QUANTITATIVE MASS SPECTROMETRY

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Overview

- Introduction: Why choose mass spectrometry for quantitative analysis?
- Background: Instrumentation, workflow
- Assay development: Step by step, hypothetical project
- Quantitative LC-MS/MS assay
- Common issues:
  - Matrix effect
  - Internal standard (IS)
  - Method validation
  - Analyte stability
- Project summary

Quantitative mass spectrometry

Applications:

- Drug discovery
  - pharmacokinetic studies
- Environmental analysis
  - pesticides and herbicides in fruits
  - contaminants in water
  - BPA leaching from plastic baby bottles
- Protein expression
- Differential analysis

Sample types

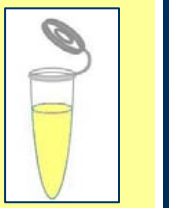
- Small molecules, MW 100 – 2000 Da:
  - synthetic molecules
  - drugs
  - metabolites
  - peptides (proteins)
- Biological matrices:
  - plasma, serum, erythrocytes
  - cerebrospinal fluid (CSF)
  - urine
  - bile
  - cell culture media
  - plant and animal tissues (e.g. leaf, brain, liver)

MS vs. UV detection

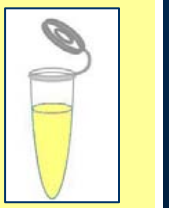
- Advantages:
  - Sensitive – targeted analysis
  - Selective
    - detection of specific (m/z) in complex biological matrix
    - additional level of selectivity achieved with MS/MS
  - Structural information - unique fragmentation pattern
  - Fast – automated, higher throughput
- Disadvantage:
  - Expensive
  - …or is it?

