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\textsubscript{18} and -C\textsubscript{8} mostly
- Mobil phase: Water + MeOH/MeCN/THF
  - Additives/buffer solution
Normal phase chromatography

Stationary phase is more polar than mobile phase

Mobile phase

- Mixture of organic solvents
- Does not contain water

Hydrophobicity is important!
Stationary phase

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

Analytes

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

Retention

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

HILIC

Hydrophilic interaction chromatography

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
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

Size Exclusion Chromatography (SEC)

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.

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.
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

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.

Example of chromatogram

Example of calibration curve

Stationary phase is prepared

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

Ion (Exchange) Chromatography
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 I/E, but RP can also be used.

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:
  - $R-K^+ + X^+ \rightleftharpoons R-X^+ + K^+$ (cation exchange)
  - $R-Cl^- + X^- \rightleftharpoons R^- + Cl^-$ (anion exchange)
- Increasing the concentration of counterion (i.e. $K^+$ and $Cl^-$) decreases the analyte retention

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

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

Acids occur in different forms depending on the eluent pH

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

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

Salt effect on retention

- Retention depends on the anion (cation)
  - Counter ions have different strength of displacing the analyte
- Strong counterions decreases analyte retention more than weak counterions with the same concentration
  - F⁻ (weak)<OH⁻<CH₃COO⁻<Cl⁻<SCN⁻<Br⁻<CrO₄²⁻<I⁻<SO₄²⁻ (strong)
  - Li⁺ (weak)<H⁺<Na⁺<K⁺<Rb⁺<Cs⁺<Mg²⁺<Ca²⁺<Ba²⁺ (strong)

Organic solvent as an additive

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

Method development

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

- Eluent component B
  - Buffer + salt (e.g. K₂SO₄)
  - 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.

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 not be used. This also limits column length and flow rate.

Ion exchange can be characterized:

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

Apparatus of ion-exchange chromatography

Suppressor-column

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

  - Needs frequent regeneration
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 sulfonates
  - 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.
Surface is covered with positive charge!

Concentration of ion-pair reagent on the stationary phase depends on:
1. ion-pair reagent concentration in eluent
2. properties of ion-pair reagent

Very little ion-pair reagent bond to stationary phase. Analyte retention is weak.

A lot of ion-pair reagent in the mobile phase that also compete for analytes charge.

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 -
Diastero(iso)mer

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

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

Chiral separation

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

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

Brush-type CSP

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

This oxygen cannot give hydrogen bond with stationary phase