EVERYTHING YOU WANTED TO KNOW ABOUT INTERNAL STANDARDS, BUT WERE TOO AFRAID TO ASK

PART 1: WHAT, WHY AND HOW

RUSSELL GRANT

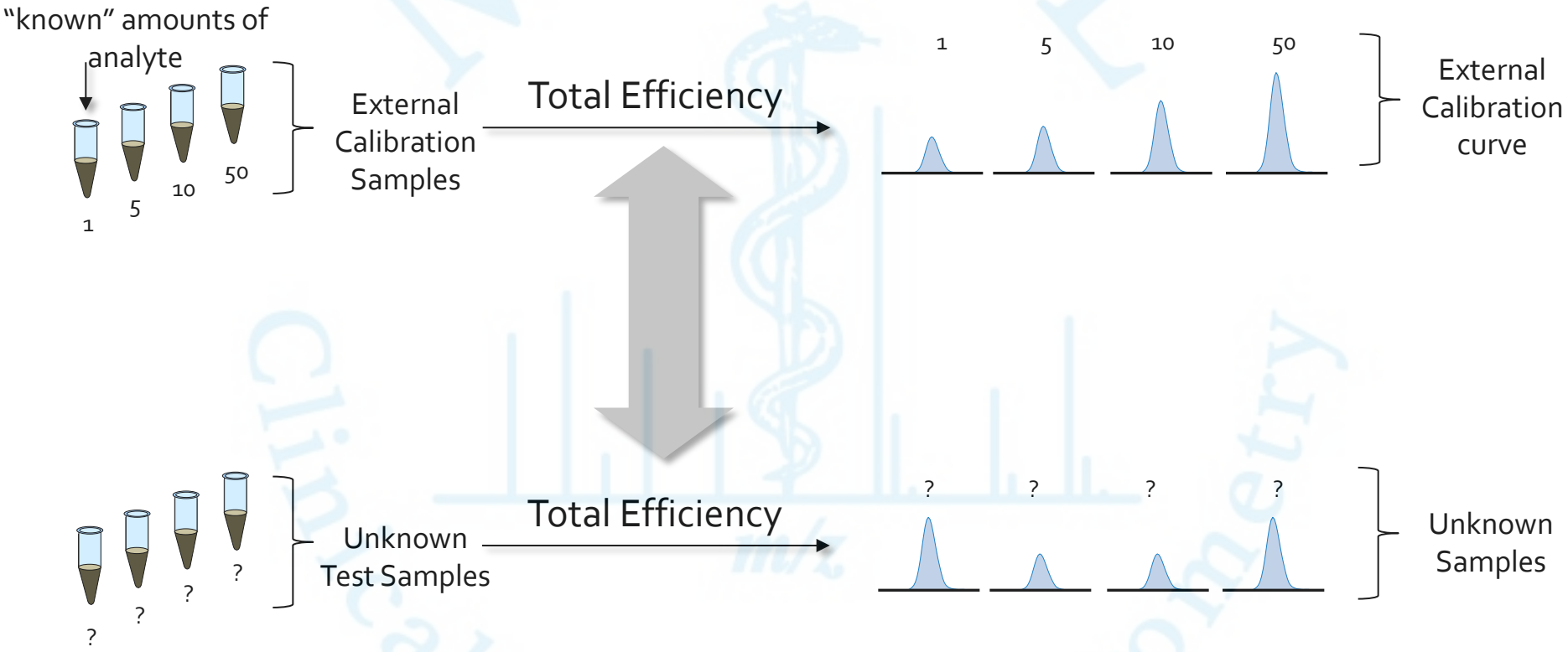
LABORATORY CORPORATION OF AMERICA® HOLDINGS, BURLINGTON, NC USA

Disclosures

- None

2

External Calibration

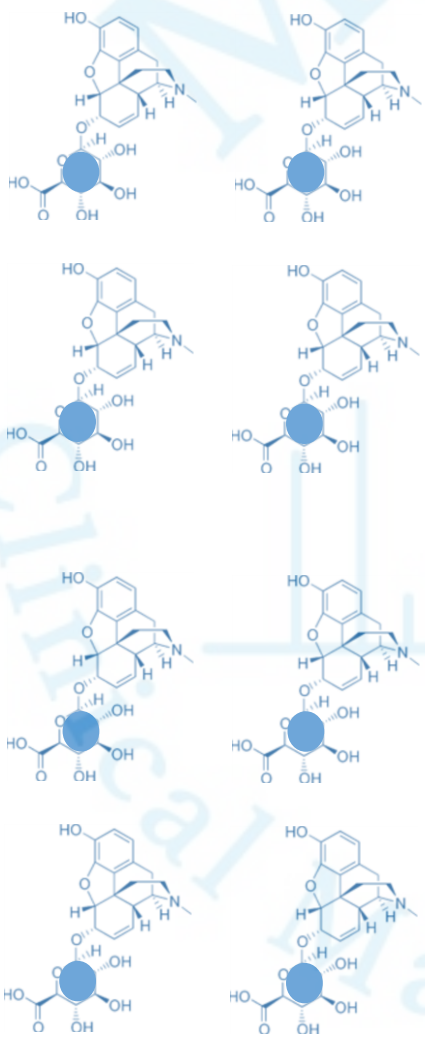


Analyte must have identical matrix effects and extraction efficiency (i.e., total efficiency) between calibrators and samples for accuracy

External Calibration cannot solve recovery differences

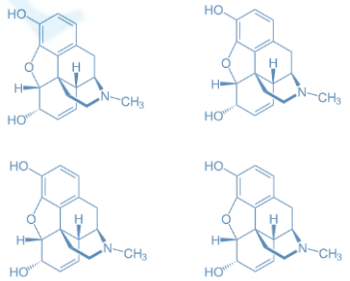
CALIBRATORS

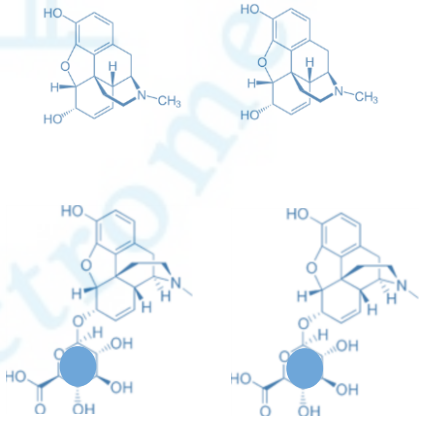
SAMPLES



Hydrolysis
(100% Efficiency)

Hydrolysis
(50% Efficiency)





