Unknown Screening using UPLC/TOF MS

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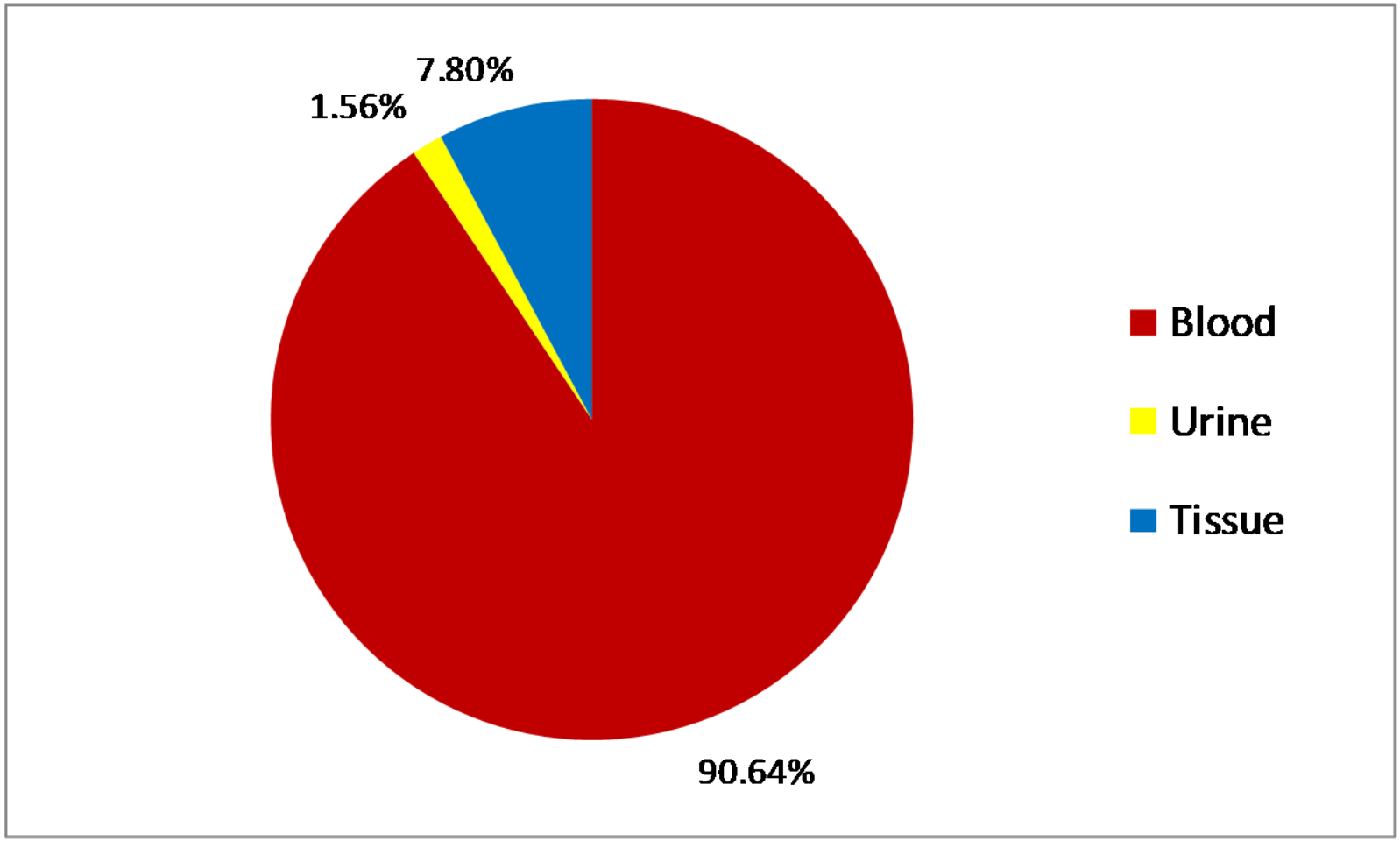
Agenda

- Introduction
  - General unknown screening (GUS) at AIT
  - Technologies for GUS
- Technical Aspects of our method
  - Intro to TOF-MS
  - Developing the method
  - Running the method
    - QC
    - Extractions
    - Calibration
  - Side by side comparison with GC/MS
- Results Interpretation
- Case Studies

Our applications for broad spectrum testing

- Postmortem toxicology
- Drug facilitated sexual assault
- Suspected poisoning with unknown substances
- Crime scene evidence
- Compliance/non-compliance – prescription meds
- Impairment/Human Performance toxicology
- Therapeutic Drug Monitoring/Management
- Meconium testing

