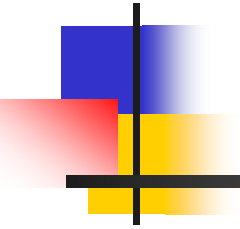


Evaporative Light Scattering Detection in Application to Lipid Separation by HPLC. Part I: Theory of operation



Igor Sinelnikov, MSc



What is HPLC?

- High Performance Liquid Chromatography (HPLC) is a form of column chromatography used to separate, identify, and quantify compounds

Mobile Phase



Pump

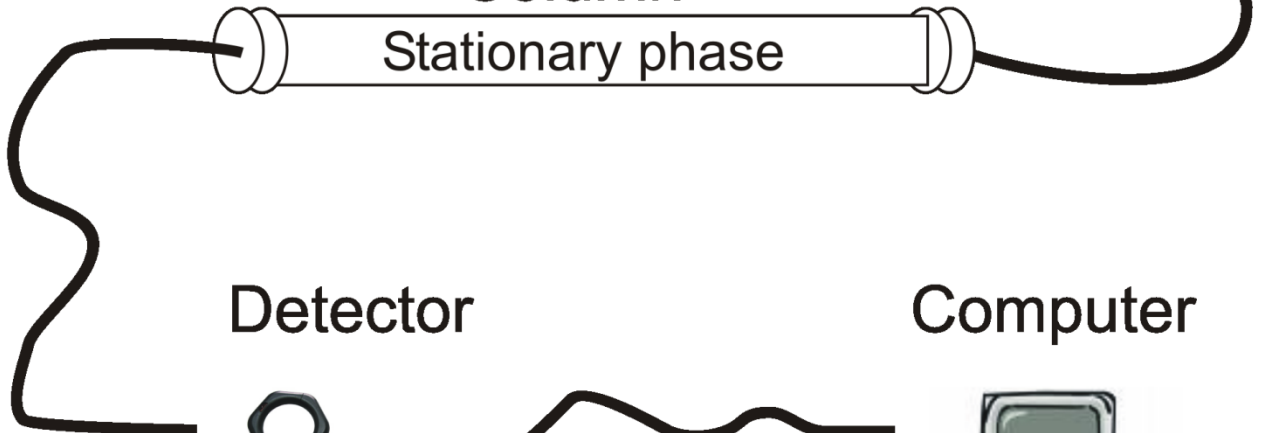


Sample



Column

Stationary phase



Detector



Computer



Two the most common types of HPLC



■ Normal phase HPLC

- polar stationary phase – silica columns
- non-polar aprotic organic solvents
(e.g. THF, hexane, CHCl_3 , CH_2Cl_2 , etc.)

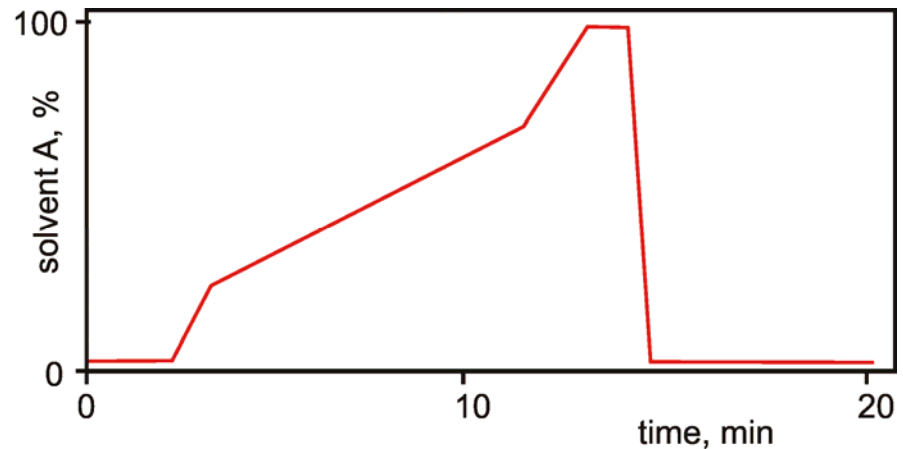
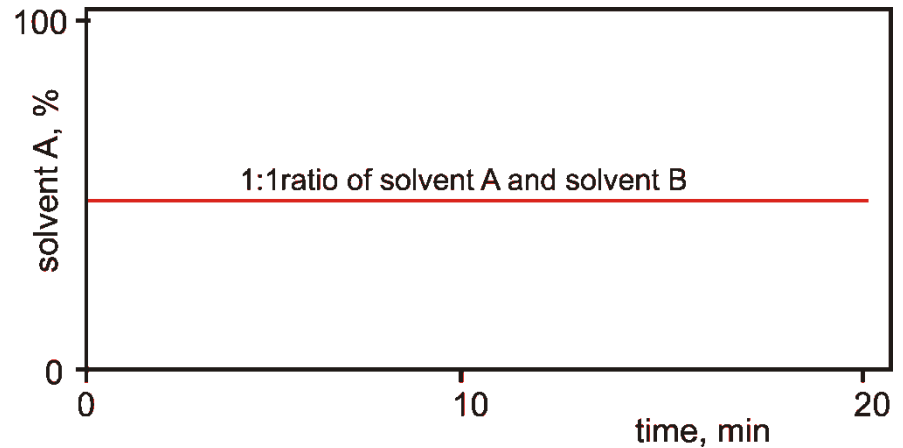
■ Reverse phase HPLC

