Measuring Drug Concentrations:
Analytical Tools available today and tomorrow

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Why we measure drugs

- Therapeutic Drug Monitoring/Management
  - Aiding the clinician in management of drug therapy
    - Blood concentration is a surrogate marker of response
  - Maintaining concentrations within narrow therapeutic range
- Monitoring compliance with prescribed medications
  - Pain Management
  - Failure of therapy

- Postmortem toxicology
  - Fatality investigations
- Impairment/Human Performance toxicology
  - Drugs/Alcohol & driving
  - Workplace accidents
- Forensic urine drug testing
  - Criminal justice system
  - Pre-employment screening
  - Transportation industry

Goals of this presentation/Outline

- By the end of this presentation you should be able to discuss:
  - Analytical tools useful in the measurement of drugs in biological specimens
  - GC & GC/MS
  - HPLC & HPLC/MS
  - HPLC/MS/MS
  - Ion Trap HPLC/MS/MS

Past Analytical Tools

- Colorimetry
- UV/Vis Spectrophotometry
- Spectrofluorimetry
- Bioassay

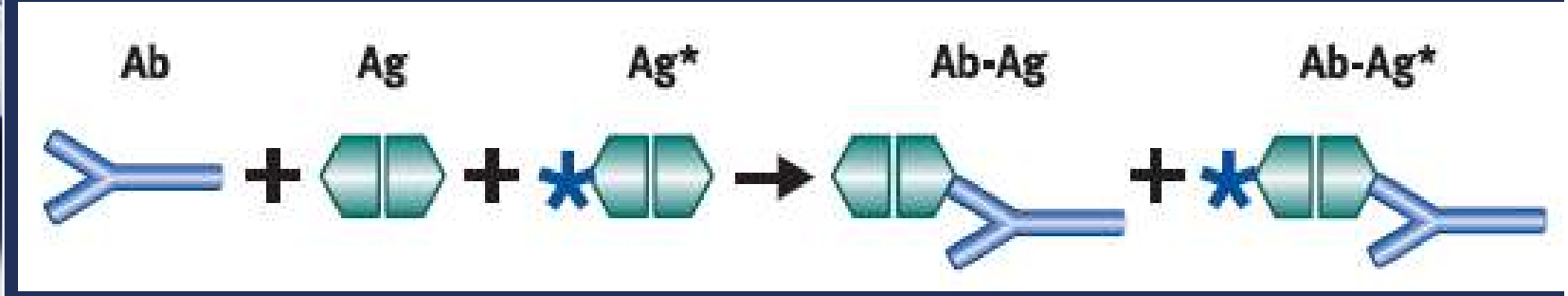
- As stand alone methods, most have been abandoned
  - Lack specificity and/or sensitivity
  - Very labor intensive
  - Lithium is an exception – still frequently measured by atomic absorption spectrophotometry

Present Analytical Tools

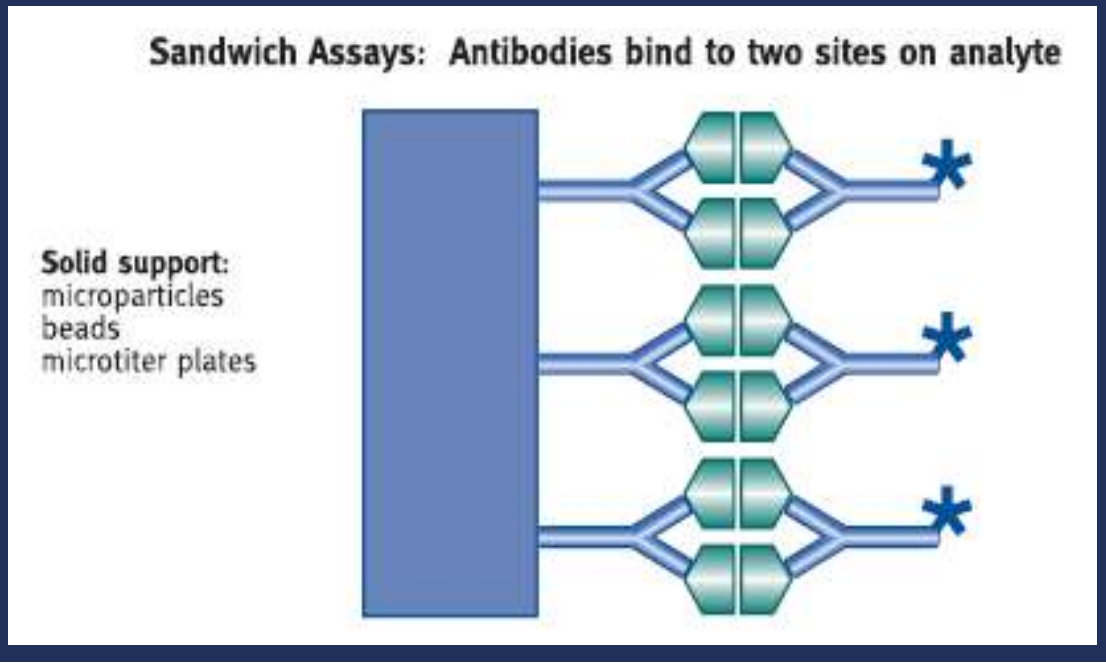
Immunoassay

- Uses antibodies to identify and measure a compound
  - Drug or antibody
- Multiple variations in use
  - ELISA, FPIA, EMIT, CEDIA….
- Precursor to modern Immunoassay – RIA in 1950s
  - First use – measure insulin antibodies in diabetics
  - Evolved to measure numerous hormones in humans
  - 1977 Nobel Prize in Medicine awarded to Dr. Rosalyn Yalow for RIA development

