 Box



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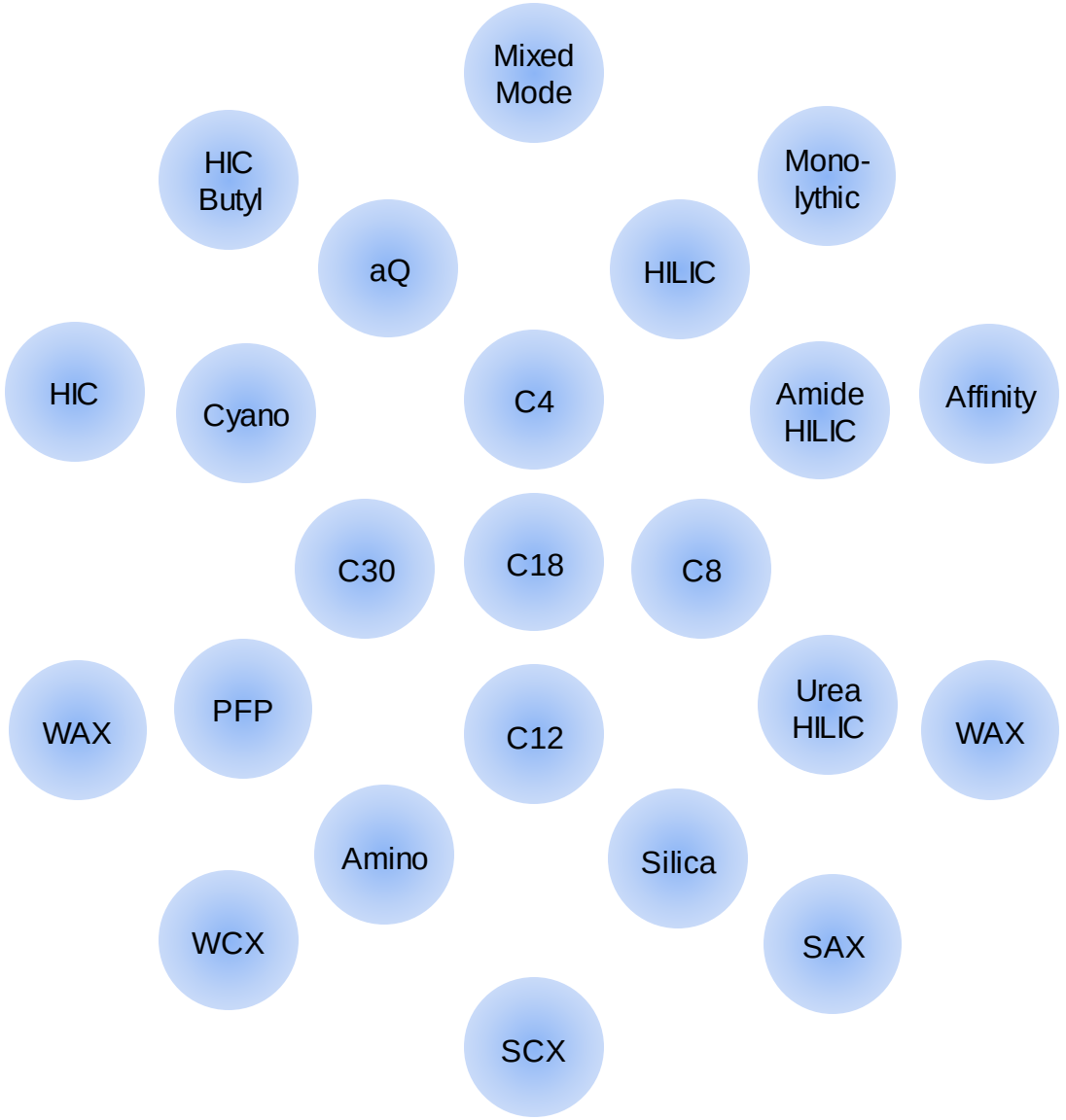
Introduction – “Unlimited" Choice of Stationary Phase



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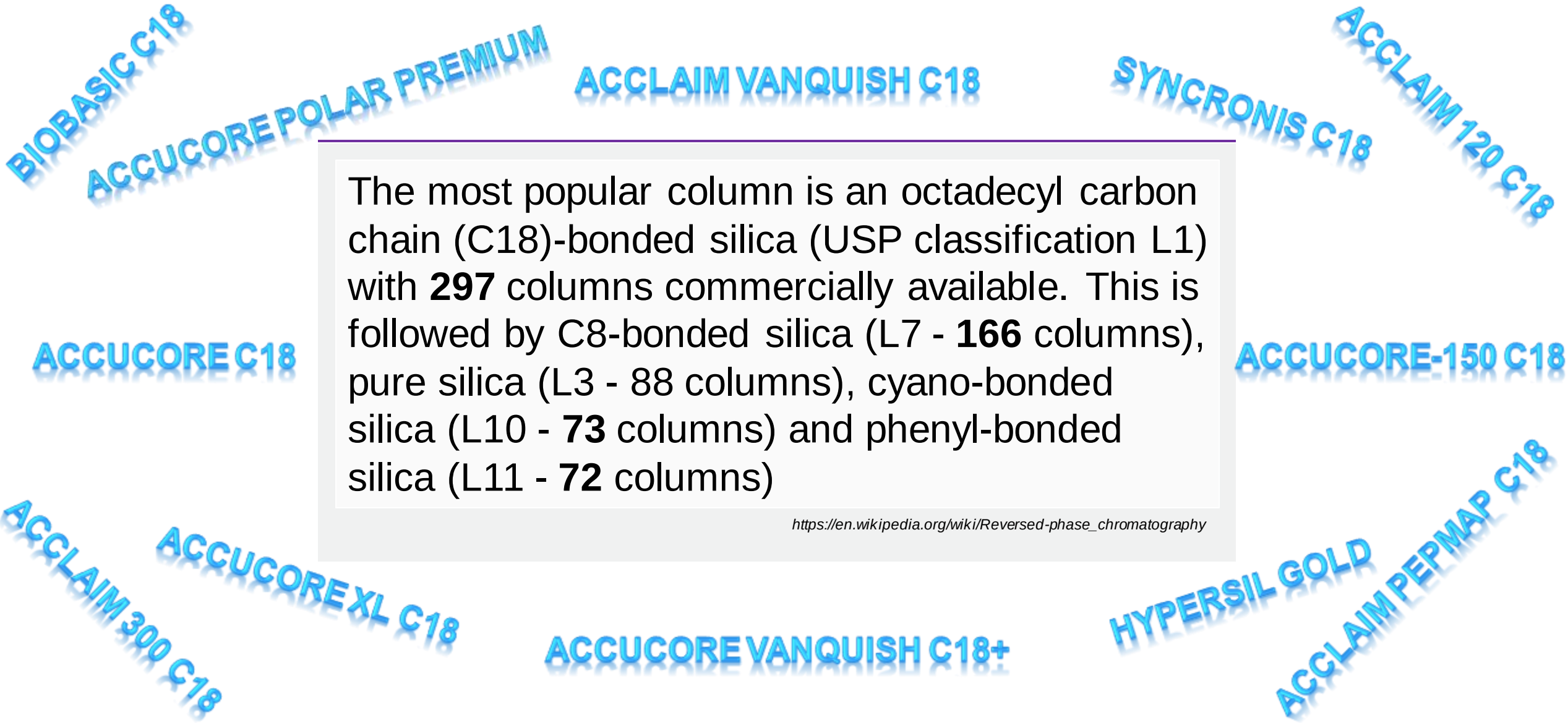
And an Almost Unlimited Choice in C18 as well



The most popular column is an octadecyl carbon chain (C18)-bonded silica (USP classification L1) with **297** columns commercially available. This is followed by C8-bonded silica (L7 - **166** columns), pure silica (L3 - 88 columns), cyano-bonded silica (L10 - **73** columns) and phenyl-bonded silica (L11 - **72** columns)

https://en.wikipedia.org/wiki/Reversed-phase_chromatography

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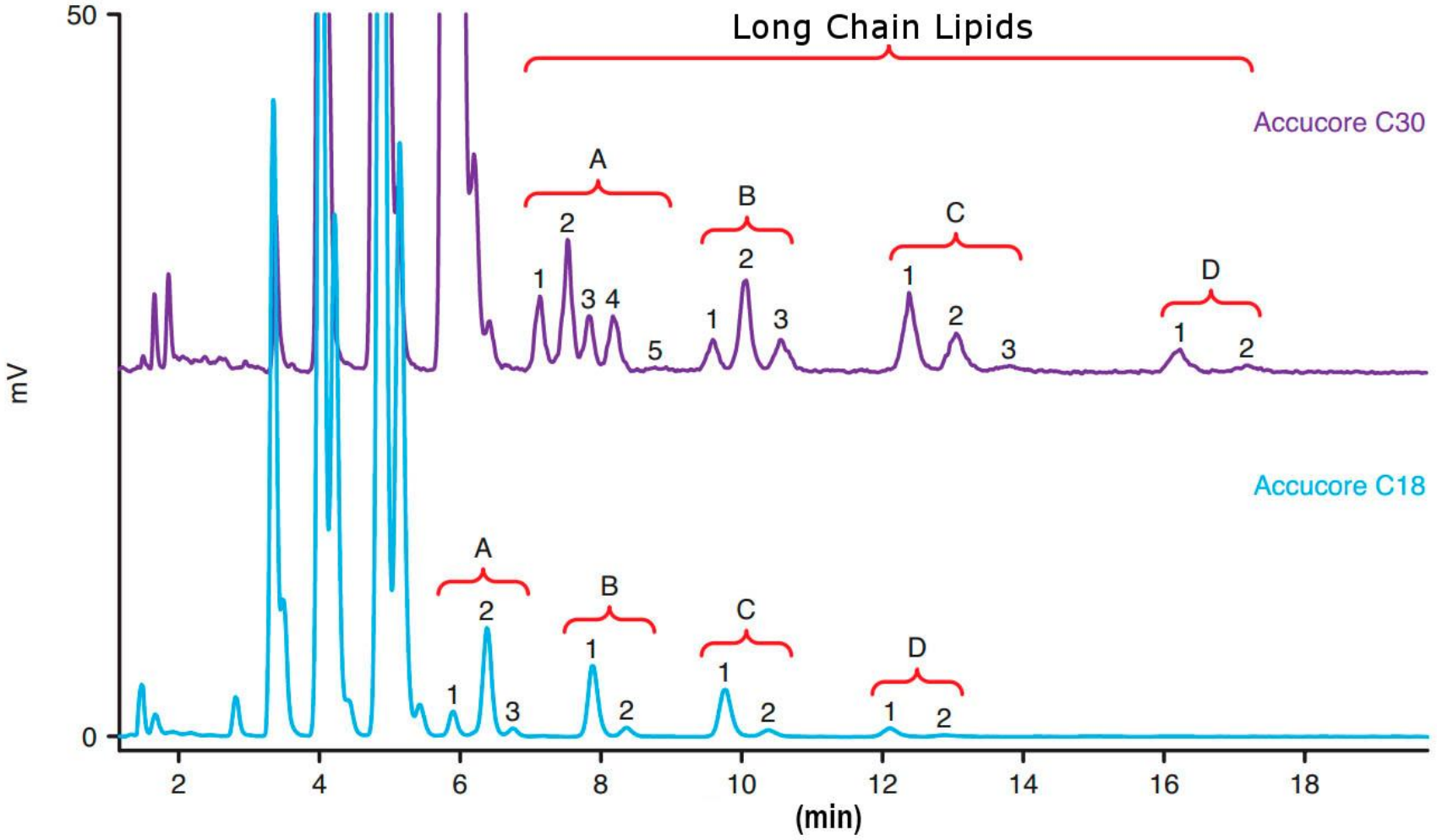
But C18 Is Not The Only Answer



