

# *Reverse/Normal Phase Chromatography*

# *Modes of Chromatography*

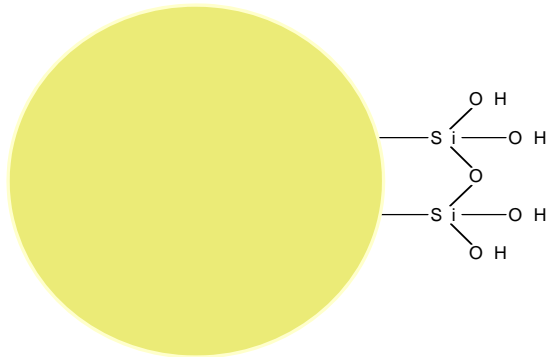
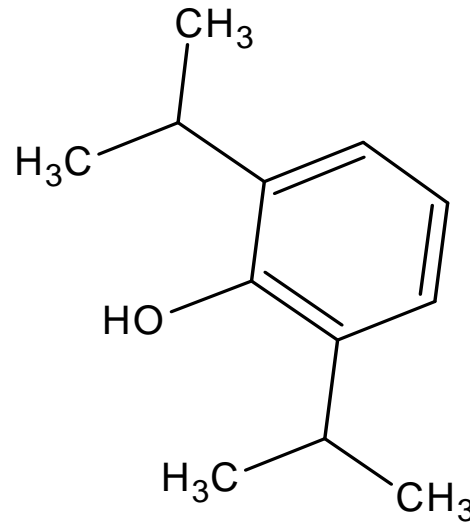
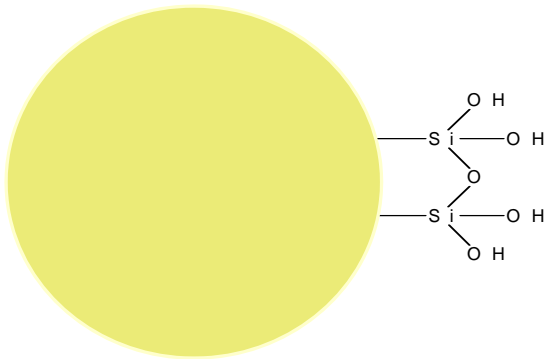
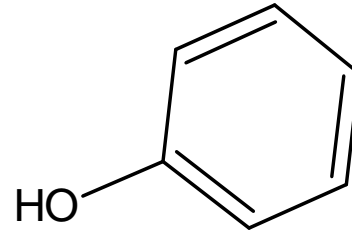
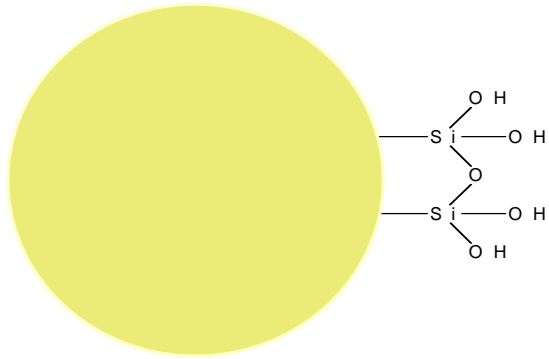
HPLC columns can be categorized into four major modes.

- Normal Phase Chromatography
- Reverse Phase Chromatography
- Ion Exchange Chromatography
- Size Exclusion Chromatography
- Reversed-phase chromatography (RPC) is used in 70–80% of all HPLC applications, RPC columns for small molecules are the focus of this discussion.

# Normal Phase

- The stationary phase is more polar as compared to Mobile phase.
- The degree of elution in this chromatography will be from non polar to mid polar.
- The polar compounds will have a strong retention on column and will be eluted in a long time or it will be strongly retained by column.

# Normal Phase Chromatography



Hexane/Chloroform



# *Normal Phase = Straight phase*

Stationary Phase	Mobile Phase
Silica	Hexane, with 0-100% modifiers
Alumina	-Iso Propyl ether
Zirconia	-Iso Propyl Chloride
Charcoal	-CHCL3
	-CH2Cl2
	Iso Propanol/ Ethanol

