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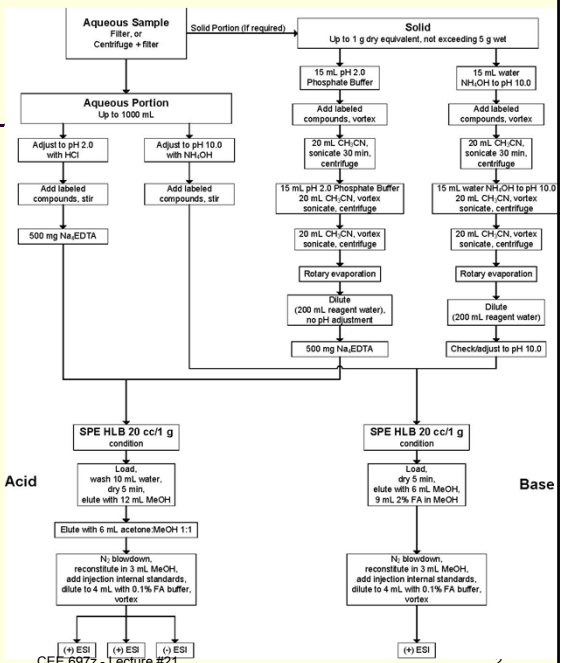
CEE 697z
Organic Compounds in Water and Wastewater

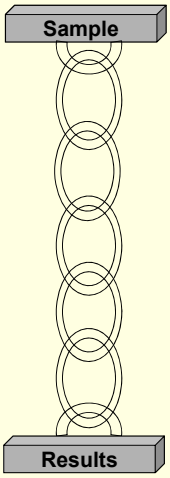
PPCP Analysis
October 27, 2014
Lecture given by Kaoru Ikuma, Ph.D.

EPA Method 1694

- Pharmaceuticals and personal care products in water, soil, sediment, and biosolids by HPLC/MS/MS
- Four analytical groups



The Analytical Chain of LC/MS



Sample Treatment

Separations

Ionization/Interfaces

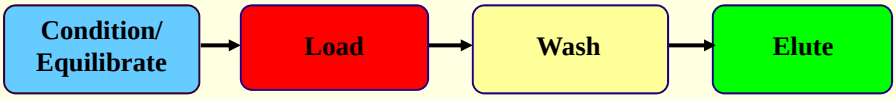
Mass Analysis

Data/Interpretation

Step 1
Sample treatment: Extraction

Solid Phase Extraction

- Extraction of organic contaminants from water and adsorb onto solid phase to concentrate
- Sample pretreatment method used to quantitatively analyze contaminants with Liquid Chromatography/Mass Spectroscopy
- Solid phase contained in cartridges or barrels



Extraction Technologies

- Off-line Solid-Phase Extraction (liquids)
  - Cartridges (syringe, sep-pak)
  - Disks
  - 96-well plates
  - Solid-phase microextraction
- On-line Solid-Phase Extraction (liquids)
  - Prospekt cartridges
- Accelerated Solvent Extraction (solids)
  - Sorbents?

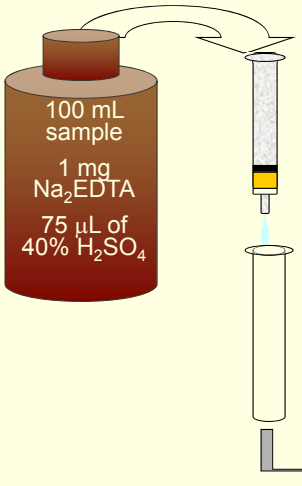
Slide courtesy of Meyer et al., USGS

Solid-Phase Extraction

- Many Types of Materials
- C2-18 on Silica backbone with varying linkages
- Polymers also with hydrophillic-lipophilic functional groups
- Anion Exchange (WC, SC, WA, SA)
- Mixed Mode
- Immunnoaffinity
- Many manufacturers

Slide courtesy of Meyer et al., USGS

SPE Example



60 mg HLB
Condition: 2 mL MeOH
3 mL 0.5 N HCl
1 mL H_2O

Load Sample

Wash: 1 mL water

Elute: 5 mL MeOH into test tube

Concentrate: N2 to 125 µL

Analyze: ESI(+)

Slide courtesy of Meyer et al., USGS

Typical Concentration Factors: Environmental SPE

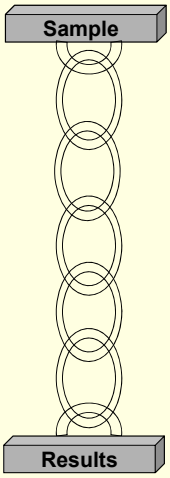
Sample Vol. (ml)	Extract Vol. (µL)	Concentration Factor
100	100	1000
1,000	500-1000	1000-2000
1 µg/L		1-2 mg/L

Slide courtesy of Meyer et al., USGS

Off-line Manual SPE Method

- 500 mL to 1000 mL sample size concentrated to 1 mL
- One-time use HLB extraction cartridges
- 6 hour extraction method time
- 16 min instrument run time
- Prior to LC/MS/MS