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Comparing Accucore C18 and Accucore C30 Columns

Thermo Scientific™ Accucore™ C30

T1: Hydrophobic Interactions

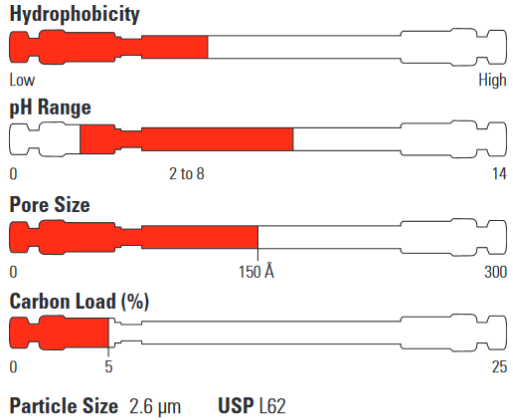
		Parameter		Term
	HR	Hydrophobic Retention	Retention of compounds based on their hydrophobicity	k'
	HS	Hydrophobic Selectivity	Separation of compounds that have similar structure, but differ slightly in hydrophobicity	α
	SS	Steric Selectivity	Separation of compounds that have similar structure, but differ in shape	α
SiO H---X	HBC	Hydrogen Bonding Capacity	Separation related to degree of end capping	α

T2: Secondary Interactions Under Neutral pH

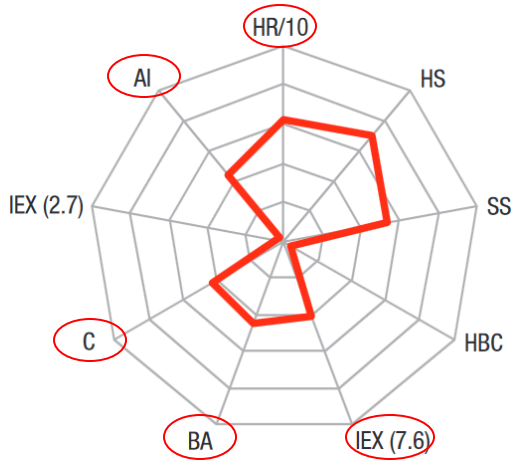
		Parameter		Term
SiO NH_2X	BA	Base Activity	Peak shape for basic analytes resulting from total silanol activity (all dissociated at pH 7.6)	t_f
M---X	C	Chelation	Peak shapes for chelating analytes resulting from silica metal content	t_f
SiO--- X+ pH 7.6	IEX(7.6)	Ion Exchange Capacity (pH 7.6)	Separation between basic and neutral compounds resulting from total silanol activity (all dissociated at pH 7.6)	α

T3: Secondary Interactions Under Acidic pH

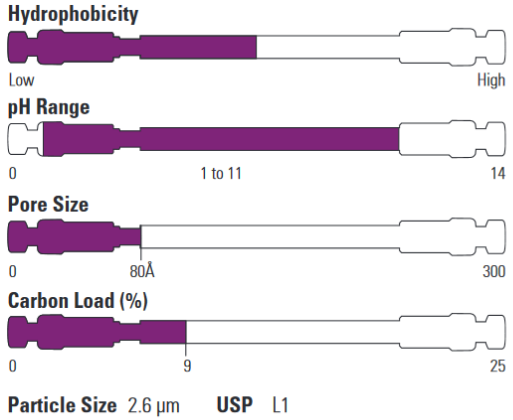
		Parameter		Term
SiO OOCHX	AI	Acid Interaction	Interactions resulting in poor peak shape for acidic analytes	t_f
SiO--- + X pH 2.7	IEX(2.7)	Ion Exchange Capacity (pH 2.7)	Separation between basic and neutral compounds resulting from acidic silanol activity	α



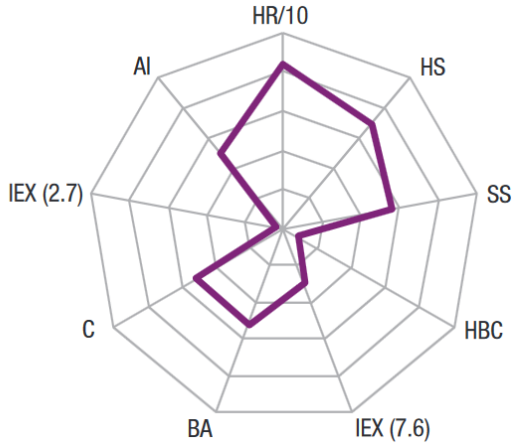
Particle Size 2.6 µm **USP** L62



Accucore C18



Particle Size 2.6 µm **USP** L1



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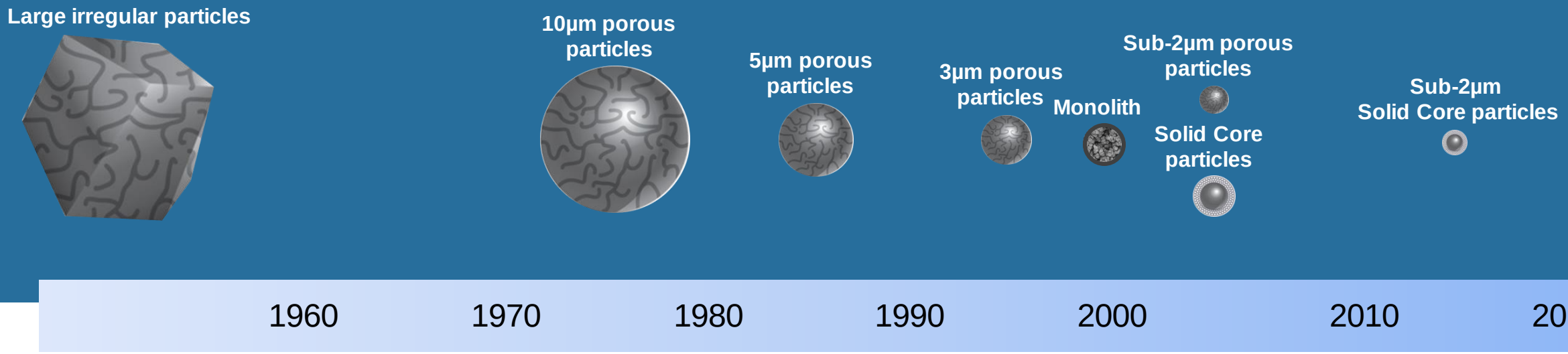
Historical Overview in Stationary Phases



Over the years, stationary phase particles have become:

- Spherical
- Smaller
- Available with solid core and porous layer
- Can be replaced by monolithic design.

Resulting in better columns and better separations

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What are we looking for when selecting a column?

- Need retention between analyte and column:
  - Reverse phase, hydrophobic interactions
  - Polar compounds – HILIC, Ion exchange
- Column needs to differentiate between similar molecules:
  - Look to the differences in the analytes to help with the choice of separation column
- Column needs to be stable in conditions being used:
  - Overloading
  - pH effects
    - remember: silica dissolves above pH 7
  - Temperature effects

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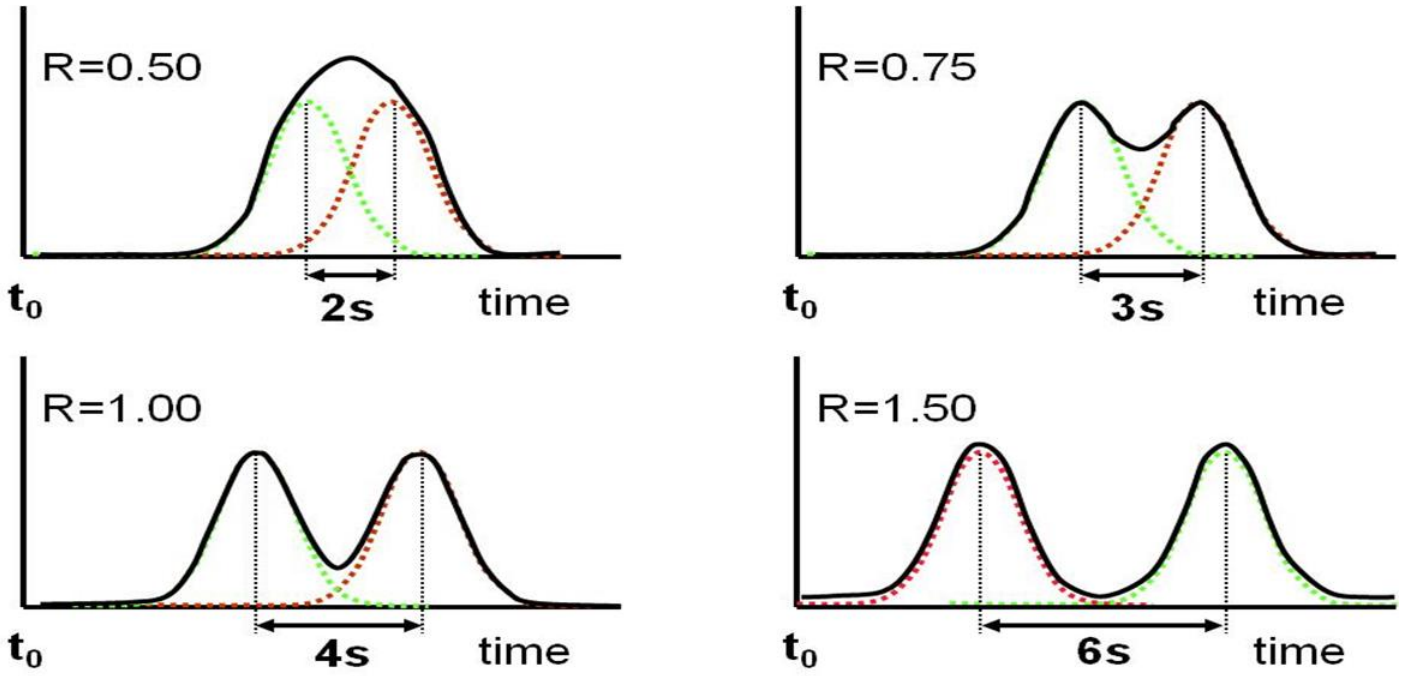
Basics in Column Selection – Resolution

- Resolution:
  - The degree of separation between two adjacent peaks
  - Higher R, better separation
- Resolution ≥ 1.50:
  - Separation quality good enough to accurately measure peak area or peak height of each peak
- Key-factors in resolution:
  - Retention
  - Efficiency
  - Selectivity.