# *Eluotropic Series*

## *Based on Polarity*

Water

Methanol

**i-Propanol**

Ethylacetate

Chloroform

CH<sub>2</sub>Cl<sub>2</sub>

Toluene

Benzene

Hexane

Heptane

Isooctane

Solvent strength



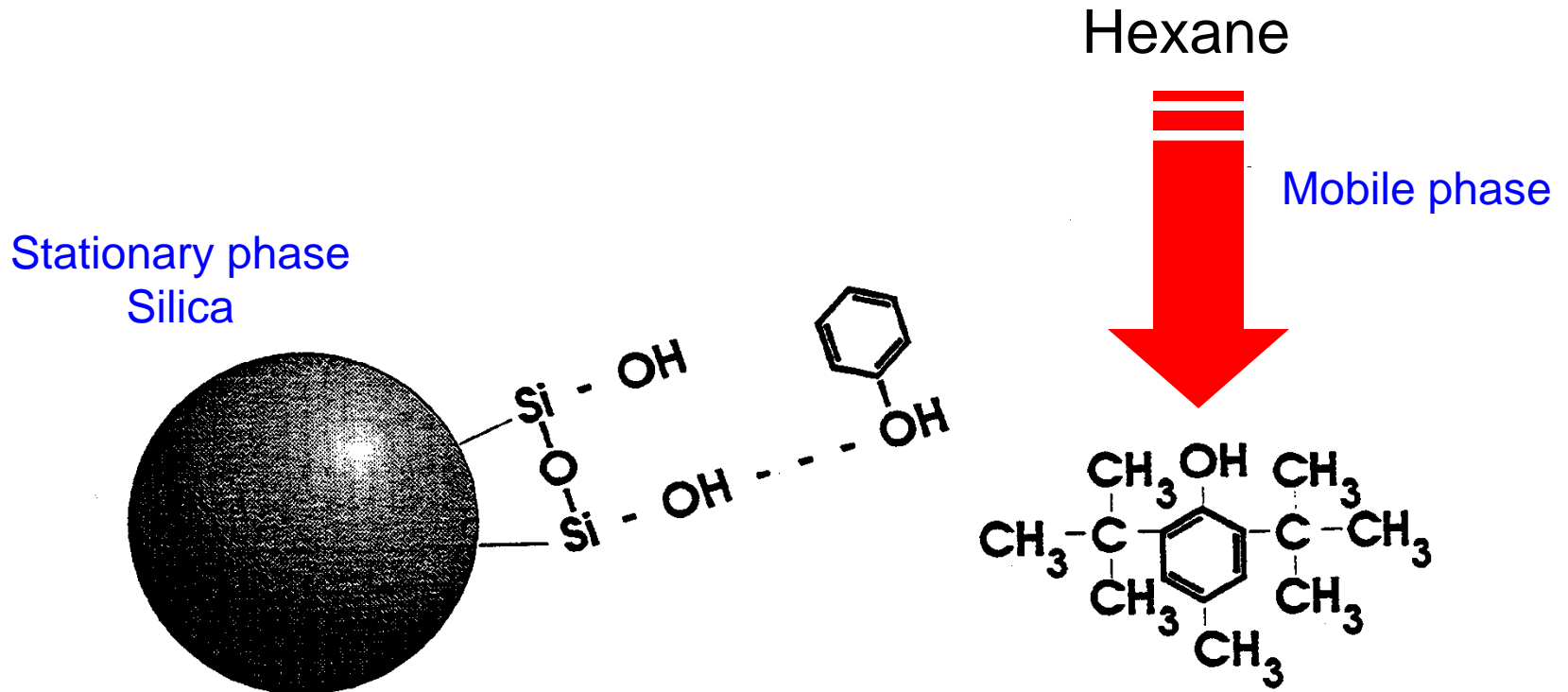
# U.V. Cut-offs for some Common Solvents

*Remember Solvents chosen can affect detection!!*

<u>Solvent</u>	<u>UV Cutoff</u>	<u>Solvent</u>	<u>UV Cutoff</u>
Water	180	N-Heptane	197
Methanol	205	Cyclohexane	200
N-Propanol	205	Carbon tetrachloride	265
Acetonitrile	190	Chloroform	245
THF	225	Benzene	280
Acetone	330	Toluene	285
Methyl acetate	260	Methylene chloride	232
Ethyl Acetate	260	Tetrachloroethylene	280
Nitromethane	380	1,2-Dichloroethane	225

*All wavelengths reported in nm.*

# Mechanism of Normal-Phase Chromatography, Adsorption



Elution order :