- non-polar stationary phase – C8 or C18
- polar organic solvents and water
(e.g. methanol, IPA, acetonitrile, etc.)

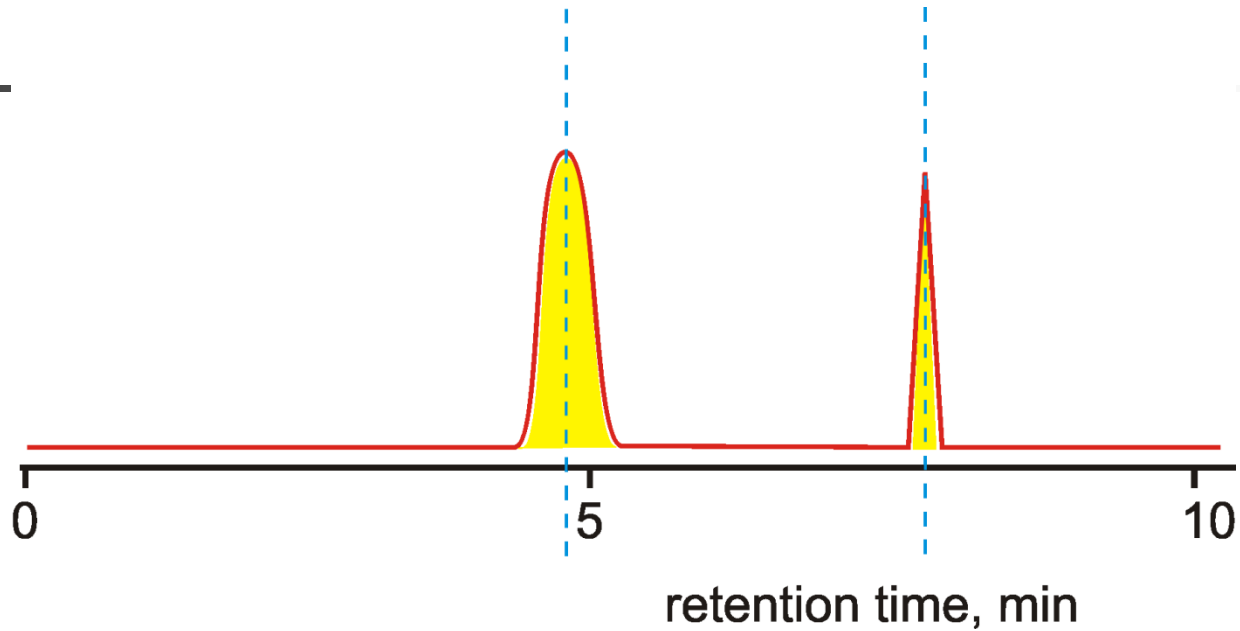
Mobile phase flow modes

- **Isocratic flow**
 - mobile phase composition is constant with time

- **Gradient flow**
 - mobile phase composition changes with time



Information obtained from HPLC



- Retention time
- Area under a peak or "peak area"



Information obtained from HPLC

- Retention time is characteristic for a compound
(It varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used)
- Peak area is proportional to the amount of sample
(Usually the proportionality is linear depending on the type of a detector)



