An Introduction to Liquid Chromatography Mass Spectrometry

Dr Kersti Karu

email: kersti.karu@ucl.ac.uk
Office number: Room LG11

Recommended Textbook:
Qualitative and quantitative measurements for many fields: clinical, environmental, food, geographical regions, forensic and etc.
Lecture Overview

- Rate theory of HPLC van Deemter equation
- Principle of LC analysis
  - HPLC subclasses
  - Stationary phases in HPLC
  - LC columns
- Liquid chromatography-mass spectrometry (LC-MS)
  - Separation process in reversed-phase liquid chromatography
  - Electrospray ionisation (ESI) source
  - Taylor cone formation occurs both at the end of the ESI tube and as the droplets disintegrate to release ions.
  - Quadrupole ion trap (QIT) analyser
  - Injection of ions into an orthogonal TOF analyser
  - Principle of orthogonal TOF analysers with V and W geometries
  - Orbitrap analyser
  - Tandem mass spectrometry
- LC-MS application sterolomics analysis
- Brief introduction to phytosterol analysis
Principles of LC-MS analysis

Basic components of an HPLC system

- HPLC flow rate 1-2 mL/min, 4.6 mm ID column, 3- or 5-μm silica-based particles, 5-30 μL injection.
- Capillary LC flow rate 200 μL/min, 1 mm ID column, 1.5- to 3-μm silica-based particles, 1-5 μL injection
- Nano LC flow rate 200 nL/min, 0.1 mm ID column, 3-μm silica-based particles, 0.1-2 μL injection
- UHPLC systems (ultra-high-pressure liquid chromatography) 0.3 - 1 mL/min, 1.5- to 3-μm silica-based particles capable of pumping at 15,000 psi.
Principle of separation in HPLC

In HPLC, analytes are separated based on their differential affinity between a solid stationary phase and a liquid mobile phase. The kinetics of distribution of analytes between the stationary and the mobile phase is largely diffusion-controlled.

To minimise the time required for the interaction of the analytes between the mobile phase and the stationary phase two criteria should be met. (1) the packing material should be small and as uniformly and densely packed as possible. This criterion is met by uniformly sized spherical particles and results in a smaller $A$ value in the van Deemter equation. (2) the stationary phase should be effectively a thin uniform film with no stagnant pools and provide a small $C$ value. Because molecular diffusion in liquids is small, the $B$ term is small.
The C-term in the van Deemter equation contains both mobile-phase and stationary phase contributions ($C_m$ and $C_s$).

The plate height

$$H = A + \frac{B}{\bar{u}} + C_m \bar{u} + C_s \bar{u}$$

Except at very low mobile-phase velocities, the longitudinal diffusion term $B$ is negligible because the diffusional coefficient is very small in the LC. With present uniform spherical particles of very small particle size (<5 µm) that are tightly and uniformly packed, the contribution of $A$ term is also very small.

$$H = C_m \bar{u} + C_s \bar{u}$$

The term $C_s$ is relatively constant at a given $k$ (the retention factor); and $C_m$ includes stagnant mobile-phase transfer (in the pores of the particles).

Column efficiency is related to particle size. For well-packed HPLC columns, $H$ is about two to three times the mean particle diameter.

$$H = (2 \text{ to } 3) \times d_p$$
**Normal phase chromatography** utilises a polar stationary phase and relatively non-polar to intermediate polarity solvents such as hexane, tetrahydrofuran.

**Reversed phase chromatography** utilises non-polar stationary phase C18 and polar hydroorganic solvents, ACN, MeOH, water.

**Hydrophilic interaction chromatography (HILIC)** water is adsorbed on a hydrophilic surface to provide the partitioning process. This mode of separation suited for highly polar water soluble analytes. ACN-water is commonly used as the eluent system, in which water is the strong eluent.

**Size exclusion chromatography** molecules are separated based on their size. The stationary phase is largely occupied by pores. Molecules that are larger than the largest pores cannot enter any pores and hence are “excluded” from the pores and come out in the void volume. Molecules that are smaller than the smallest pores, in contrast, can explore the entire space in the stationary phase and come out last.

**Ion exchange chromatography** - ion exchange particles carry fixed positive or negative charges, a sulfonic acid type resin, for example, has $\text{SO}_3^-\text{H}^+$ groups where $\text{H}^+$ groups can be exchange for other cations (cation exchange resin). The electrostatic interaction is governing factor in ion exchange affinities and hydrophobic interactions play also significant role.