- ▶ Least polar elutes first
- ▶ Most polar elutes last



# Normal-Phase Chromatography

## Example

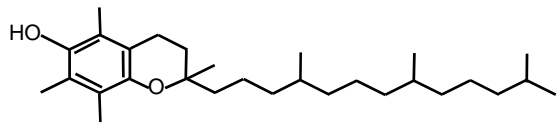
Column : Silica

Mobile phase : Hexane 99.5%

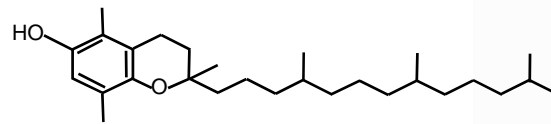
2-propanol 0.5%

Detection : Fluorescence ex. 290, em. 330

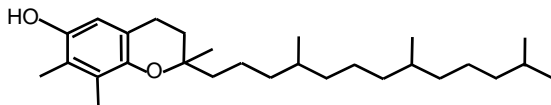
Sample : Vitamin E analogous



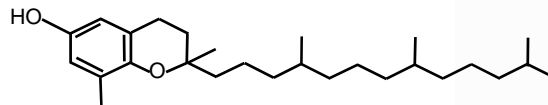
$\alpha$  -Tocopherol



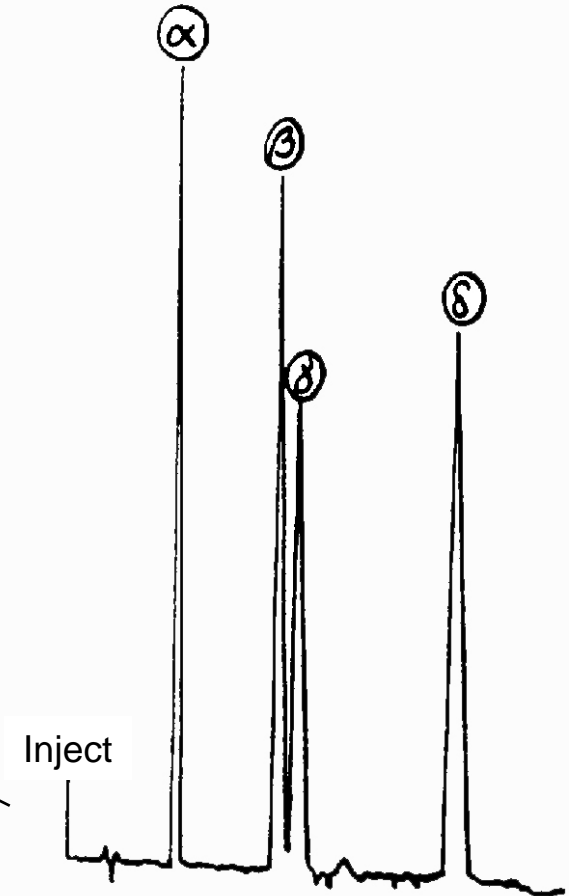
$\beta$  -Tocopherol



$\gamma$  -Tocopherol



$\delta$  -Tocopherol

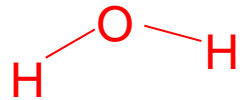


# Polarity Scale

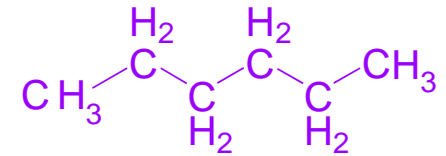
## Mobile & Stationary Phase

POLAR

NON-POLAR

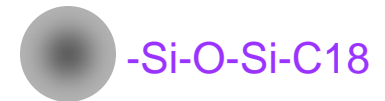
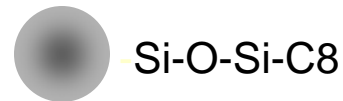
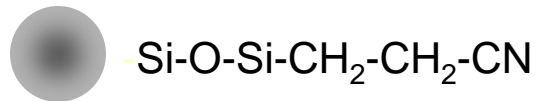
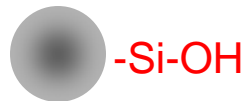


Mobile phases:



Water - Acetonitrile - THF - Hexane

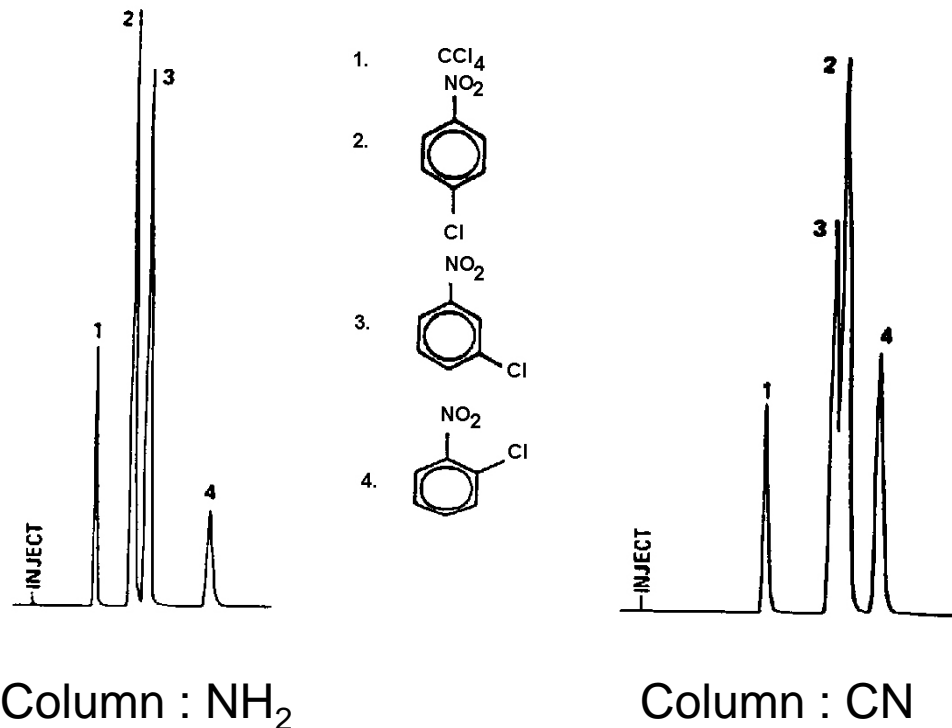
Stationary phases:



Silica - CN (Nitril) - C<sub>8</sub> - C<sub>18</sub> (ODS)