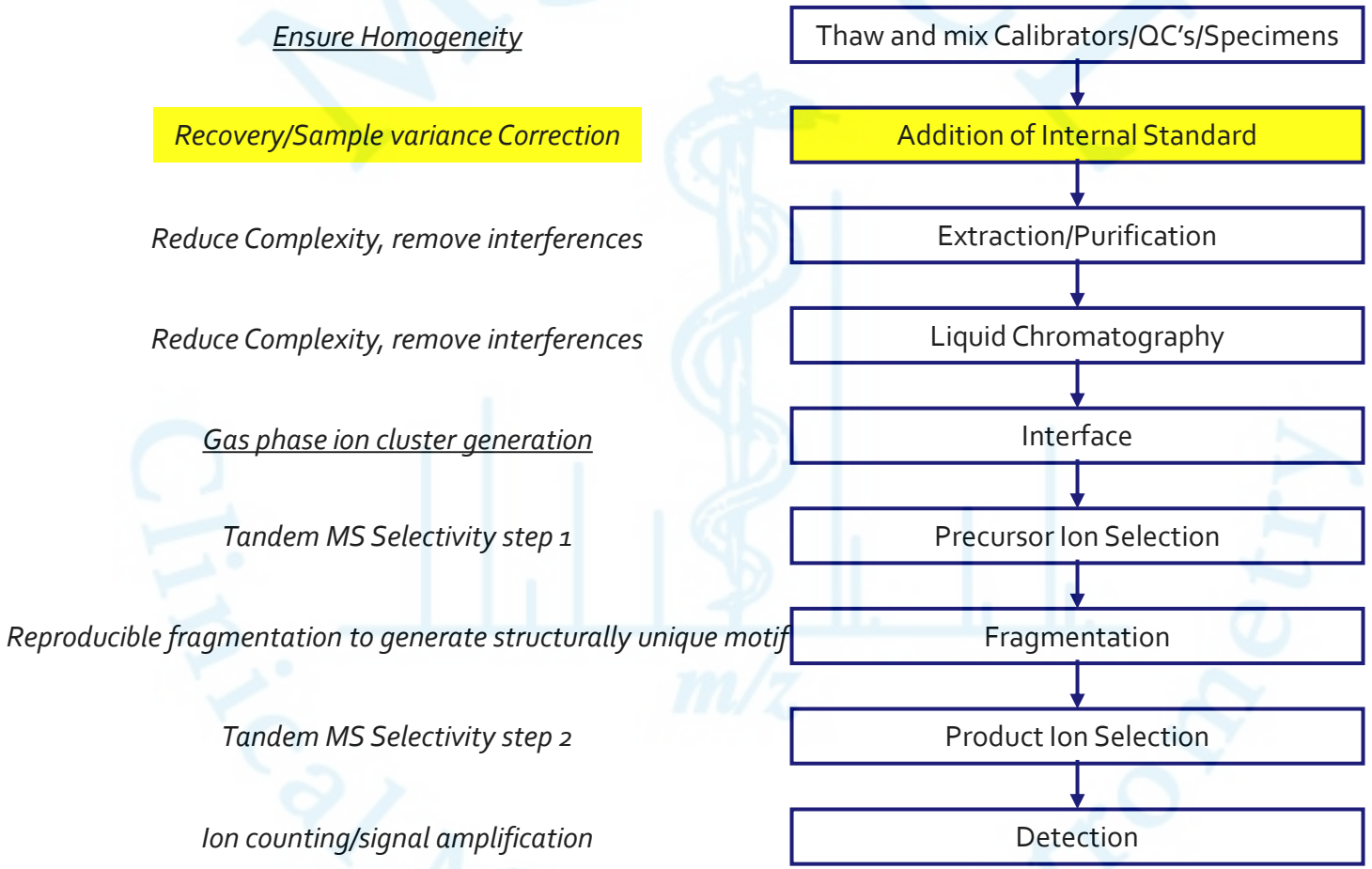
-50%
Bias

What is an Internal Standard

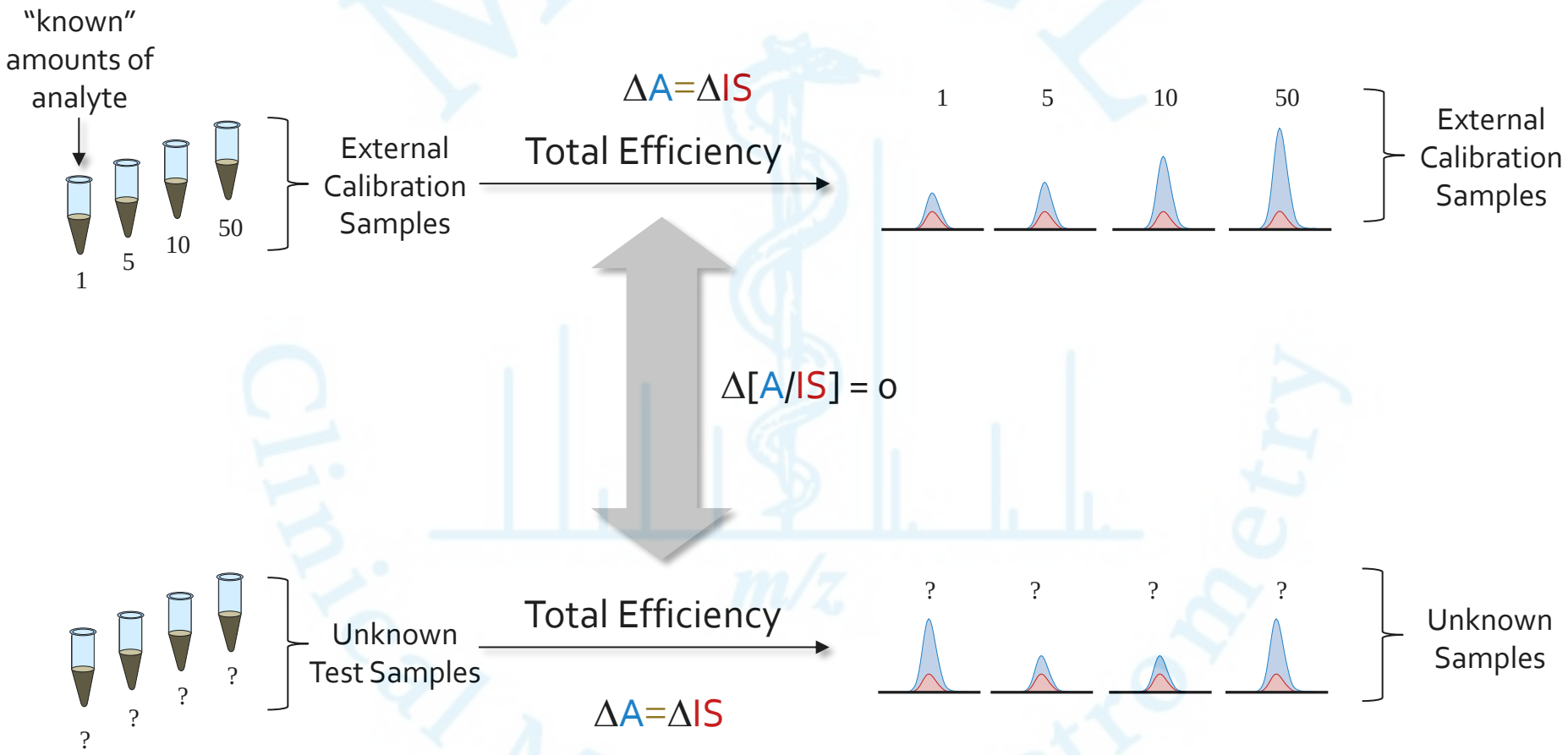
An internal standard in analytical chemistry is a chemical substance that is added in a constant amount to samples, the blank and calibration standards in a chemical analysis.

Used to correct for the loss of analyte during sample preparation, injection and ionization

When should IS be added?



External Calibration with Internal Standardization

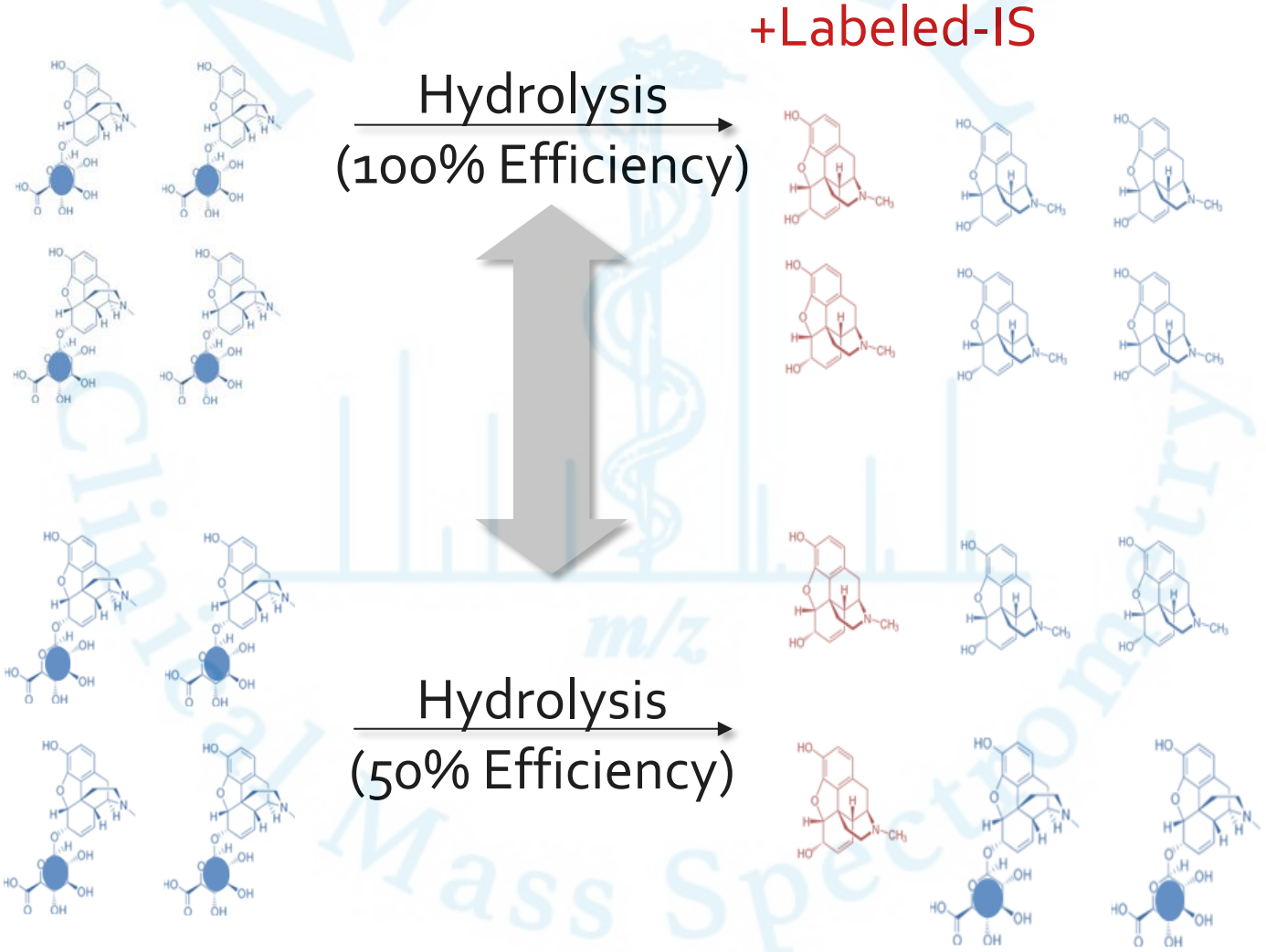


Difference in the matrix effects and extraction efficiency (total efficiency) incurred by the Analyte between calibrators and samples should be identical to the difference incurred by the Internal Standard