- Competitive Immunoassays
  - Labeled reagent antigen and unlabeled analyte from patient sample compete for antibody



- Non-competitive Immunoassays
  - Analyte from patient sample binds to reagent antibody



- Advantages
  - Technically simple – can be carried out in hospital labs or physician's office labs
  - No extraction
  - Amenable to automation
  - High throughput with short analysis times
  - Quantitative (non-forensic)
- Disadvantages
  - Not suitable as stand alone test for forensic purposes
  - Lengthy method development process
  - Not available for all drugs
  - Lack of specificity
    - Interference from endogenous compounds
    - Cross reactivity with metabolites or related compounds

Chromatography

- Thin Layer Chromatography
- Gas Chromatography
- Liquid Chromatography

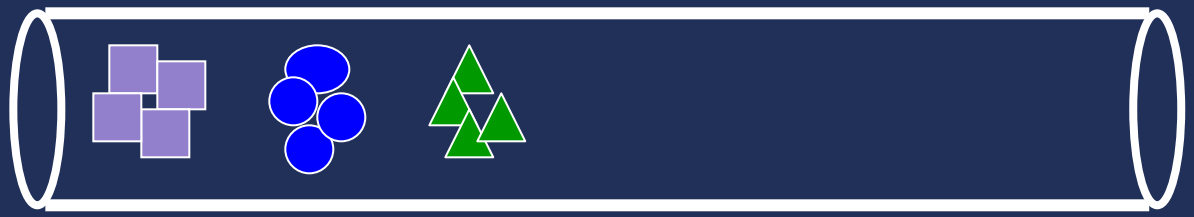
- All involve separation of matrix components
  - Interaction with/affinity for mobile and stationary phases
  - 1952 Nobel Prize in Chemistry for Partition Chromatography (Martin & Synge)

Flow of Mobile Phase

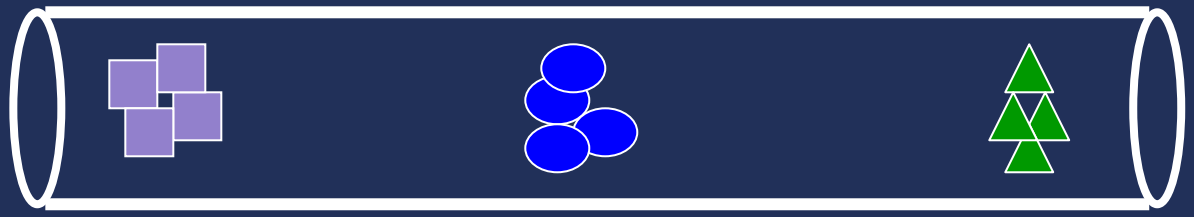
Injector → Detector

T=0

T=5′



T=10′



Most Interaction with Stationary Phase Least

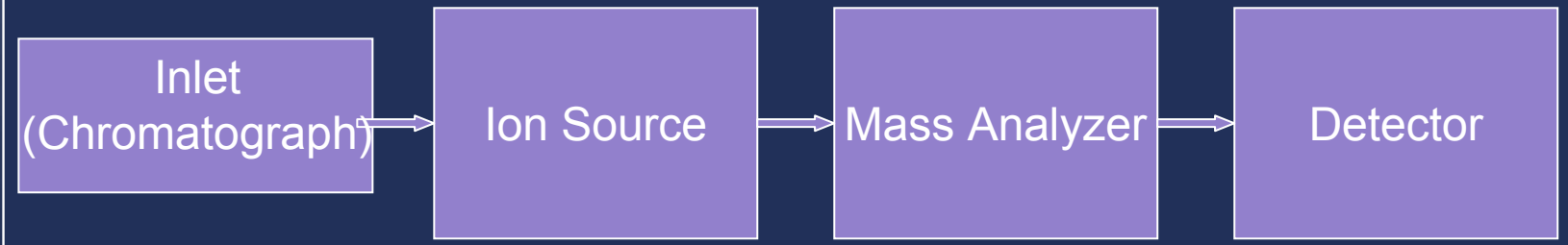
Gas Chromatography

- Sample introduced into the GC and immediately vaporized
- Analytes enter liquid coated capillary GC column
  - Contained in oven for temperature control
- “Carrier gas” flow carries analytes through column
- Compounds separated from each other on column
  - Isothermal or temperature gradient elution
  - Highly nonpolar analytes, cholesterol, elute late
- Detected by signal provided when target compound elutes from the column

- TCD – Thermal Conductivity Detector
  - First GC detector, 3rd most widely used now
  - Universal detector – responds to any gaseous material with a thermal conductivity different than the carrier
- FID – Flame Ionization Detector
  - Most commonly used detector
  - Responds to Hydrocarbons, C-H bonds
- NPD – Nitrogen Phosphorus Detector
  - Hot alkali salt from rubidium or cesium ionizes Phosphorus & Nitrogen containing compounds
- ECD – Electron Capture Detector
  - Electrophilic compounds remove electrons & decrease signal at detector
  - Useful for halogens
- All measure a property of the drug, not the drug itself

Gas Chromatography/Mass Spectrometry (GC/MS)

- Most widely used GC detector in forensic toxicology
- Molecular identification – measures the compound directly, not a property of the compound