Our testing mix – Q1 & Q2 2008



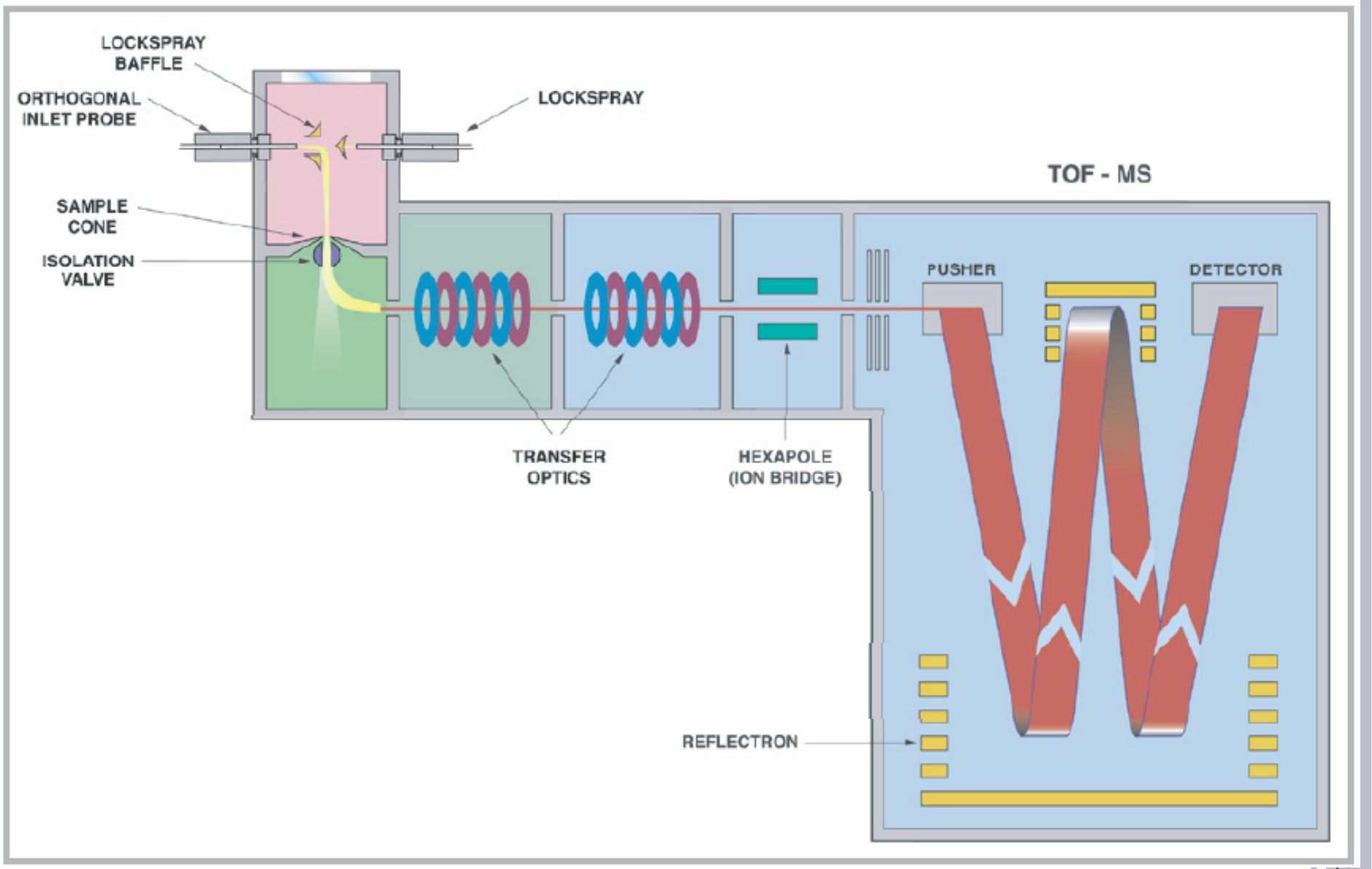
Technologies for comprehensive screens

- Immunoassay arrays
  - No sample pretreatment, automatable
  - Limited tests available, requires confirmation
- GC/MS
  - Library match using mass data
  - Extensive sample prep, limited use for non-volatile analytes
- HPLC (Remedi system)
  - Potentially larger library because of LC
  - Vendor discontinuing service, requires confirmation
- LC-MS/MS
  - “Crash & shoot” or “dilute & shoot” often possible
  - Quadrupole, ion trap, time of flight (and combinations) all in use
  - Expensive equipment, technically complex

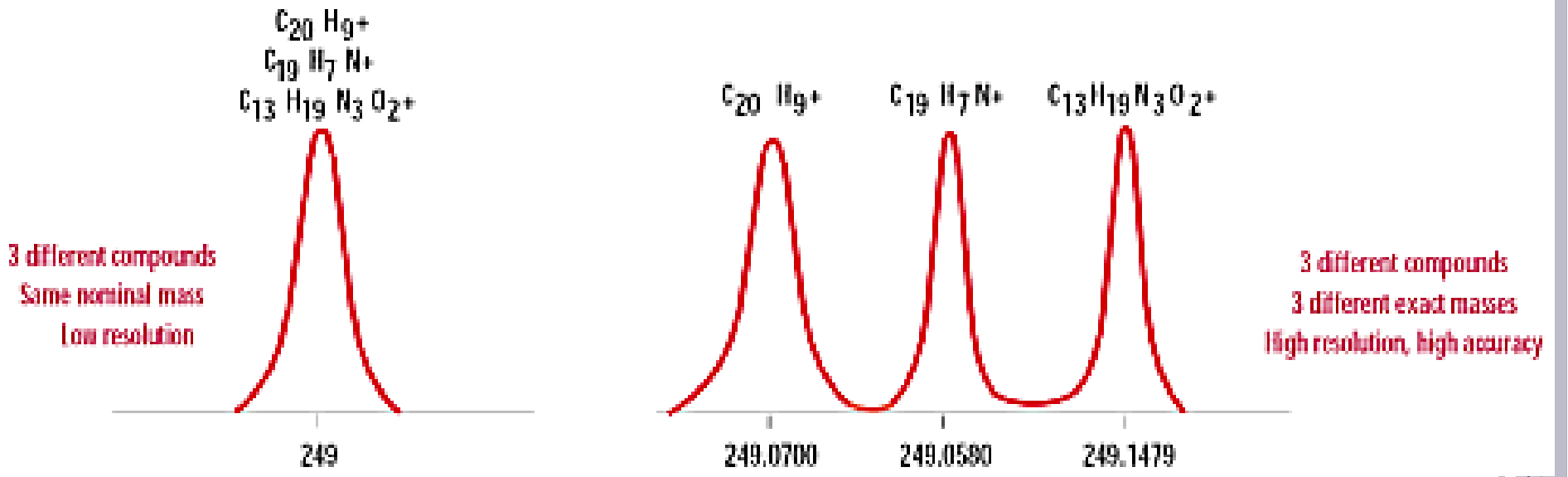
Time of Flight Mass Analyzer

- Very accurate clock!
- Ions formed in an ion source are accelerated to a high velocity by an electric field into a long 'drift tube'
- The ions pass along the tube until they reach a detector
- The time taken for an ion to traverse the analyzer is inversely proportional to its velocity, hence proportional to its mass
- Each m/z value has its characteristic time–of–flight from the source to the detector.

Time of Flight Mass Spectrometer



TOF ALLOWS HIGH RESOLUTION



Waters Acquity with LCT Premier

Library Creation

- Acquire reference standard from supplier
- Dissolve reference standard in the appropriate solvent
- Dilute solution to 1 μg/mL
- Inject reference standard on UPLC/TOFMS
- Calculate exact $[M+H]^+$
- Extract theoretical exact $[M+H]^+$ out of the TIC
- Record the retention time of the analyte
- Make a new entry in the method and add the appropriate information (i.e. analyte name, aliases, exact $[M+H]^+$, retention time, along with any integration and tolerance parameters.)