External Calibration with Poor Internal Standardization

CALIBRATORS

SAMPLES

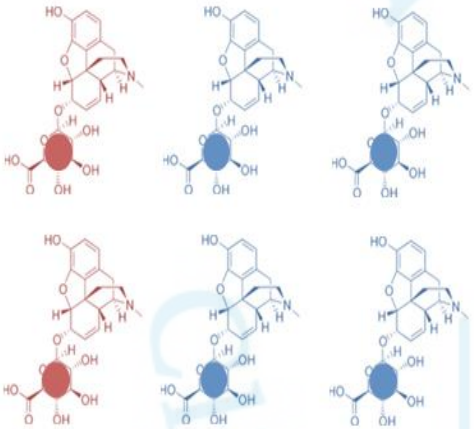


External Calibration with Good Internal Standardization

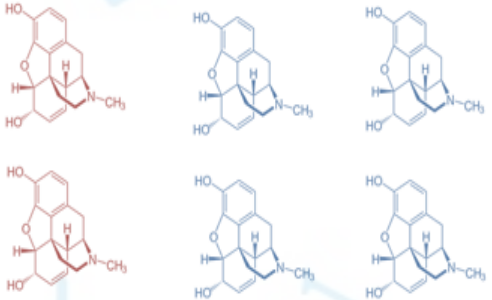
CALIBRATORS

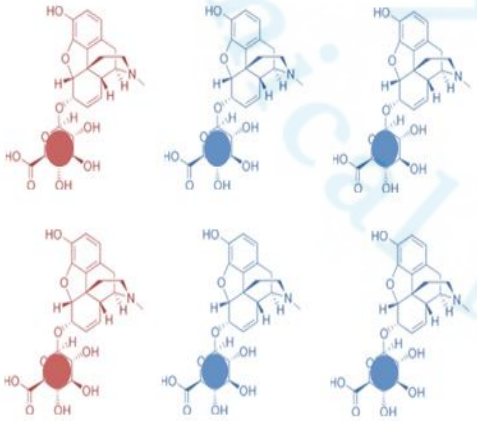
SAMPLES

+Labeled-IS

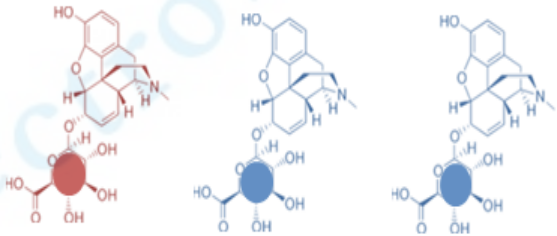


Hydrolysis
(100% Efficiency)



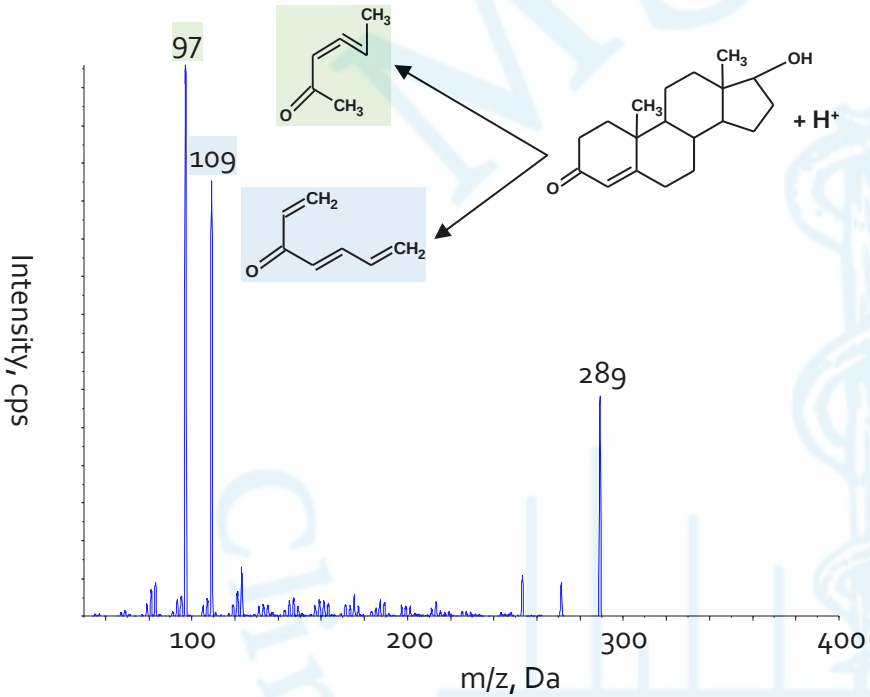


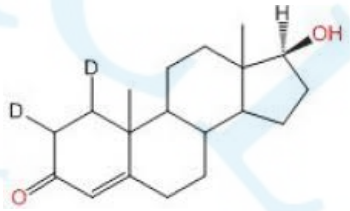
Hydrolysis
(50% Efficiency)



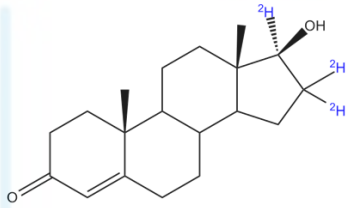
0%
Bias

What is a Good IS?…pick your favorite

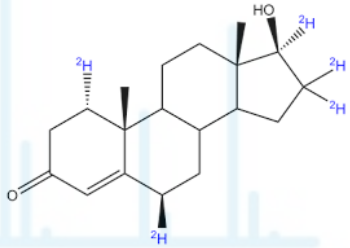




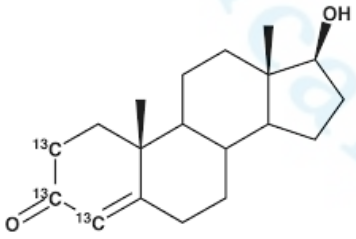
Testosterone D2
m/z 291 – 99, 111



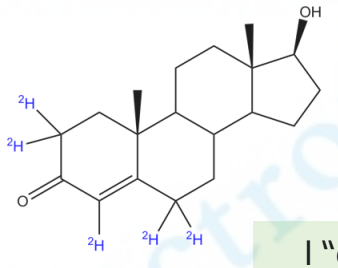
Testosterone D3
m/z 292 – 97, 109



Testosterone D5
m/z 294 – 100, 111



And now?
$^{3}C_{13}$ Testosterone
m/z 292 – 100, 112



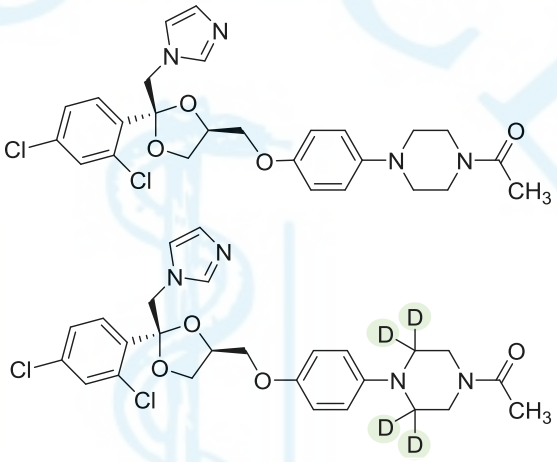
Testosterone D5
m/z 294 – 102, 114

I "guessed" at transitions for the second D5 IS - H/D Scrambling?

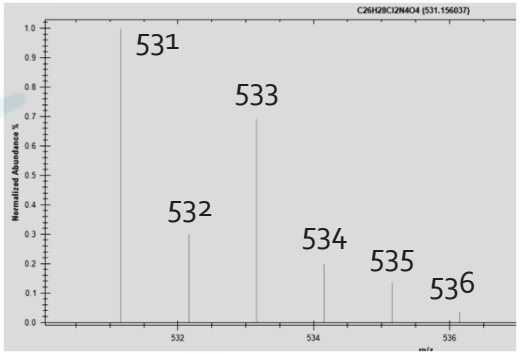
*Endocrine Abstracts (2012) **28** P30, Testosterone measurement by mass spectrometry - a tale of three internal standards Laura Owen & Brian Keevil*

Considerations for Degree of Labelling – Natural abundance

Element	Atomic Mass	Relative Abundance (%)
H	1	99.99
	2	0.01
C	12	98.93
	13	1.07
N	14	99.64
	15	0.36
O	16	99.76
	17	0.04
	18	0.20
S	32	94.99
	33	0.75
	34	4.25
	35	0.01
Cl	35	75.76
	37	24.24
Br	79	51.00
	81	49.00