**Ion exclusion chromatography** like SEC, depends on principles of exclusion to accomplish separation, all analytes elute within a finite retention window. Weak electrolytes can be separated by this technique, the dominant application area being the separation of organic acids that can be separated from strong acids and further separate according to their $pK_a$. For example column with a -SO$_3$H cation exchange resin. The sulfonate group is fully ionised and the resin matrix is negatively charged. If consider a weak acid HA, the anion A$^-$ will be excluded from the interior of the resin, but no such penetration barrier exists toward a neutral molecule. The fully ionised elute in the void volume and other elute in the order of increasing $pK_a$; acids that are largely unionised elute last.
Stationary phases in HPLC

High-purity silica particles, low in trace metal content, <10 µm to <2 µm diameter particles. Smaller diameter particles create higher back pressure, but they exhibit very little loss of efficiency at higher flow rates, permitting faster separation. For analysis of small molecules, polypeptides and many proteins, and for very high molecular weight proteins, particles with respective pore sizes of 60 - 150 Å; 200 - 300 Å; and 1,000 - 4,000 Å are used to allow the analyte to penetrate the pores. Most HPLC is performed in the liquid-liquid partition mode, the stationary phase is bonded to the support particles. Microporous particles are commonly used, the pores being permeable to the analytes and the eluting solvent (c). Most of the mobile phase moves around the particles. The solute diffuses into the stagnant mobile phase within the pores to interact with the stationary phase, and then diffuses out into the bulk mobile phase. The use of small particles minimises the path length for diffusion and hence band broadening.
Silica particles have surface silanol groups, SiOH. Silanol groups provide polar interaction sites. The reaction with monochlorosilane R(CH₃)₂SiCl, where R =CH₃(CH₂)₁₆-CH₂- will lead to “C18 silica” stationary phase. The extent to which silanol groups are functionalised depends on the chain length of the functionalising agent. The extent of functionalisation is expressed in terms of wt %C (as obtained by elemental analysis).

**Macroporous-micro/mesoporous structures packings.** Pores smaller then ~100A° are referred to as micropores while those larger than ~1000 A° are regarded as macropores. Pores of intermediate size are mesopores (d). Mostly used for analysis of large molecules.

**Nonporous packings (b)** silica in very small particle size (1.5 µm) as there is no pores, the pore diffusion and longitudinal diffusion limitations disappear. With very small particles, the diffusion distance from the mobile to the stationary phase is short; column efficiency is virtually flow rate independent. However, the pressure needed to attain a certain flow rate thorough a given column varies inversely as the square of the particle diameter (the pressure is 1000% higher when the column is packed with 1.5 µm than with 5 µm particles). Nonporous packings have a lower surface area, limiting how much sample can be injected on column and exhibit lower RT.

**Bonded phases (a)** the packing material is composed of an inner fused or non porous particle core and a porous outer particle shell. Analytes interact with the outer shell, reducing resistance to mass transfer and providing superior separation efficiency. Particle size as small as 1.3 µm used.
**A monolithic column** constitutes of a single solid rod that is thoroughly permeated by interconnecting pores. As with perfusion packings, they have a bimodal pore structure. Macropores, which act as flow-through pores, are about 2 µm in diameter. The silica skeleton contains mesopores with diameters of about 130 Å. It can be surface modified with stationary phase like C18. The rod is shrink-wrapped in a polyetheretherketone (PEEK) plastic holder. The surface area of the mesopores is about 300 m²/g, and the total porosity is 80%. The column exhibits a van Deemter curve close to that for 3.5-µm packed particles, but with a pressure drop ~40% of the packed column run at the same flow rate. Polymeric monoliths are available in capillary formats from 0.1 to 1 mm in diameter and up to 250 mm in length.