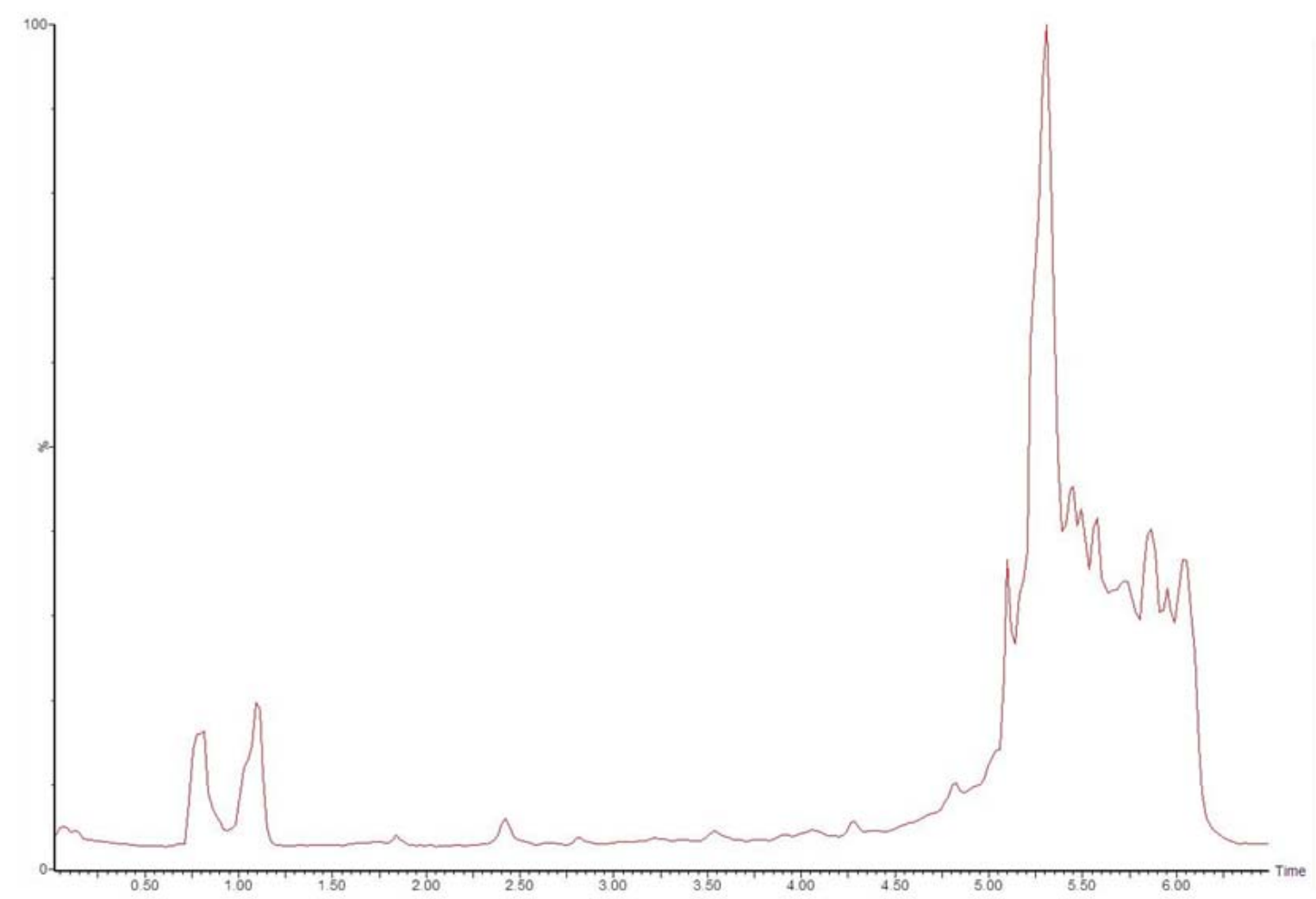
Partial Analyte Library – entire library contains >300 compounds

Anesthetics - Lidocaine, Procaine, Midazolam, Ketamine, Bupivacaine, Mepivacaine, Ropivacaine, Pramoxine, Thiopental, Methohexital
Antibiotics – Azithromycin, Piperacillin, Chloramphenical, Ciprofloxacin, Ceftriaxone, Tilmicosin, Clindamycin
Anticonvulsants – Primidone, Valproic Acid, Lamotrigine, Zonisamide, Phenytoin, Levetiracetam, Gabapentin, Felbamate, Carbamazepine, Topiramate
Antidepressants – Fluoxetine, Sertraline, Amitriptyline, Bupropion, Escitalopram/Citalopram, Venlafaxine, Duloxetine, Fluvoxamine, Phenelzine, Trazodone
Antipsychotics – Olanzapine, Risperidone, Paliperidone, Aripiprazole, Quetiapine, Chlorpromazine, Haloperidol, Loxapine, Pimozide, Ziprasidone
Cardiovascular – Verapamil, Propranolol, Mexiletine, Papaverine, Atenolol, Metoprolol, Quinidine, Warfarin, Amiodarone, Furosemide, Diltiazem
Hallucinogens – Phencyclidine, Lysergic Acid Diethylamide, Mescaline
Narcotics – Alfentanil, Dextromethorphan, Meperidine, Propoxyphene, Buprenorphine, Fentanyl, Sufentanil, Alfentanil, Pentazocine, Tramadol
NSAIDs – Ibuprofen, Naproxen, Piroxicam, Fenoprofen, Flurbiprofen, Tolmetin, Diclofenac, Ketoprofen, Sulindac, Indomethacin
Opiates – Morphine, Codeine, Diacetylmorphine, 6-Monoacetylmorphine, Hydrocodone, Hydromorphone, Oxycodone, Oxymorphone
Sedative and Hypnotics – Orphenadrine, Promethazine, Zolpidem, Flunitrazepam, Methaqualone, Glutethimide, Pentobarbital, Eszopiclone/Zopiclone
Stimulants – Amphetamine, Methamphetamine, MDMA, MDA, Methylphenidate, Benzphetamine, Methcathinone, Caffeine, Nicotine, Cotinine
Miscellaneous – Tolbutamide, Chlorpropamide, Trihexyphenidyl, Betamethasone, Dexamethasone, Oxymetazoline, Strychnine, Methocarbamol, Metoclopramide, Donepezil