# Normal-Phase Chromatography on Different Columns

Aminopropyl- and Cyanopropylcolumns: Less polar different selectivity



Mobile phase : Hexane

# *Normal-Phase Chromatography*

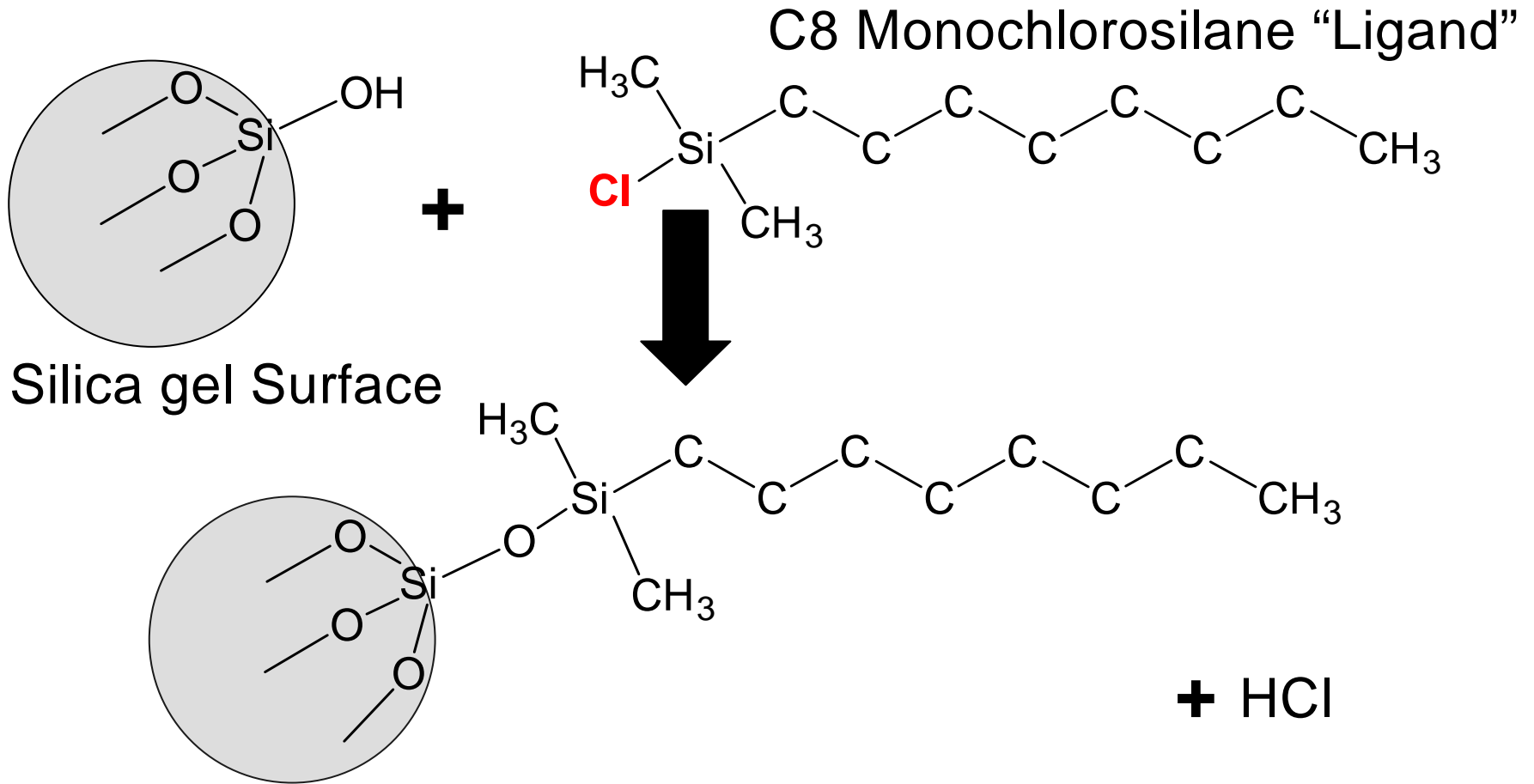
## Typical mobile phases

- Hexane
- Methyl-*t*-Butyl-Ether (MTBE)
- Ethyl acetate
- Methylene chloride

## Typical stationary phases

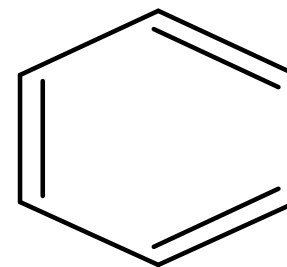
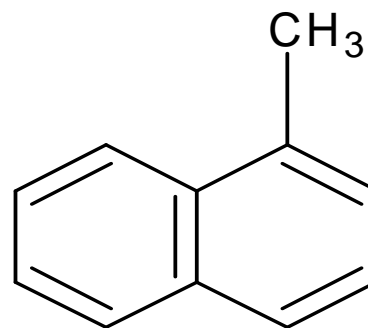
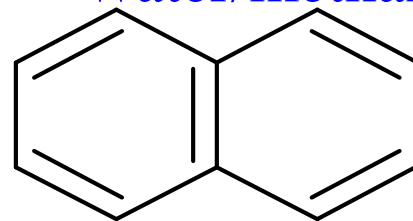
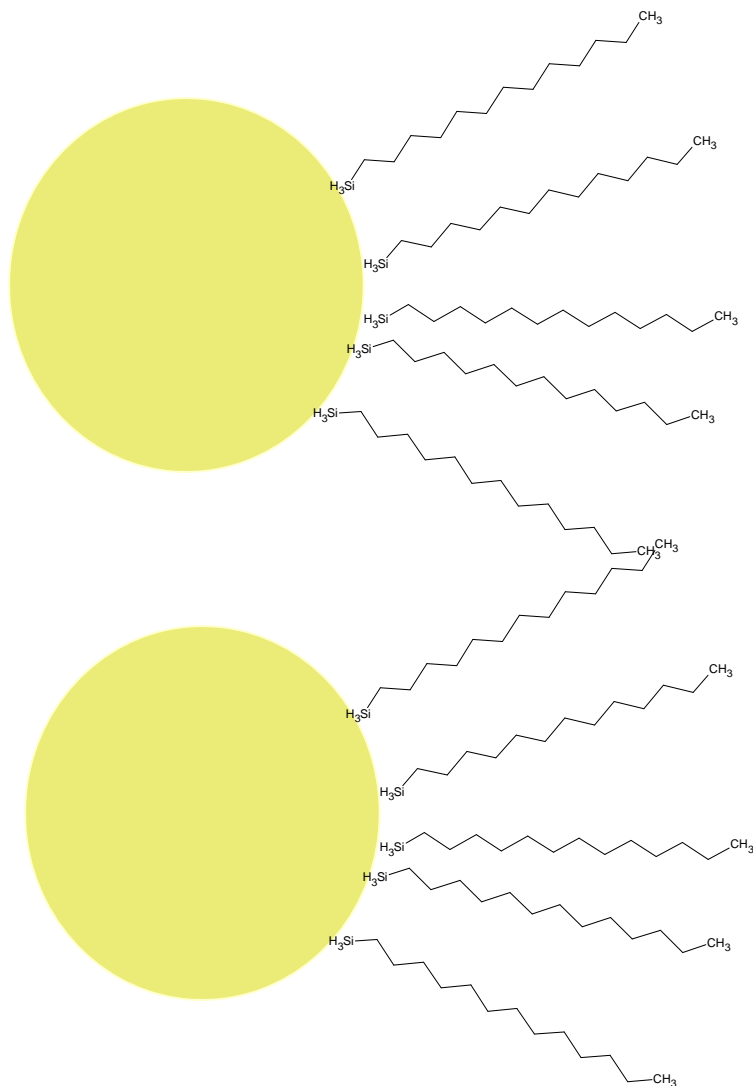
- Silica
- Amino (NH<sub>2</sub>)
- Cyano (CN)
- Diol
- Alumina