**Stationary phases for hydrophilic interaction chromatography (HILIC)** three types of HILIC phases: neutral, charged and zwitterionic. The neutral HILIC phase contains amide or diol functionalities bonded to porous silica. A charged phase exhibits strong electrostatic interactions and may consist of bare silica or amino, aminoalkyl or sulfonate functionalities bonded to porous silica. The separation selectivity between different analytes can be favoured by the electrostatic interactions that are contributing to the retention with the charged HILIC stationary phases. A zwitterionic bonded phase, a $\text{O}_3\text{S(CH}_2)_3\text{N}^+(\text{CH}_3)_2\text{CH}_2^-$ zwitterionic group is the functional entity bonded to a porous silica support, or a polymeric support for greater pH range.
Sample injection system

LC Columns
A reversed phase LC column contains porous particles coated with an organic stationary phase, e.g., a C\textsubscript{18} hydrocarbon chain. The mobile phase carries analytes around and through the particles. The order of elution is determined by the length of time individual analytes partitioned in the stationary phase. Compounds elute in reverse order of their polarity, the most polar compounds elute earlier.
Two concentric steel tubes carry the liquid and nebulising gas. After nebulisation, additional gas is used to dry the droplets. As the droplets shrink the charge density increases to the point at which the droplets are no longer stable and ions are released from the liquid matrix into the vapour phase. Ions enter the mass analyser at an angle to reduce the entrainment of neutral molecules.

At low flow rates, such as in nanospray ESI, spontaneous evaporation is sufficient to eliminate the need for the drying gas.
Stable nebulisation of the liquid flow occurs when a Taylor cone is established at the tip of the ESI source. As the droplets are dried the charge density increases resulting in droplet disintegration either because of Coulombic forces or by distortion to form a Taylor cone from which ions are released.
Ions injected into the trap are held by rf and dc voltages placed on the hyperbolic ring electrode and end caps. Ions move in a complex sinusoidal path where there are crossover points. Space-charge effects occur at the crossover points of the ion paths when too many ions are present. By changing the voltages progressively ions with different $m/z$ values are ejected sequentially onto the detector, generating spectra. MSMS fragmentation takes place when a specific $m/z$ is isolated in the trap and excited in the presence of a neutral gas, providing MS/MS data. The isolation/fragmentation cycle can be repeated to obtain MS$^n$ data.
The basic principle of TOF analyser:- pulses of ions are accelerated from the ion source into the analyser tube, and the time for an ion to travel through a field-free region to the detector is measured. The time-of-flight for the passage of an ion in a TOF analyser is a function of momentum, and therefore its $m/z$. The acceleration voltage and (consequently) the kinetic energy (momentum), is the same for all ions. Thus those with the lowest $m/z$ will travel fastest and arrive at the detector first, followed by the sequential arrival of ions with successively higher $m/z$.

Ions are generated continuously in API sources, thus, the individual packets of ions required in TOF analysis must be excised from the ion beam, and accelerated orthogonally into the analyser using a pulsed pusher voltage. Only part of the continuous ion beam can be sampled as the excised ion packet must traverse the analyser before another set of ions can be introduced. The duty cycle of the orthogonal injection process limits the sensitivity of orthogonal TOF instruments.
Ions arriving from the source are accelerated orthogonally into the analyser by a pulsed voltage from the pusher. Ions separate based on their momenta. As they travel through the analyser the lightest, fastest, ions (lowest \( m/z \)) will arrive at the detector first.

Some horizontal momentum imparted in the source remains so that ions travel at an angle into and out of the reflectron thus attaining a characteristic ‘V’ trajectory. Increasing the distance that the ions travel improves mass resolution, e.g., by using ‘W’ geometry.
Ions, typically from ESI and APCI, are collected in a specialised component called the C-trap and then injected into the orbitrap as high-speed pulses. Ions are injected at an angle and offset from the centre of the trap. The momentum of the ions causes them to orbit around, and oscillate along, the central spindle-like electrode. The lateral oscillation of the ions along the inner electrode induces a transient (image) current in the split outer electrode. The recorded image current is interpreted using Fourier transform analysis to provide $m/z$ values and intensities.
Quadrupoles as ion guide (q) the rf voltages can be applied to the quadrupole, but without being combined with a dc field. Under these conditions all ions, e.g. those emerging from the ion source that enter the q are transmitted, regardless of their $m/z$ values.

Ions are produced in an atmospheric pressure ionisation (API) source. The quadrupole can be used in broad bandpass (q) mode, to pass all ions to the time-of-flight (TOF) analyser, or in narrow band pass (Q) mode to select an ion with a specific $m/z$ for collision induced dissociation (CID). The CID cell also has a q function by which the fragments formed are constrained and transferred to the TOF analyser. CID fragment ions are separated and collected in the TOF analyser.
The LIT-orbitrap combination can be used for both MS and MS^n experiments because the LIT has its own detector. Additional fragmentation at a different collision energy can be undertaken in a second collision cell. Products from either collision cell are injected, via the C trap, into the orbitrap where transients are collected for subsequent FT analysis.