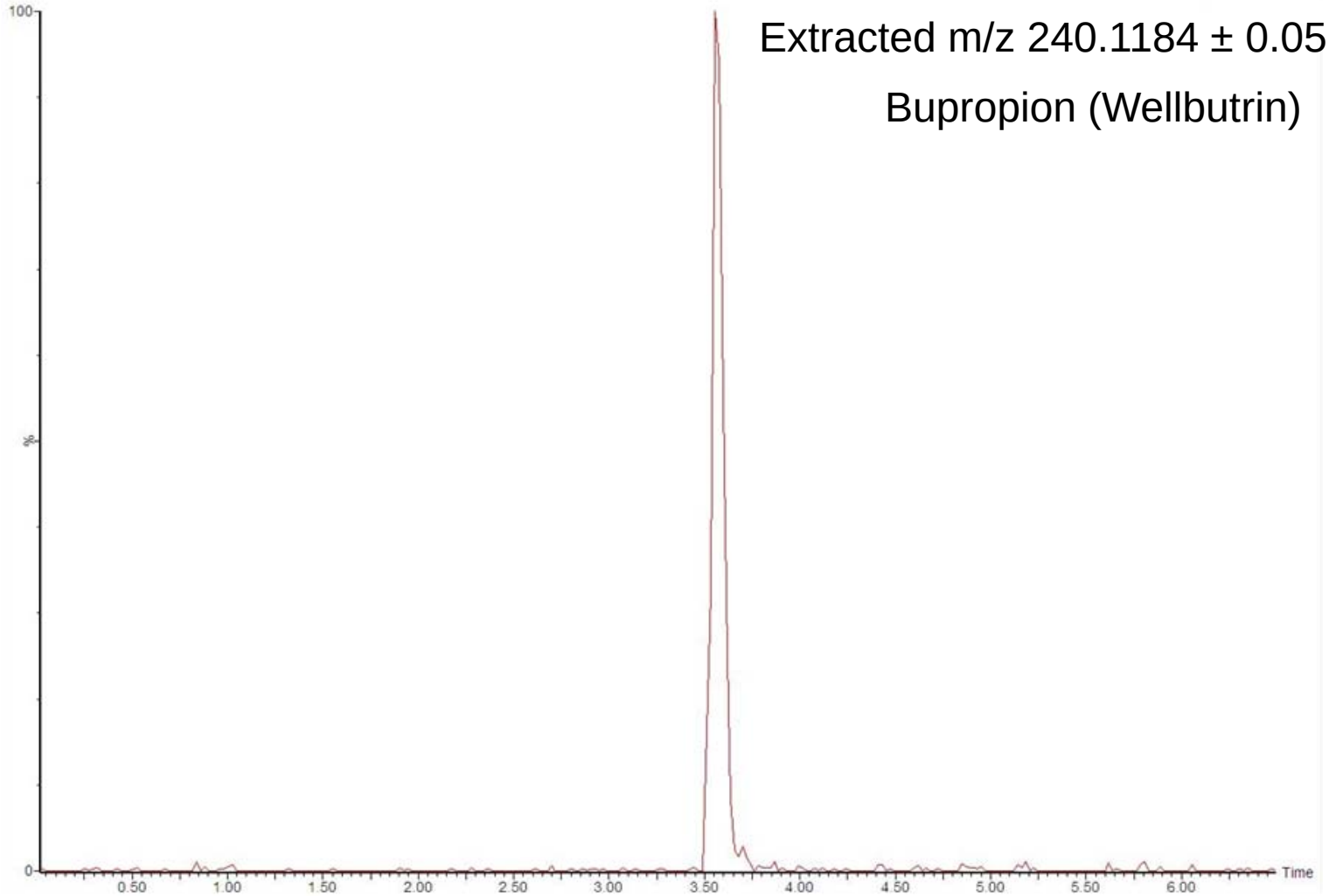
Quality Control

- Each batch of specimens
  - 7 positive quality control samples that include all analytes in custom library – processed exactly like actual specimen
  - 1 negative quality control sample – processed exactly like actual specimens
  - Internal standard (proadifen)added to all specimens
  - Discarded red cells reconstituted with saline
- Daily calibration with sodium formate over entire mass range
- Continual calibration with lockspray feature
  - Every 24 data sets – Leucine-Enkephalin infused
  - Linear time *vs* m/z relationship allows for single point calibration

