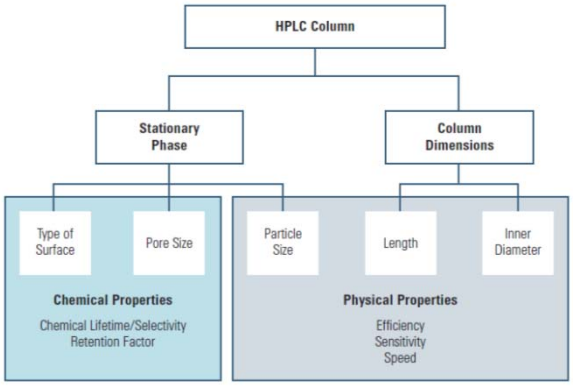
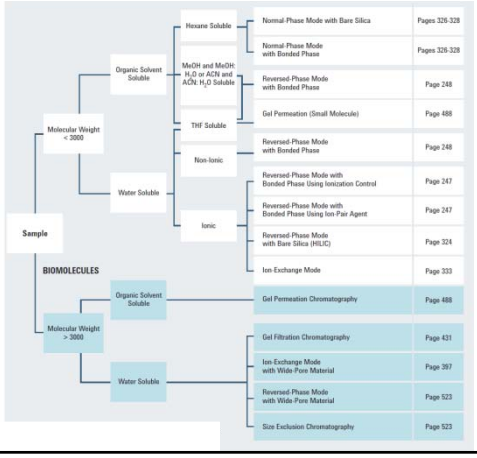
Types of liquid chromatography



We focus on the stationary phase chemistry:

- Normal and reversed phase
  - Ion-pair chromatography
- Size exclusion chromatography
- Chiral chromatography
- Ion chromatography



Normal and reversed phase chromatography

Reversed phase chromatography

- Most common type of chromatography
  - Main topic for the rest of the semester
- Stationary phases: -C_{18} and –C_8 mostly
- Mobil phase: Water + MeOH/MeCN/THF
  - Additives/buffer solution

Reversed phase chromatography

Analytes are very strongly bond to the stationary phase and do not elute

Analyted do not show any retention.

Analyte

All compounds are eluted very fast. No separation.

Eluent elutes only very polar compounds from the column

Eluent

Statsionary phase

Non-polar

Polar

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Normal phase chromatography

Analyte bonds very strongly to stationary phase and elutes very slowly

Analyte

Eluent interacts with stationary phase too strongly and therefore analytes elute too fast.

Eluent

Stationary phase

Non-polar

Polar

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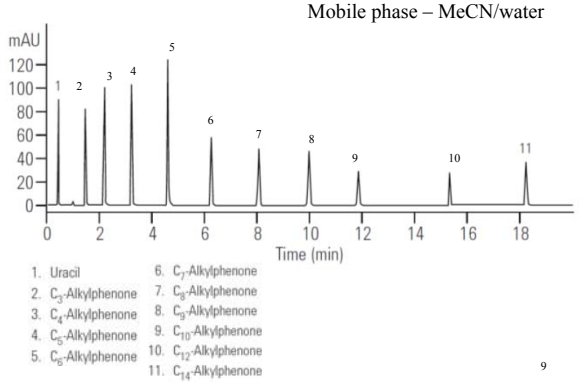
Mobile phase – MeCN/water



1. Uracil
2. C_3-Alkylphenone
3. C_4-Alkylphenone
4. C_5-Alkylphenone
5. C_6-Alkylphenone
6. C_7-Alkylphenone
7. C_8-Alkylphenone
8. C_9-Alkylphenone
9. C_{10}-Alkylphenone
10. C_{12}-Alkylphenone
11. C_{14}-Alkylphenone

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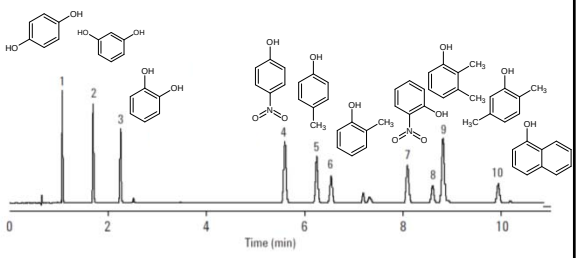
Mobile phase: MeCN/water + 0.1% formic acid



Hydrophobicity is important!