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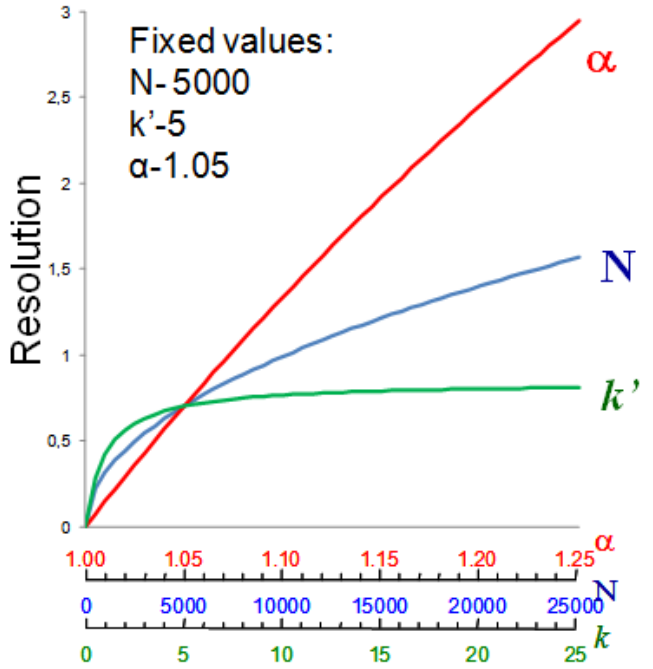
Resolution – Retention, Efficiency and Selectivity

- Retention or capacity factor(*k*):
  - Amount of time spend by an analyte interacting with the stationary phase
  - High *k* value indicates more retention
- Selectivity or separation factor (α):
  - Ability to 'chemically' distinguish between sample components
  - Greatest impact on resolution
- Efficiency (*N*):
  - The efficiency of a chromatographic peak is a measure of the dispersion of the analyte band as it travels through the HPLC system and column
  - The plate number is a measure of the peak dispersion on the HPLC column, which reflects the column performance

Efficiency Retention Selectivity

$$R = \frac{\sqrt{N_2}}{4} \cdot \frac{k_2'}{k_2' + 1} \cdot \frac{\alpha - 1}{\alpha}$$



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Make Sure to Match the Column and the System

UHPLC is now not limited to small molecules
Biomolecular analysis can benefit from the higher resolution and faster analysis this technique offers.

Thermo Scientific™ Viper™ Fingertight Fittings
MP35N 100um i.d. Finger tight and zero dead volume.

Vanquish Parallel Binary Pump
Independent piston drives and variable stroke volume, 1500 bar. – ultra low baseline noise

LightPipe™ Technology in the Vanquish DAD Detector
Provides you with an unmatched detection experience. Ultra-wide dynamic range, high sensitivity

Sampler
Zero maintenance ceramic injection valve, loop pre compression, 4 x 96 well plates, charger.

Oven
Adiabatic and forced air. 30cm column, active eluent preheating, column switching, ceramic valves.

Thinking Outside of the C18 Box

- Reversed Phase is the most popular form of chromatography:
  - C18 covers 80-90% of Reversed Phase
  - Your trustworthy C18 columns is typically the first choice to target new analytes
  - Retention is primarily based on the analyte's hydrophobicity
  - Polar mobile phase: Water / Methanol (MeOH) / tetrahydrofuran (THF) / Acetonitrile (ACN)

- Thinking outside the box with the Thermo Scientific™ Hypersil GOLD™ family of columns:
  - 12 different chemistries
  - Multiple dimensions
  - Ideal to demonstrate the different separation possibilities

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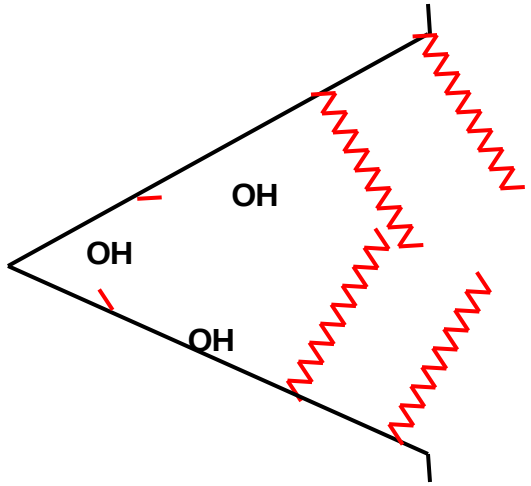
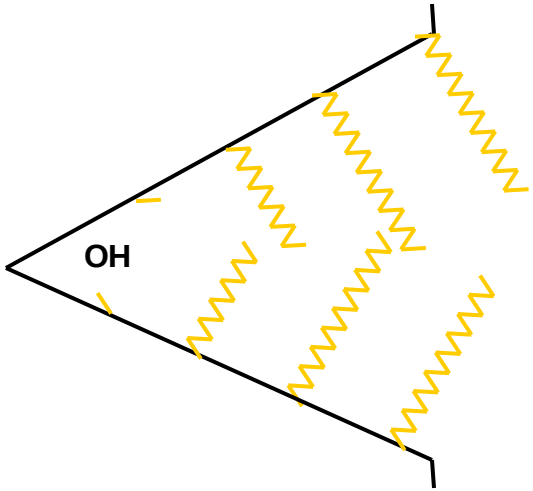
Hypersil GOLD UHPLC and HPLC Column Family

Hypersil GOLD columns are manufactured using a proprietary approach to bond C18 and other reagents in such a way that the phase has very few residual silanols contributing to the observed chromatography. This significantly reduces peak tailing whilst maintaining true reversed phase retention.



"Traditional" C18



Hypersil GOLD

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Hypersil GOLD – Common Selectivity Options

- **Hypersil GOLD** for outstanding peak shape using generic gradients with C18 selectivity
- **Hypersil GOLD C8** offers similar selectivity but with less retention
- **Hypersil GOLD aQ** for challenging reverse phase separations employing highly aqueous mobile phases
- **Hypersil GOLD PFP** offers alternative selectivity, particularly for halogenated and substituted aromatics
- **Hypersil GOLD CN** for both reversed and normal phase separations
- **Hypersil GOLD Phenyl** offers alternative selectivity and is particularly suitable for aromatic and moderately polar compounds

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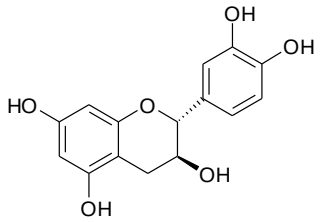
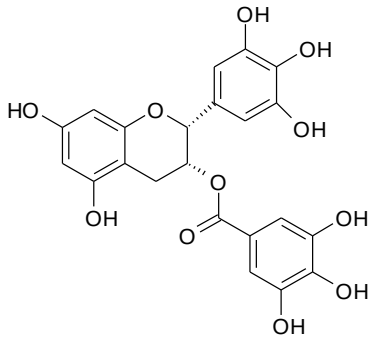
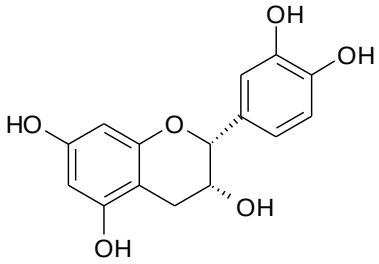
Catechins – A Good Example Suite