The Analytical Chain of LC/MS



Sample Treatment

Separations

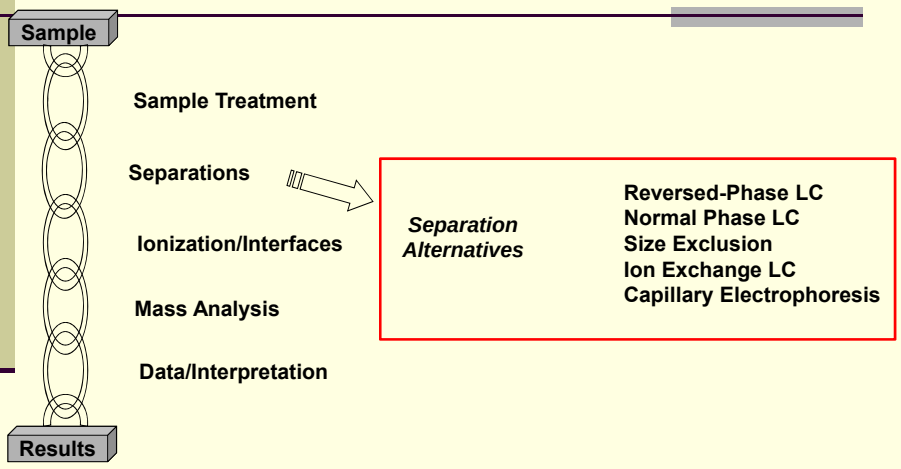
Ionization/Interfaces

Mass Analysis

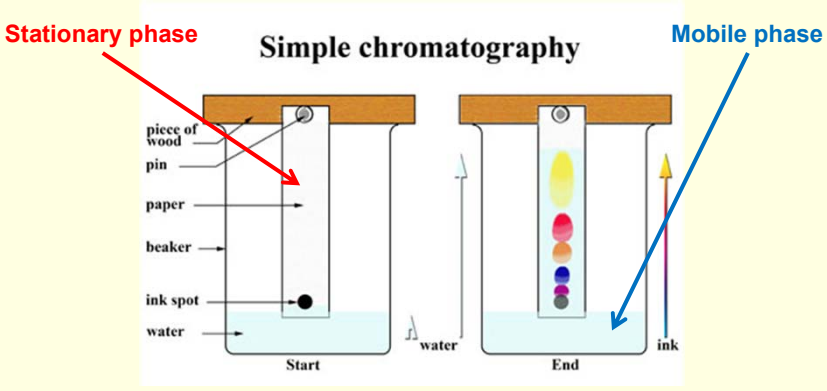
Data/Interpretation

Step 2
Separation:
Liquid chromatography

The Analytical Chain of LC/MS

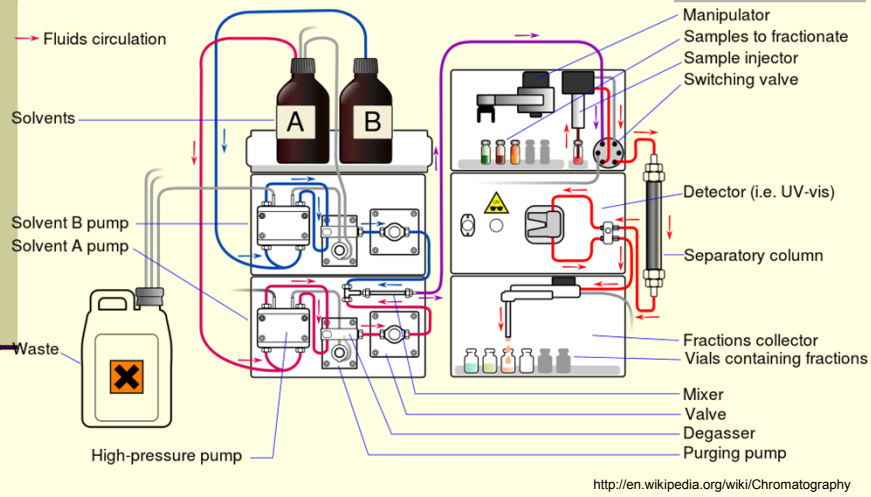


What is Chromatography ?



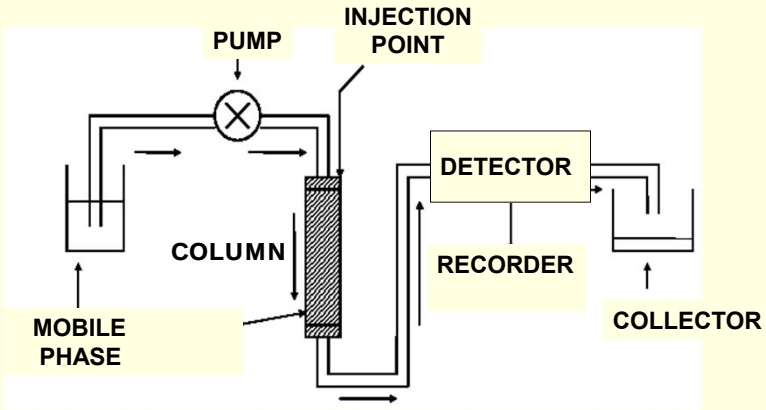
http://www.micromountain.com/sci_diagrams/sci_app/sci_app_pages/ctography_lab_eng.htm