# Reversed Phase Chromatography



# Reversed phase

Water/methanol



# *Eluotropic Series*

Solvent strength

**Water**

**Methanol**

**Acetonitrile**

**THF**

i-Propanol

CH<sub>2</sub>Cl<sub>2</sub>

Benzene

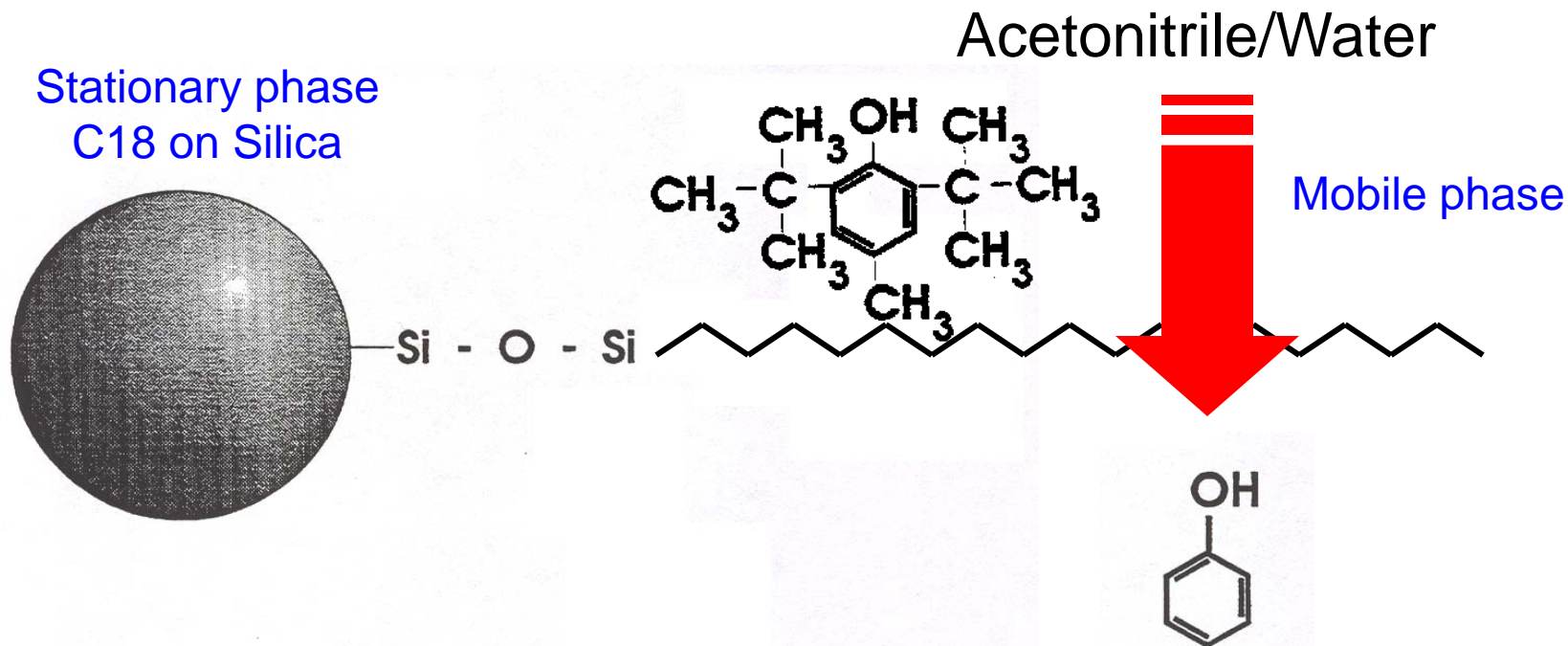
Hexane

Heptane

Isooctane



# Mechanism of Reversed-Phase Chromatography, Partition



Elution order :