Analysis and data processing overview

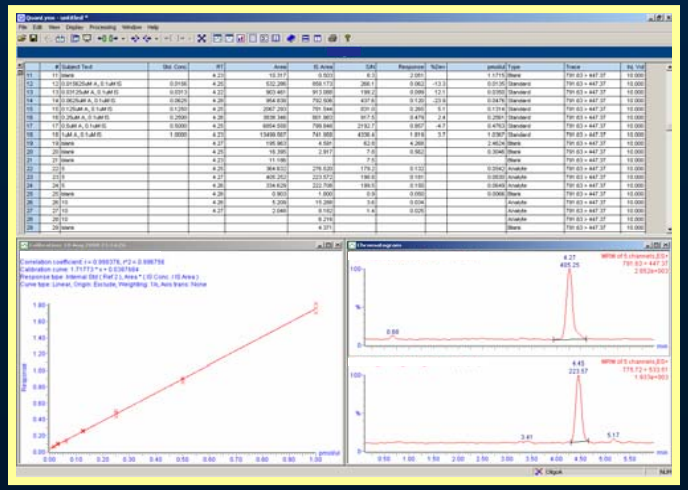
A) Analysis workflow



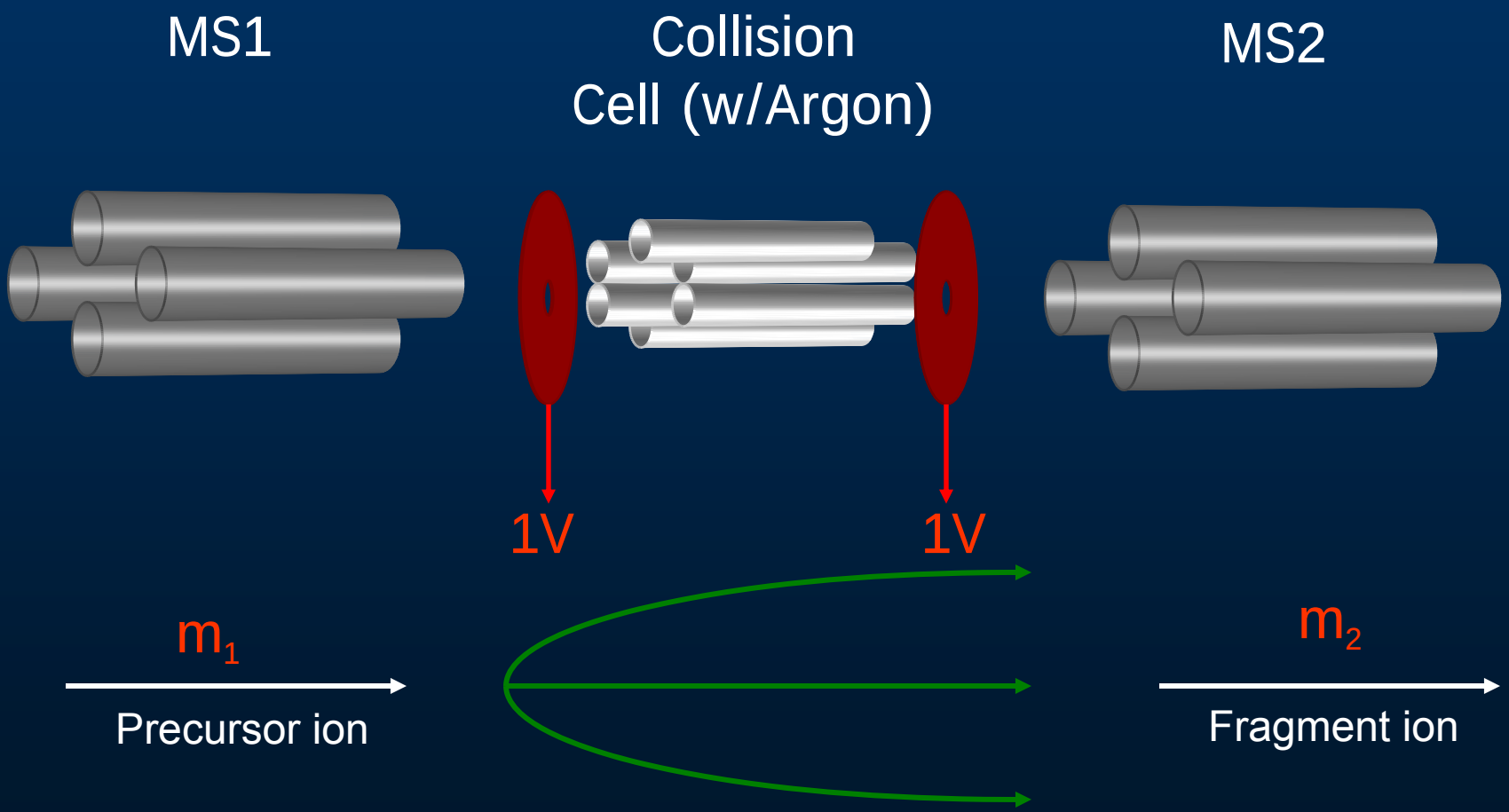
X,Y,Z
+
IS



B) Data processing



Triple quadrupole analyzer

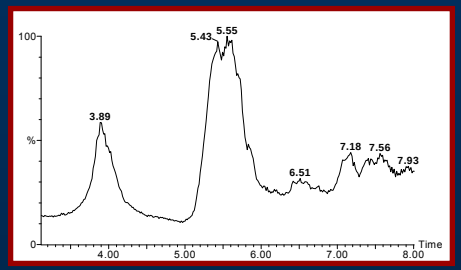


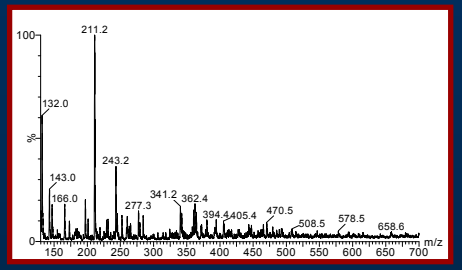
Scheme from Water Quattro Premiere Training