Ketoconazole Q1 Scan



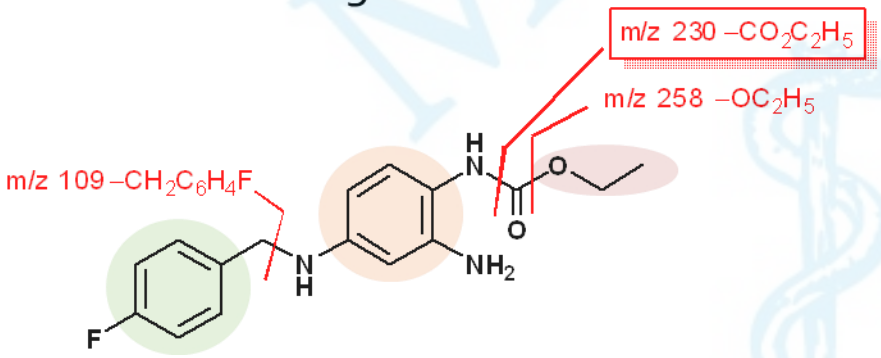
Deuterons and Isotopes	*m/z*	Relative Abundance Ketoconazole	Isotopic Distribution D4-Ketoconazole
0	531.156	100	0.01
1	532.159	30	0.04
2	533.154	69	0.24
3	534.156	20	5.91
4	535.152	14	60.47
5	536.154	4	27.66
6	537.156	0	5.67
7	538.158	0	1.23

"Walk the Isotopes" and use *m/z* 537 for D4-IS

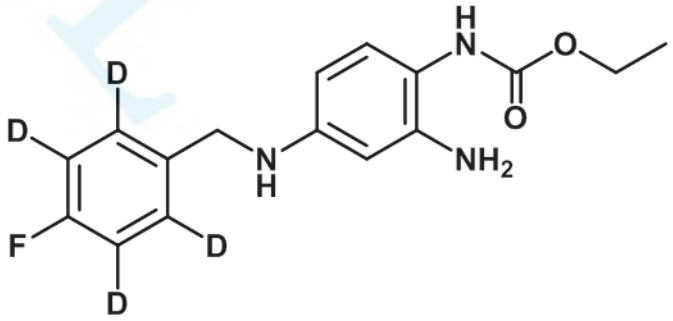
Courtesy of Mitzi Rettinger, MilliporeSigma

Considerations for Position of Labelling

Retigabine



D_4-Retigabine



Labelling positions:

- OC_2D_5 or OCH_2CD_3 - loss during MS fragmentation?
- Central phenyl ring - H/D exchange during synthesis?
- 4-fluorophenyl ring - Lower scrambling , H/D exchange

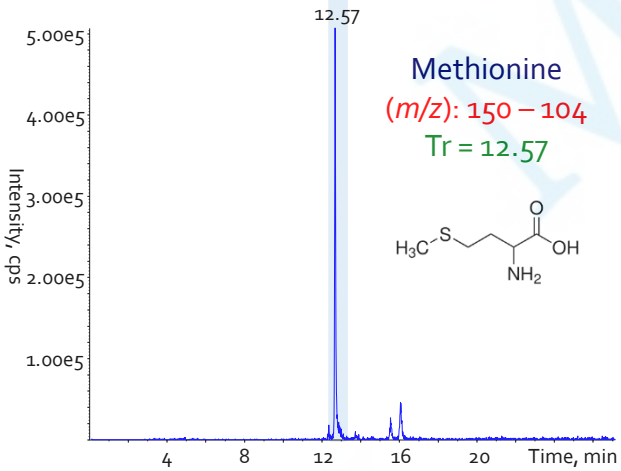
Isotopic Distribution (%)	
D_0	0.011
D_1	0.277
D_2	6.593
D_3	9.363
D_4	82.800
D_5	0.804
D_6	0.153
D_0/D_4	0.013%

D_0/D_4 ratio – adequate selectivity

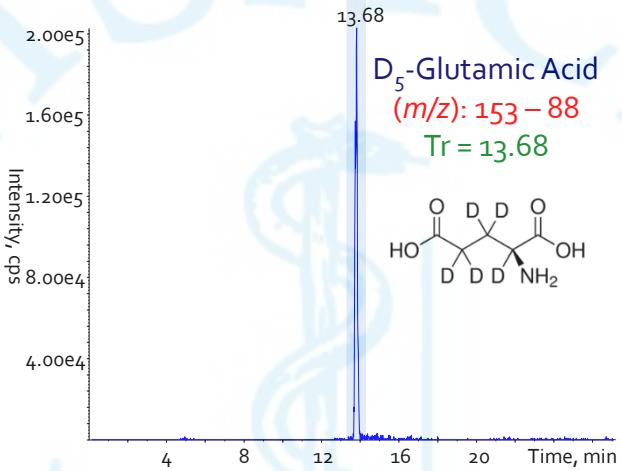
Selection of Internal Standards for LC-MS.MS
https://www.cerilliant.com/Shoponline/OpenDocument.aspx?DocumentId=389

Courtesy of Mitzi Rettinger, MilliporeSigma

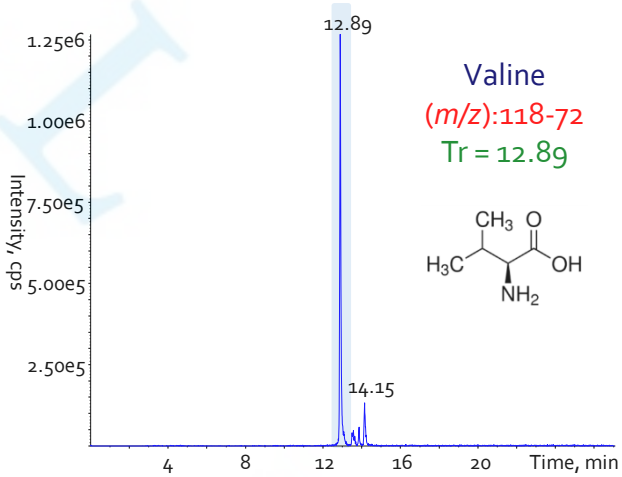
Structurally Unique in specimens and between each other