High performance liquid chromatography (HPLC)



http://en.wikipedia.org/wiki/Chromatography

HPLC Instrument Basics



Types of HPLC Phases

- **Adsorption**
  - **Normal Phase – polar bed, non polar mobile phase (n-hexane, tetrahydrofuran)**
  - **Reverse Phase – non-polar bed w/ polar mobile phase (methanol, water, acetonitrile mixture)**
  - *** most common**
- **Ion Exchange**
  - **Stationary bed ionically charged surface, opposite to sample ions**
  - **Use with ionic or ionizable samples**
  - **Stronger charge = longer elution time**
  - **Mobile Phase – aqueous buffer**
- **Size Exclusion**
  - **Column material precise pore sizes**
  - **Large molecules first, then small**

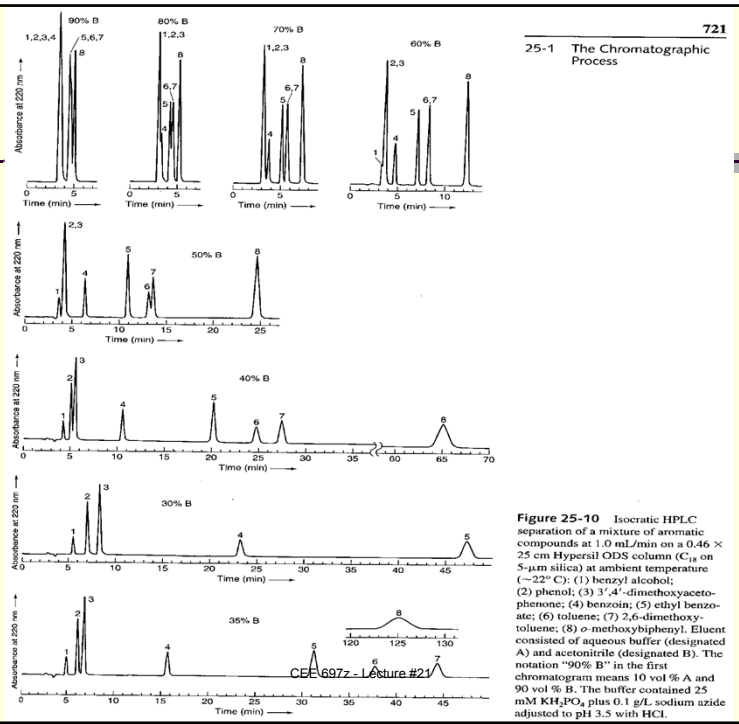
Mobile Phase / Eluent

- Purity **- Low viscosity**

- Detector compatibility **- Chemical inertness**

- Solubility of sample **- Price**

- All solvents “HPLC grade”
  - **Filtered using 0.2 µm filter**
  - **Extends pump life**
  - **Protects column from clogs**
- Solvent Degassing / Purging
  - **Displacement w/ less soluble gas**
  - **Vacuum application**
  - **Heat solvent**



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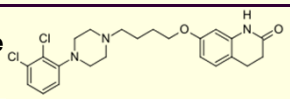
25-1 The Chromatographic Process

Figure 25-10 Isocratic HPLC separation of a mixture of aromatic compounds at 1.0 mL/min on a 0.46 × 25 cm Hypersil ODS column (C_{18} on 5-μm silica) at ambient temperature (~22° C): (1) benzyl alcohol; (2) phenol; (3) 3′,4′-dimethoxyacetophenone; (4) benzoin; (5) ethyl benzoate; (6) toluene; (7) 2,6-dimethoxytoluene; (8) *o*-methoxybiphenyl. Eluent consisted of aqueous buffer (designated A) and acetonitrile (designated B). The notation "90% B" in the first chromatogram means 10 vol % A and 90 vol % B. The buffer contained 25 mM KH_2PO_4 plus 0.1 g/L sodium azide adjusted to pH 3.5 with HCl.

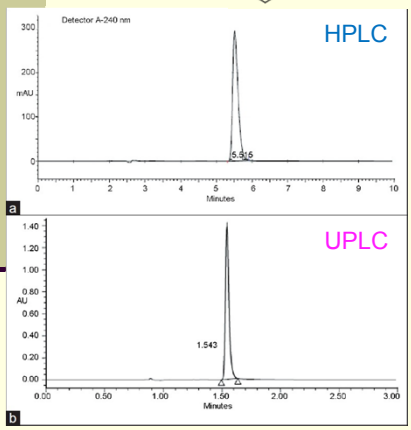
HPLC Columns (stationary phase)

- Stainless steel
- Common sizes:
  - 10,12.5, 15, 25 cm long
  - 4.6 mm i.d.
- Length for optimum separation dictated by theoretical plates needed for good resolution
- Filled with stationary phase material (typically particles of ~5 μm)

HPLC vs. UPLC (ultra performance LC)

Aripiprazole 

Dopamine agonist, used as a second generation antipsychotic drug



Validation experiment	HPLC method	UPLC method
Specificity	No interference to analyte peak	No interference to analyte peak
LOQ	0.1 µg/ml	0.05 µg/ml
LOD	0.05 µg/ml	0.01 µg/ml
Linearity and range		
Co-relation coefficient	R^2=0.999	R^2=0.999
Regression equation	Y=31252X+17480	Y=32511X-25877
Method precision (n=6)	%RSD=1.07	%RSD=0.77
Intermediate precision (*n*=6)	%RSD=0.23	%RSD=0.98
Accuracy (recovery %)	99-101	99-100
Robustness	Highly robust method	Highly robust method
Total analysis time	10.0 min	3.0 min