Basic schematic of a Mass Spectrometer

Ionization Techniques

- Electron ionization (EI)
  - Most common technique
  - Analytes pass through source containing electron beam
  - High energy causes fragmentation of molecule
  - Fragmentation pattern highly reproducible
  - Large libraries exist
- Chemical Ionization (CI)
  - Electrons ionize a reagent gas
  - Ionized gas transfers proton to analyte ⇒Molecular ion ($M + H^+$)
  - Considered “Soft” Ionization
  - Sensitive, useful for molecules that fragment into non-definitive ions

Quadrupole Mass Analyzer

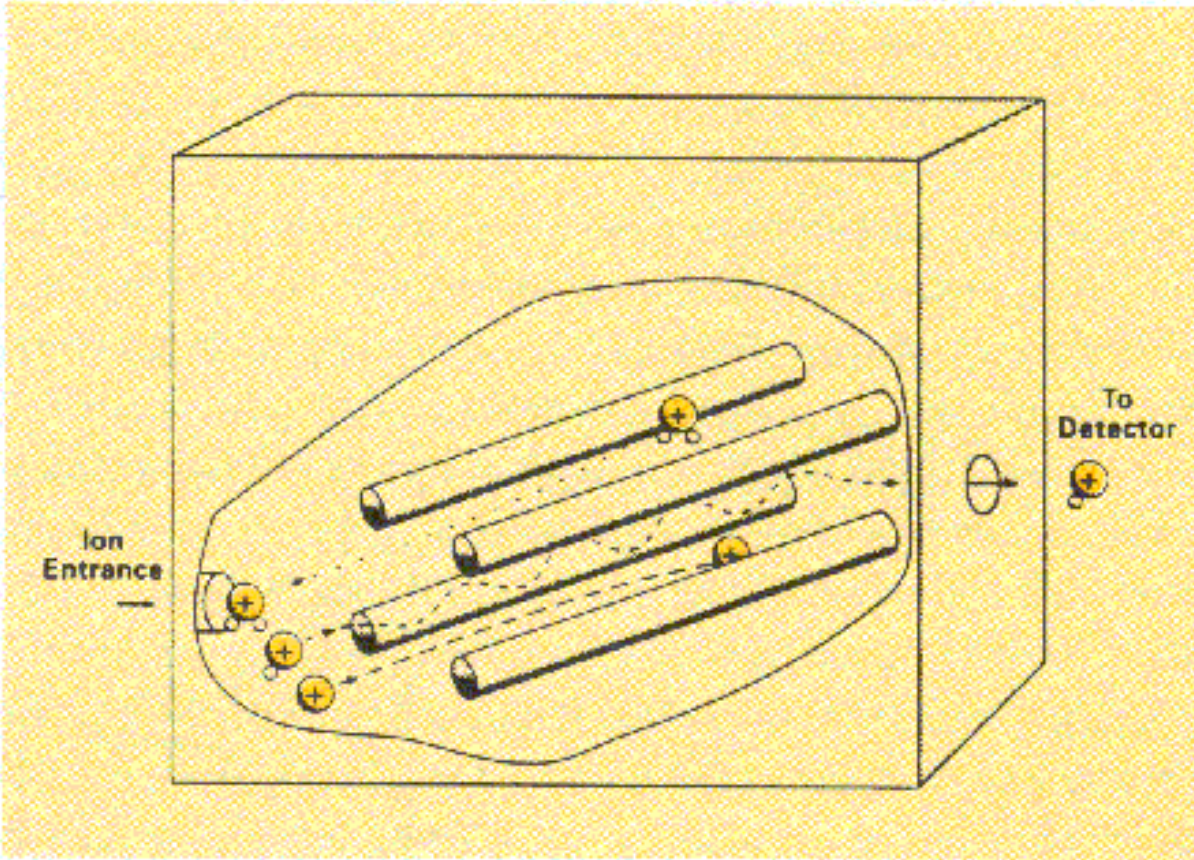


Figure 5
The quadrupole mass filter. Ions with the selected mass-to-charge ratio pass through the analyzer to be collected at the detector, while ions with other m/z values collide with or escape between the rods.