Methionine
(*m/z*): 150 – 104
Tr = 12.57



D_5-Glutamic Acid
(*m/z*): 153 – 88
Tr = 13.68

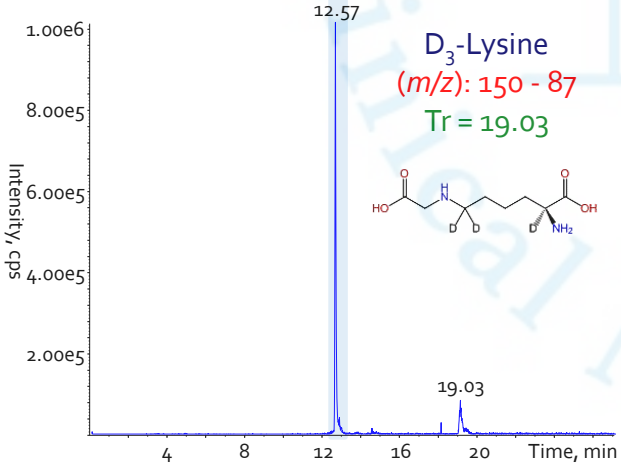


Valine
(*m/z*):118-72
Tr = 12.89

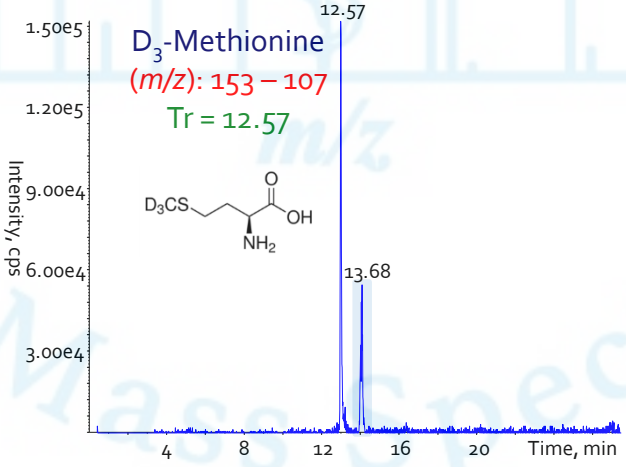
Isobaric

Isobaric

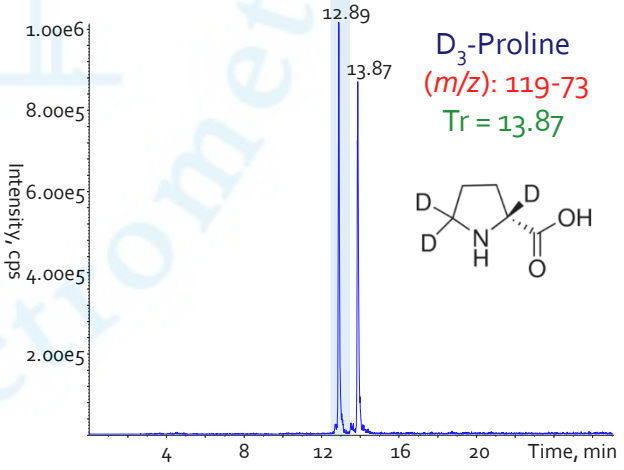
Isotopic



D_3-Lysine
(*m/z*): 150 - 87
Tr = 19.03

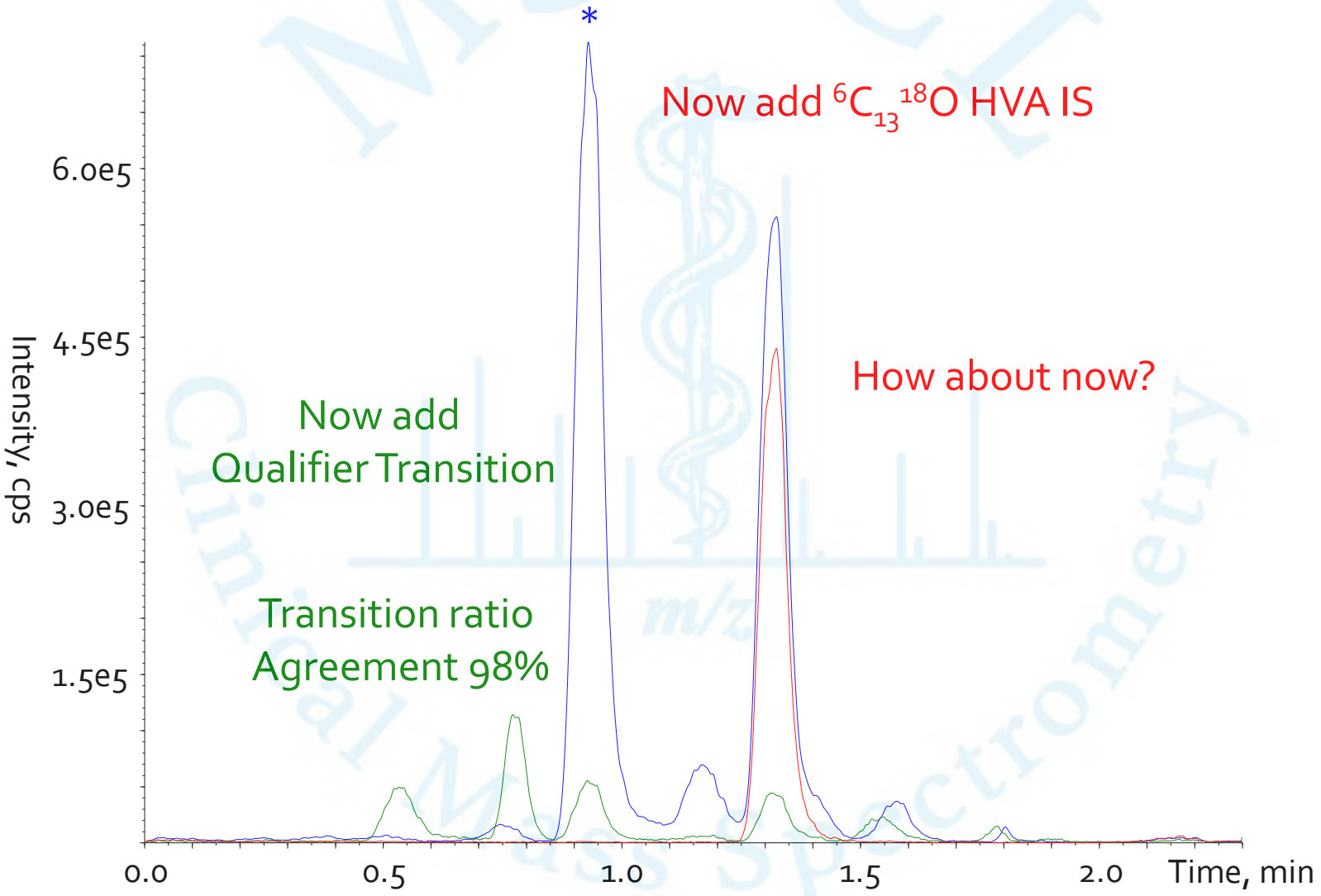


D_3-Methionine
(*m/z*): 153 – 107
Tr = 12.57

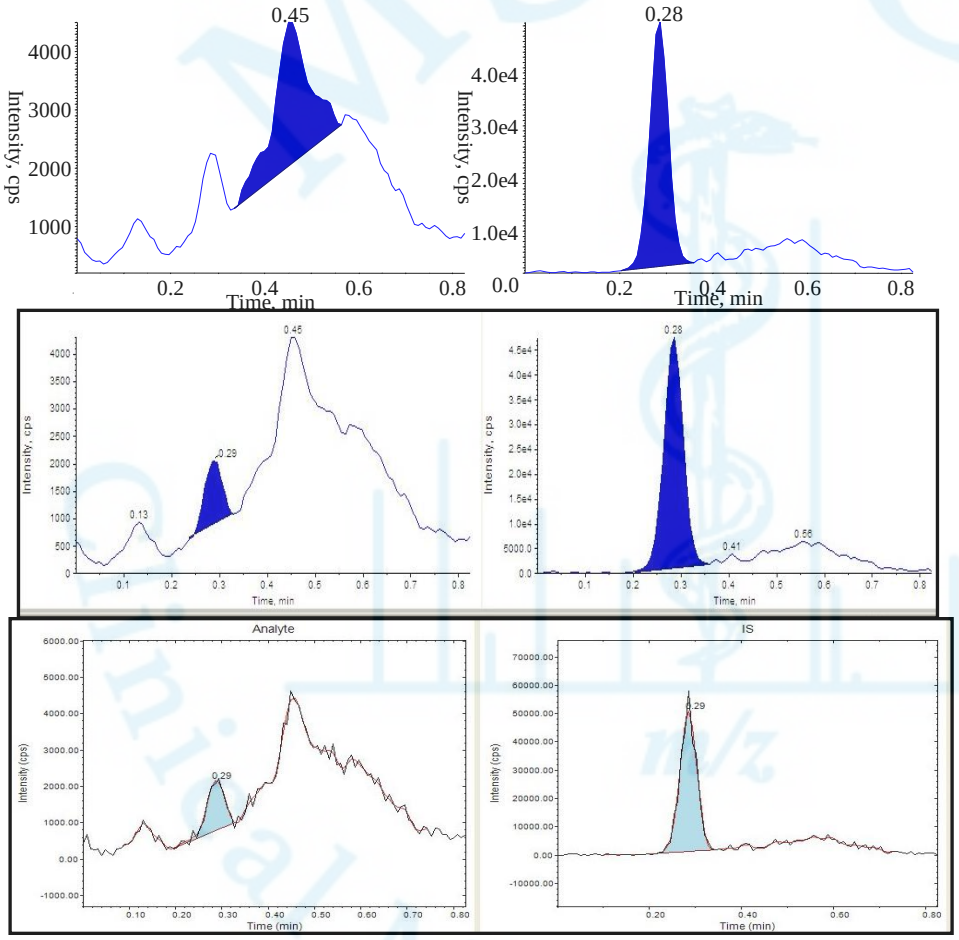


D_3-Proline
(*m/z*): 119-73
Tr = 13.87

What do you think about the * peak at 1 minute for HVA?



Using IS to inform Peak Picking parameters



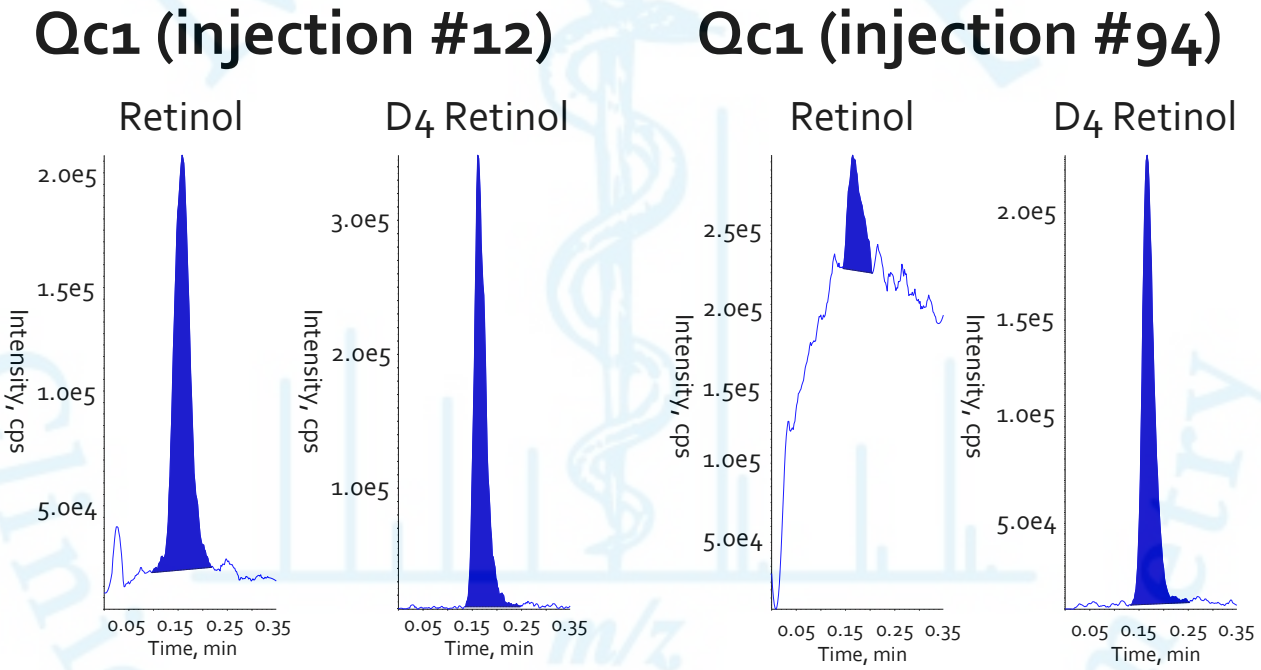
Analyst:
Auto Integration

Analyst:
Manual integration

Ascent:
Automatic optimization of quantitation parameters

Manual adjustment of parameters is labor intensive and introduces human bias
Set relative agreement (Analyte and IS) in integration methods "narrowly"