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Normal phase chromatography

Stationary phase is more polar than mobile phase

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Mobile phase

- Mixture of organic solvents
  - Does not contain water

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Stationary phase

- Anorganic adsorbent
  - Silica gel
  - Aluminium oxide
- Polar bonded phases
  - Cyano
  - Diol
  - Amino

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Analytes

- Neutral substances
- Problems occur with ionic substances
  - Mobile phase additives

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Retention

- Is described as adsorption process
  - Stationary phase is covered with solvent molecules
  - Analyte retention occurs due to displacement of solvent molecules

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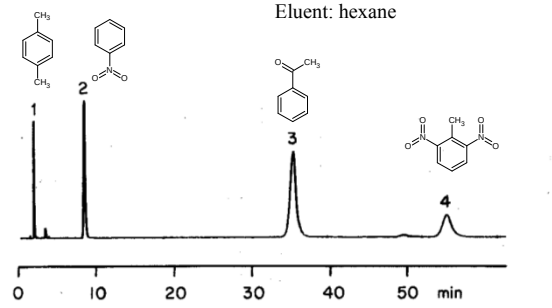
Stationary phase: silica gel
Eluent: hexane



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HILIC

Hydrophilic interaction chromatography

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Problem

- Very polar compounds
  - In normal phase chromatography bond too strongly with the stationary phase and elute very slowly from the column
  - In reversed phase chromatography no retention occurs and different analytes can not be separated
- If compound is ionic ion-chromatography can be used

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HILIC chromatography:

- Same stationary phases as in normal phase chromatography
- Same eluents (MeCN/water) as in reversed phase chromatography
- Mechanism is explained via liquid-liquid partitioning
- Stationary phase is covered with water layer, while the mobile phase contains less water
- Analyte partitions between the water layer and the mobile phase
- Polar compounds show higher retention and elute later

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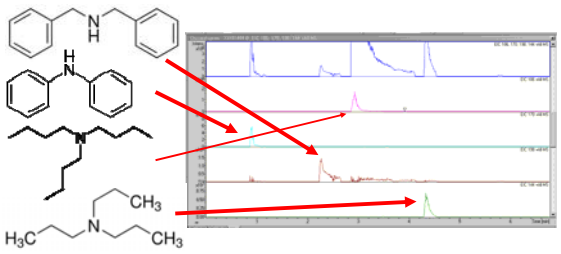
Example



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Size Exclusion Chromatography (SEC)

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Introduction

- In Size Exclusion Chromatography (SEC) two modes are used:
  - Gel Filtration uses water-based eluent. Mostly used for protein separation.
  - Gel Permeation Chromatography uses and organic solvent. Used for synthetic polymer separation.

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SEC principles of separation

- Depending on their size, molecules can enter the pores of stationary phase.
  - 1. Very large molecules can't fit into the pores and elute early.
  - 2. Small molecules can enter and move within the pores freely.
  - 3. Middle sized molecules can enter the pores partially. The smaller the molecule the longer it takes to elute.

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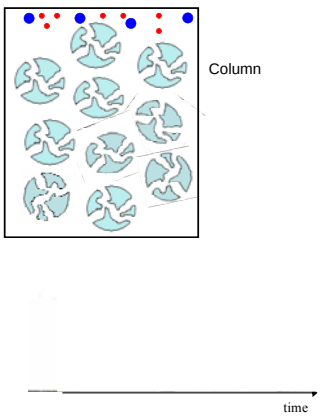

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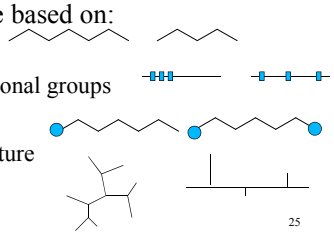
SEC properties

- Separation is based on the hydrodynamic volume of the compounds (size).
- Allows to separate based on:
  - Chain length
  - Location of functional groups
  - Functional groups
  - Geometrical structure



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SEC obtained information

- Retention time→ average molecular weight
- Peak shape→ molecular weight distribution

- Calibration is done with the same type of polymer as being analysed
- Sample and calibration mixture are dissolved in the same solvent.