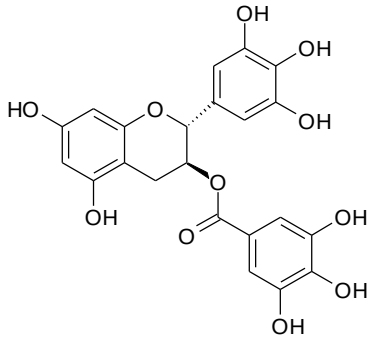
1. Catechin



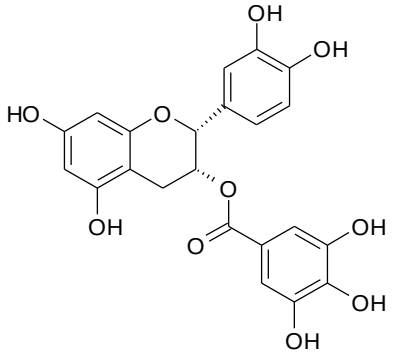
2. Epigallocatechin Gallate



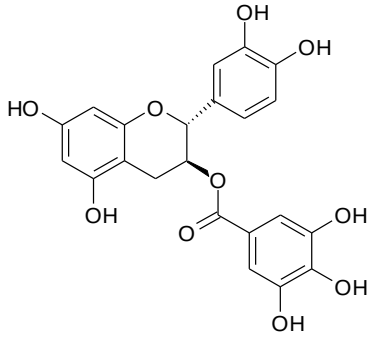
3. Epicatechin



4. Gallocatechin Gallate



5. Epicatechin Gallate



6. Catechin Gallate

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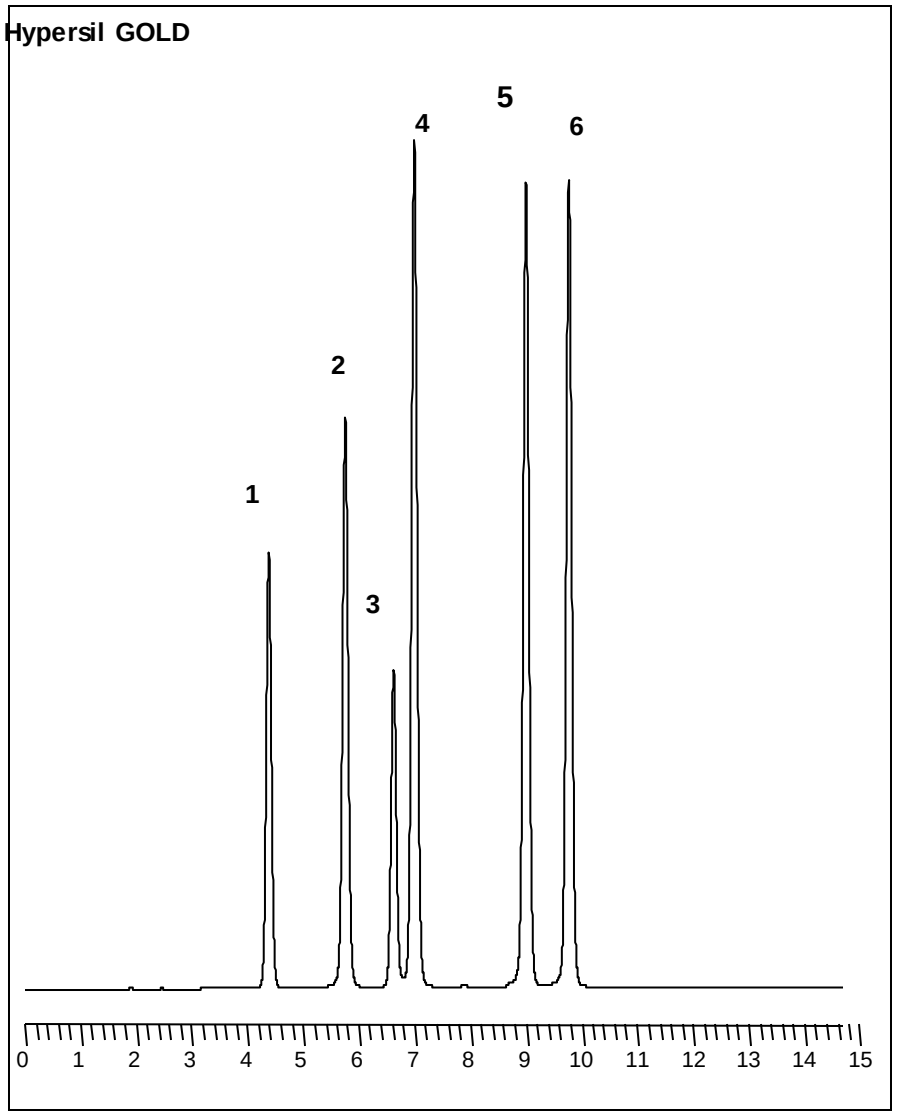
Catechins on Hypersil GOLD



- Predictable elution order based on analyte hydrophobicity
- Outstanding peak shape

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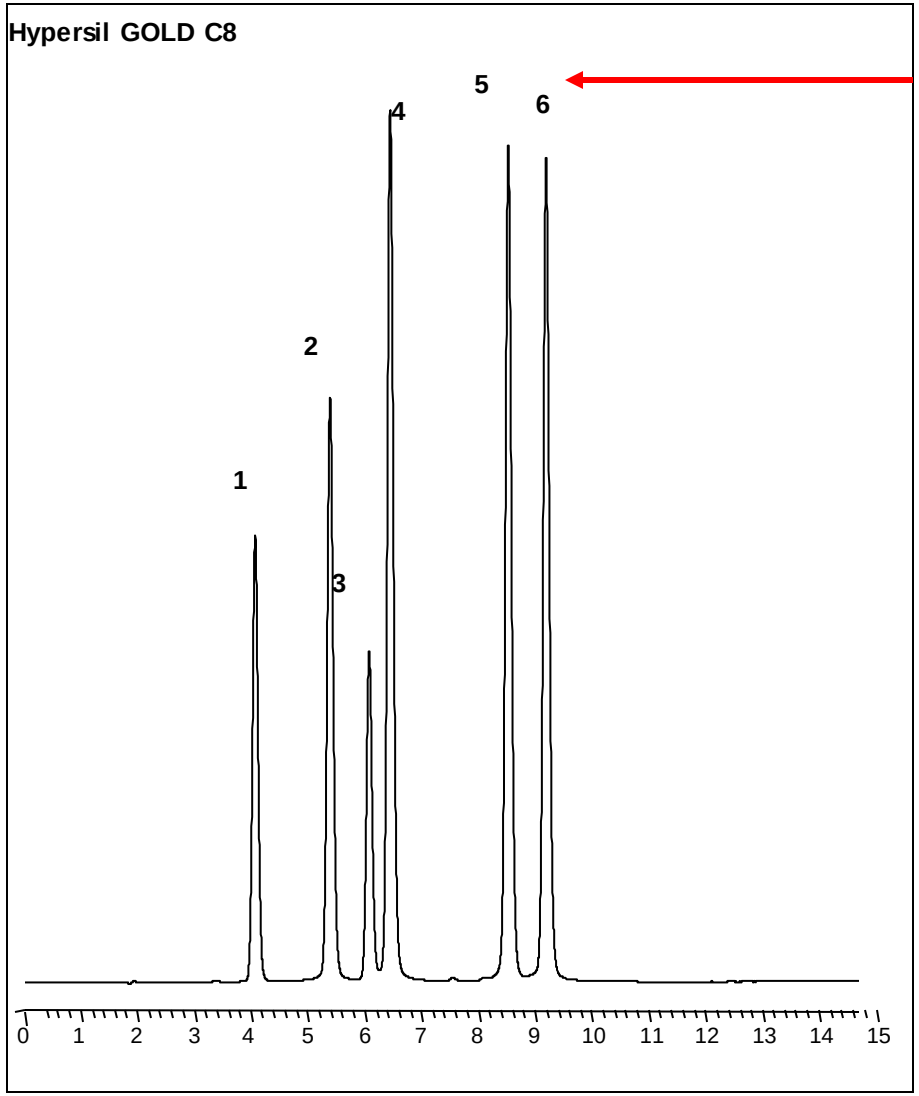
Catechins on Hypersil GOLD C8



- Reduced retention for all analytes
- Same elution order

H252-008

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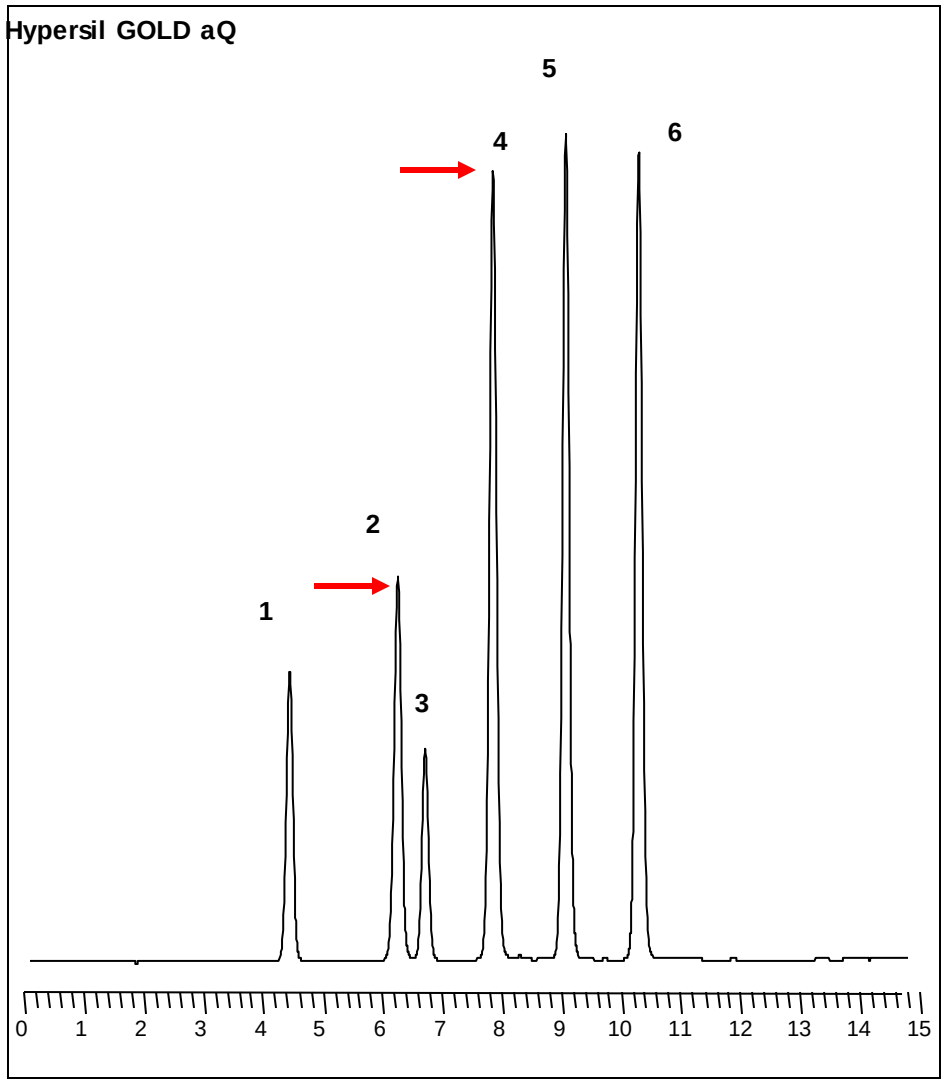
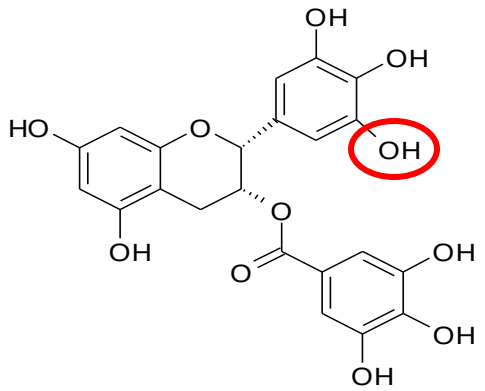
Catechins on Hypersil GOLD aQ