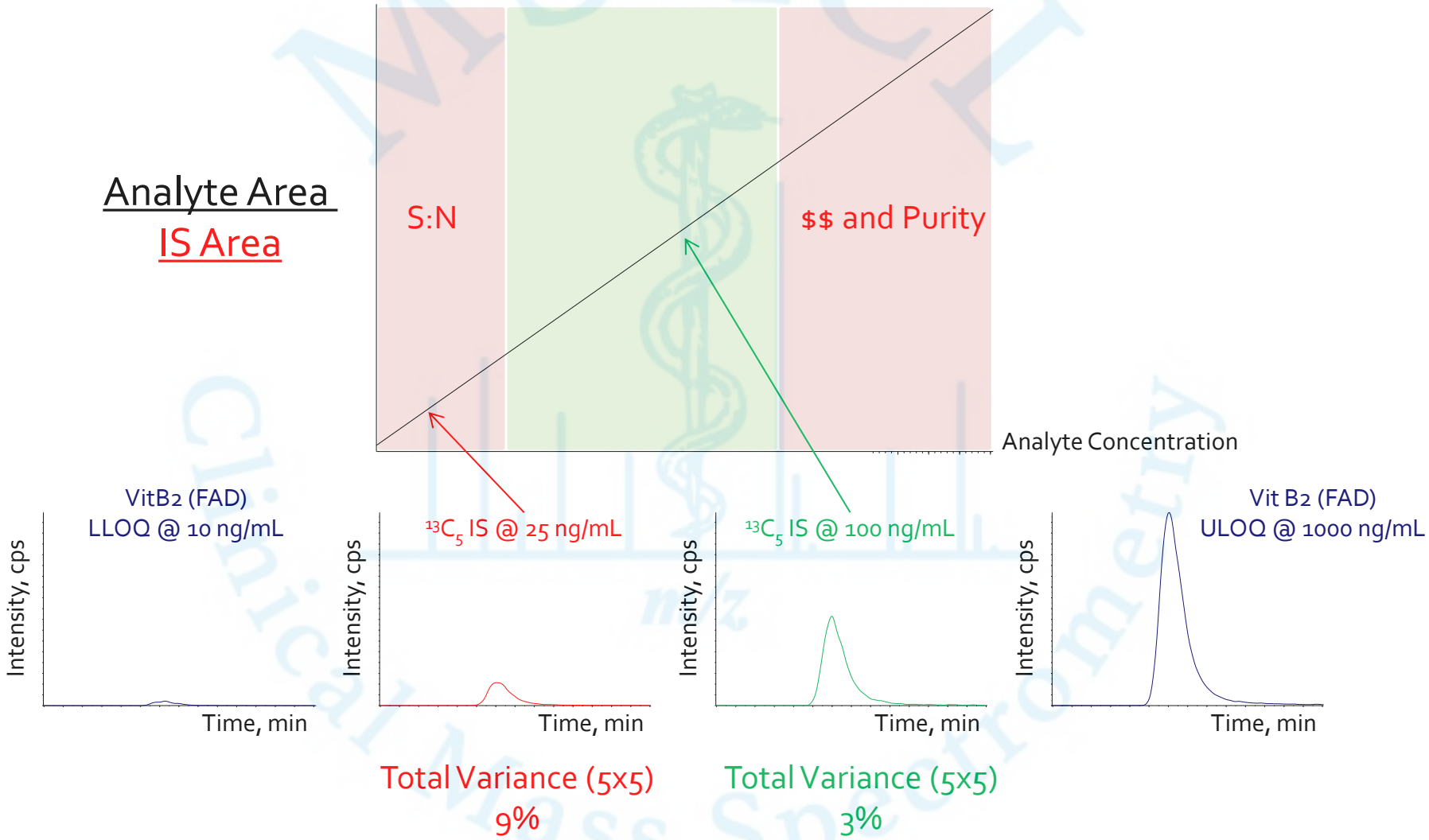
What Does the IS Tell You Qualitatively



Good IS informs analyte retention time and peak shape

Enables diagnosis of problems – guides correction

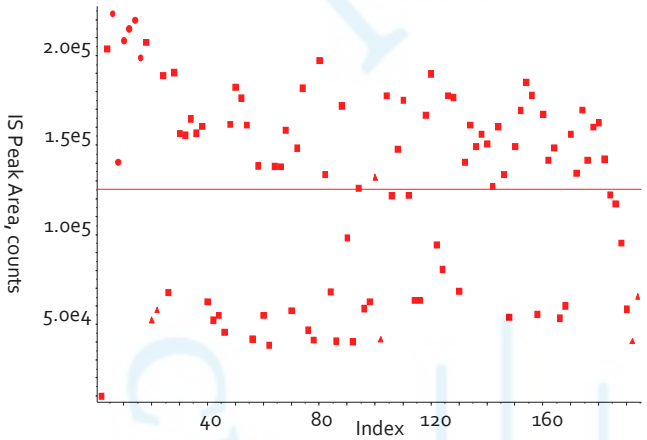
How much IS should you add?



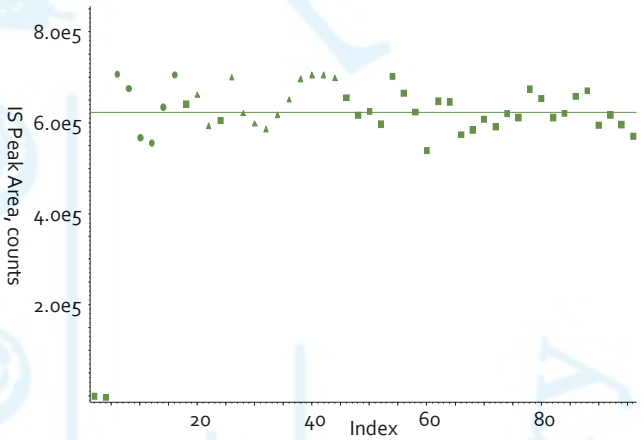
How much? 100-fold range – Mid point, 1000-fold range 10 – 25 x LLOQ

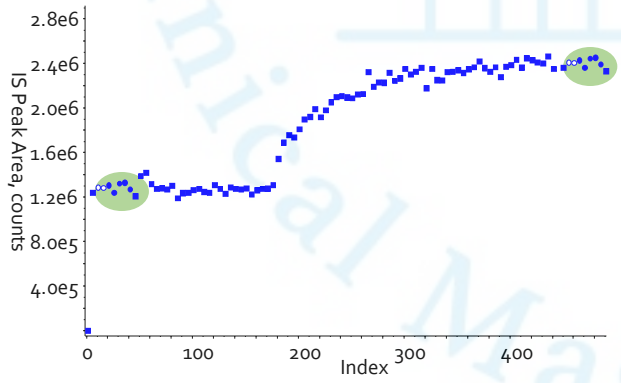
How should you add IS? – be precise, precisely!

Reverse T_3 Manual SPE
First time ever



Reverse T_3 Automated SPE
First time ever





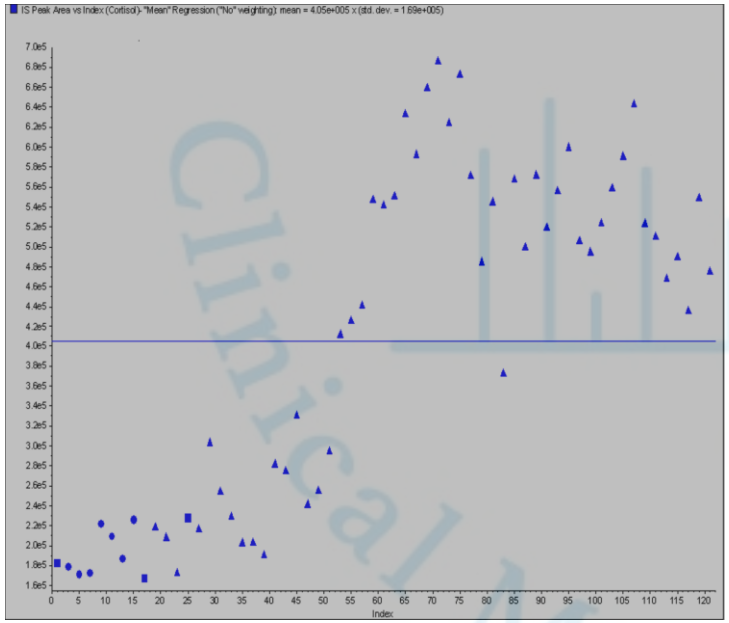
D_5 Phenylalanine trend – second LC channel started (different solvent chemistry)

Calibrators re-injected = System drift (not prep)