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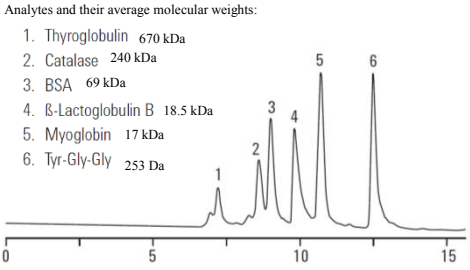
Example of chromatogram



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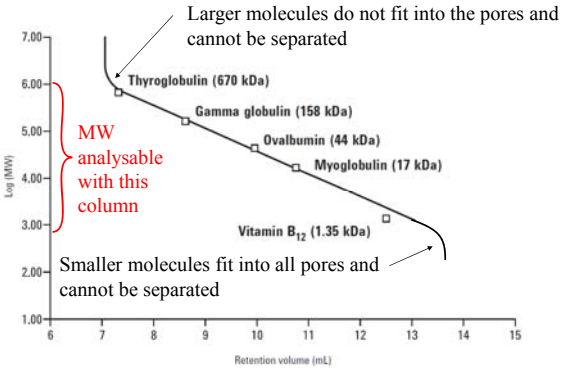
Example of calibration curve



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Stationary phase is prepared

- Gel Filtration
  - Dextran (linear glucose polymer)
  - polyacrylamide
  - Agarose
- Gel Permeation
  - Extensively cross-linked copolymer of polystyrene and divinylbenzene

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Ion (Exchange) Chromatography

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Terminology

- Both terms IEC – ion exchange chromatography and IC – ion chromatography are used
  - Sometimes as synonyms
- IEC covers the processes that occur on the surface of ion exchange resin
- IC is the chromatography of ion separation and detection.
  - Mostly conductivity cell is used as a detector.
  - Separation is usually carried out with IE, but RP can also be used.

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IEC Retention

- Stationary phase is covered with charged groups:
  - amine, quaternary ammonium – positively charged
  - sulfonate, carboxylate – negatively charged
- Retention is based on the following equilibria:
  - $R^-K^+ + X^+ \Leftrightarrow R^-X^+ + K^+$ (cation exchange)
  - $R^+Cl^- + X^- \Leftrightarrow R^+X^- + Cl^-$ (anion exchange)
- Increasing the concentration of counterion (i.e. K^+ and Cl^-) decreases the analyte retention

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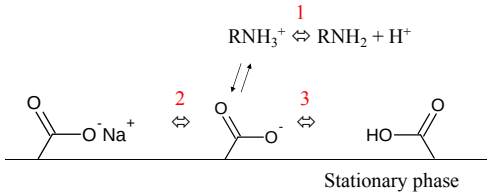
1. Is influenced by eluent pH and analyte pKa
2. Is influenced by counterion concentration and properties
3. Is influenced by eluent pH and Stationary phase properties



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Why IC?