SRM vs. Full Scan MS and SIM

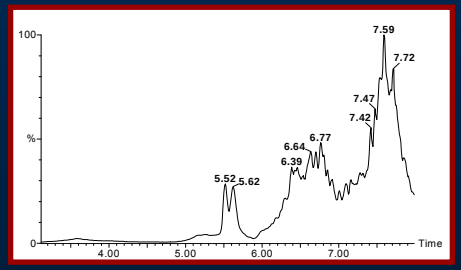
Chromatogram Spectrum

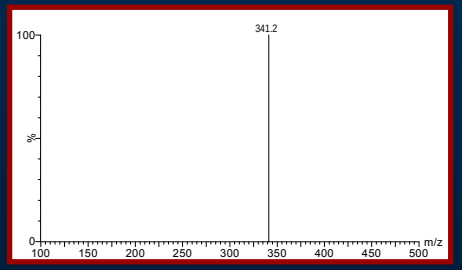
Full scan MS



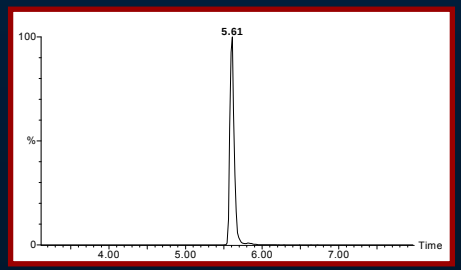


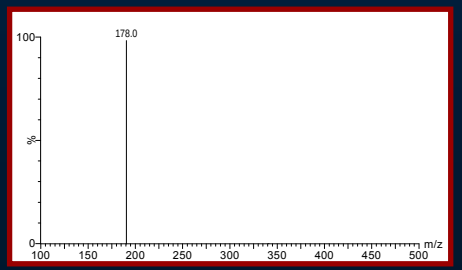
Selected Ion Monitoring (SIM)





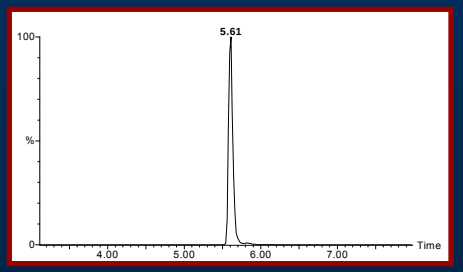
Selected Reaction Monitoring (SRM)

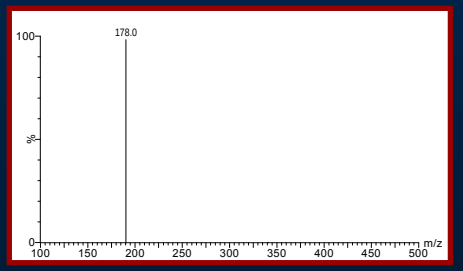




Advantages of SRM scanning mode

- MS/MS provides higher sensitivity and selectivity, enabling
  - Less extensive sample preparation
  - Greater sensitivity via increased selectivity
  - Use of shorter HPLC columns
  - Use of shorter run times and higher sample throughput
- Translates into time and effort savings, plus a more sensitive method