Total Ion Chromatogram



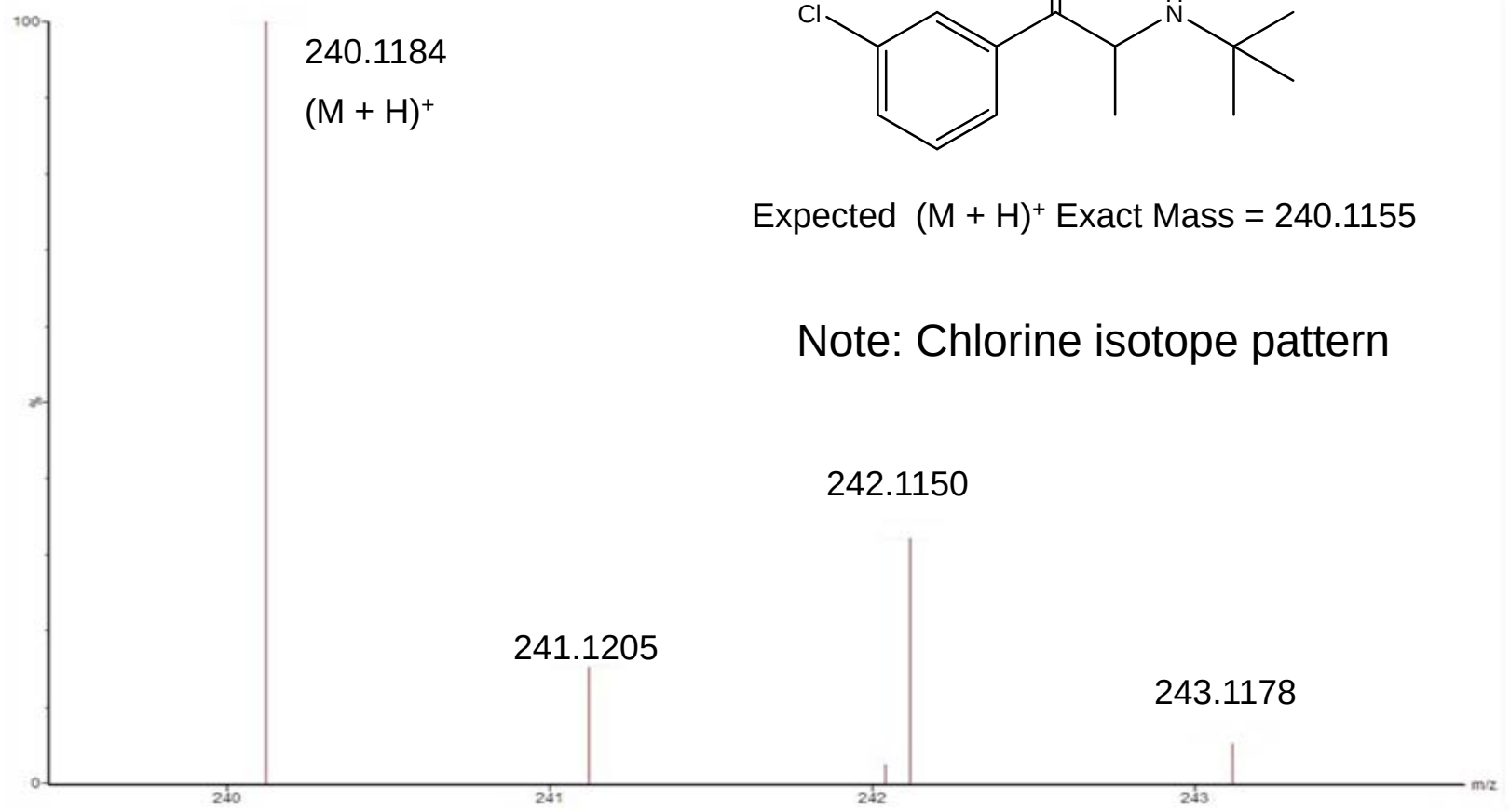
Extracted ion chromatogram



Extracted m/z 240.1184 ± 0.05 amu

Bupropion (Wellbutrin)

Mass Spectrum



Expected $(M + H)^+$ Exact Mass = 240.1155

Note: Chlorine isotope pattern

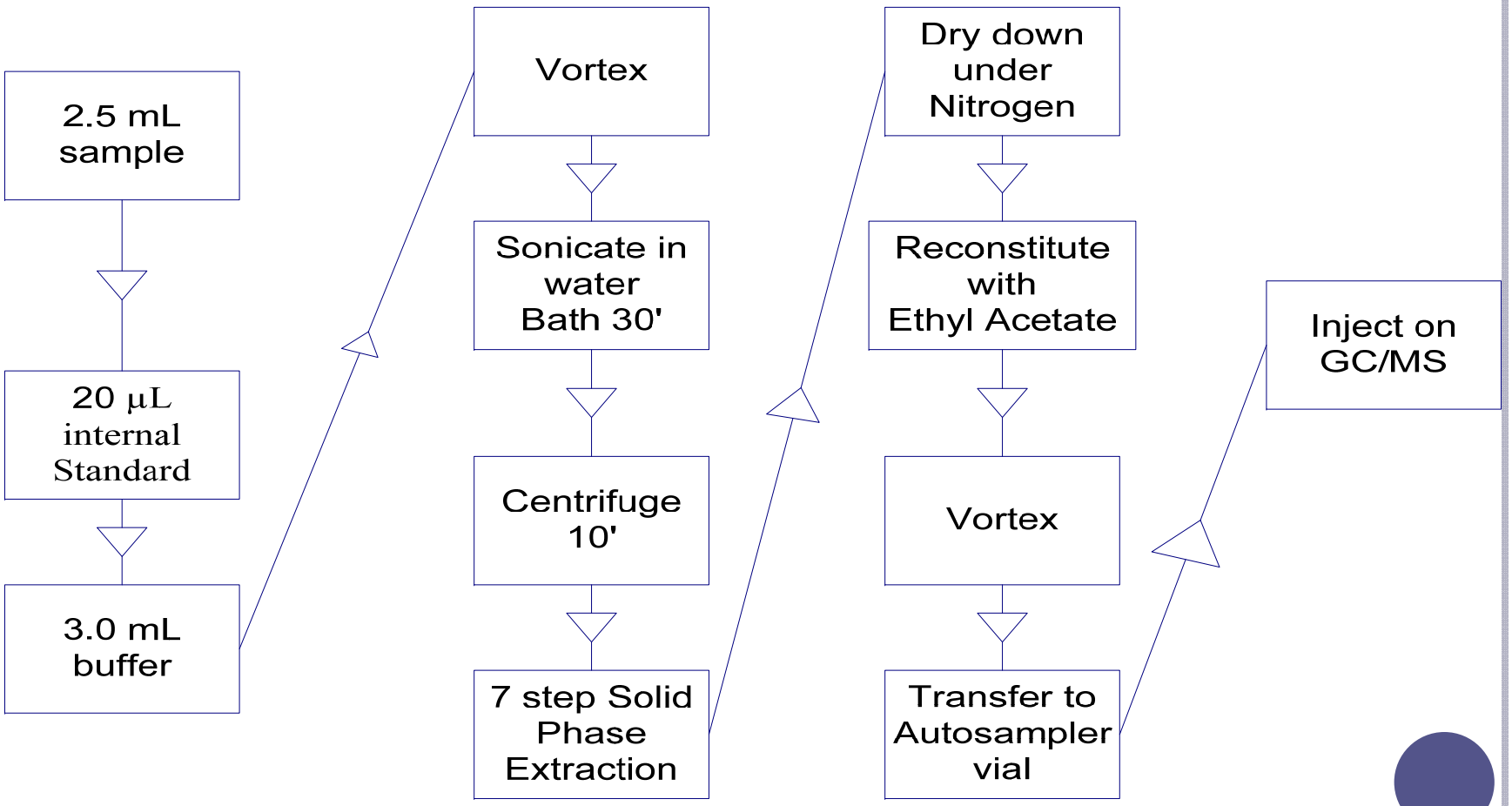
'Old' Screen Approach

- ELISA screen for 13 drug classes
- GC/MS screen for therapeutics and other drugs of abuse
- Methodology limits # of drugs that can be detected
  - Predominantly fragments (not always molecular ion), sensitivity problems for many analytes