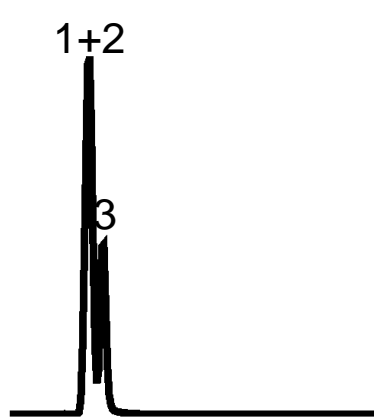
- ▶ Most polar elutes first
- ▶ Least polar elutes last



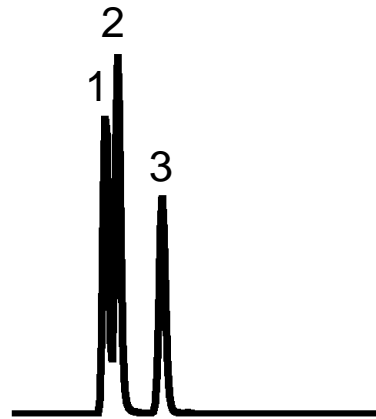
# Reversed-Phase Chromatography

## Example

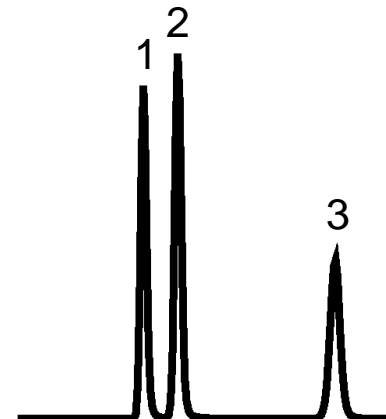
What chromatographic parameter has primarily changed ?