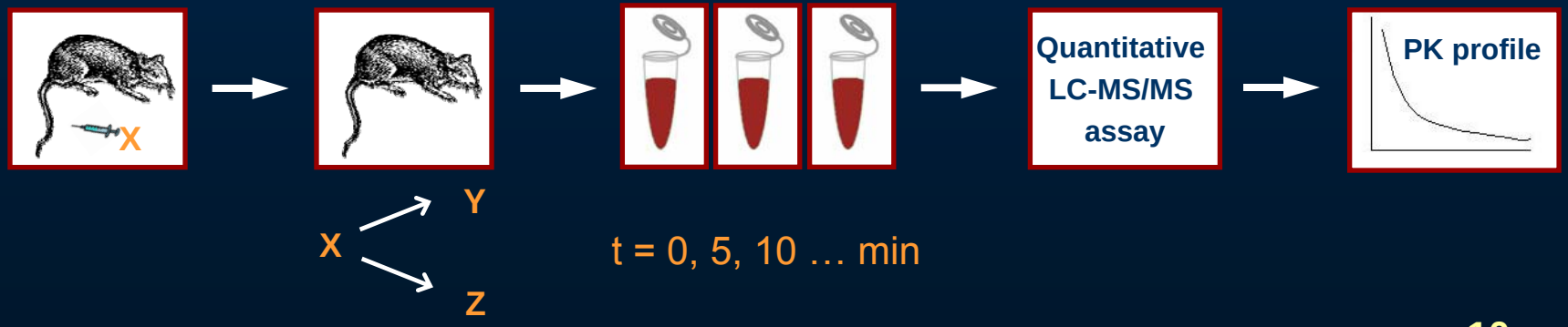
Hypothetical project

A scientist is working on a new drug candidate X

- Compound X is administered to rats
- Compound X is metabolized to Y and Z
- Blood samples are collected at 6 time points

Scientist wants to know

- how fast the drug is eliminated from the body of the rat
- PK profiles for metabolites Y and Z – potential toxicity of Z

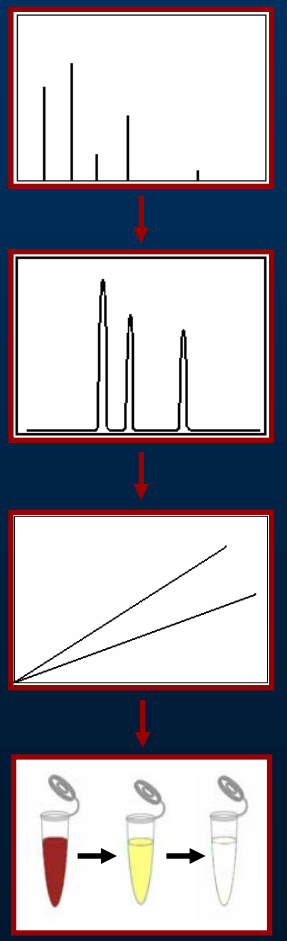


Before we start – checklist

Questions:	Our project:
• What are the analytes? (MW, structure, solubility, stability)	X=400, Y=416, Z=416
• Sample types? (matrices)	Serum
• Desired/required LLOQ and calibration range?	1 fmol up to 10 pmol on column
• Is an IS available?	Isotopically labeled X is expensive, 8 week lead time
• Purpose of developing the assay? (preliminary studies, confirmation of findings from different methods, publication)	Preliminary data on PK of X, Y, Z
• Available funding and timelines?	Limited funding, 3-4 weeks

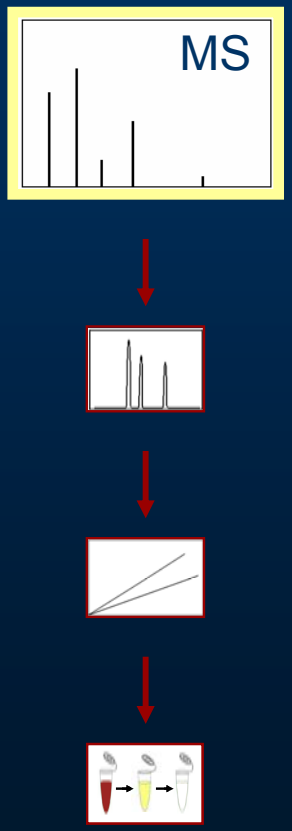
Assay development outline

- Mass spectrometry (MS)
- Liquid chromatography (LC)
- LC-MS/MS method optimization and characterization
- Sample preparation