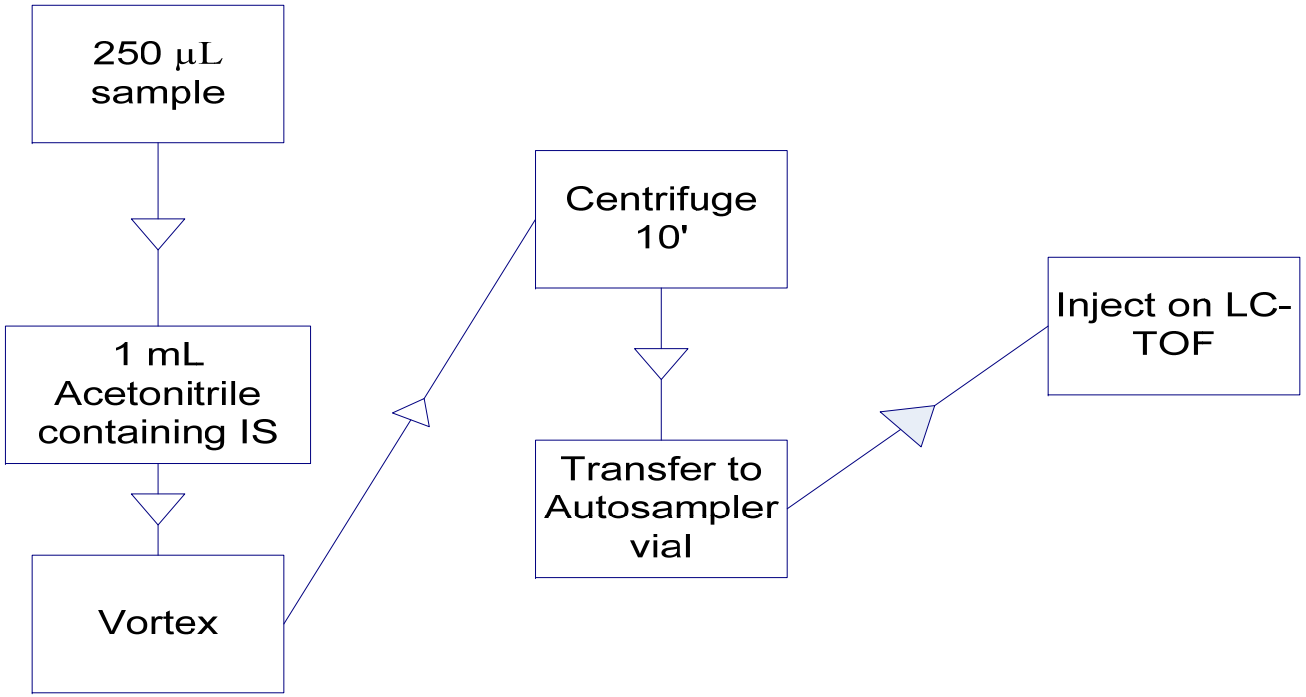
'New' Screen Approach

- ELISA screen for 2 drug classes (opiates, cannabinoids)
- UPLC/TOF screen for expanded number of therapeutics, drugs of abuse – ~300 drugs currently in library
- Performed on Waters LCT Premier (UPLC/TOF)

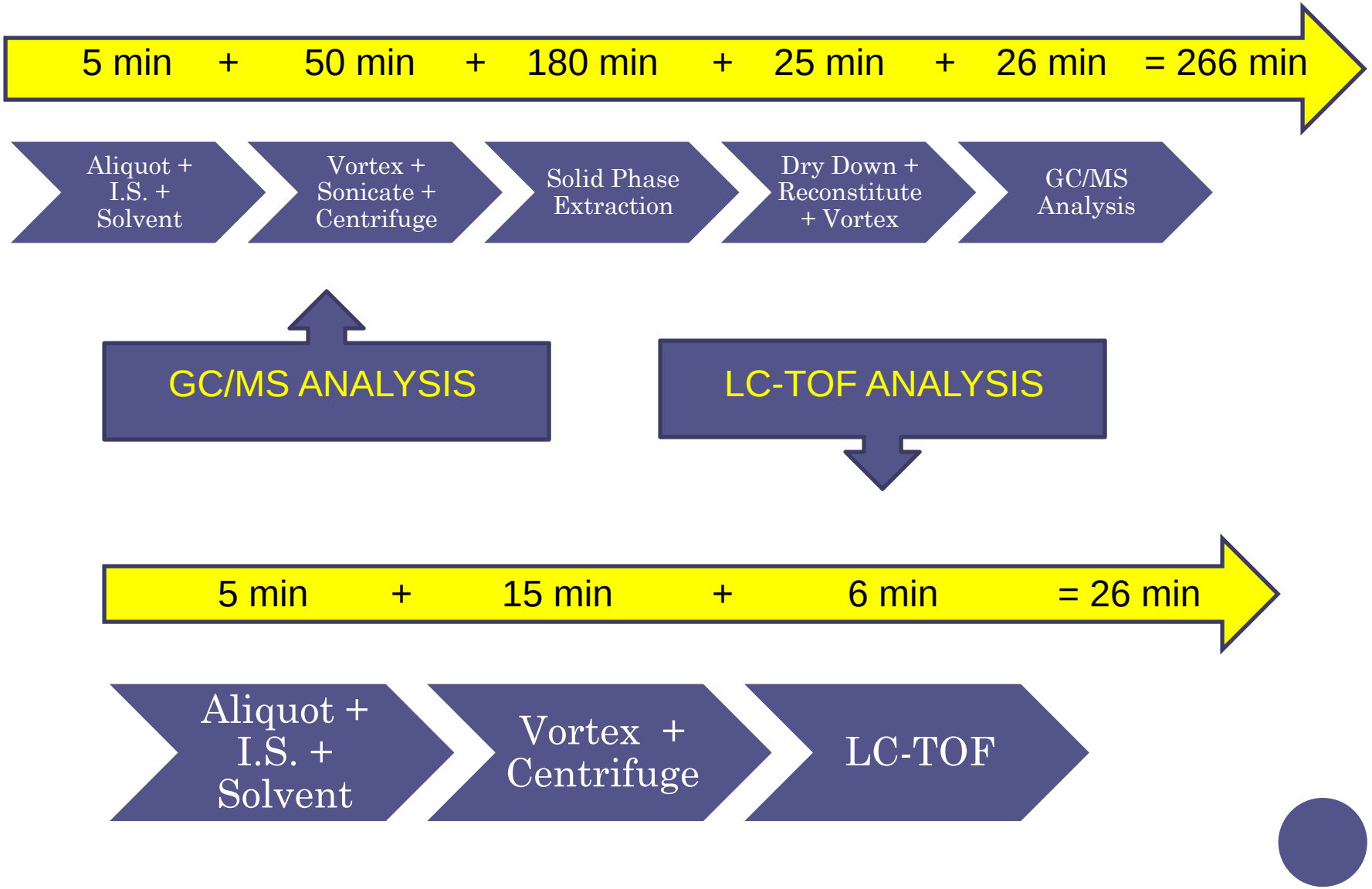
GC/MS Extraction Flow Chart



TOF Extraction Flow Chart



Timeline Comparison



"In-Production" numbers - 53 sample 'parallel' batch

TOF Data

- Extraction Time is approximately 1.5 hours
- Run Time (Minutes) on TOF: 9 minutes per sample
- Total Run Time (Minutes) on TOF: 477 minutes
- 477 minutes = 7.95 hours