Conventional detectors in HPLC

- **Refractive Index (RI)** detectors measure the ability of sample molecules to bend or refract light
 - two flow cell design
 - not compatible with a gradient flow

- **Ultra-Violet (UV)** detectors measure the ability of a compound to absorb UV light
 - UV transparent mobile phases are required
 - will not detect UV-inert compounds



Limitations of RI and UV detectors

- UV and RI are not compatible with a wide range of solvents
- RI detection is not gradient compatible
- Different analytes produce different UV responses depending on their extinction co-efficient



Evaporative Light Scattering detectors – ELSD

- ELSD can outperform traditional detectors when analysing non-chromophoric samples by HPLC
- Can detect anything that is less volatile than the mobile phase
- ELSD is universal and compatible with a wide range of solvents



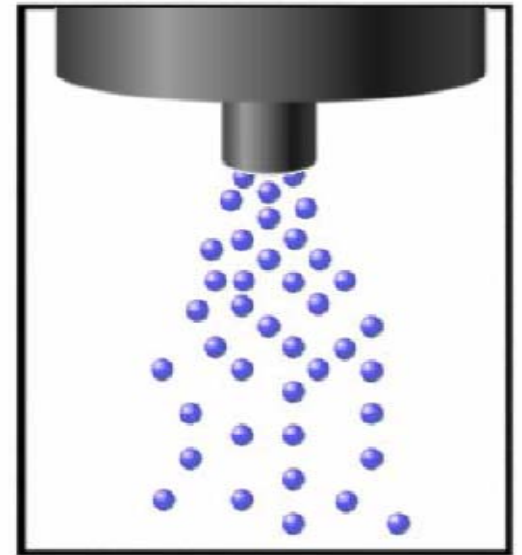
ELSD: Principle of operations

- The ELSD employs three distinct stages:
 1. Nebulisation
 2. Evaporation
 3. Detection



Nebulisation

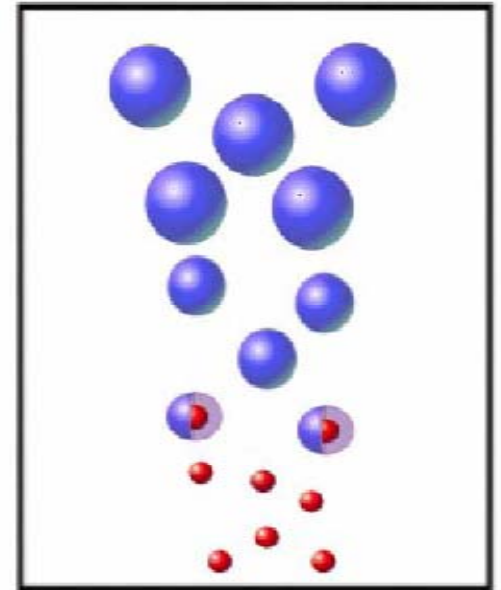
- Eluent flow mixed with N₂ or Air in a chamber
- Efficient nebulisation:
 - stable droplet plume;
 - uniform droplet size;
 - temperature independently controlled





Evaporation

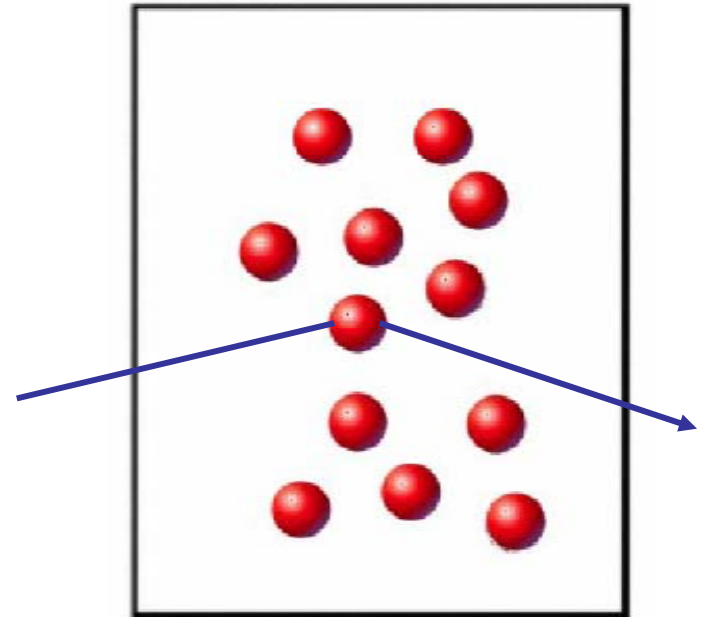
- Droplets pass through heated drift tube
- Removes mobile phase to leave particulate form of analyte
- Temperature set according to analyte and controlled by user