Mobile phase :  
MeOH/H<sub>2</sub>O 80/20

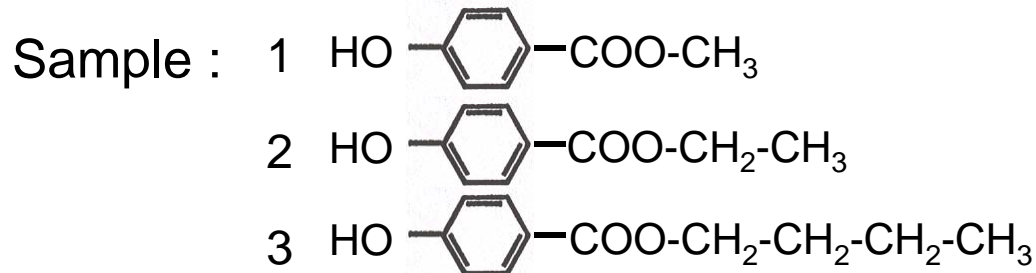


Mobile phase :  
MeOH/H<sub>2</sub>O 60/40



Mobile phase :  
MeOH/H<sub>2</sub>O 45/55

Column : C18

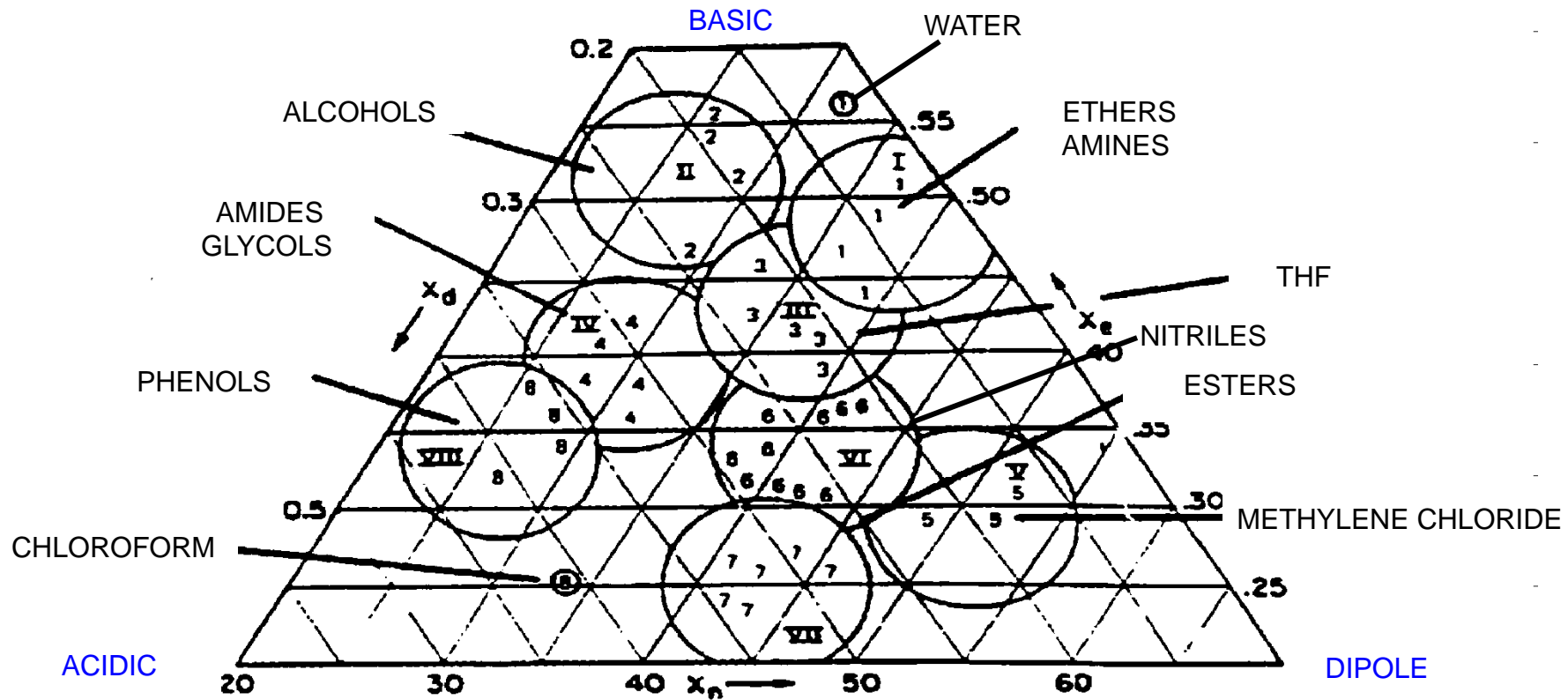


# Classification of Solvents

## Polarity Index - Selectivity Group

Polarity Index	Solvent	Selectivity group
9.0	Water	9
6.6	Methanol	2
6.2	Acetonitrile	6
5.2	Ethanol	2
4.8	Dioxane	6
4.3	Chloroform	9
4.3	2-propanol	2
4.3	Ethyl Acetate	6
4.2	THF	3
3.9	Butanol	2
3.4	Methylene Chloride	5
2.9	Ethyl Ether	1
2.3	Toluene	7
1.8	Triethyl Amine	1
0.0	Hexane	0

# Classification of Solvents



# Reversed-Phase Chromatography

## Typical mobile phases

- Water
- Methanol
- Acetonitrile
- Tetrahydrofuran (THF)

## Typical stationary phases

- C18 (ODS)
- C8
- C4
- Phenyl
- Amino (NH<sub>2</sub>)
- Cyano (CN)

# *Separation Based on Polarity*

- Normal/straight phase chromatography
  - ◆ The “original” type - used by Tswett
  - ◆ Polar stationary phase
  - ◆ Non-polar mobile phase
- Reversed-phase chromatography
  - ◆ Most commonly used today
  - ◆ Non-polar stationary phase
  - ◆ Polar mobile phase

- **HILIC** - **H**ydrophilic **I**nteraction **C**hromatography
  - Term coined in 1990 to distinguish from normal-phase\*
- HILIC is a *variation* of normal-phase chromatography without the disadvantages of using solvents that are not miscible in water
  - “Reverse reversed-phase” or “aqueous normal-phase” chromatography
- Stationary phase is a POLAR material
  - Silica, cyano, amino, diol
- The mobile phase is highly organic (> 80%) with a smaller amount of aqueous mobile phase
  - Water (or the polar solvent(s)) is the strong, eluting solvent

\*Alpert, A. J. *J.Chromatogr.* 499 (1990) 177-196.

<u>Solvent</u>
Water
Methanol
Ethanol
Isopropanol
Acetonitrile
Acetone
Tetrahydrofuran

Strongest



Weakest

In **HILIC**, utilizing a less polar solvent can **increase** the retention of polar analytes.