GC/MS Data

- Extraction Time is approximately 4 hours
- Run Time (Minutes) on GC/MS: 26 minutes per sample
- Total Run Time (Minutes) on GC/MS: 1,378 minutes
- 1,378 minutes = 22.97 hours

UPLC-TOF Broad-Spectrum Screens

- Savings of 2.5 hours prep time
- Savings of 15 hours run time
- Elimination of 11 of our 13 ELISA tests
  - Opiates, THC still performed by ELISA
- Elimination of labor- and cost-intensive solid phase extraction
- Analytes can be run semi-quantitatively, saving confirmations
  - Acetaminophen

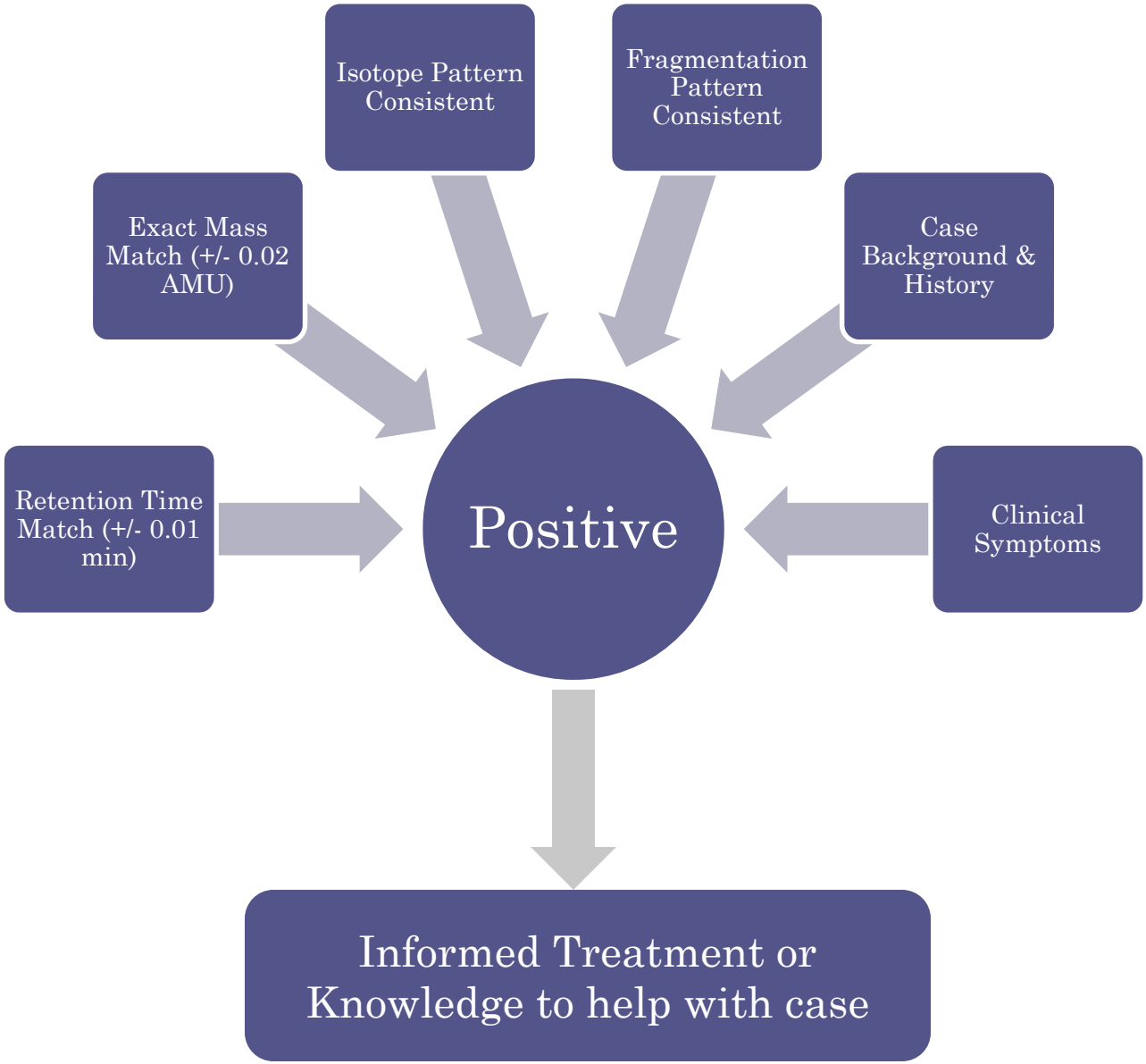
Parallel Specimens Comparison

Number	GC/MS and ELISA Results	UPLC/TOFMS Results
11	Acetaminophen, Cyclobenzaprine, Opiates	Acetaminophen, Cyclobenzaprine, Morphine, Citalopram
20	Opiates, Cocaine	Morphine, 6-MAM, Codeine, Cocaine, Benzoylecgonine
30	Promethazine, Methadone, Barbiturates, Opiates	Promethazine, Methadone, EDDP, Phenobarbital, Morphine, Flurazepam, Desalkylflurazepam, Methocarbamol, Hydroxyzine
49	Lidocaine	Lidocaine, Amiodarone
64	Promethazine, Methadone	Methadone, EDDP
83	Negative	Carbamazepine
87	Fluoxetine	Fluoxetine, Norfluoxetine, Flecainide
107	Ibuprofen, Lamotrigene, Amitriptyline, Nortriptyline, Bupropion, Diphenhydramine, Chlorpheniramine, Promethazine, Methadone	Ibuprofen, Lamotrigene, Amitriptyline, Nortriptyline, Bupropion, Diphenhydramine, Chlorpheniramine, Promethazine, Methadone, Propranolol, Buspirone, Topiramate
110	Negative	Carbamazepine, Carbamazepine-10,11-epoxide
114	Venlafaxine, Norvenlafaxine, Cocaine	Venlafaxine, Norvenlfaxine, Benzoylecgonine, Topiramate
115	Ibuprofen, Verapamil, Opiates, Benzodiazepines	Ibuprofen, Verapamil, Desmethylverapamil, Diazepam, Nordiazepam, Morphine, Codeine, Sildenafil