Ion Trap Mass Analyzer

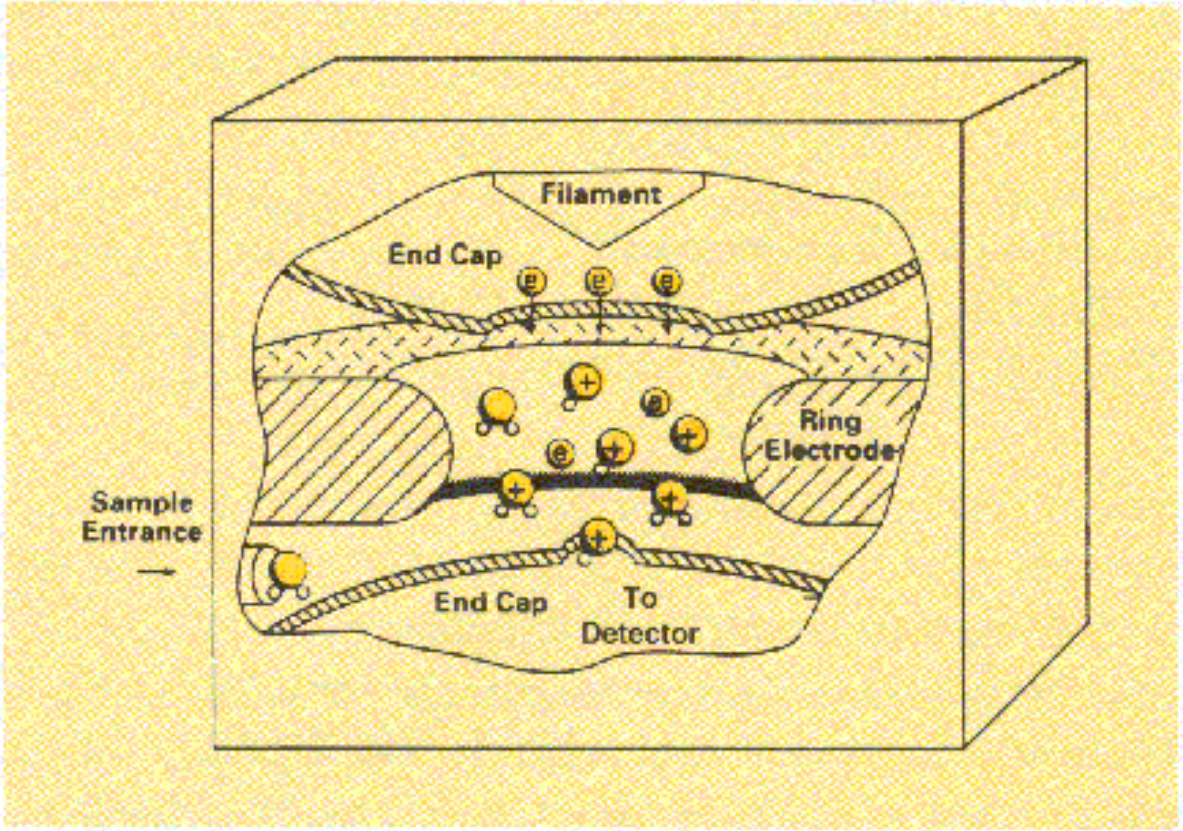


Figure 6 Quadrupole ion trap mass spectrometer. Ionization and mass sorting occur in the space bounded by the ring and cap electrodes. Alternatively, sample ions can be formed externally and then injected through an end cap opening.

Date Collection Modes

- Scan
  - Looking for all ions within specific m/z ratios
  - Ideal for unknowns
- SIM (Selected Ion Monitoring)
  - Looking for specific ions
  - More sensitive
  - Ideal for targeted analyte measurement

- Ion trap has greater sensitivity in scan mode
- Quadrupole has greater sensitivity in SIM mode

High Performance Liquid Chromatography (HPLC or LC)

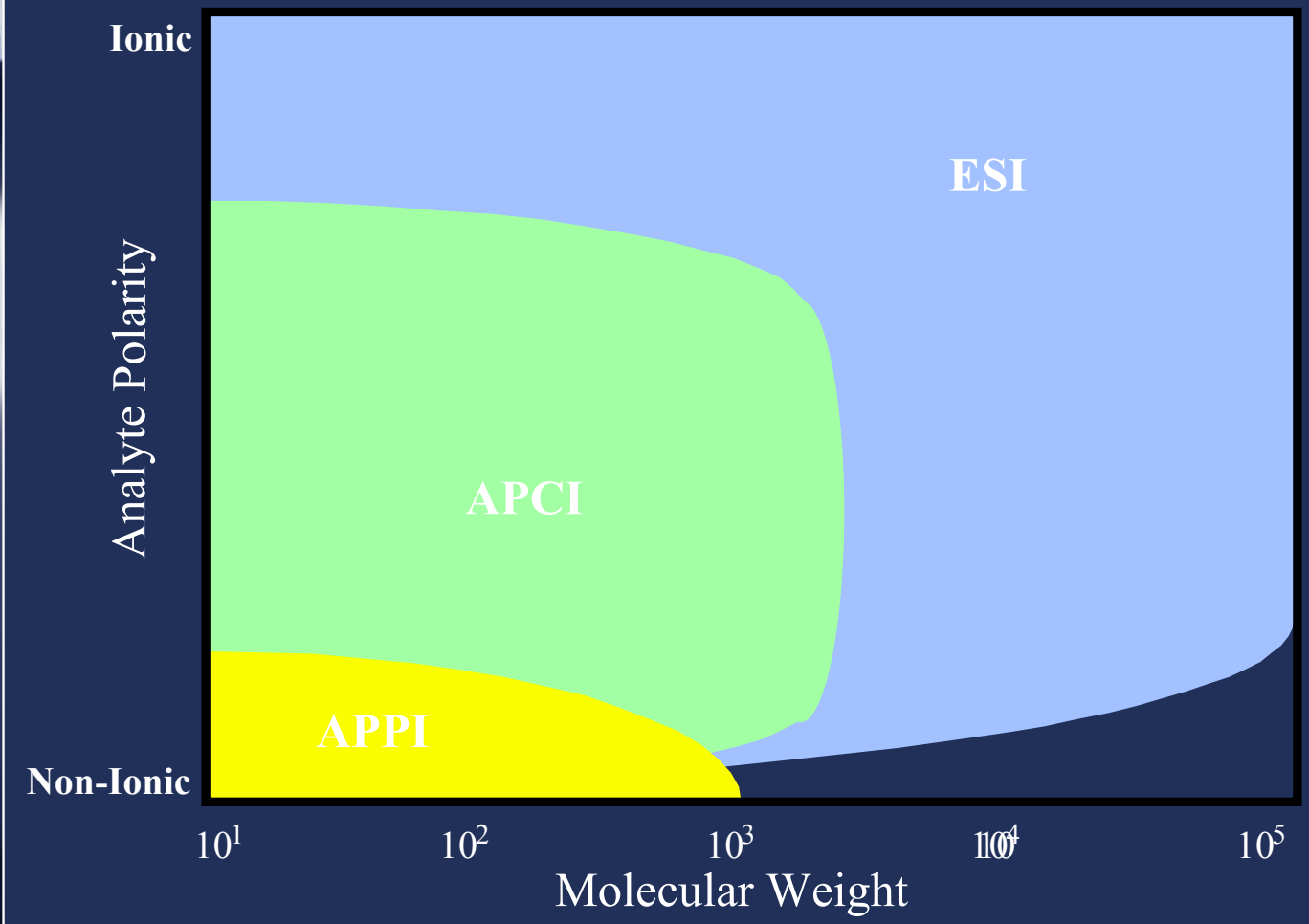
- Applicable to greater variety of analytes
- Separation of compounds
  - Non-polar stationary phase (C_8 or C_{18})
  - Polar mobile phase (aqueous)
  - “Reverse Phase”
- Isocratic or gradient elution
  - Increase strength (polarity) of mobile phase