- Why not use Reversed Phase Chromatography (Ion-Pair Chromatography)?
- Detection
  - Conventional detectors are blind to most inorganic (and some organic) compounds – conductivity cell
  - For MS-detection eluent additives should be volatile, ion-pair reagents (i.e laurylesulfate) usually are not
- Preparative Chromatography
  - Non-volatile ionpair-reagents are problematic
- Suitable as the first step in multidimensional chromatography

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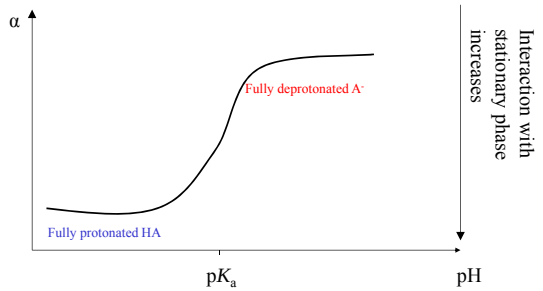
Acids occur in different forms depending on the eluent pH



Why acids/bases can not always be determined with reversed or normal phase chromatography?

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Eluent pH

- Analytes are usually acids or bases
- Ion Exchange is most effective when stationary phase and analyte are charged oppositely
- Derive retention dependency
  - For weak and strong cation/anion exchanger
  - For strong/weak acid/base

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Weak and Strong ion exchange resins

- Cation exchange
  - WCX and SCX – weak/strong cation exchange
- Anion exchange
  - WAX ja SAX– weak/strong anion exchange
- Strong are applicable 2<pH<12
- Weak resins lose charge at some pH
- Weak are seldom used
  - To modify selectivity
  - To decrease selectivity

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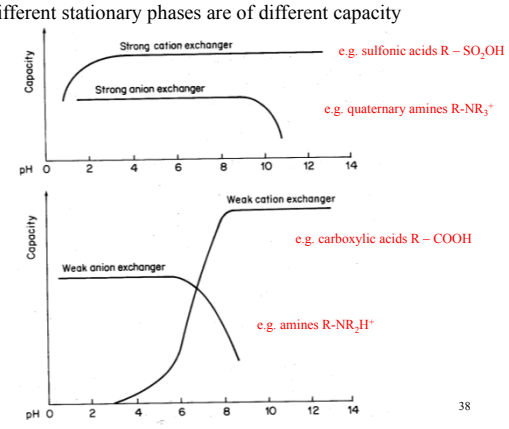
Different stationary phases are of different capacity



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pH effects

- Silica based columns can not be used pH>8 and pH<1.
- SCX and SAX with OH^- may catalyze some reactions
  - e.g. ester hydrolyses

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Salt effect on retention

- Retention depends on the anion (cation)
  - Counter ions have different strength of displaceing the analyte
- Strong counterions decreases analyte retention more then weak counterions with the same concentration
  - F^- (weak)<OH^-<CH_3COO^-<Cl^-<SCN^-<Br^-<CrO_4^-<NO_3^-<I^-<SO_4^{2-}(strong)
  - Li^+(weak)<H^+<Na^+<NH_4^+<K^+<Rb^+<Cs^+<Mg^{2+}<Ca^{2+}<Ba^{2+} (strong)

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Organic solvent as an additive

- Organic solvent decreases retention
- MeOH and MeCN can be used to alter selectivity

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Method development

- Column
  - SAX for acidic and anionic compounds; SCX for basic and cationic compunds;
- Eluent
  - Start with water based buffer solution.
  - pH>6 for SAX and pH<6 for SCX.
  - If analyte pK_a is known pH>pK_a for anion exchange and pH<pK_a for cation exchange should be used
  - concentration 20...50 mM

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Method development

- Eluent component B
  - Buffer + salt (e.g. K_2SO_4)
  - Test gradient 0...100% B
- If analyte does not elute
  - Increase temperature
  - Add MeOH
  - Use weak ion exchange resin
- If retention is ok (0.5<k<20)
  - Adjust selectivity with modifying salt, pH and organic additive.

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Ion exchange resins

- Synthetic organic polymers

  Most often used resins. Usually copolymers of styrene and divinylbenzene containing appropriate functional groups.

  The biggest advantage is that synthetic polymers can be used in a wide pH range (0-13). Therefore also weak acids/bases can be determined.

  The biggest disadvantages is softness – high pressure can nt be used. This also limits column lenght and flow rate.