Detection

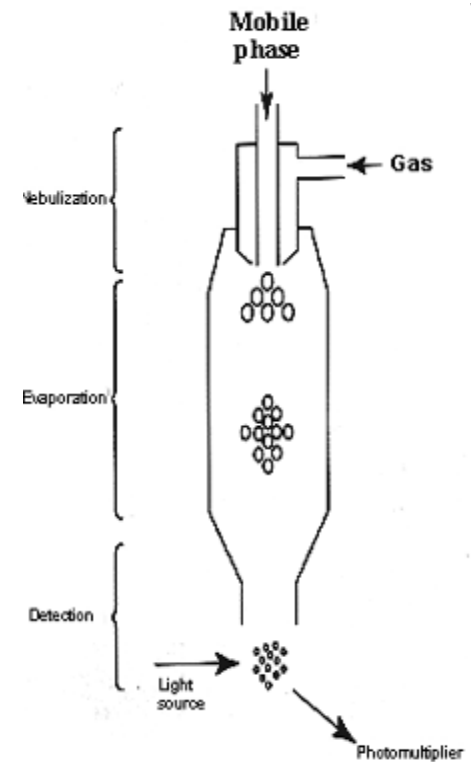
- Particles irradiated with light source (Laser or LED)
- Particles scatter light according to their size (mass sensitive)
- Scattered light is detected by photomultiplier at fixed angle from incident light (usually 45°)



ELSD

Characteristic properties of ELSD

- ❖ Low background noise (no solvent peaks)
- ❖ Reproducibility (in the $1\mu\text{g}$ range, STD $\sim 1\%$)
- ❖ Low band broadening (short transit time)
- ❖ "Near" linear response (instrument and concentration dependant, smart choice of standards)
- ❖ Sensitivity (1-50 ng of sample)





Advantages of ELSD: Theory

- Universal - responds to all compounds in the mobile phase
- Not dependent on spectroscopic properties of analyte
- Not susceptible to baseline drift during gradient elution, temperature or solvent pump fluctuations
- ELSD compatible with a much wider range of solvents compared to Refractive Index detector
- No interference from solvent front peaks (enables fast analysis)
- Flow rates up to up to 5ml/min can be achieved with no affect on baseline stability
- Ideal for High Throughput Screening and quantification



What are Lipids?

- Lipids are broadly defined as any fat-soluble (lipophilic), naturally-occurring molecule, such as fats, oils, waxes, cholesterol, sterols, fat-soluble vitamins (such as vitamins A, D, E and K), monoglycerides, diglycerides, phospholipids, and others.
- The main biological functions of lipids include energy storage, acting as structural components of cell membranes, and participating as important signaling molecules.

(adapted from Wikipedia)



Lipid classification

- 8 major categories

Fatty Acyls [FA]

Glycerophospholipids [GP]

Sterols [ST]

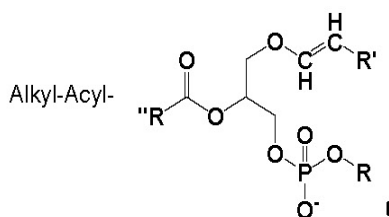
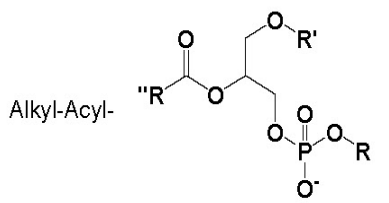
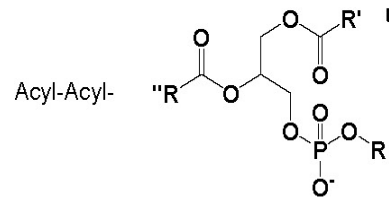
Saccharolipids [SL]

Glycerolipids [GL]