In the LIT-ICRMS, ions from the LIT traverse a set of extended optics into the ICR cell located within a superconducting magnet. A ramped rf voltage pulse is used to coalesce different m/z values and move them close to the receiver plates where transients are collected for subsequent FT analysis.
High resolution improves accurate mass measurement

In unit resolution, the measured peak may actually be an average of several unresolved isobaric species.

It is difficult to determine exactly the center point of a broad peak.

The center point of a narrow peak can be determined precisely.

Isobaric ions have the same nominal mass but different empirical formulae, and thus different accurate masses. Isobaric peaks can be resolved by high resolution.
Cyclopentanoperhydrophenanthrene skeleton

Natural steroids have two methyls

5α-Cholestane structure with IUPAC numbering showing four fused-ring isoprene structure
Steroids in Brain

- 25% body’s cholesterol is present in brain
- Cholesterol formed *de novo*
- 1st step of cholesterol metabolism formation of an oxysterol
24S-Hydroxycholesterol

Cholesterol

Cerebrum
5-15 ng/mg
7-8 µg/mg
0.7-2.1 µg/mg

Plasma
70 ng/ml
2 mg/ml
35 ng/mg

Liver
0.07 ng/mg 24S-Hydroxycholesterol
0.7 µg/mg Cholesterol
0.10 µg/mg 24S-Hydroxycholesterol/Cholesterol

Cerebellum
5 ng/mg
8 µg/mg
0.6 µg/mg

Cerebrospinal fluid
2.5 ng/ml
3.0 µg/ml
830 ng/mg
Oxysterols in brain

ng/mg

ng-pg/mg
• Transport forms of cholesterol
• Oxysterols are ligands for LXR
• Oxysterols implicated in neurodegenerative disease (AD)
• Oxysterols formed by reaction with O$_3$ or ROS as a result of inflammation

Björkhem et al Curr Opin Lipidol 2002 13 247
Analytical Strategy

• Extraction: 1\textsuperscript{st} $\text{C}_2\text{H}_5\text{OH}$, 2\textsuperscript{nd} $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$

• Separation: Straight phase

• Derivatisation:

• LC-ES-MS\textsuperscript{n}
• Specificity for 3β-OH
• Enhance Solubility
• Enhance Sensitivity
• x 100

Smith & Brooks J Chromatogr 1974 101 373
Wang et al Anal Chem 2006 78 164
nano-ESI Mass Spectrum

C⁵⁻⁳β,24S-diol
C⁴⁻²⁴S-ol-3-one GP hydrazone

Ion Current: 8x10⁷

Relative Abundance, %

m/z 534

\([M]^+\)

m/z 403

\([M+H]^+\)

m/z 385

\([M+H-H_2O]^+\)

C⁴⁻²⁴S-ol-3-one GP hydrazone

\([M]^-\)
Direct infusion ESI into the QTOF

100 mg brain
Inject ~ 0.1 mg

- Cholesterol
- Hydroxycholesterol
- Dehydrocholesterol
- Dihydroxycholesterol

Mass-to-charge (m/z) values:
- Cholesterol: 518
- Hydroxycholesterol: 534
- Dehydrocholesterol: 333, 373, 550
- Dihydroxycholesterol: 665
C^4-24S-ol-3-one GP hydrazone

m/z 534 [M]^+ → MS^2

m/z 455
[M-79]^+

m/z 427
[M-79-H_2O]^+

m/z 437
[M-79-CO]^+

m/z 394
[M-79-H_2O]^+

m/z 409
[M-79-CO]^+

m/z 455
[M-79]^+
m/z 534 [M]^+ → m/z 455 [M-79]^+ →

C^{4-24S-ol-3-one GP hydrazone}

m/z 455 [M-79]^+ → m/z 151 *b1-12

m/z 151 *b1-12 → m/z 163 C^{4-24S-ol-3-one GP hydrazone}

m/z 163 *b3-C_2H_4 → m/z 177 [M-107]^+

m/z 177 *[f-H] → m/z 437 [M-79-H_2O]^+

m/z 353 *b2 HN CH_2 *N → m/z 409

m/z 367 *e HN CH_2 *N → m/z 427

m/z 394 *f HN CH_2 *N → m/z 437 [M-79-H_2O]^+
Chromatography

- LC-MS\textsuperscript{n}
- Ultimate 3000 HPLC – LTQ\textsuperscript{duo} ion trap MS
- PepMap C\textsubscript{18} Column (3 μm particles, 180 μm x 150 mm)
- 800 μL/min
- (A) 50% MeOH, 0.1% FA
- (B) 95% MeOH, 0.1% FA
- 30%(B) \rightarrow 80%(B) \rightarrow (80%) \rightarrow 30%(B)
  