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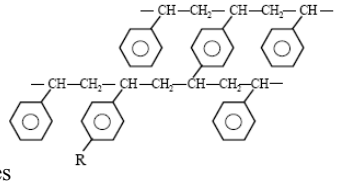
Ion exchange resins

- Synthetic organic polymers

  Crosslinked polystyrene polymer:



- Oxides

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Ion-exchange

Ion-exchange can be characterized:

- Selectivity
  - Stationary phase
  - Analytes charge
  - Solvated ions volume
  - Analytes polarizability
  - Ion-exchanger capacity
  - Functional groups on the stationary phase

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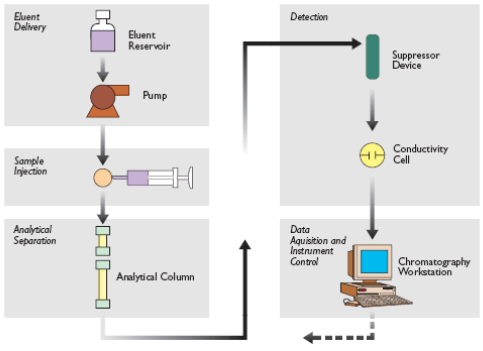
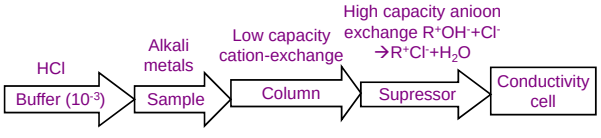
Apparatus of ion-exchange chromatography



Suppressor-column

- Eluent high conductivity hinders usage of conductivity detector
- Suppressor-column helps to remove ions:



- Needs frequent regeneration

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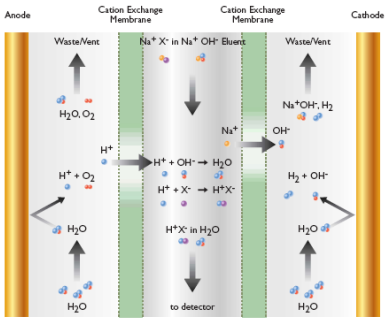
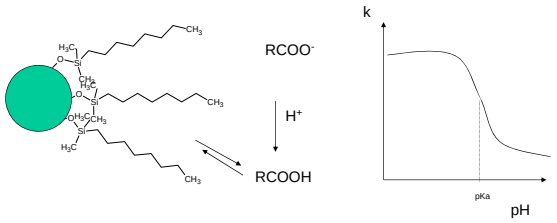
Membran suppressor



Common applications

- Determination of inorganic ions
  - Mostly anions
- Amino acids, peptides and proteins
- Nucleic acids

Ion-pair chromatography



Therefore: Retention of carboxylic acids depends on the eluent pH.

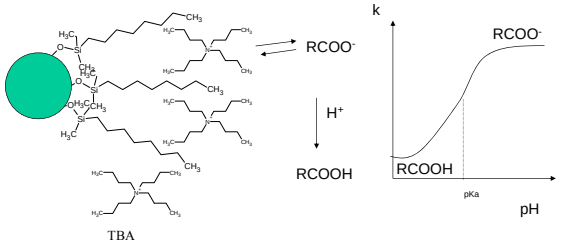
Same stands for weak bases.

Mobile phase

- Ion-pair reagent
  - Opposite charge to the analyte
  - Long hydrophilic change
    - Bonds with reversed phase
- Alkyl solfonates
  - For cation determination
- Tetraalkylammonium salts
  - For anion determination

Mobile phase

- Optimization
  - pH
  - Concentration of ion-pair reagent
- In case of gradient both components have to contain ion-pair reagent with the same concentration.



TBA

Surface is covered with positive charge!