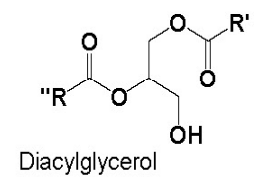
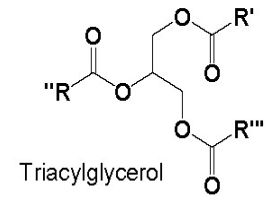
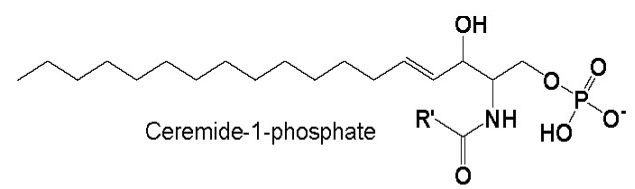
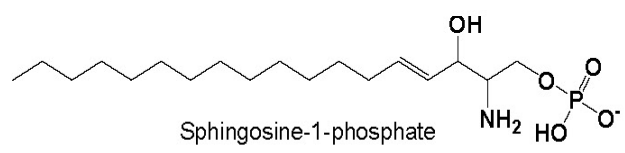
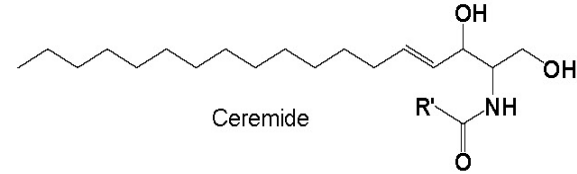
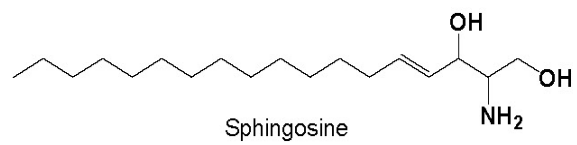
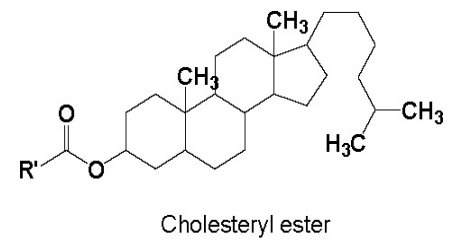
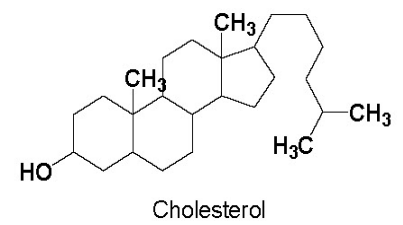
Sphingolipids [SP]

Prenol Lipids [PR]

Polyketides [PK]



Residue -R	Sub-class	Residue -R	Sub-class
—H	Phosphatidic Acid (PA)		Phosphatidyl-serine (PS)
	Phosphatidyl-ethanolamine (PE)		Phosphatidyl-glycerol (PG)
	Phosphatidyl-choline (PC)		Phosphatidyl-inositol (PI)



R = Head groups of phospholipids
 R', R'', R''' = Varying fatty acid residues



Lipids are important because they are:

- the main structural component of biological membranes
- vital part of the cell signaling processes
- essential for life and important indicators/potential biomarkers in nutrition and health sciences



Difficulties in lipid analysis

- very large structural diversity
(estimated ~2000-3000 lipids in human metabolome)
- very large differences in concentration profiles
- solubility issues
- some lacks the “chromophore” groups for detection



Application of ELSD in HPLC separation of lipids

- irrespective to spectroscopic properties of analytes
- compatible with aprotic and protic organic solvents and aqueous mobile phase
- sensitivity in the order of 1-10ng per injection
- wide dynamic range
- “nearly” linear response;
- can be used in tandem with LC-MS

ELSD - Ideal complement to LC-MS

Sample Mixture of known 1:1 ratio

LC-MS
results show ratio to be
3:1

UV-Vis
results show ratio to be
10:1

PL-ELS 2100
results show ratio to be
1:1
(Response independent
of optical properties)

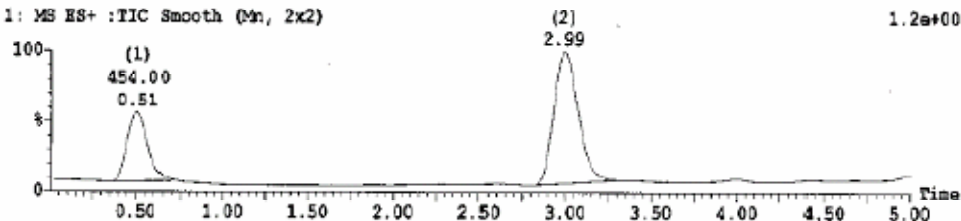
Sample Report:

Sample 1 Vial 1:

Date 14-Jul-2003 Time 11:25:22 Description TestM

1: MS ES+ :TIC Smooth (Mn, 2x2)

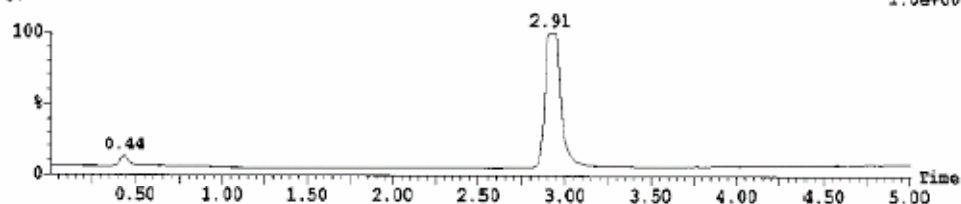
1.2e+008



Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1	Found	0.51	8e+006	30.40	0	6e+007	454.00
2		2.99	2e+007	69.60	0	1e+008	

UV

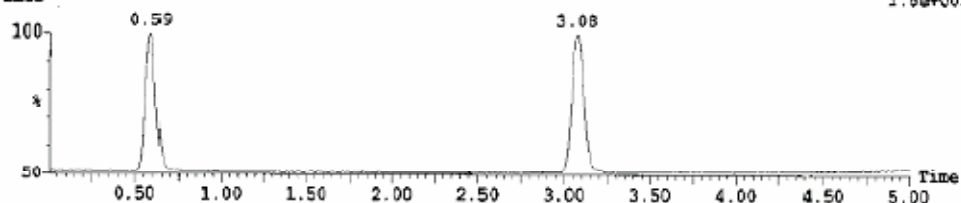
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Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
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ELSD