Thakkar, et al., 2011

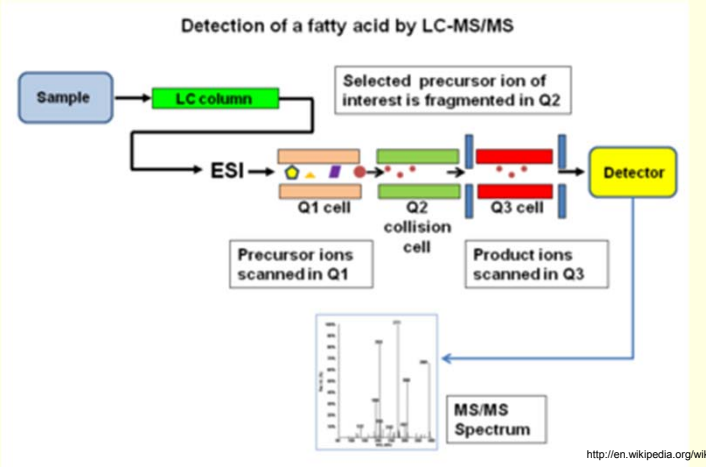
What is LC/MS/MS ?

- It is Liquid chromatography coupled with Mass Spectrometer
- The discussion is restricted to the available instrument by Waters, Milford, MA (Micromass Quattro micro API Mass Spectrophotometer)

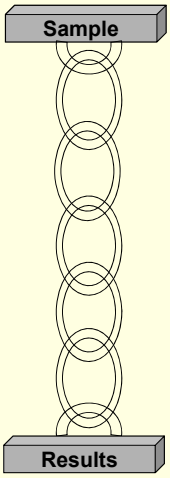
Power of LC/MS/MS

- MS provides exceptionally clean product (fragment) ion chromatograms for quantification
- The signal-to-noise (S/N) ratio is optimized
- Useful for the rapid screening of complex samples where analytes of interest are known
- Compound identity confirmation can be achieved with MS/MS using the product ion scan mode
- By detecting a specific product ion (precursor ion mode) or charged fragments resulting from a neutral loss (neutral loss mode), you can classify a compound of interest

General Principle of Operation of LC/MS/MS



The Analytical Chain of LC/MS



Sample Treatment

Separations

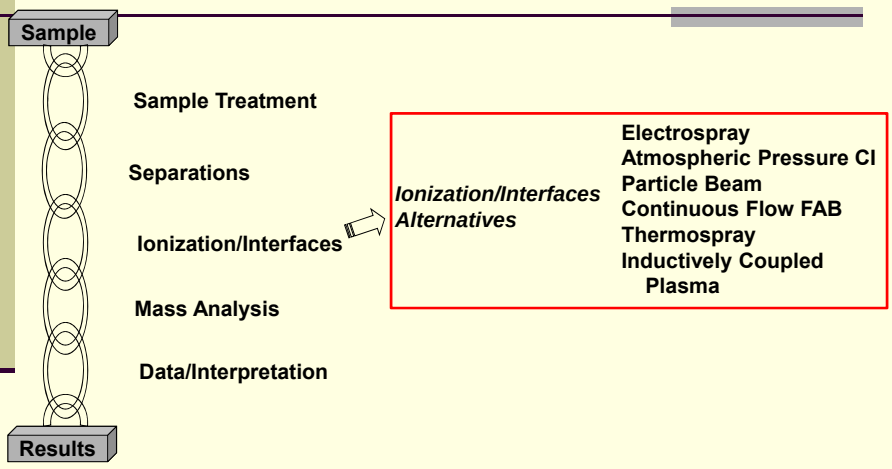
Ionization/Interfaces

Mass Analysis

Data/Interpretation

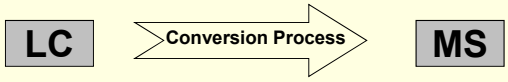
Step 3
Interface and ionization

The Analytical Chain of LC/MS



Interface and Ionization

❑All interface/ionization combination must convert dissolved analyte eluting from a separation system into gas-phase ions at reduced pressure.



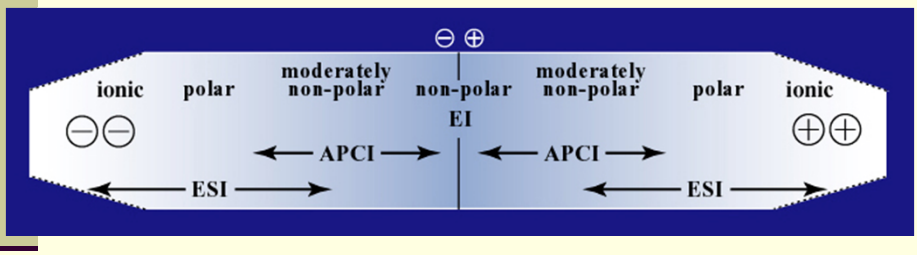
State-of-matter:	Liquid-phase	Evaporation	Gas Phase
Pressure:	Atmospheric	Pressure Reduction	High Vacuum
Charge State:	Neutral(Ionic)	Ionization	Ionic

Ionization Source

Broad range of atmospheric pressure ionization (API) sources

- **Electrospray (ESI) probe** – the most widely used API technique for sensitive, general analysis of polar & ionic comp.
- **Atmospheric Pressure Chemical Ionization (APCI) probe** – ionization capabilities for less polar & neutral chemical species
- **IonSABRE™ APCI** – excellent sensitivity for less polar & nonpolar analytes, especially at higher liquid flow rates
- **ESCi™ Multi-Mode Ionization** – combines ESI and APCI in the same analysis
- **APPI™/APCI Dual Ionization** – provides APCI in simultaneous operation with photoionization (PI)
- **MUX-technology™** – provides the ability to multiplex four sample streams into a single Waters Micromass mass spectrometer