- UV Detection
  - Fixed or variable wavelength
  - Applicable to wide range of analytes
  - Careful solvent selection (UV cutoff)
  - Potentially high background – many compounds absorb
- Diode array Detection
  - UV beam sent through flow call
  - Captures multiple spectra of flow cell contents
  - Data can be searched against library
- Fluorescence Detection
  - Naturally fluorescent or derivatized
  - Highly Sensitive
  - Limited use/suitability
- Measure a property of the drug, no identification

LC/MS

- Liquid effluent presents problems
  - Vacuum can't keep up with volume of gas
- Atmospheric Pressure Ionization (API)
  - Electrospray Ionization (ESI)
    - Charged droplets evaporate, charged molecules drawn into Mass Spectrometer
    - Part of 2002 Nobel Prize in Chemistry
  - Chemical (APCI)
    - Chemically charged solvent passes charge to analyte
  - Photo (APPI)
    - Photons directly ionize analyte
- Mass Analyzers similar to GC/MS

API Polarity/MW Domains



GC/MS & LC/MS: A Comparison

	GC/MS	LC/MS
Applicability	Limited, ~20% of drugs	Wide
Specificity (metabolites, etc.)	High	High
Speed of Analysis	Medium - Fast	Slow - Medium
Capital cost (equipment)	Medium (~$80K)	High (>$150K)
Reagent costs (consumables)	Low	Medium – High
Skill required	Medium – High	Medium – High
Development effort	Low – medium, many published methods	Medium - High
Specimen Prep	Medium - High	Low - Medium

Future Analytical Tools

- ChromaLynx® Software for Single Stage LC/MS
- Triple Quadrupole Mass Spectrometry (Tandem MS)
- Ion Trap/Quadrupole Tandem MS

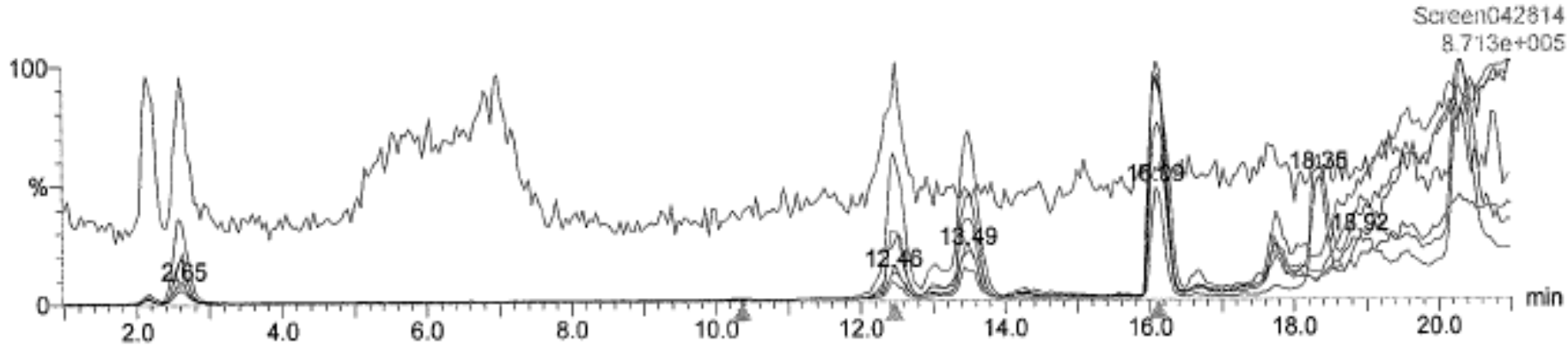
- All are currently in limited use, use is increasing

LC/MS with ChromaLynx

- Ideal for analysis of unknowns
- Uses existing instrumentation
  - Single quad ZQ or Triple quad Quattro Micro
- Collects large amounts of data – every 0.25 sec:
  - Scans from 100 – 700 m/z
  - Voltage change in 15V increments (15 – 90V)
  - 7 functions – 6 positive, 1 negative
- Highly specific!!
- Preloaded library, updatable

Method : C:\MassLynx\ChromaLynx_Training.PRO\METHDB\Basic_Identify_Method_1N3P.idm