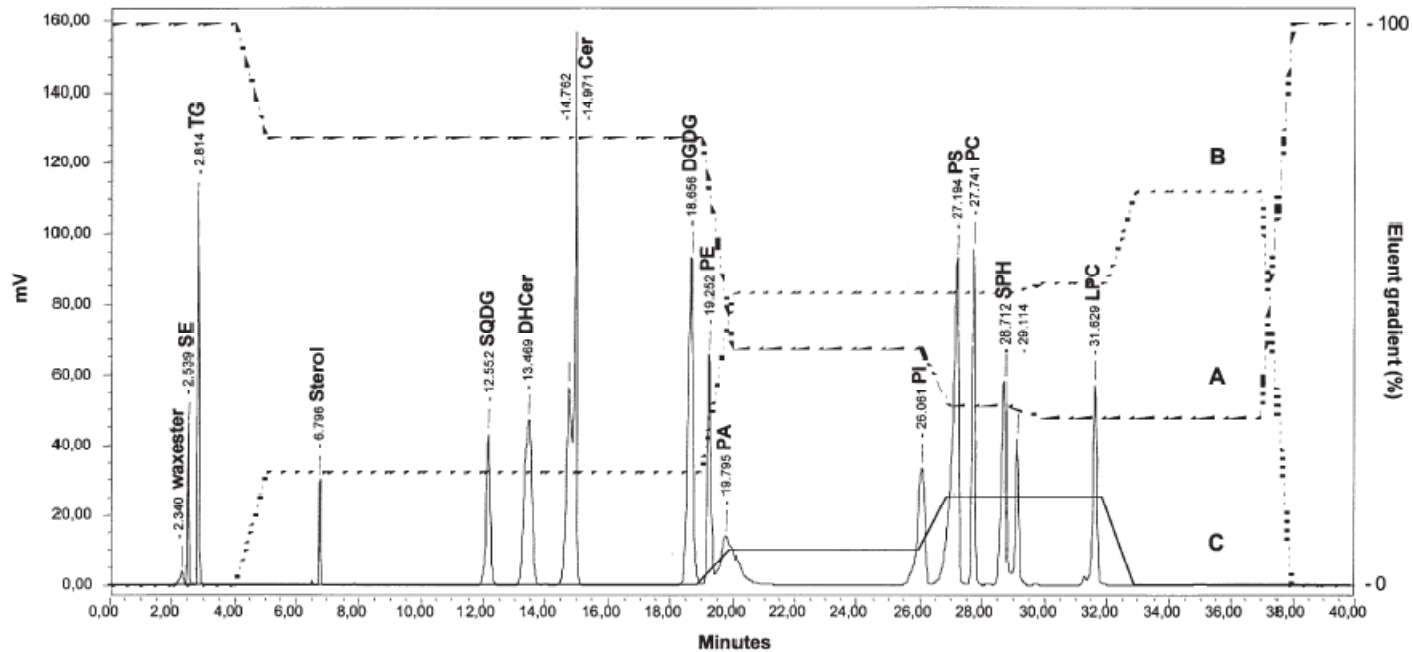
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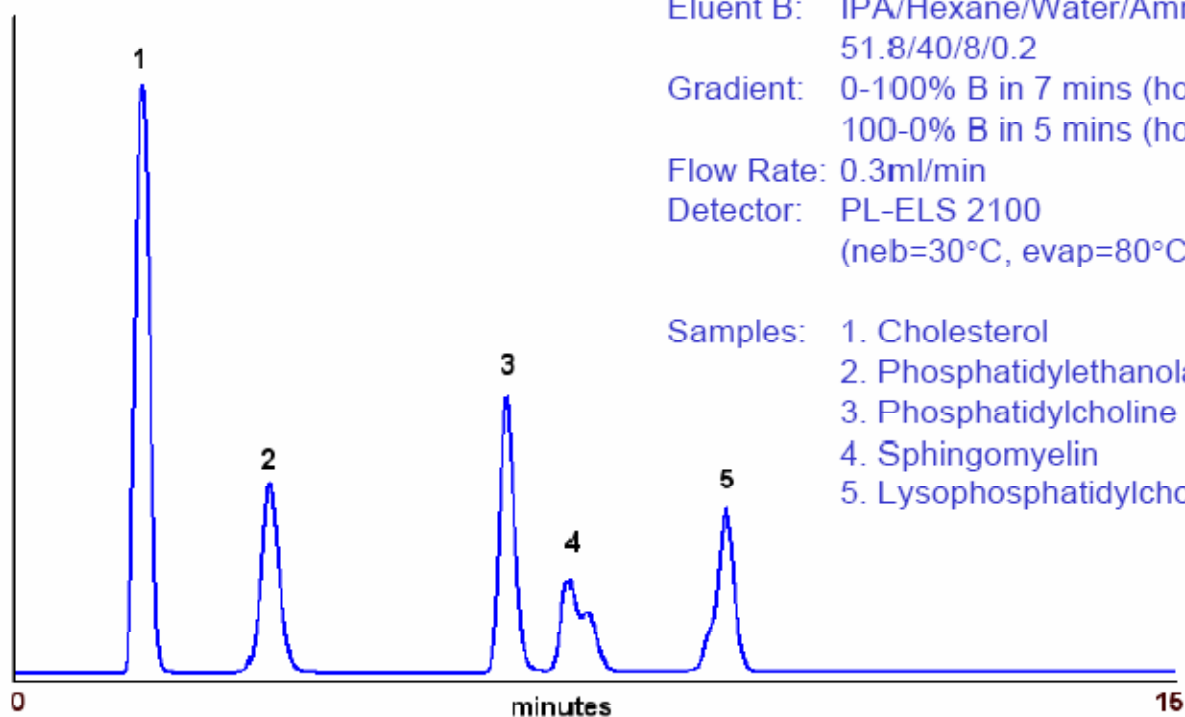
Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
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Examples of lipid separation using ELSD HPLC

Lipid class separation (up to 14 categories/classes)



Examples of lipid separation using ELSD HPLC



Column: Lichrospher DIOL 5 μ m, 150x2.1mm
Eluent A: IPA/Hexane/Water/Ammonia Hydroxide
57.8/40/2/0.2

Eluent B: IPA/Hexane/Water/Ammonia Hydroxide
51.8/40/8/0.2

Gradient: 0-100% B in 7 mins (hold 8 mins)
100-0% B in 5 mins (hold 10mins)