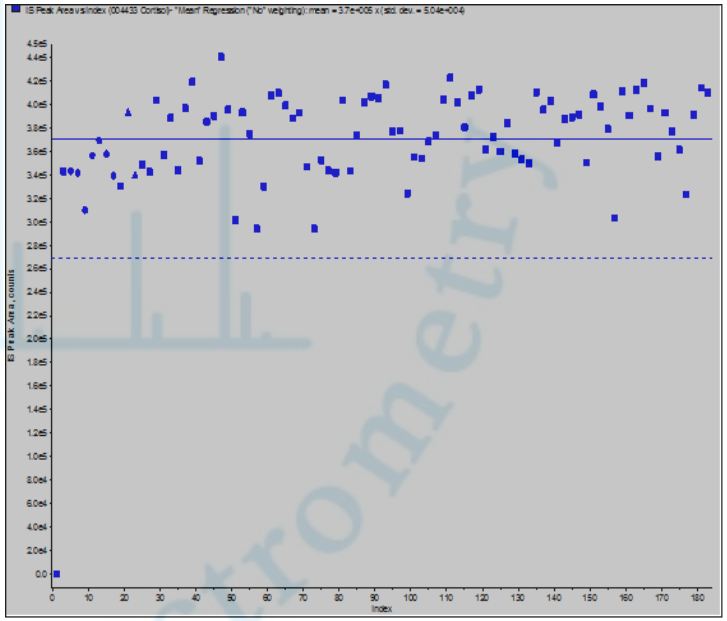
Addition and/or Recovery Variance of IS makes outlier detection Impossible

IS Peak Area Trend: Outliers and Drift

Tecan Liquid handler IS addition Using same 8 tips with aqueous D4-Cortisol **Drift** across run observed



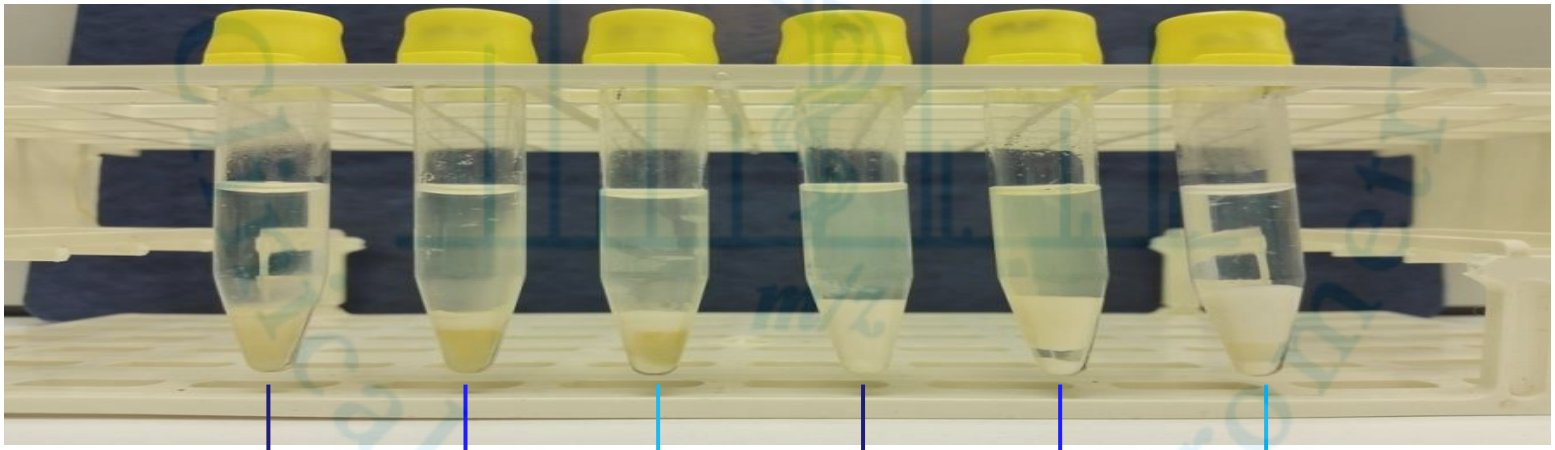
With 0.1% BSA (aq) D4-Cortisol solution and Pre-wetted tips (x3) prior to dispensing **IS peak area CV = 8.6%**



IS peak area precision enables outlier detection

How should you add IS? Protein Precipitation issues…

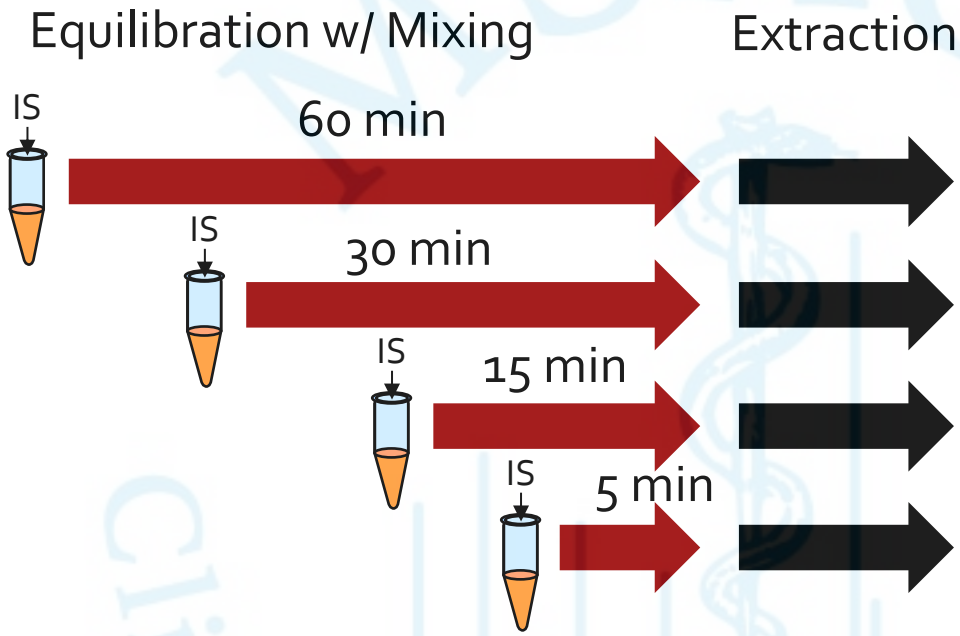
Acetone, Acetonitrile, Ethanol, Isopropanol, Methanol



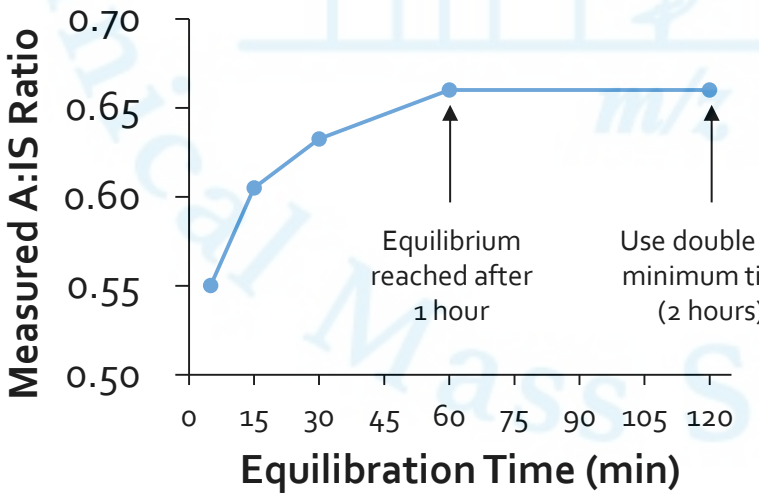
ACN $(NH_4)_2SO_4$ $ZnSO_4$ MeOH $(NH_4)_2SO_4$ $ZnSO_4$

Precipitation techniques are FAST, Q: IS recovery same as analyte?

How to evaluate IS addition?



Aliquots of sample pool equilibrated with IS for decreasing amounts of time prior to extraction in parallel (i.e., reverse timing)