If a Confirmatory run is desired…

- Single or tandem quadrupole mass spectrometry is common – with ionization conditions sufficient to create fragmentation – fragmentation pattern provides what is effectively a "chemical fingerprint" pattern for identification
- TOF is a good option
  - Same extraction, different aliquot of sample
  - In-source fragmentation by increasing the cone voltage
  - Allows a spectral match using fragmentation pattern along with accurate mass measurement

Results Interpretation



Case Study 1 – Determination of an Unknown Analyte

- History
  - Male found deceased on a farm outside of a barn. A puncture wound is seen on the decedent's wrist. A syringe is found in close proximity to the victim. The syringe and the deceased's postmortem specimens (blood, urine, vitreous fluid) were all sent to the laboratory for screening.
- Results
  - The syringe was rinsed with Methanol:DI Water. The syringe rinse and postmortem specimens were run according to the outlined screening method.
  - Urine specimen was negative on immunoassay array
  - Blood specimens were negative for all drugs of abuse and therapeutic drugs contained within the accurate mass library BUT an unknown analyte peak at 3.57 minutes with a mass of 869.57 was observed in both specimens.

Case Study 1 – Determination of an Unknown Analyte

- Investigation
  - Research was conducted to find a drug or chemical that had a mass equal to 869.57 Daltons.
  - It was found that a macrolide antibiotic drug for use in cattle, sheep, and goats (Tilmicosin, trade name Micotil®) fit the requirement.
  - A reference standard was obtained from a supplier and spiked in blood.

It's a match!

Figure 3

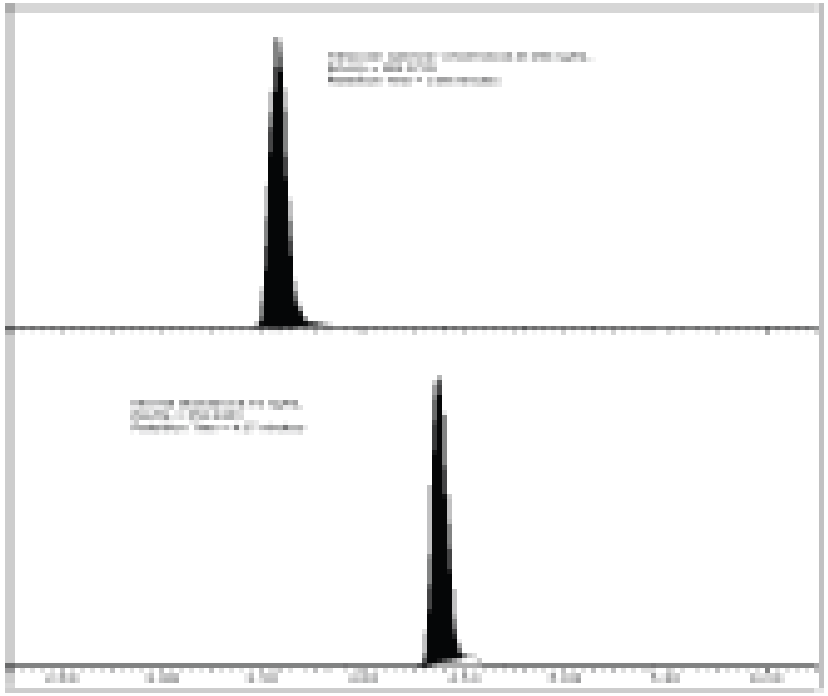
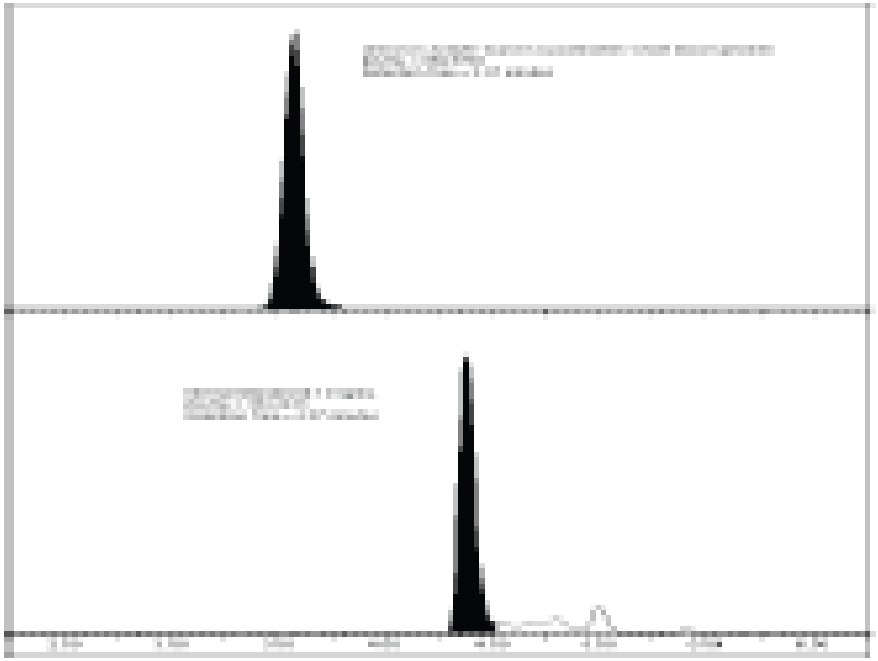


Figure 5



Reference Standard

Whole Blood

Case Study #2 – "Eye See Clearly Now" – Identification of Visine (tetrahydrozoline)

- History
  - Two siblings (infant and toddler) seen in the emergency room
  - Case history and initial investigation led to the suspicion of ingestion of Visine (tetrahydrozoline)
- Results
  - Urine specimen submitted to lab for identification of drugs and other chemical substances – including tetrahydrozoline
  - Screening analysis negative for drugs of abuse
  - Screening analysis positive for tetrahydrozoline – confirmed with standard
- Investigation
  - Litigation still pending