# HILIC "Reversed reversed Phase"

## Sugar standards

High Performance Carbohydrate  
Analysis Column

75/25 CH<sub>3</sub>CN/H<sub>2</sub>O

1.4 ml/min

M410 dRI

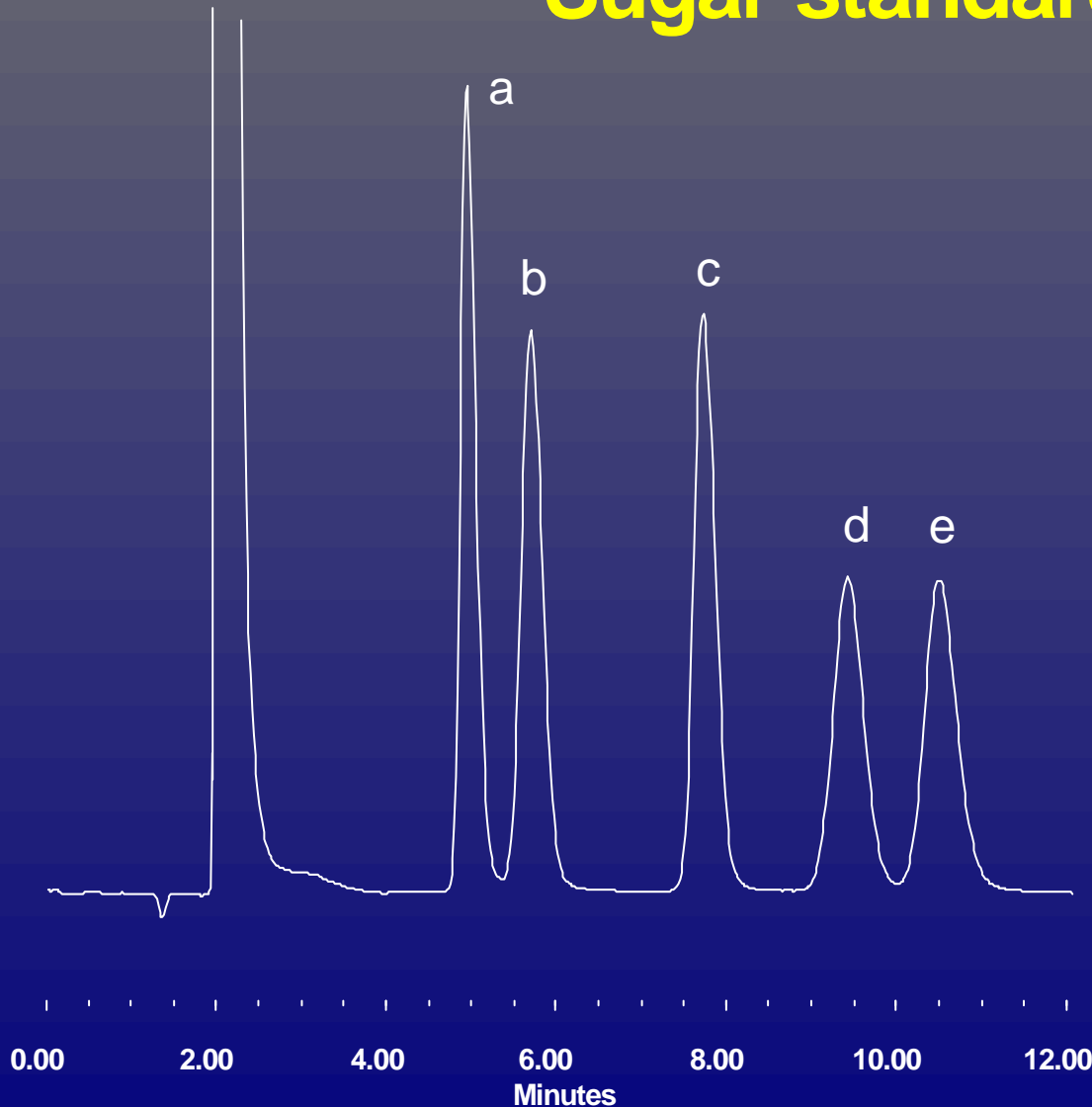
a- fructose

b- glucose

c- sucrose

d- maltose

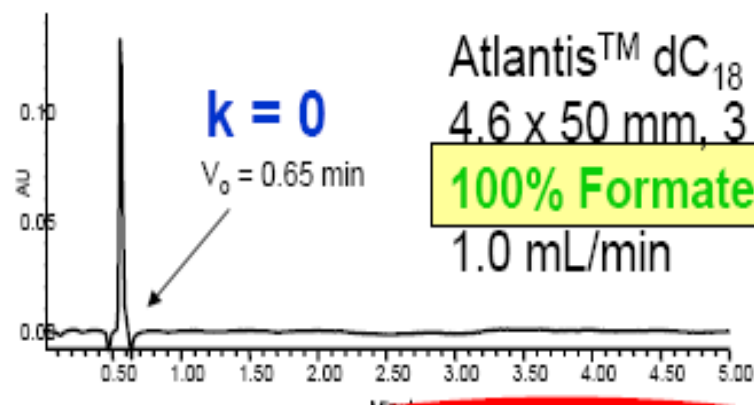
e- lactose



Waters  
22,477



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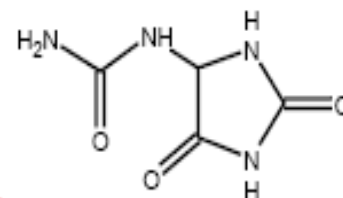


Atlantis™ dC<sub>18</sub>

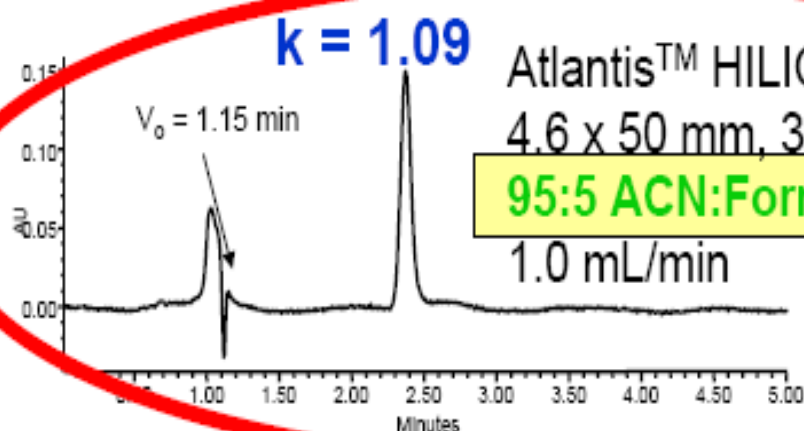
4.6 x 50 mm, 3 μm

**100% Formate Buffer, pH 3**

1.0 mL/min



Allantoin



Atlantis™ HILIC Silica

4.6 x 50 mm, 3 μm

**95:5 ACN:Formate Buffer, pH 3**

1.0 mL/min

HILIC offers retention when there is no retention by reversed-phase.