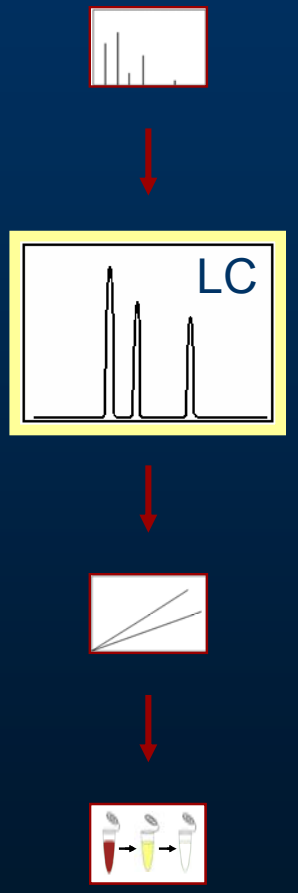
Method development - mass spectrometry

- Acquisition of MS and MS/MS spectra for standard solution of the analyte
  - 10-50uM, (20uM X, 20uM Y, 5uM Z)
  - direct infusion
- Precursor ion – fragment ion MS parameters optimization
  - most efficient ionization, $M+H^+$ ions observed for X, Y and Z; Y – in-source fragmentation (loss of H_2O)
  - most efficient fragmentation
- Method set up and test run
  - X: m/z 401 → 152
  - Y: m/z 417 → 300
  - Z: m/z 417 → 278



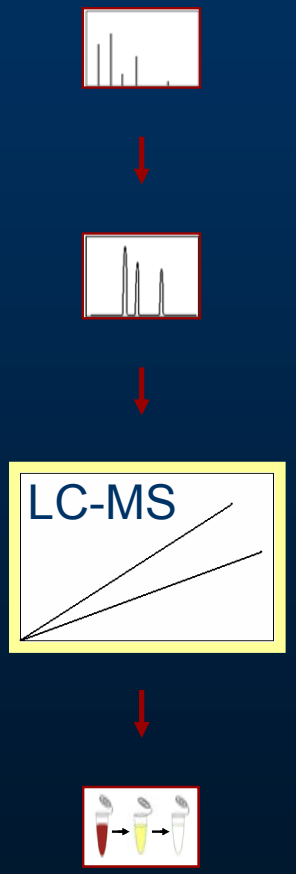
Liquid chromatography

- HPLC column
  - RP type column (C18, NH_2, SCX etc.)
  - Retention (weak)
  - Separation (X and Y co-elute, need to be separated because of in-source fragmentation of Y)
- Mobile phase
  - Gradient elution (water, acetonitrile)
  - MS compatible solvents and buffer modifiers (20 mM ammonium formate pH 4)
- Gradient
  - Shortest runs possible (usually 4-8 min. per injection) (7 min.)



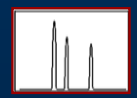
LC-MS method optimization & characterization

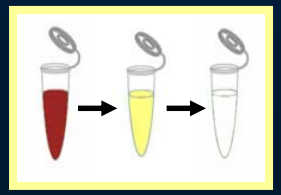
- Calibration curve (linear 0.2 fmol – 5 pmol)
- Limit of detection (LOD)
  (X: 0.2 fmol; Y, Z: 2 fmol)
  - 3:1 signal-to-noise ratio
- Limit of quantitation (LOQ)
  - 10:1 signal-to-noise ratio
- Sample matrix interferences
  - analysis of blank sample matrix spiked with pure standard (some interference)
- Carryover (none)
- Stability of the analyte (?)



Sample preparation

- Objectives:
  - Isolating analyte from matrix
  - Removing contaminants, desalting
  - Concentrating analyte
  - Reconstituting in appropriate LC-MS compatible reagent
- Extraction methods:
  - dilution
  - protein precipitation (cold, acidic methanol)
    - Methanol
    - Acetonitrile
  - Solid Phase Extraction (SPE)
    - C18
    - Ion exchange (e.g. SCX)
  - Liquid-liquid extraction (e.g. MTBE, diethyl ether)
  - Combination of two or more of the above