- Same elution order
- Extra retention of analytes 2 and 4:
  - Gallocatechins have an extra OH group to interact with polar endcapping



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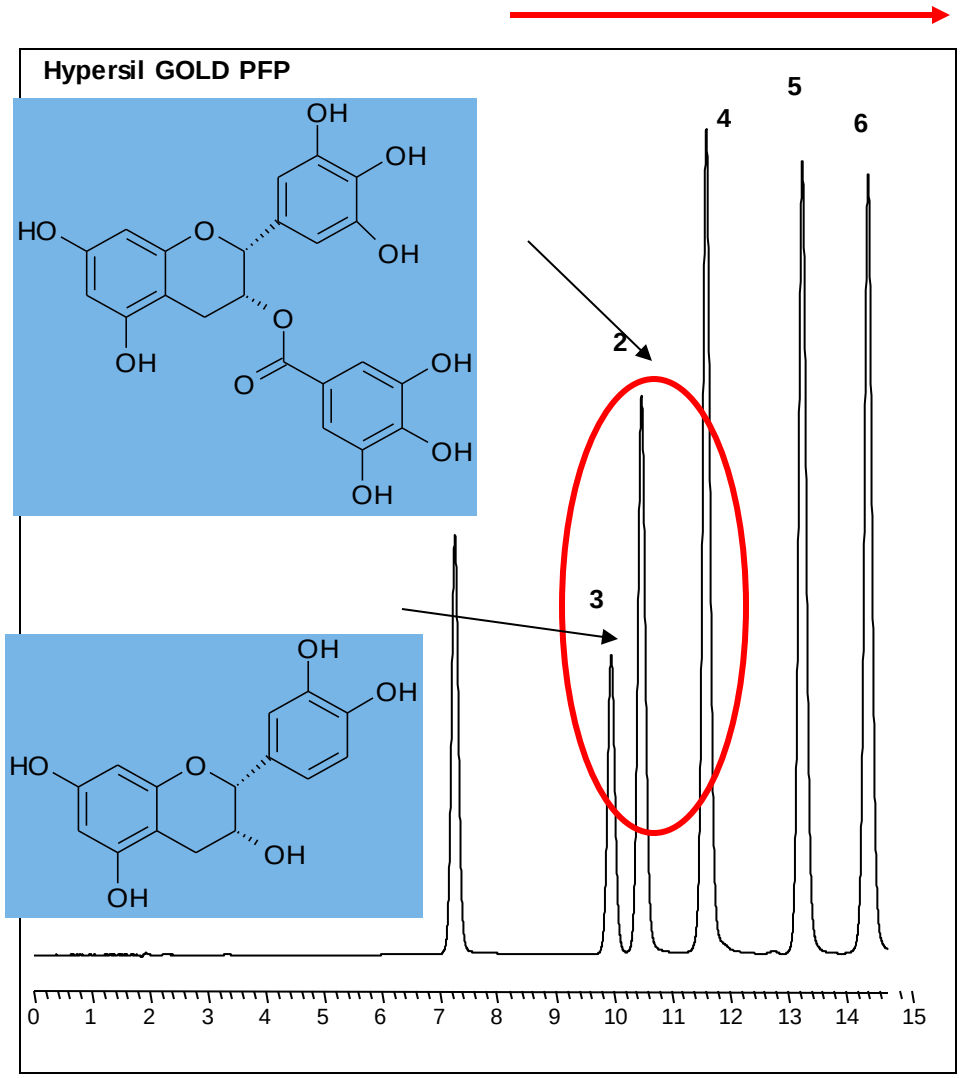
Catechins on Hypersil GOLD PFP



H254-007

- Extra retention for all six analytes
- 2, 3 reverse elution order:
  - Extra aromatic ring epigallocatechin gallate (2) gives enhanced retention on PFP

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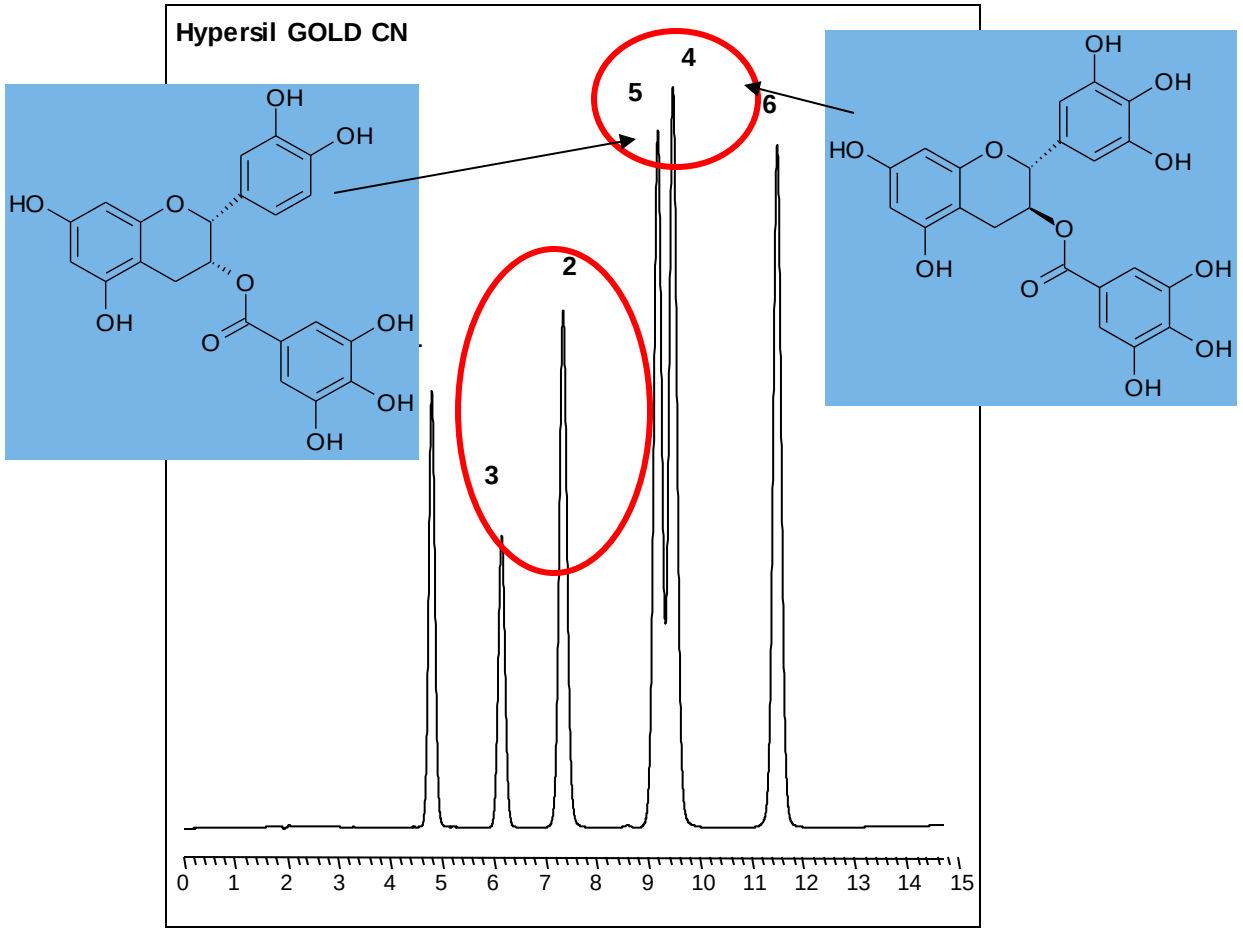
Catechins on Hypersil GOLD CN



- More retention than Hypersil GOLD
- 2,3 and 4,5 reverse elution order
  - additional hydroxy group in gallocatechin gallate (4)

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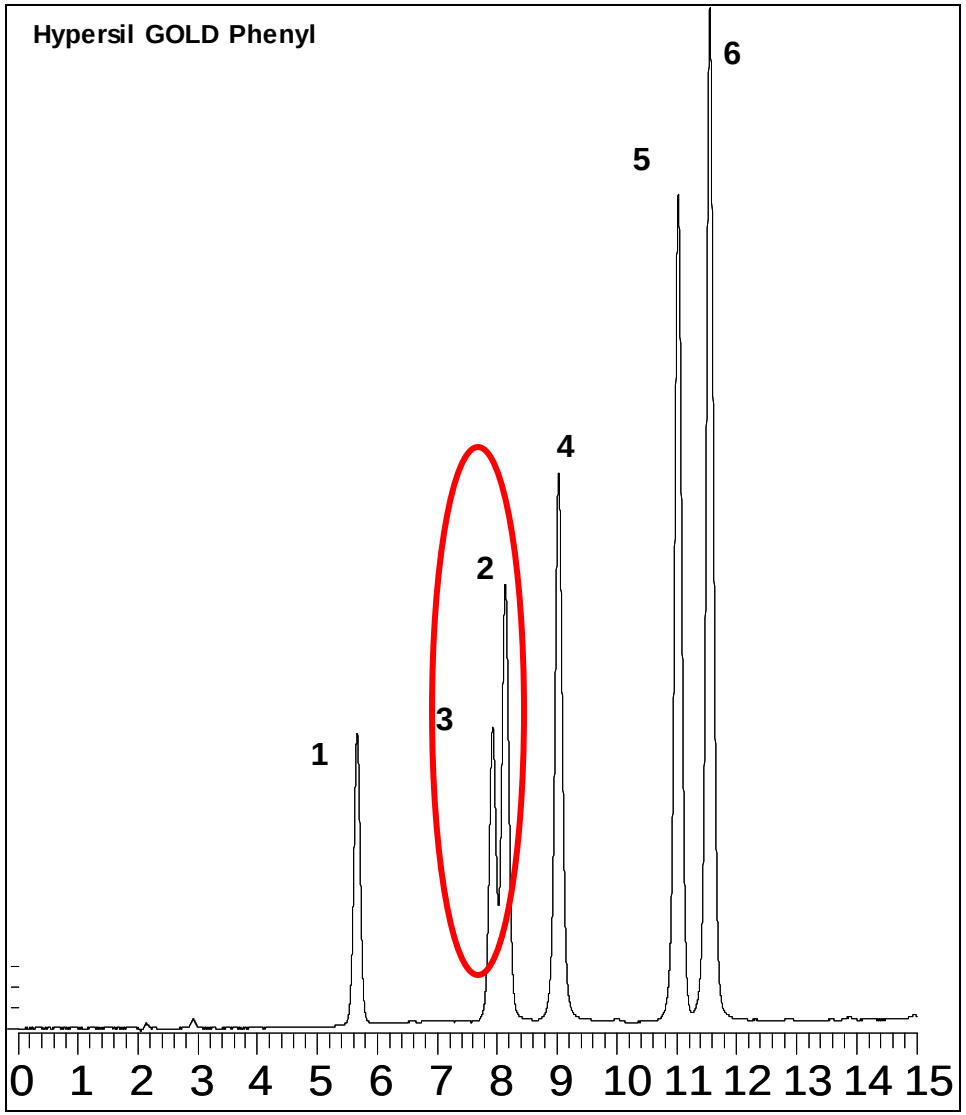
Catechins on Hypersil GOLD Phenyl