Name: Screen042814, ID: 40007167



Candidate: Caffein, RT: 10.36 (10.20), Average Match: 319.8
Status: Positive

Scan	Function	Ret. Time	Rel. Ret. Time	Abundance	Rel. Abundance	Compound Name
220	1	10.3402		86253	0.000	Caffein
221	2	10.3809		208558	0.000	Caffein
220	3	10.3595		624086	0.001	Caffein

Candidate: Cocaine, RT: 12.47 (13.10), Average Match: 554.5
Status: Positive

Scan	Function	Ret. Time	Rel. Ret. Time	Abundance	Rel. Abundance	Compound Name
265	1	12.4657		2018450	0.010	Amoxapine-8-hydroxy
264	2	12.4195		27410022	0.026	Cocaine
265	3	12.4705		34707252	0.028	Cocaine
265	4	12.4773		21001352	0.028	Cocaine
265	5	12.4823		3460580	0.004	Antipyrine
265	6	12.4769		3107261	0.004	Bromazepam

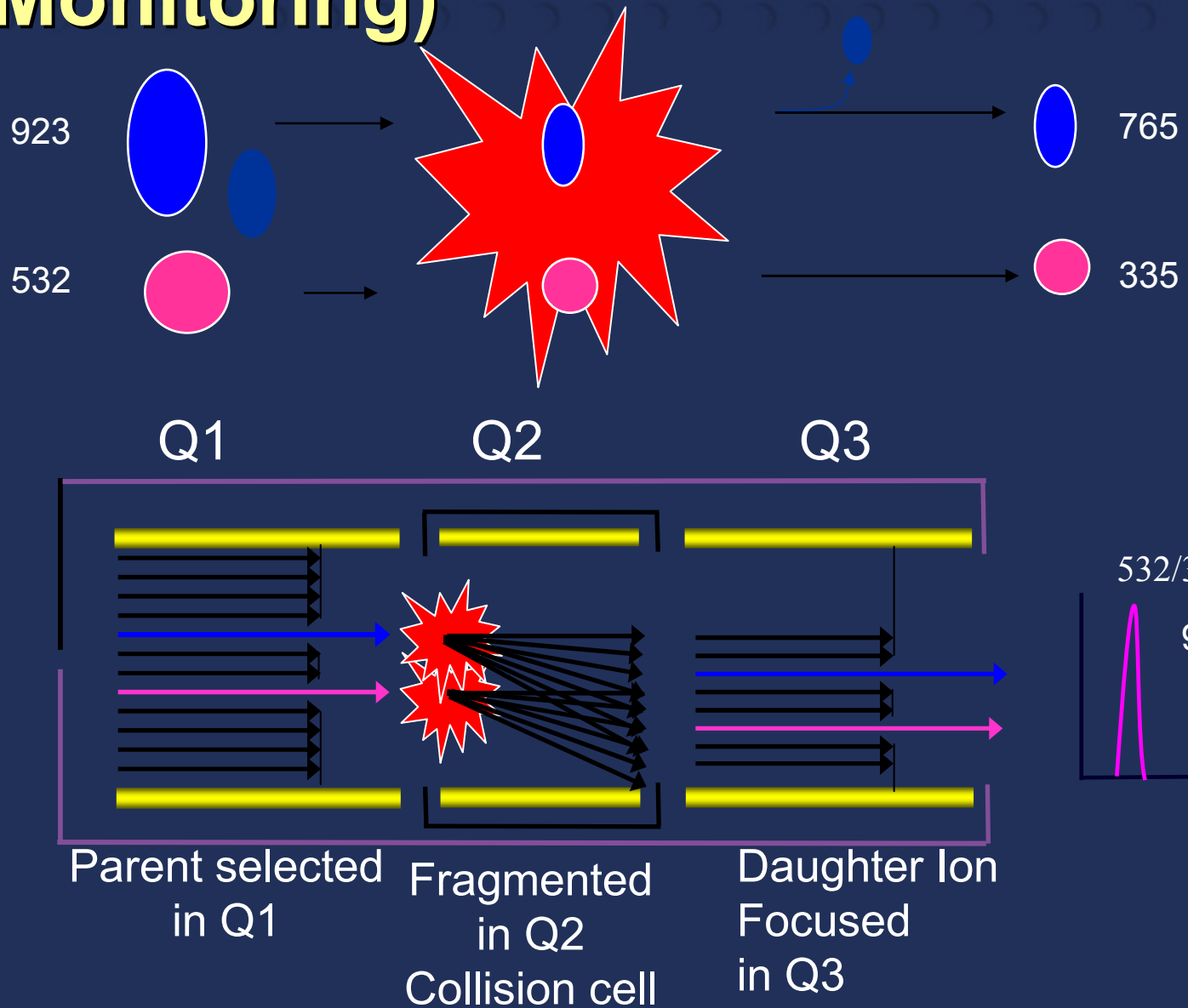
Cost Savings using ChromaLynx

- Our current comprehensive drug screen in blood
  - Panel of 13 ELISA assays
  - GC/MS screen using basic/acidic/neutral SPE
- ~700 specimens per month
- Comprehensive drug screen using ChromaLynx
  - Panel of 3 ELISA assays (Opiates, Oxycodone, PCP)
  - Same SPE extraction
  - Same instrument time (~26 minutes/sample)
- Approximately $0.90/ELISA test
  - (0.90 X 10) X 700 samples = $6300 savings per month!!