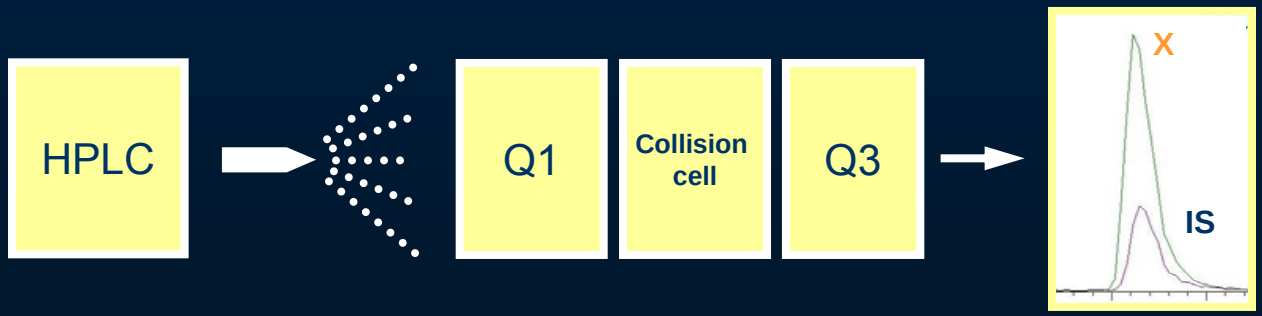





Quantitative LC-MS/MS assay

Each set of analyzed samples contains the following:

- 6-8 point calibration curve sets (in triplicate). A calibration curve set is usually run at the beginning, end and middle (at least one) of the sample set
- 1 QC per 10 samples (in triplicate)
- n# of samples (in triplicate)
- blank injections if necessary