- Extra retention for all analytes
- Less retention than PFP
- 2, 3 reverse elution order:
  - More aromatic rings give enhanced retention

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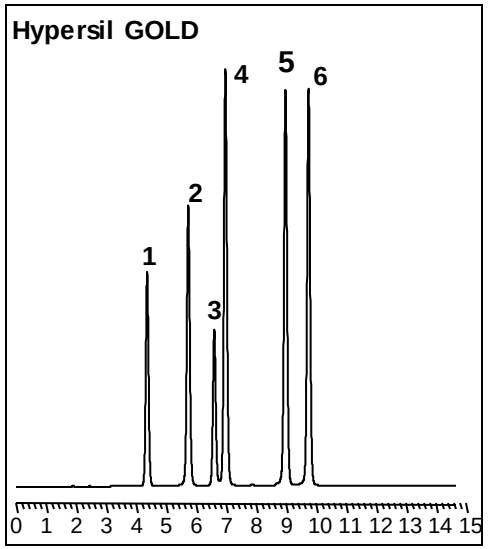
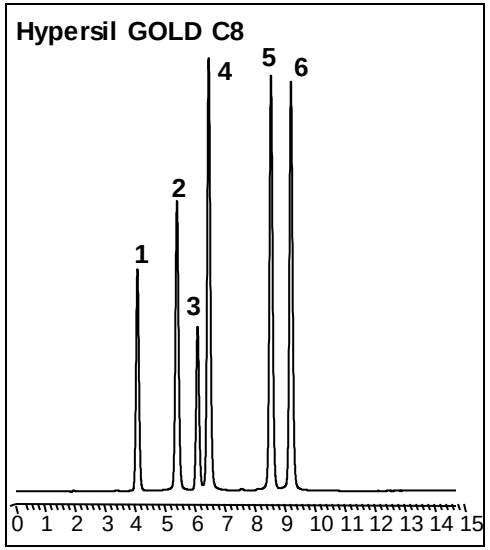
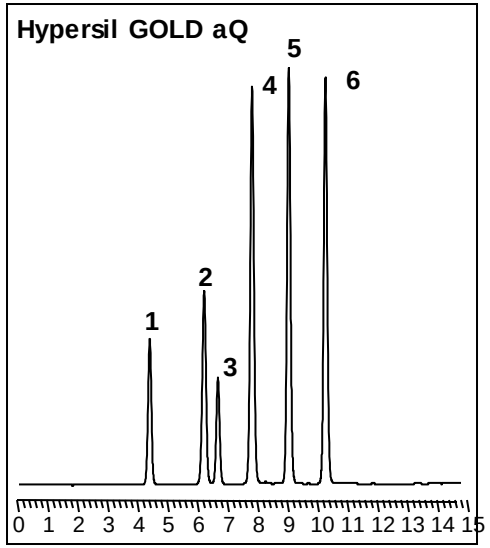
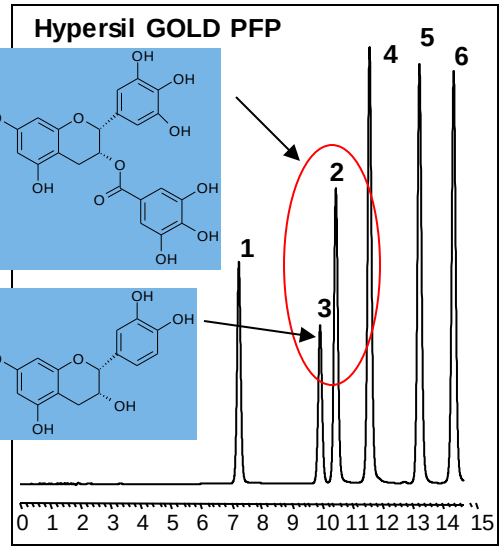
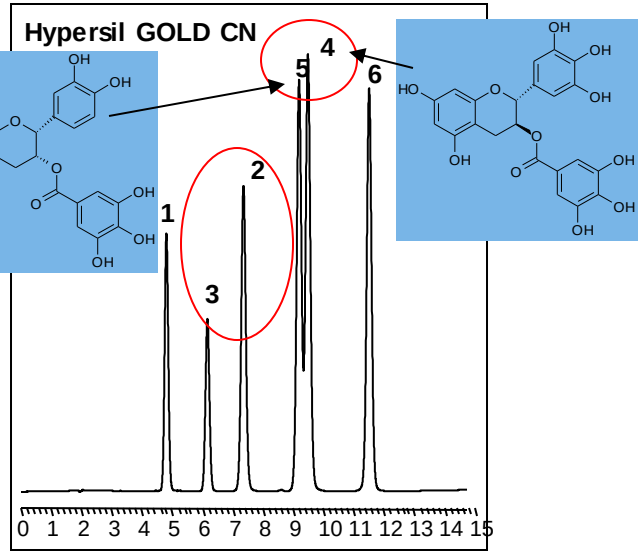
Hypersil GOLD family comparison

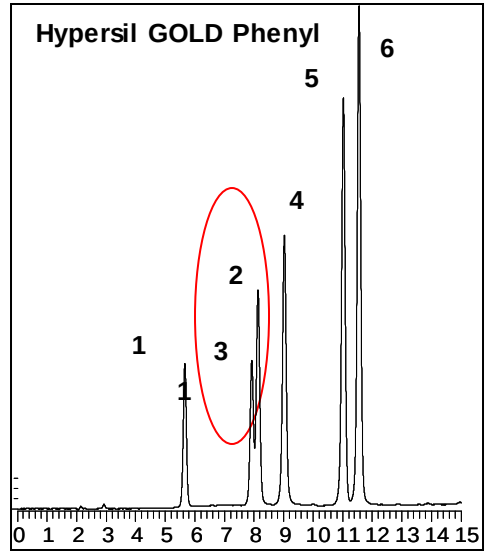












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Hypersil GOLD family – extending the versatility and choice

- **Hypersil GOLD C4**
  - Short alkyl chain length, low hydrophobicity column for less retention
- **Hypersil GOLD Amino**
  - demonstrates excellent chromatographic properties in three modes: weak anion exchange, reversed phase and normal phase.
- **Hypersil GOLD AX**
  - Separate proteins, peptides, anionic species and polar molecules
- **Hypersil GOLD SAX**
  - Highly stable silica-based quarternary amine strong anion exchange column, designed for aqueous mobile phase
- **Hypersil GOLD Silica**
  - High efficiency column for non-polar and moderately polar organic compounds by normal phase chromatography

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Thinking even further outside of the C18 Box

Specialty application columns:

- Pharmaceutical applications
- Food Safety control
- Environmental applications
- Polymeric columns for DNA/RNA molecules
- HILIC or Hydrophilic Interaction Chromatography columns

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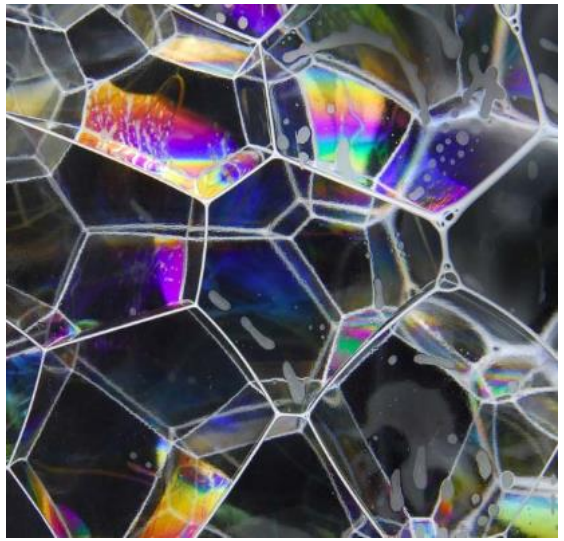
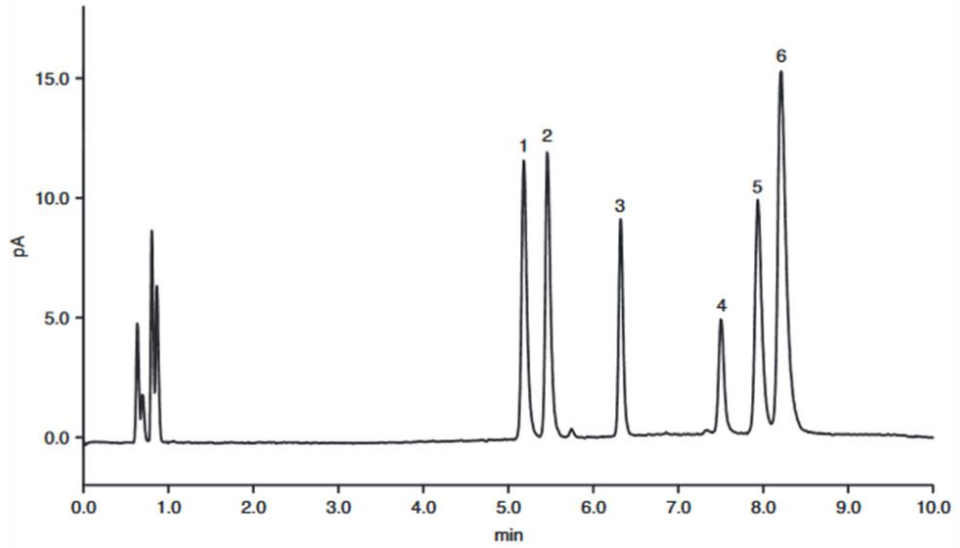
Separation of Industrial, Agricultural and Pharmaceutical Surfactants