Triple Quadrupole Mass Spectrometry: LC/MS/MS

- First Quadrupole (Q1)
  - Selects the molecular ion (parent)
  - Directs it to the collision cell
- Collision Cell (q2)
  - Low flow of Argon or Nitrogen is collision gas
  - Collision of molecular ion with argon causes disintegration into daughter ions
- Second Quadrupole (Q3)
  - Selects daughter ions based on characteristic mass/charge ratio
  - Send selected daughters to detector

MS/MS – MRM (Multiple Reaction Monitoring)



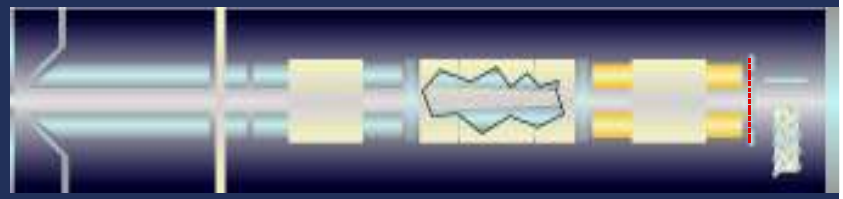
LC/MS/MS

- Advantages
  - Very high selectivity
  - Works with “dirty” samples
  - Less stringent chromatography/separation requirements
- Disadvantages
  - High capital costs ($250K)
  - Lack of standardized methods