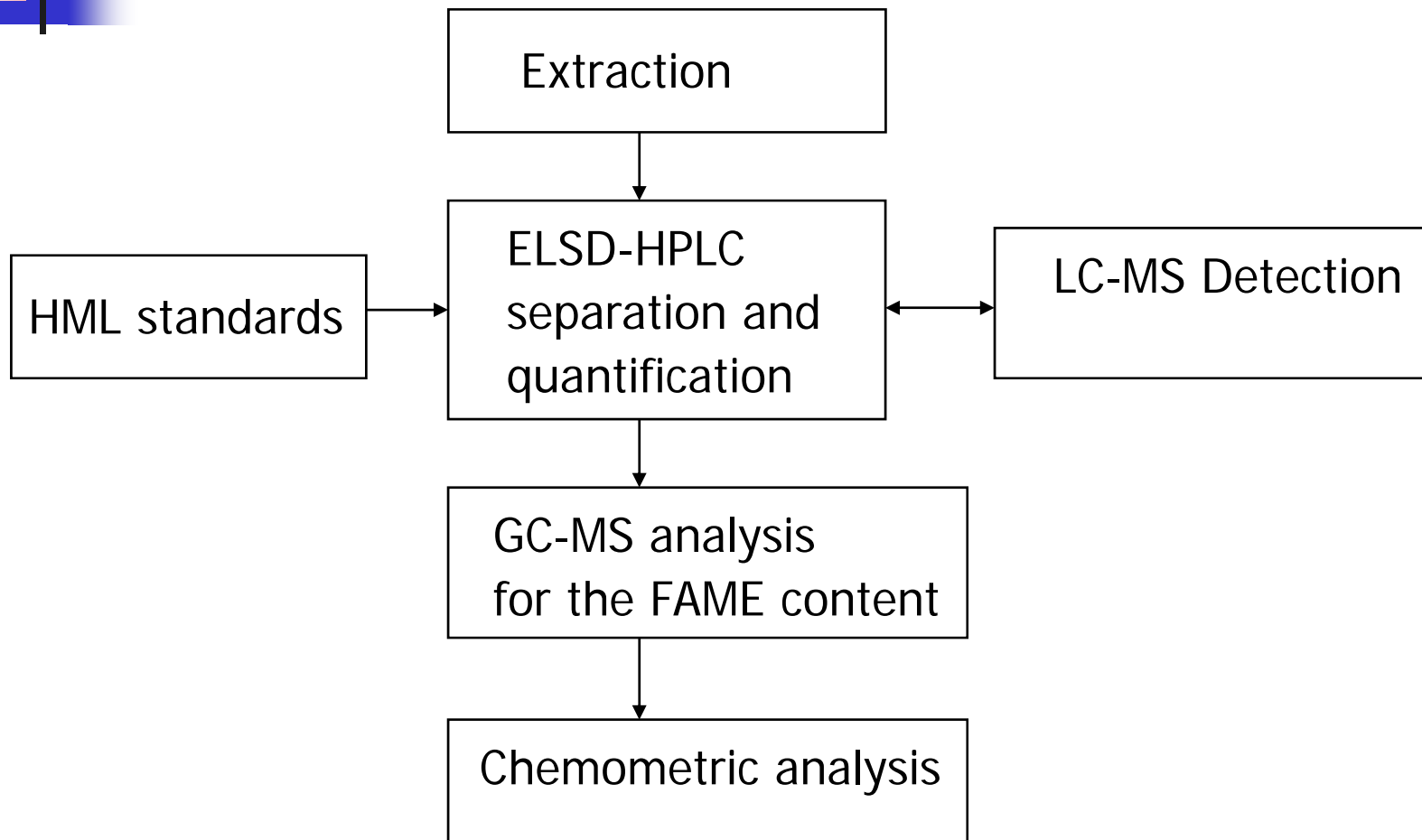
Flow Rate: 0.3ml/min

Detector: PL-ELS 2100
(neb=30°C, evap=80°C, gas=1.0 SLM)

Samples: 1. Cholesterol
2. Phosphatidylethanolamine
3. Phosphatidylcholine
4. Sphingomyelin
5. Lysophosphatidylcholine

(adapter from www.polymerlabs.com)

Current work flow in lipid analysis



Disappointments

standard mixture:
column:
mobile phase:

Uracil, Acetophenone, Anisole, Toluene
250mmx4.6mm Si, Phenomenex, 5um
ACN 65%, water 35%