- Acclaim Surfactant Plus
  - Mixed mode columns using Reversed Phase and Anion Exchange Chromatography
- Controlled via mobile phase ionic strength, pH, and/or organic solvent type and composition
  - Resulting in a specific elution order of cationic, nonionic, amphoteric and anionic surfactants



Separation of dodecyltrimethylammonium bromide (1), dodecylpyridinium chloride (2), benzalkonium chloride (3,4), cetyltrimethylammonium bromide (5) and cetylpyridinium chloride (6)



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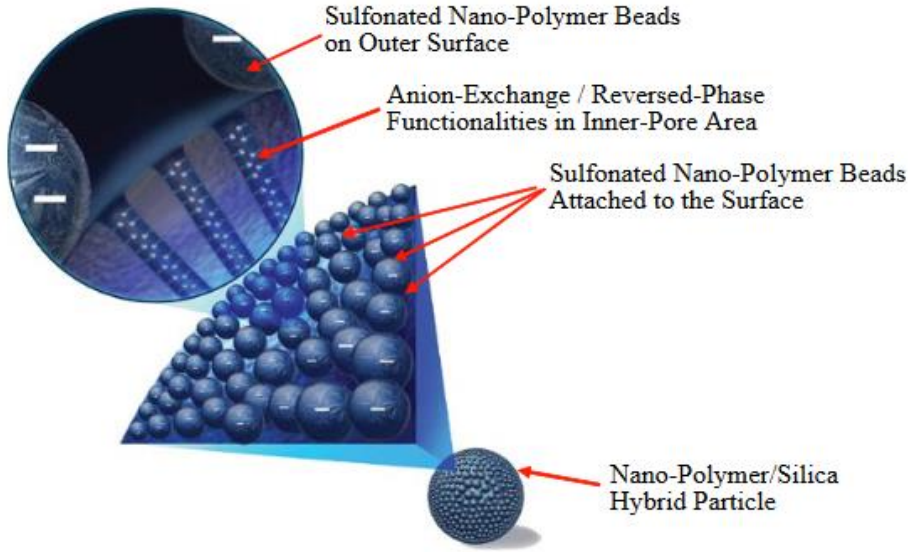
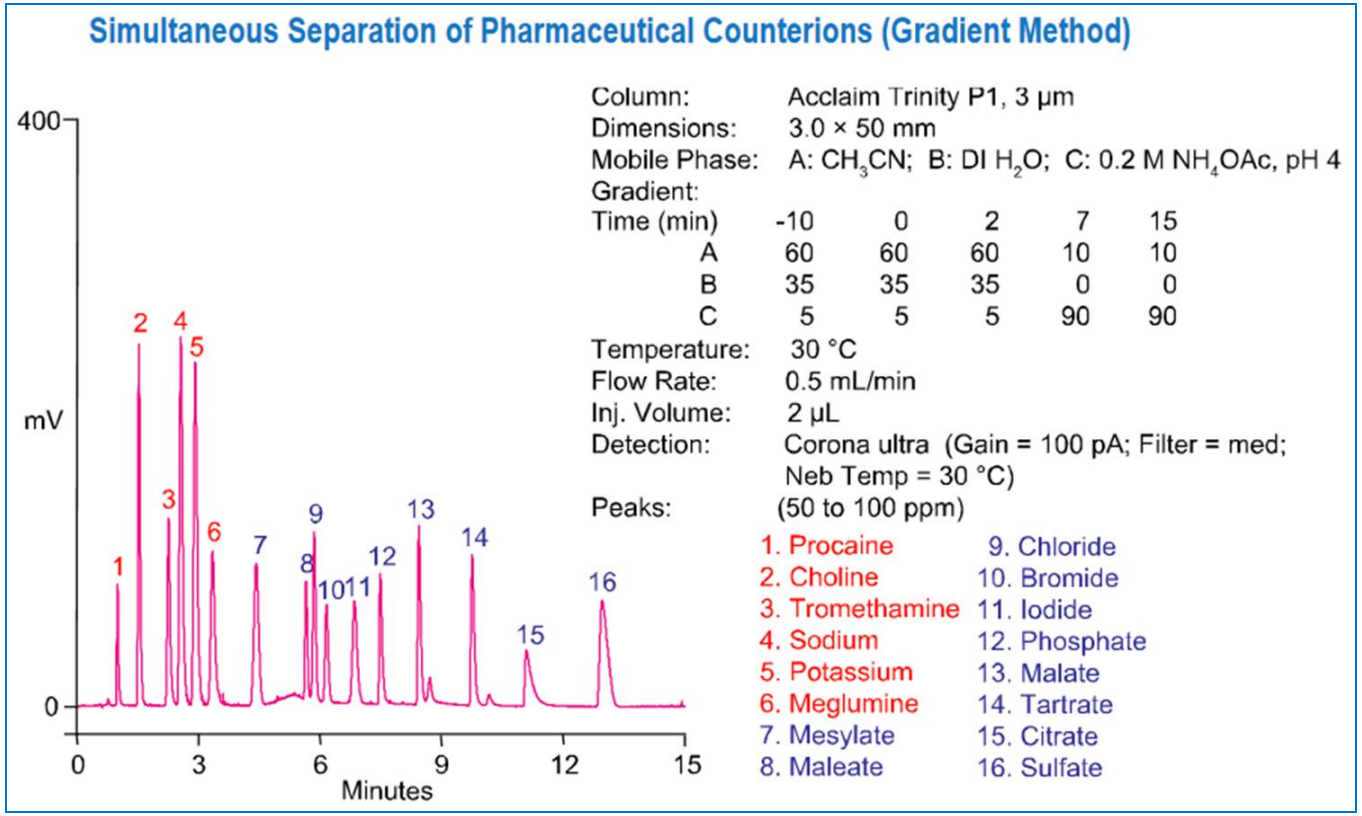
Determination of Active Pharmaceutical Ingredient and Counterions

- Acclaim Trinity P1
  - Mixed-mode chemistry – Nanopolymer Silica Hybrid
    - Reversed Phase, Anion Exchange and Cation Exchange
  - Ideal for simultaneous separation of drugs and their counterions





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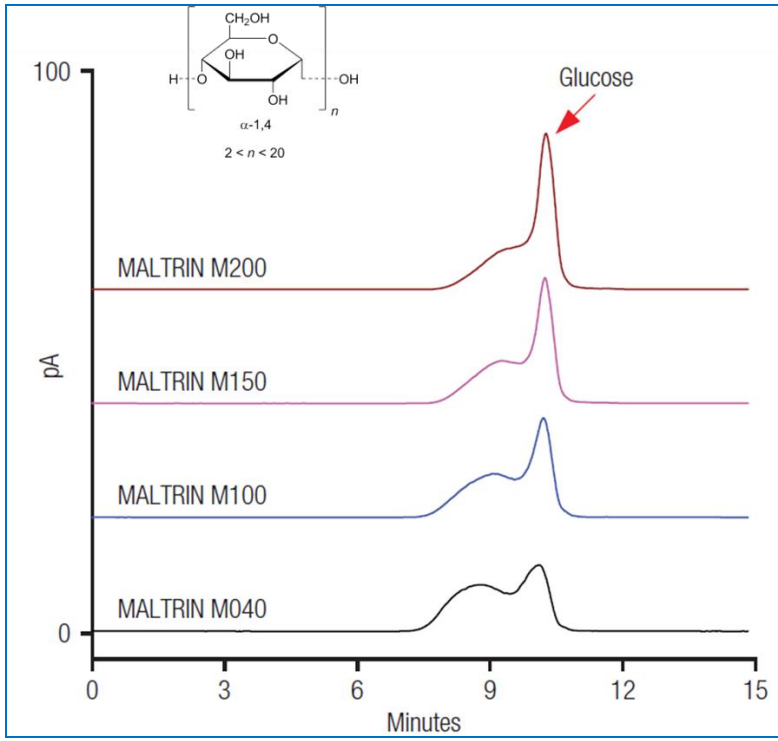
Different Degrees of Hydrolysis on Maltodextrins on Acclaim SEC-1000 column

- Acclaim SEC
  - Polymeric (Polymethacrylate) size exclusion
  - Acclaim SEC-300
    - nominal pore size of 300Å, for separating in the MW range of 100 to 50,000 Dalton
  - Acclaim SEC-1000
    - Nominal pore size of 1000Å, for separating in the MW range of 100 to 1,000,000 Dalton
  - Designed for separation of water-soluble polymers and oligomers, e.g. polyethylene glycols, polyvinyl alcohols, polyvinyl pyrrolidones, dextrans, polyacrylic acids, etc.