Quadrupole vs. Ion Trap

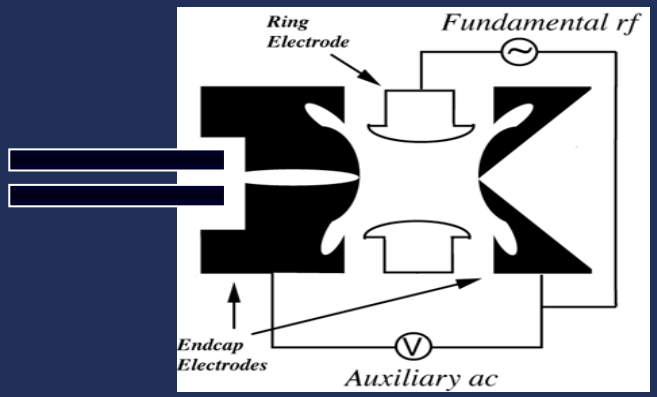
Triple Quadrupole MS

- Great for quant (MRM)
- Broad Dynamic range
- Very selective scans

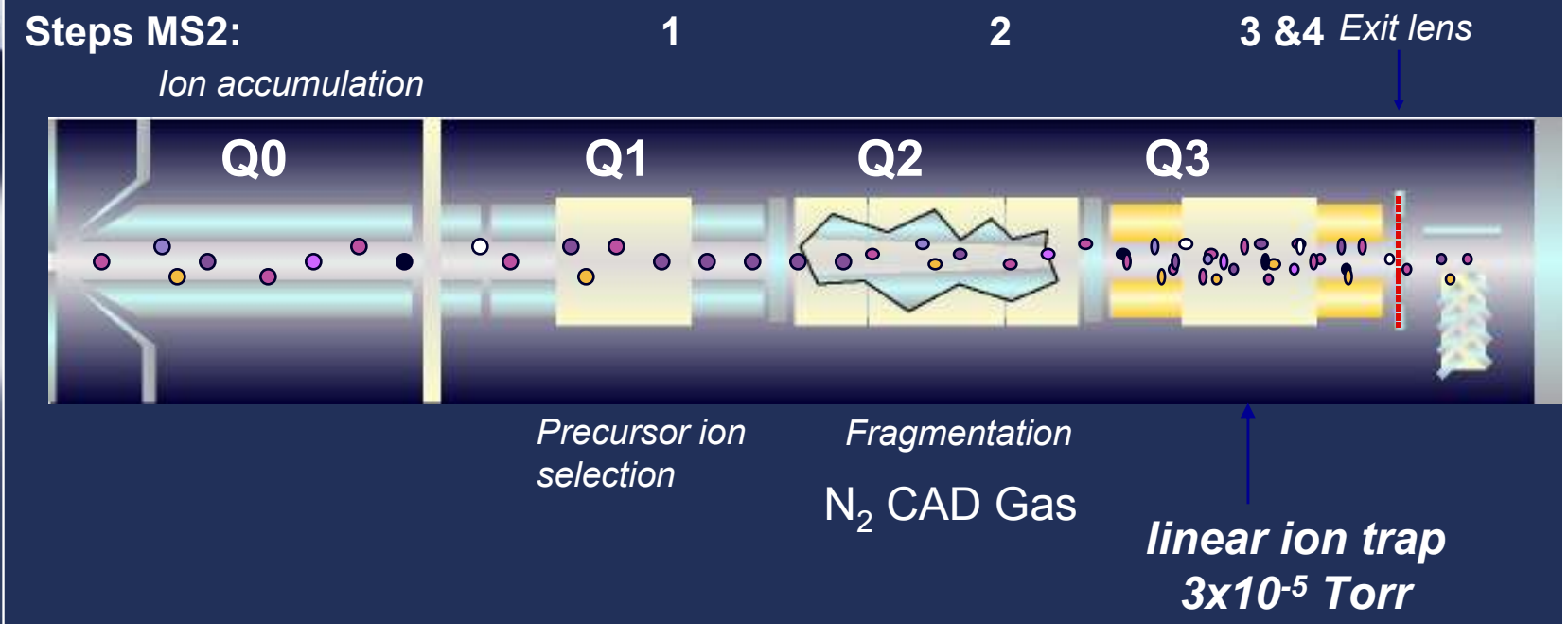


Ion Trap MS

- Poor Quantitation
- Only product ion scans
- Good Sensitivity

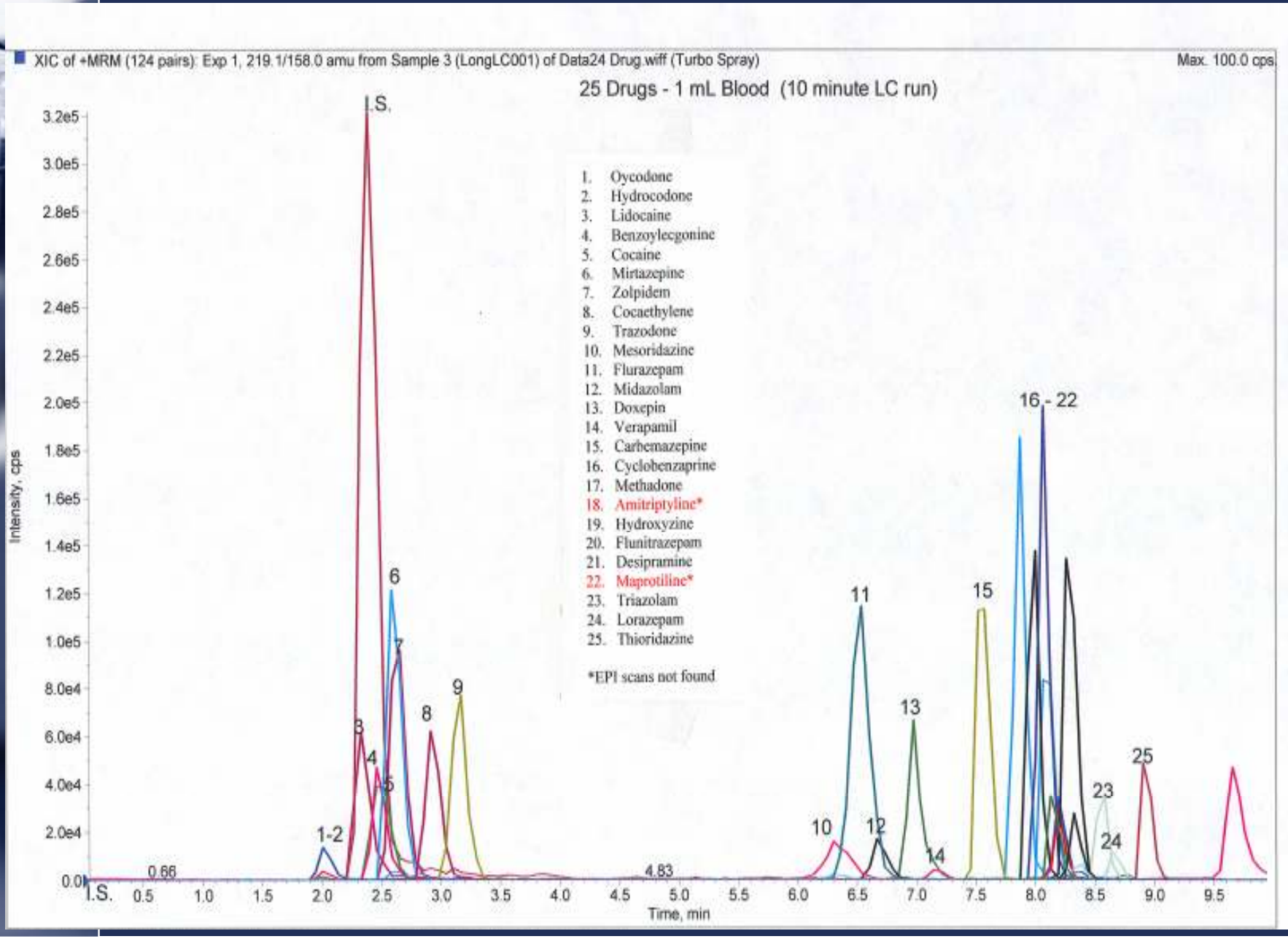


Q TRAP® System - Hybrid



1. Precursor ions selection in Q1
2. Fragmentation in Q2 – monitoring of ~100 transitions
3. Trap products in Q3-short
4. Mass scan

Drug Analysis in Biological Fluids



QTrap® Advantages

- Increased sensitivity & selectivity
  - Essentially MS^3
- Provides ID, confirmation & quantitation in one run
  - MRM for quantitation
  - MS/MS to scan for unknowns
- Full fragmentation for library comparison
- Reduced sample preparation
- High throughput, reduced analysis times

Analysis of Urine Opiates

- Analytes measured
  - Morphine, Codeine, Hydrocodone, Hydromorphone, Oxycodone, Oxymorphone
- Current Method
  - Hydrolysis
  - SPE
  - GC/MS in SIM
  - ~10 minutes total run time (injection to injection)
- Q-Trap Method
  - Hydrolysis
  - “Dilute & Shoot”
  - ~5 minute total run time
- Significant savings in terms of labor, reagent/SPE cartridges, instrument time

LC/MS Systems used in our Laboratory

System	Company	Type	Application
HPLC-ZMD	Waters	Single quadrupole	TDM Drug Screens (ChromaLynx)
HPLC-ZQ	Waters	Single quadrupole	TDM
HPLC – Quattro Micro	Waters	Triple quadrupole	TDM, Blood and Urine DOA – single drug/class
HPLC - Q-Trap	Agilent/ABI	Triple quadrupole with Ion Trap	Drugs of Abuse in Urine & Blood