Results – QuanLynx report

Analyte and IS area

Signal-to-noise ratio

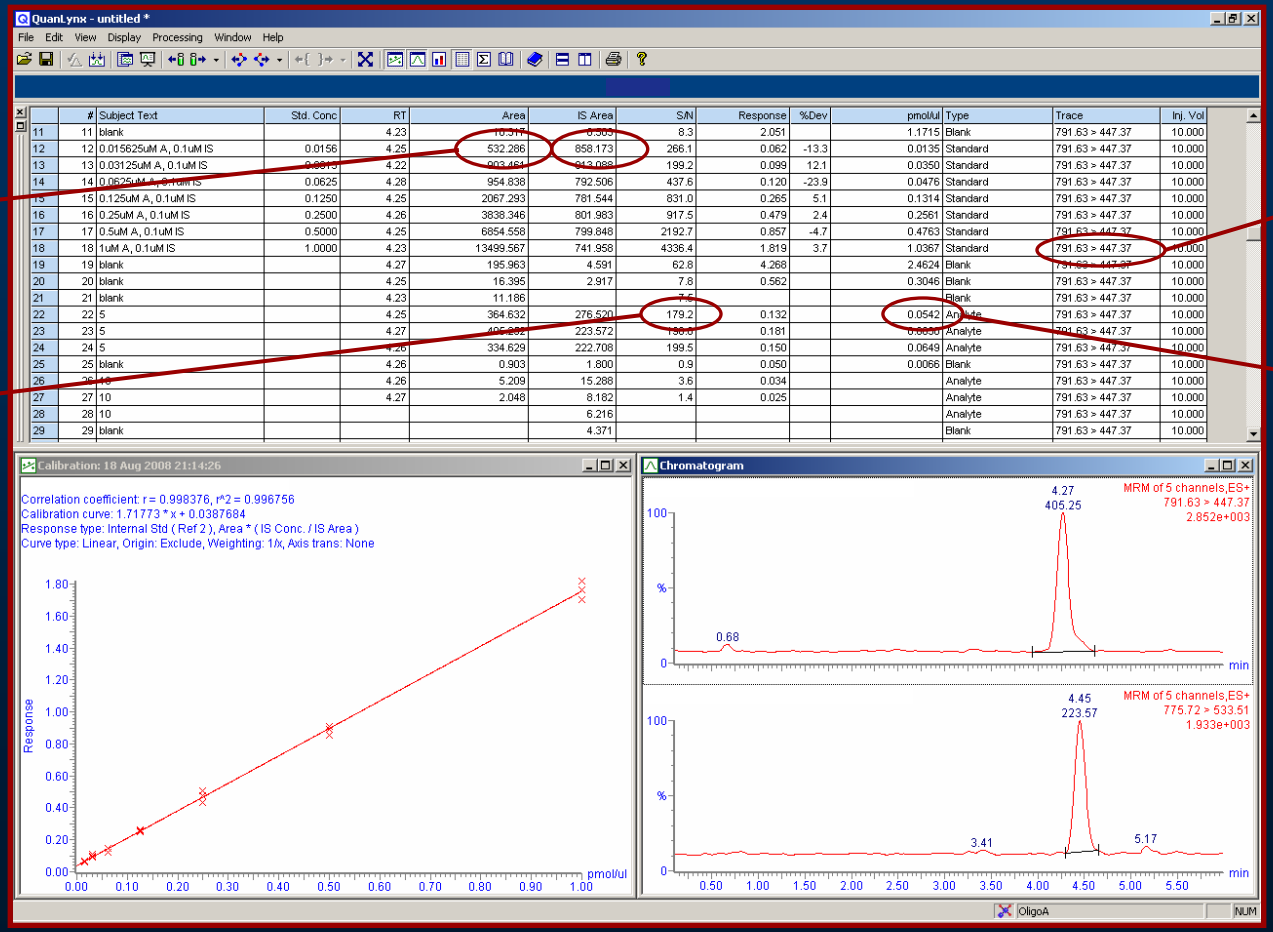
Calibration curve

SRM trace

Calculated concentration

Analyte

IS



#	Subject Text	Std. Conc	RT	Area	IS Area	S/N	Response	%Dev	pmol/ul	Type	Trace	Inj. Vol
11	blank		4.23	16.317	6.365	8.3	2.051		1.1715	Blank	791.63 > 447.37	10.000
12	0.015625uM A, 0.1uM IS	0.0156	4.25	532.286	858.173	266.1	0.062	-13.3	0.0135	Standard	791.63 > 447.37	10.000
13	0.03125uM A, 0.1uM IS	0.0313	4.22	903.464	813.088	199.2	0.099	12.1	0.0350	Standard	791.63 > 447.37	10.000
14	0.0625uM A, 0.1uM IS	0.0625	4.28	954.838	792.506	437.6	0.120	-23.9	0.0476	Standard	791.63 > 447.37	10.000
15	0.125uM A, 0.1uM IS	0.1250	4.25	2067.293	781.544	831.0	0.265	5.1	0.1314	Standard	791.63 > 447.37	10.000
16	0.25uM A, 0.1uM IS	0.2500	4.26	3838.346	801.983	917.5	0.479	2.4	0.2561	Standard	791.63 > 447.37	10.000
17	0.5uM A, 0.1uM IS	0.5000	4.25	6854.558	799.848	2192.7	0.857	-4.7	0.4763	Standard	791.63 > 447.37	10.000
18	1uM A, 0.1uM IS	1.0000	4.23	13499.567	741.958	4336.4	1.819	3.7	1.0367	Standard	791.63 > 447.37	10.000
19	blank		4.27	195.963	4.591	62.8	4.268		2.4624	Blank	791.63 > 447.37	10.000
20	blank		4.25	16.395	2.917	7.8	0.562		0.3046	Blank	791.63 > 447.37	10.000
21	blank		4.23	11.186		7.5				Blank	791.63 > 447.37	10.000
22	5		4.25	364.632	276.520	179.2	0.132		0.0542	Analyte	791.63 > 447.37	10.000
23	5		4.27	405.252	223.572	196.6	0.181		0.0650	Analyte	791.63 > 447.37	10.000
24	5		4.26	334.629	222.708	199.5	0.150		0.0649	Analyte	791.63 > 447.37	10.000
25	blank		4.26	0.903	1.800	0.9	0.050		0.0066	Blank	791.63 > 447.37	10.000
26	10		4.26	5.209	15.288	3.6	0.034			Analyte	791.63 > 447.37	10.000
27	10		4.27	2.048	8.182	1.4	0.025			Analyte	791.63 > 447.37	10.000
28	10				6.216					Analyte	791.63 > 447.37	10.000
29	blank				4.371					Blank	791.63 > 447.37	10.000

Most common challenges

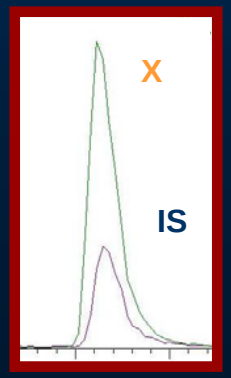
- Matrix effects
- Internal standard (IS)
- Method validation