Ionization-Continuum Diagram



Slide courtesy of Meyer et al., USGS

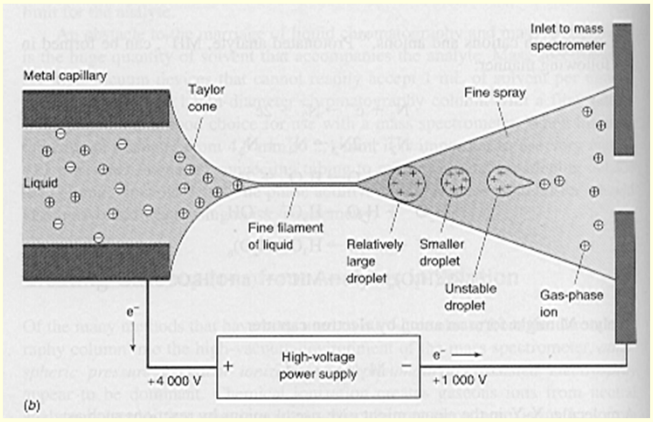
Electrospray (ES)

- In an electrospray interface, the column effluent of LC is nebulized into an atmosphere-pressure ion source.
  - ES is composed of a hollow needle with a high electrical potential through which the effluent flows (1-10uL/min).
  - The high field at the tip of the needle produces a cone shaped liquid meniscus from which a spray of highly charged droplets emerges.
  - Subsequent evaporation of the droplets results in ion formation.

Ionization in Electrospray

- Ionization of the solute in solution.
- Nebulize the solution and charge the droplets.
- Desolvation of the droplets by evaporation.
- Desorption of the solution ions to gas phase ions.

Slide courtesy of Meyer et al., USGS



From: Harris, 1999

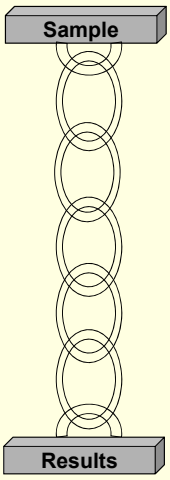
ESI types

- Positive
  - Use volatile proton donor (e.g., 0.1% formic acid)
- Negative
  - Use volatile proton acceptor (e.g., 0.3% NH_4OH)

Matrix Effects

- Suppression
- Enhancement
- Mostly occur in ESI

The Analytical Chain of LC/MS



Sample Treatment

Separations

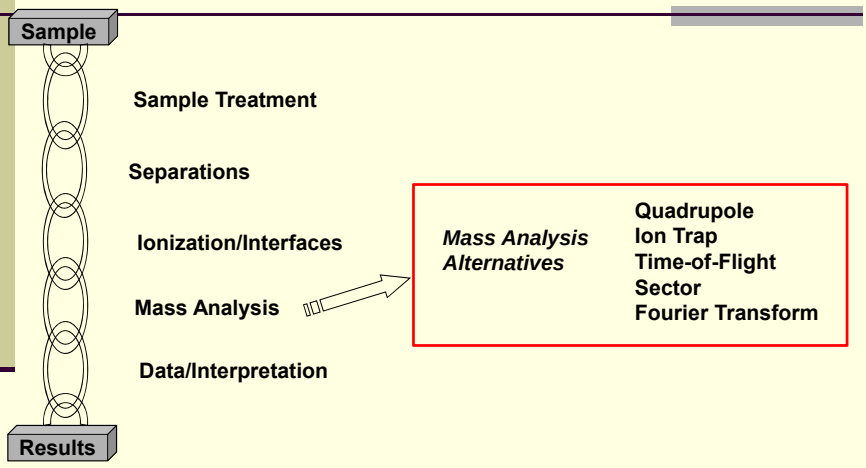
Ionization/Interfaces

Mass Analysis

Data/Interpretation

Step 4
Mass analysis: Mass spectrometer

The Analytical Chain of LC/MS



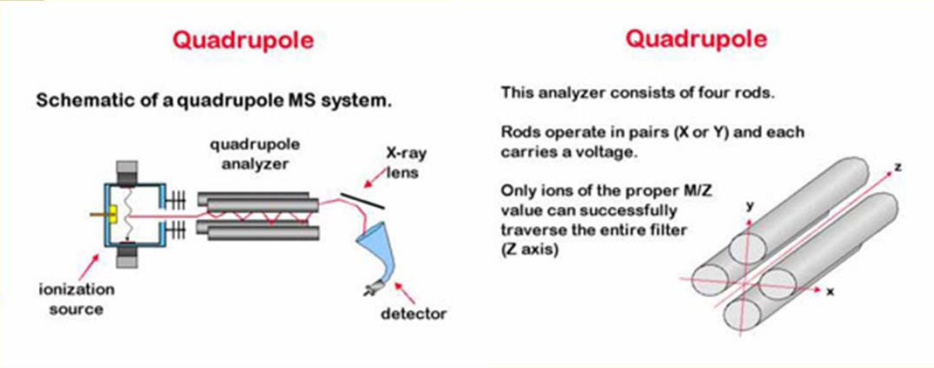
Types of MS

- 4 Types commonly used in environmental analysis
  - Magnetic Sector MS
  - Quadrupole MS
  - Ion-trap MS
  - Time of Flight MS
- Others
  - Fourier Transform Ion Cyclotron Resonance MS (FT-ICR)