  15min \hspace{1cm} 10min \hspace{1cm} 10min
capLC - MS³
Chromatography

- LC-MS\textsuperscript{n}
- Surveyor HPLC – LTQ-Orbitrap
- Hypersil GOLD Column (1.9 µm particles, 50 x 2.1 mm)
- 200 µL/min
- (A) 50% MeOH, 0.1% FA
- (B) 95% MeOH, 0.1% FA
- 20%(B) $\rightarrow$ 80%(B) $\rightarrow$ (80%) $\rightarrow$ 20%(B)
  
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A (%)</th>
<th>B (%)</th>
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<tbody>
<tr>
<td>7</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
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LC-MS$^n$ Strategy ($m/z$ 300 - 600)

Data From LTQ-Orbitrap (Exact Mass RIC ±5ppm)
Reconstructed ion chromatogram (RIC) for m/z 534

7.35

RT: 0.00 - 17.00

m/z = 534.4027 - 534.4081 F:
FTMS + p ESI Full ms [320.00-600.00] MS
ESI_Unisil2_070206_fr

7.65

Exact Mass: 534.4054

C_{34}H_{52}N_{3}O_{2}^+

Exact Mass: 534.4054

C_{34}H_{52}N_{3}O_{2}^+
**534 → MS²**

24S_OH_C #51  RT: 5.45  AV: 1  NL: 5.99E6
F: FTMS + p ESI Full ms2 534.40@30.00 [ 145.00-550.00]

M-79

$\text{C}_{29}\text{H}_{47}\text{N}_{2}\text{O}_{2}^+$

Exact Mass: 455.36320

M-107

$\text{C}_{34}\text{H}_{52}\text{N}_{3}\text{O}_{2}^+$

Exact Mass: 534.40540
24(S)-hydroxycholesterol

24S_OH_C #67-80  RT: 6.36-7.10  AV: 14  NL: 2.03E6
F: FTMS + p ESI Full ms3 534.40@30.00 455.40@30.00 [125.00-550.00]
The prenatal diagnosis of Smith-Lemli-Opitz syndrome from amniotic fluid
Sterol analysis for the prenatal diagnosis of Smith-Lemli-Opitz syndrome

- **Cholesterol**: 518 → 439
- **Desmosterol**: 516 → 437
- **7-DHC**: 516 → 437

Graph showing time (min) on the x-axis and %RA on the y-axis with peak times of 25.83, 24.46, and 23.90.
0.002-0.01 μL amniotic fluid injected on-column

(a) Ion Count 6.7E6
Ion Count 6.7E6
Ion Count 9.85E4
Ion Count 9.85E4
MS² : 518→439
MS² : 516→437

(b) Ion Count 5.58E6
Ion Count 5.58E6
Ion Count 6.27E6
Ion Count 6.27E6
MS² : 518→439
MS² : 516→437
Mean ratio of 7/8-DHC to cholesterol for 18 amniotic fluids from SLOS-affected and non-affected pregnancies. Eighteen samples were subjected to oxidation/GP-derivatisation reactions in triplicate and were analysed by cap-LC-MS^n.
β-sitosterol
5-cholesten-24β-ethyl-3β-ol

Stigmasterol
5,22-cholestadien-24β-ethyl-3β-ol

Campesterol
5-cholesten-24α-methyl-3β-ol

Stigmastanol
5-cholestan-24β-ethyl-3β-ol
Analytical Strategy

- Extraction of phytosterols: C$_2$H$_5$OH
- Derivatisation with GP hydrazine
- Direct infusion ESI onto the QTOF
- LC-ES-MS$^n$
Direct infusion of a sample into the mass spectrometer

LC and Mass Spectrometry

- LC-MS: Separate components of interest, giving sharp peaks (10 s – 1 min) away from the solvent front