Acclaim SEC-1000

Dimension: 4.6 x 300 mm

Mobile Phase: 100 mM ammonium acetate pH5.0

Temperature: 25 °C

Flow Rate: 0.35 mL/min

Injection Volume: 5 µL

Detection: Corona ultra Charged Aerosol Detector

Sample: MALTRINs, 5 mg/mL each

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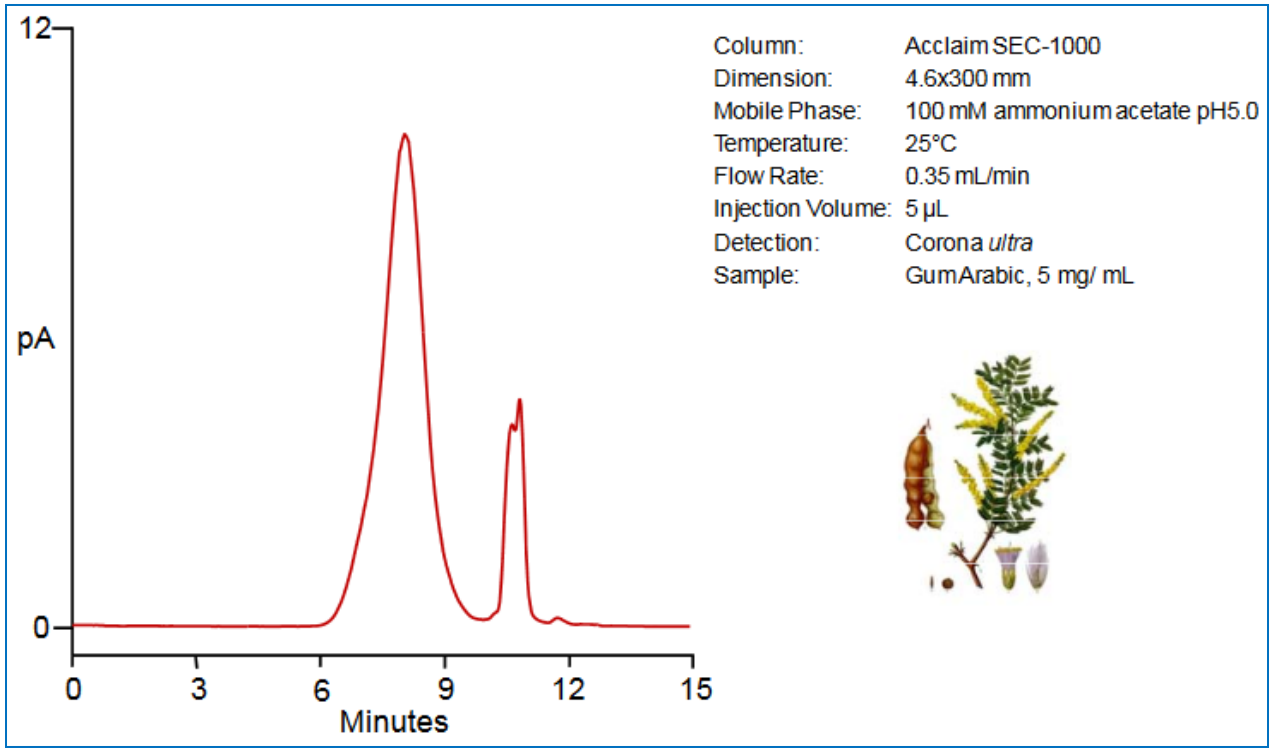
Determine the Molecular Weight Distribution of Gum Arabic by Acclaim SEC-1000

- Acclaim SEC
  - Polymeric (Polymethacrylate) size exclusion
- Gum Arabic is a complex mixture of glycoproteins and polysaccharides
- Primarily used in the food industry as a stabilizer
- Also a key ingredient in traditional lithography and is used in printing, paint production, glue, cosmetics and various industrial applications.



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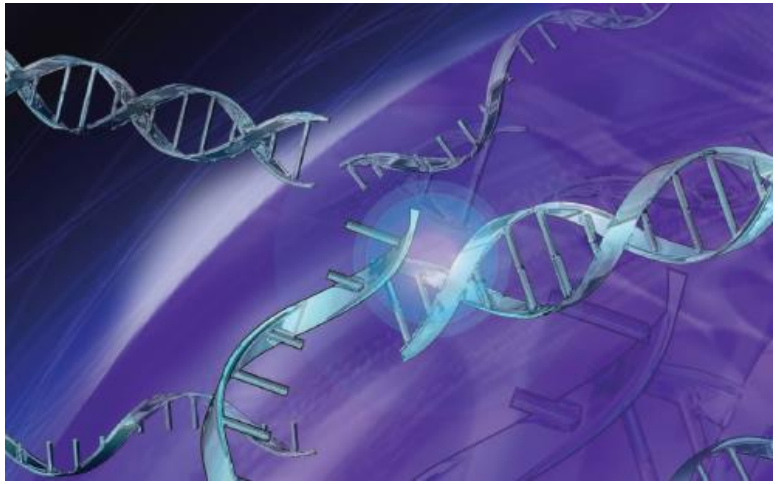
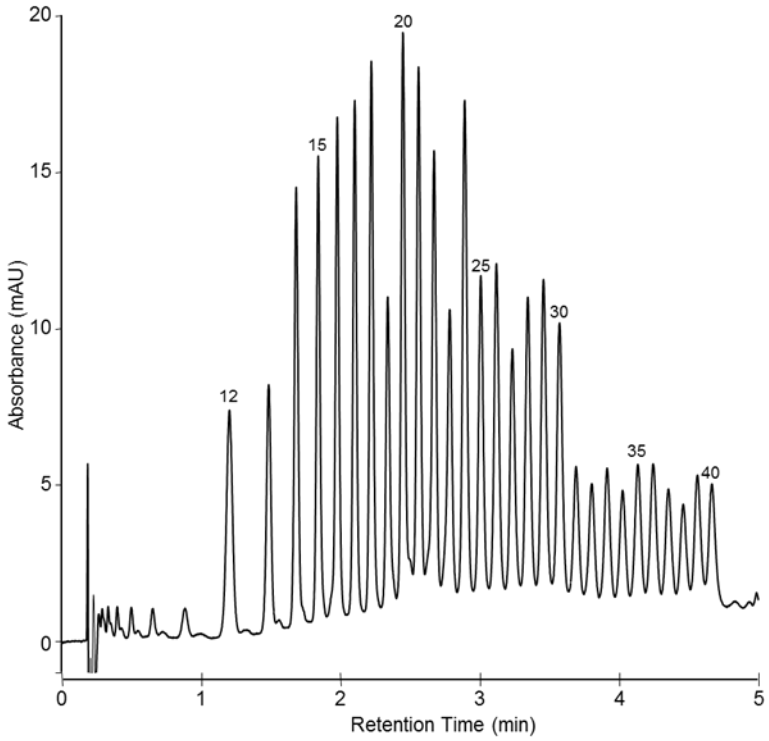
Polymeric Columns for DNA/RNA Molecules

- Thermo Scientific™ DNAPac™ RP
  - Analysis of oligonucleotides and double-stranded (ds) DNA/RNA fragments
    - Ion-pair Reversed Phase, triethylamine (TEA) and hexylamine (HA)
  - Polymer bead, 4 µm, wide pore



Fast separation of 12-40mer deoxythymidines (dTs)



Column:	DNAPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	0.1 M TEAA in Water / Acetonitrile (25:75 v/v)
Gradient:	

Time (min)	%A	%B
-2.0	78	22
0.0	78	22
4.0	61	39
4.1	10	90
5.0	10	90

Gradient curve:	3
Temperature:	80 °C
Flow rate:	0.80 mL/min
Inj. volume:	4 µL
Detection:	UV (260 nm)
Sample:	poly dT12-40 (1 A/mL)
Peak label:	Length of DNA

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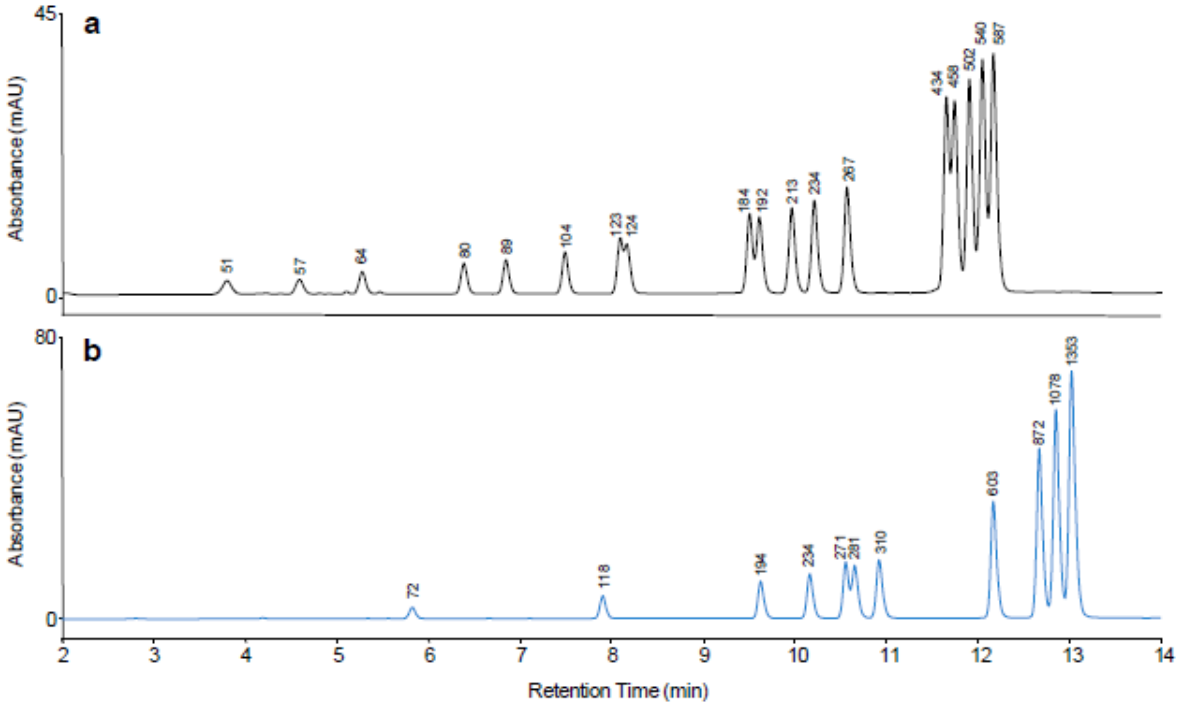
DNAPac RP – Separation of Large dsDNA Samples

Column:	DNAPac RP, 4 µm
Format:	2.1 × 100 mm
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	0.1 M TEAA, pH 7.0 in Water / Acetonitrile (75:25 v/v)

Gradient:

Time (min)	%A	%B
-8.0	64	36
0.0	64	36
12.0	31	69
12.1	5	95
15.0	5	95

Flow rate:	0.40 mL/min
Inj. volume:	5 µL
Temperature:	55 °C
Detection:	UV (260 nm)
Sample:	a. pBR322-BsuRI digest (100 µg/mL) b. ΦX174-BsuRI digest (100 µg/mL)
Peak label:	base pairs



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Conclusion

Key learnings:

- C18 is always the go-to phase
- Understand your application
- Check available literature
- Know your analytes
- Be prepared to overcome challenges before success
- Contact Thermo Fisher Scientific Technical Support for advice

www.separatedbyexperience.com/chromexpert

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Join the Fun! *Cache a Chromeleon* Game

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- If you are playing, you have earned points for attending this seminar. Be sure to scan the barcode on the desk outside the door.
- Ask booth staff for more details on how to play.

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Thank You

Please join me in the
Columns and Consumables
section of our booth where I'll
address additional comments and questions.

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