MS Quadrupole

- Most common mass analyzer
  - in use since the 1950s
- Quadrupole MS are smaller, cheaper and more rugged than magnetic sectors
- Low scan times (<100 ms) – ideal for GC or LC inlets
- Called mass filters rather than mass analyzers
  - ions of only a single mass to charge (m/q) ratio pass through the apparatus
  - separate ions based on oscillations in an electric field (the quadrupole field) using AC and DC currents

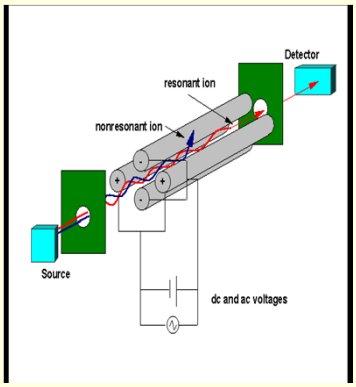
Schematic of Quadrupole



Hardy, U of Akron

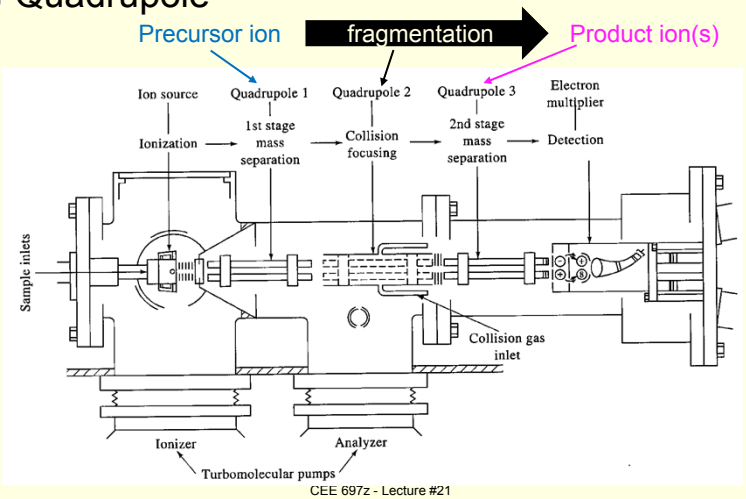
Operation of Quadrupole Mass Filter

- voltages applied to electrodes affect trajectory of ions with the m/q ratio of interest as they travel down the center of the four rods
- these ions pass through the electrode system
- ions with other m/z ratios are thrown out of their original path
- these ions are filtered out or lost to the walls of the quadrupole, and then ejected as waste by a vacuum system
- in this manner the ions of interest are separated



MS/MS

- Quadrupole

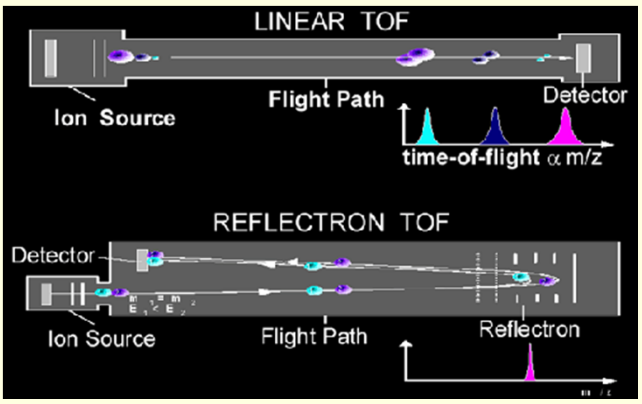


Time-of-Flight Mass Spectrometry

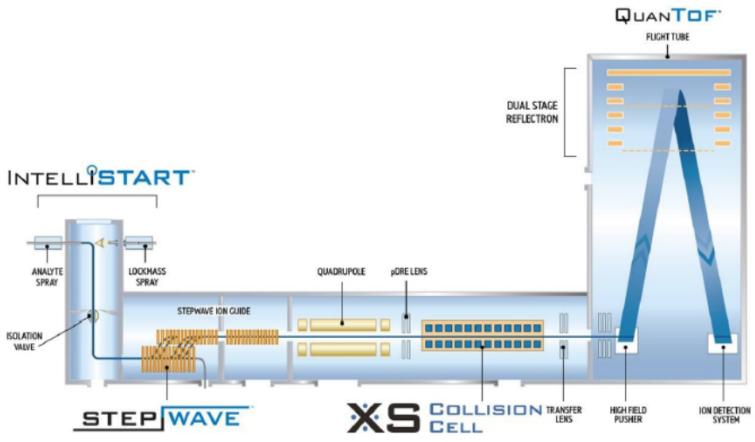
- Ionization: positive ions are produced periodically by bombardment of the sample with brief pulses of electrons, secondary ions, or in cases laser-generated photons.
- Acceleration: The ions are then accelerated by an electric field pulse of 10^3 to 10^4 V (the "pusher") that has the same frequency as, but lags behind, the ionization pulse
- Drift: The accelerated particles then pass into a field-free drift tube. The drift tube's length can range from 0.5 - 3.0 meters