Matrix effects

- Matrix effects:
  - Ion suppression
  - Interferences from metabolites
  - Signal enhancement
- Assessment of matrix effect:
  - Post-column infusion of analyte
  - Comparison of analyte in matrix-free solution vs. spiked blank matrix – extraction efficiency assessment
- Minimizing matrix effects:
  - Use isotopically labeled internal standard
  - Generate “cleaner” extract
  - Optimize HPLC method

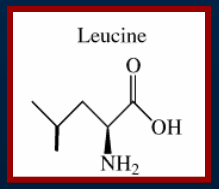
Internal standard – to use or not to use?

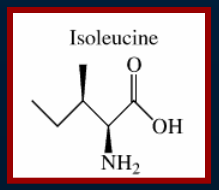
- Benefit of IS:
  - If the analyte and IS suffer the same losses and the same effects in the matrix, matrix effects and sample losses cancel when we take the ratio of IS to analyte
- IS compensates for common analyte losses:
  - Protein binding
  - Analyte absorption on surfaces
  - Extensive sample manipulation
  - Degradation
  - Evaporation
  - Autosampler variability



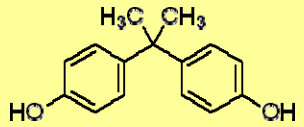
Selecting an Internal Standard

- IS criteria:
  - Maximum similarity in physical, chemical and chromatographic properties
  - Mass difference >4 Da
- IS choices:
  - Isotopically labeled (expensive, 8 week lead time)
  - Chemical isomer, e.g. leucine & isoleucine
  - Chemical analogue, e.g. methyl- or chloro- derivative (methyl- and chloro- derivatives available; methyl- not good)

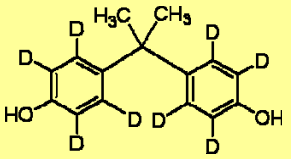




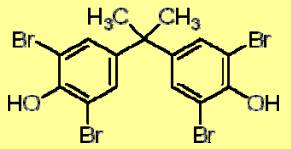
BPA options (Sigma)



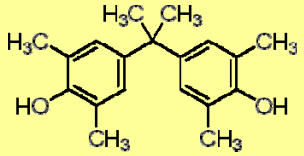
Bisphenol A



deuterated



bromo-



methyl-

Method Validation

- Detection capability:
  - LOD – signal to noise ratio 3:1
  - LOQ – signal to noise ratio 10:1
- Calibration curve – linear dynamic range
- Precision, accuracy and recovery
- Selectivity
- Stability of analyte (and matrix):
  - Short-term, long-term
  - Low and high concentrations
  - Analyte in sample solvent and in raw matrix
  - Dry extract, reconstituted standard/extract
  - Freeze/thaw cycles

Summary of hypothetical project

- MS – SRM transitions:
  - X: m/z 401 → 152
  - Y: m/z 417 → 300; most intense transition was m/z 417 → 401
  - Z: m/z 417 → 278
- HPLC:
  - C18 column
  - in order to retain polar analytes, ammonium formate used as mobile phase modifier
  - 7 min. gradient necessary to achieve separation of X and Y
- LLOQ of X, Y and Z: 0.5 fmol
- Calibration curve range: 0.2 fmol to 10 pmol
- IS: chloro-derivative of X
- Sample preparation: protein precipitation at low pH reduced analyte-protein binding
- Full method validation was not performed – based on the PK results, X not a good drug candidate

Literature references

1. Mass Spectrometry: Principles and Applications by Edmond de Hoffmann, Vincent Stroobant
2. Trace Quantitative Analysis by Mass Spectrometry by Robert K. Boyd, Cecilia Basic, Robert A. Bethem
3. Mass spectrometry in drug metabolism and Pharmacokinetics by Ragu Ramanathan
4. HPLC for Pharmaceutical Scientists by Yuri V. Kazakevich, Rosario LoBrutto
5. LC/MS: A practical User's Guide by Marvin McMaster
6. www.ionsource.com

Acknowledgements

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