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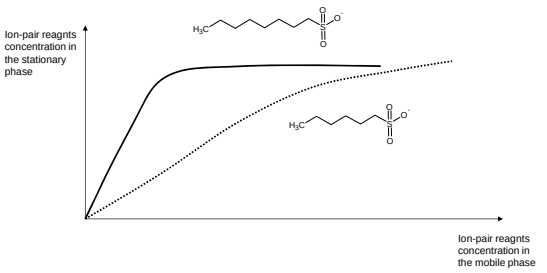
Concentration of ion-pair reagent on the stationary phase depends on:

1. ion-pair reagent concentration in eluent
2. properties of ion-pai reagent



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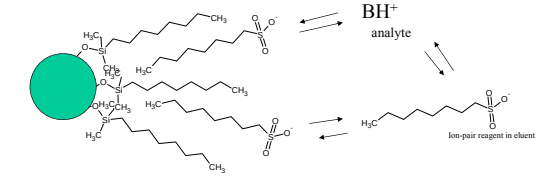

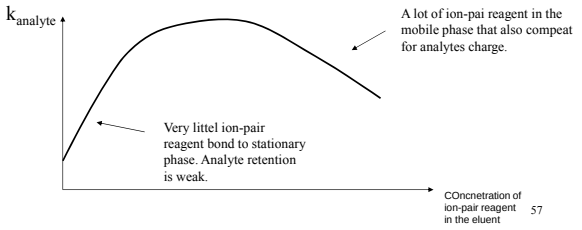

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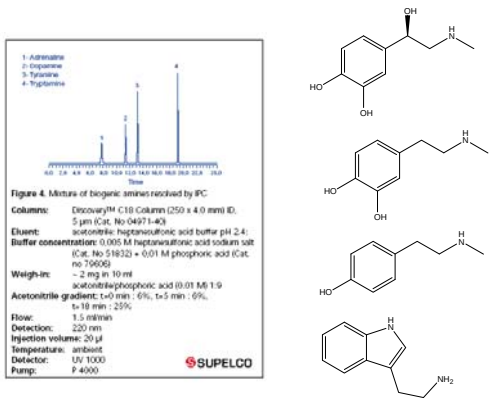

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Chiral separation

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Enantiomer

- Stereoisomers, which are
  - mirror images
  - non-superimposable
- Same physical and chemical properties, except for the direction in which they rotate the plane of polarized light
- Nomenclature
  - R and S
  - D and L
  - + and -

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Diastereo(iso)mer

- Stereoisomers, which are not
  - non-superimposable
  - Are not mirror images
- Different physical and chemical properties

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Chiral separation

- Separation has to be carried out on chiral system:
  - Chiral component in mobile phase
  - Chiral liquid stationary phase (liquid-liquid partition chromatography)
  - Chiral solid stationary phase
  - After derivatization with chiral reagent

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Chiral separation

- Separation is based on the diastereomeric complex between analyte and chromatographic system.

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Chiral solid stationary phase

- Chiral compound is bond to stationary phase (CSP – *chiral stationary phase*)
  - No one stationary phase is capable of separating all possible isomers
- Different types
  - Brush-type CSP
  - Helix-shaped phases
  - Cavity phases
  - Proteins
  - Ligand-exchange phases

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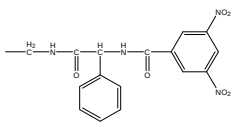
Brush-type CSP

- Most common are Pirkle type CSP
  - Designed according to 3-poind rule
  - Work in both normal and reversed phase chromatography
  - Is available in both isomeres – analytes elution order is reversible



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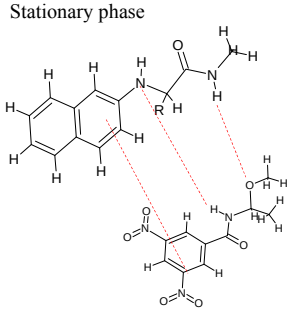
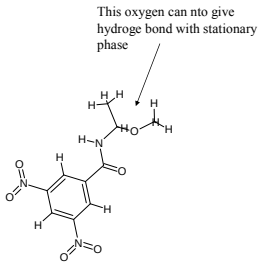
Stationary phase



S-isomer



R-isomer

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