Time-of-flight MS

- Lighter ions are subject to greater acceleration



Xevo G2-XS QTof



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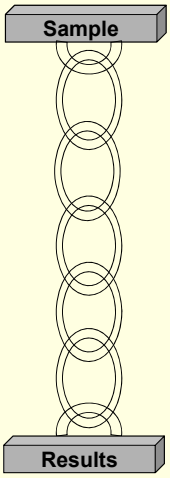
The Analytical Chain of LC/MS



Sample Treatment

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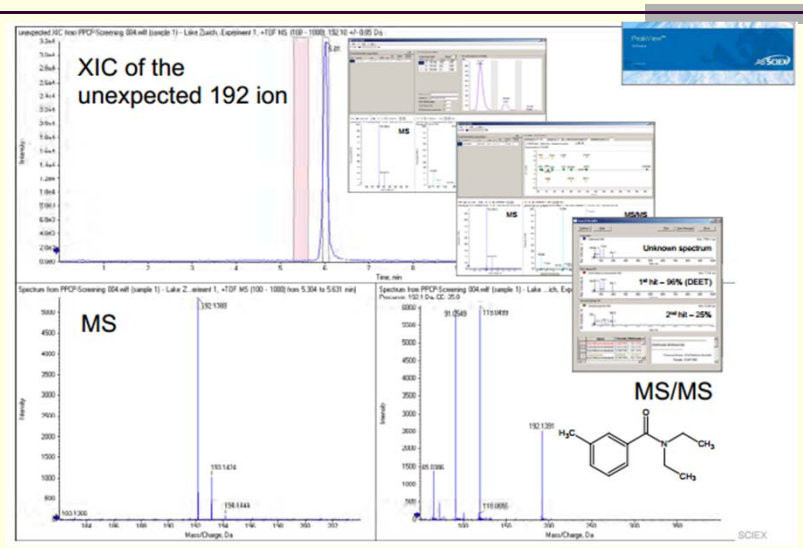
Mass Analysis

Data/Interpretation

Step 5
Data analysis and interpretation

Exact Mass, MS/MS for DEET



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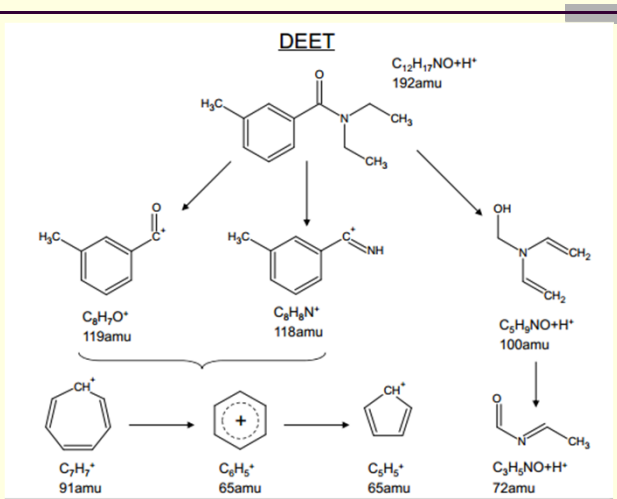
From: Schreiber et al., 2010

MS/MS Transitions for DEET



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From: Schreiber et al., 2010

QA/QC of LC/MS/MS-based PPCP analysis

Experimental design

- Method detection limit (MDL)
- Analytical sensitivity
- Calibration drift
- % Recovery

Typical MDLs and % recoveries

By LC/MS/MS (API 4000 triple quadrupole MS)

Table 1. Target compounds and their common uses, detection limits, and method recovery efficiencies.

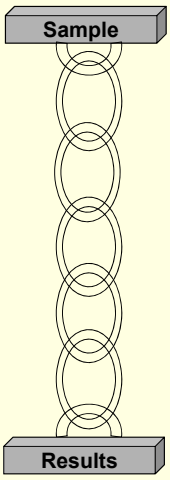
Compound	Use/function	Minimum detection limit	Percent recovery in ultra-pure water ($n = 9$)	Percent recovery in septic effluent (n)
		ng mL^{-1} in extract		
Acetaminophen	pain reliever	0.66	64.3	57.1 (4)
β-estradiol	natural hormone	0.28	91.8	87.6 (3)
Caffeine	stimulant	0.33	91.5†	76.5 (4)
Carbamazepine	antiepileptic	0.90	92.4	61.2 (6)
Carisoprodol	muscle relaxant	0.51	95.6	68.4 (6)
Chlorpropamide	antidiabetic agent	0.64	81.9	55.6 (6)
Estriol	natural hormone	0.23	85.6	87.6 (3)
Estrone	natural hormone	0.72	88.1	52.8 (3)
Ethynyl estradiol	synthetic hormone, contraceptive	0.19	90.8	80.3 (3)
Fenofibrate	blood-lipid regulator	0.86	51.9	18.4 (6)
Fluoxetine	anti-depressant	0.26	79.2	41.9 (6)
Paraxanthine	caffeine metabolite	0.31	74.1	71.0 (4)
Warfarin	anticoagulant	0.71	64.7	48.5 (6)

† $n = 6$ for caffeine.

Wilcox et al., 2009 JEQ

What is causing the lower % recoveries?

The Analytical Chain of LC/MS



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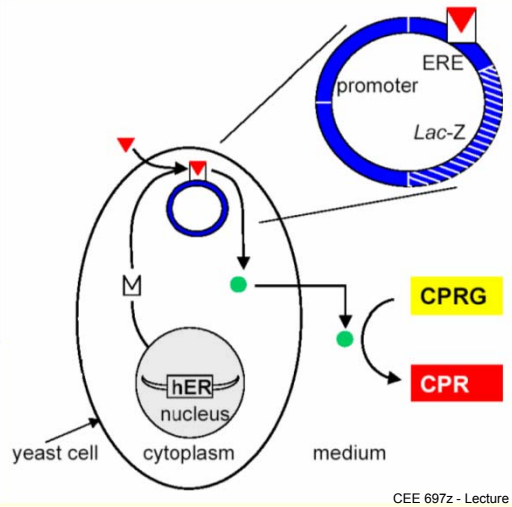
Bioassays

Biological Activity Tests

- Estrogenic and antiestrogenic activity will be assessed by measuring changes in gene expression in the Japanese medaka fish.
  - expose fish to 1-L water sample for 96 hours
  - sacrifice the fish; livers removed
    - other tissues, e.g. gonads and brain also will be removed, stored in RNAlater® and archived for potential future studies or examination of expression of other genes
  - measure vitellogen in mRNA in the liver using real time reverse transciptase PCR (Roche Light Cycler).
    - Detection limit is typically 10 femtomolar
- Vitellogenin, the precursor egg yolk protein
  - normally produced only in female fish - but
  - male fish exposed to xenoestrogens will also produce it.

The Yeast Estrogen Screen (YES) is an *in-vitro* test to measure estrogenic activity



estrogen
estrogen receptor
activated receptor
β -galactosidase

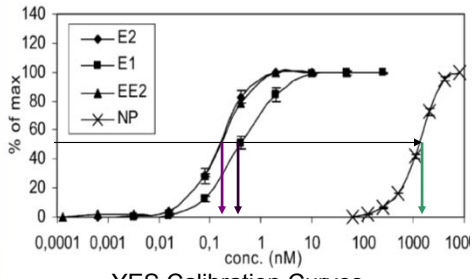
hER: human estrogen receptor gene
ERE: estrogen responsive element
lac-Z: reporter gene
CPRG: chlorophenol red-*β*-D-galactopyranoside

sensitivity:
c (E2) = 10^{-11} mol/L (2.7 ng/L)

Routledge, E.J. and Sumpter, J.P. (1996) Environ. Toxicol. Chem. 15, 241-248

Erik Rosenfeldt

Activity Assay Outputs

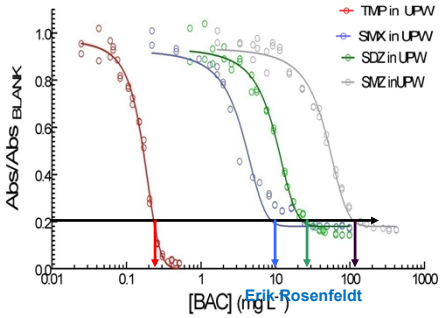


YES Calibration Curves

Compound	EC$_{50}$ (nM)	E2$_{eq}$
E2	~0.21	1.0
E1	~0.3	0.7
EE2	~0.20	1.1
NP	~1050	0.0003

~~Compound~~	~~80% Inhibition (mg/L)~~
SMX	10 (max 82%)
SDZ	~15 (max 83%)
SMZ	~102 (max 82%)
TMP	~0.15 (max 100%)

MIC Calibration Curves



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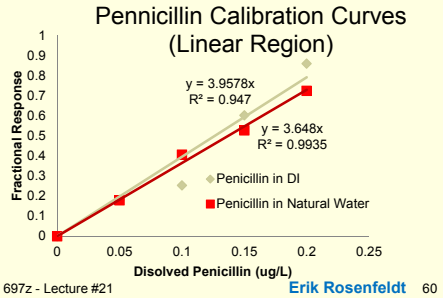
Antibiotic Challenge (ABC)

- Relies upon growth of *Bacillus stearothermophilus* spores
- Test takes 2.5 hours

Negative Positive

Erik Rosenfeldt

ABC Calibrations



Immunoassays

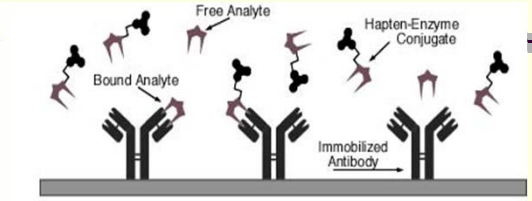
Slide courtesy of Meyer et al., USGS

Immunoassay Types

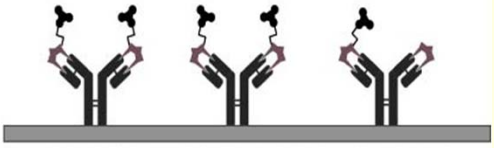
- Enzyme-Linked Immunosorbent Assays
  - Coated
    - Tubes
    - 96-well plate
  - Magnetic Particle
- Radioimmunoassay
  - H_3, C_{14}

Slide courtesy of Meyer et al., USGS

General Immunoassay Concept



Step 1. The unknown analyte in the sample competes with a known amount of hapten-enzyme conjugate.



Step 2. Excess reagents (unbound) are removed by washing the solid-phase with a buffer or distilled water.

Slide courtesy of Meyer et al., USGS

The End

- To next lecture