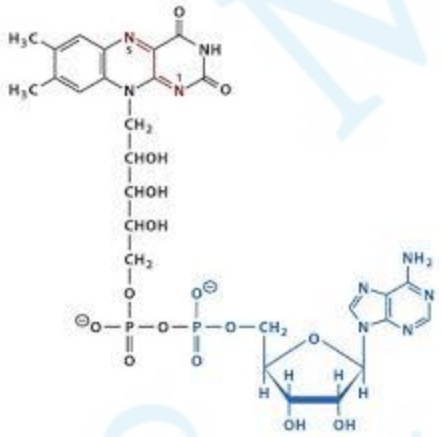


Plateau in A:IS ratio indicates IS has reached binding equilibrium with analyte

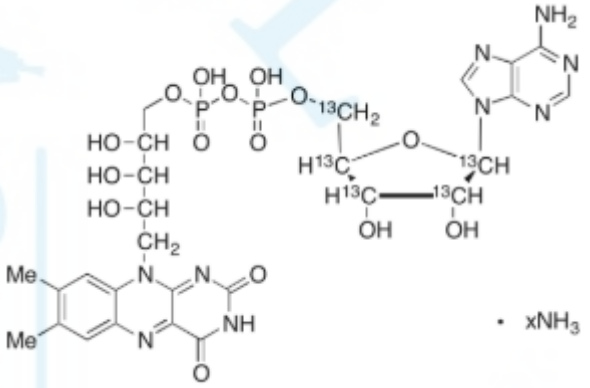
Use double the time

Vitamin B2 Flavin Adenine Dinucleotide in Whole Blood

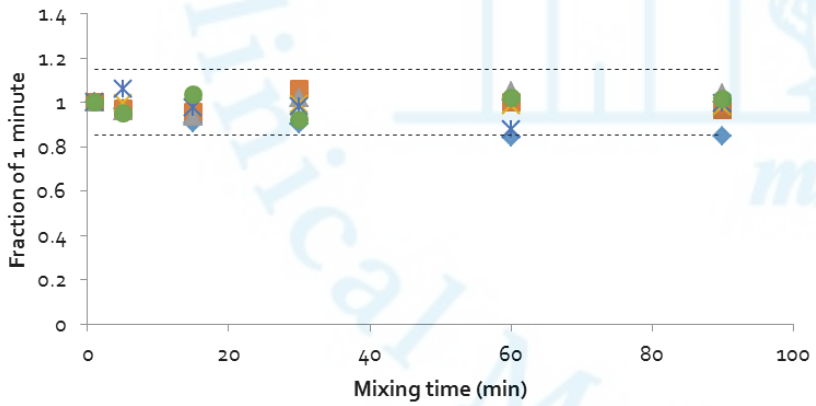
Flavine Adenine Dinucleotide



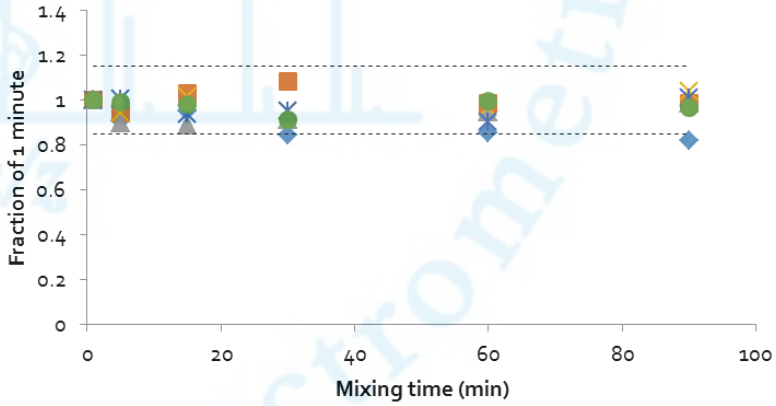
Flavine Adenine Dinucleotide -$^{13}C_5$ Ammonium Salt



FAD:IS ratio in Whole Blood versus 1 min



FAD-IS Area in Whole Blood versus 1 min



Calculate as Fractions (recovery) relative to max/min mixing time

Use Individual samples and interrogate IS peak area trend too!

WHAT Internal Standards should be

NATURE:

- Structurally unique (exogenous) – *Not observed in samples*
- Resolved (separated) from analyte(s) by MS, *but* Co-elute
    - *Stable labeled isotope (^{13}C, ^{15}N, ^{18}O, ^{2}H in order) ≥ +3 amu*
- No H/D Exchange – *check stability in solution and ion source*
- Structurally similar (analog) – *Limited recovery or ionization effect correction*
- Structurally dissimilar - *injection check at best?*

WHY Internal Standards are used

UTILITY:

- Identification of analyte retention time shift and peak shape
- Injection variance
- Normalization of recovery differences
- Normalization of ionization effects *between calibrators and samples*
- *One of the most valuable components in LC-MS/MS analytical quality*

HOW Internal Standard should be used

ADDITION:

- FIRST step after mixing/pipetting sample (*Identical biochemically to analyte*)
- Reproducibly added (*precise!*) to samples, calibrators, QC's except double blanks
- Solution ideally miscible with sample – *to correct analyte recovery if equilibrium is a concern*
- **Mixed well prior to extraction – *Equilibrated identically to analyte – you should always prove this with real samples versus calibrators***

EVERYTHING YOU WANTED TO KNOW ABOUT INTERNAL STANDARDS, BUT WERE TOO AFRAID TO ASK

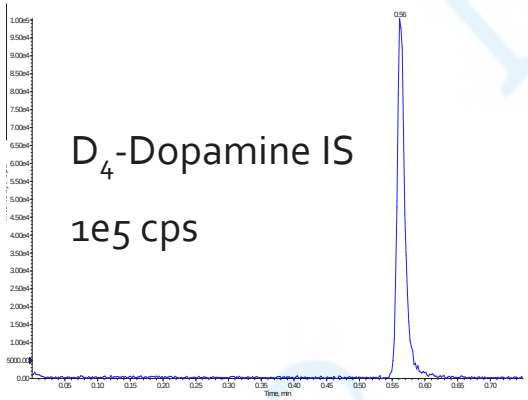
PART 2: BUT WHAT ABOUT WHEN….

RUSSELL GRANT

LABORATORY CORPORATION OF AMERICA® HOLDINGS, BURLINGTON, NC USA

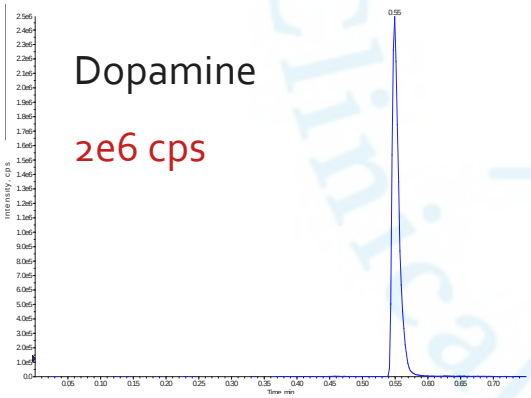
Analyte observed in IS solution

IS in Water APCI Source



D_4-Dopamine IS

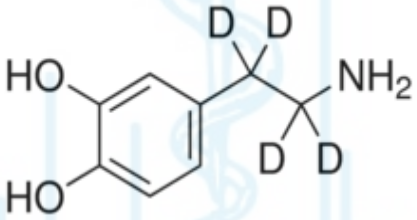
1e5 cps



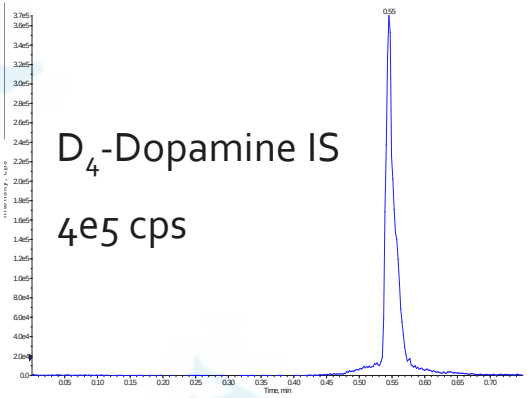
Dopamine

2e6 cps

Neat Dopamine D4 solution appeared to contain mostly Dopamine

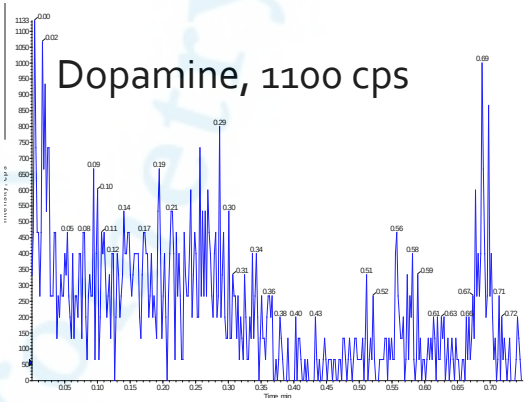


IS in Water ESI source



D_4-Dopamine IS

4e5 cps

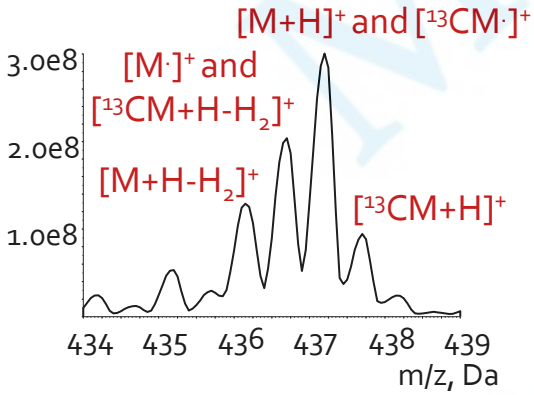


Dopamine, 1100 cps

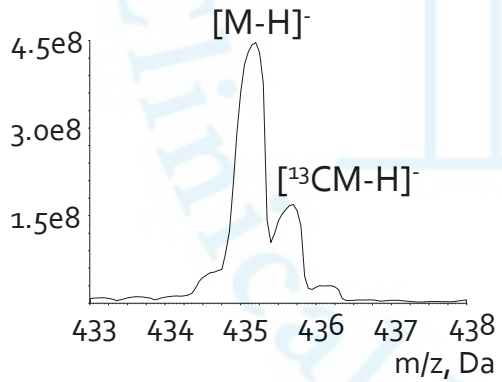
Labelling position is important - ESI confirmed purity and used for assay

LAST ON – FIRST OFF!

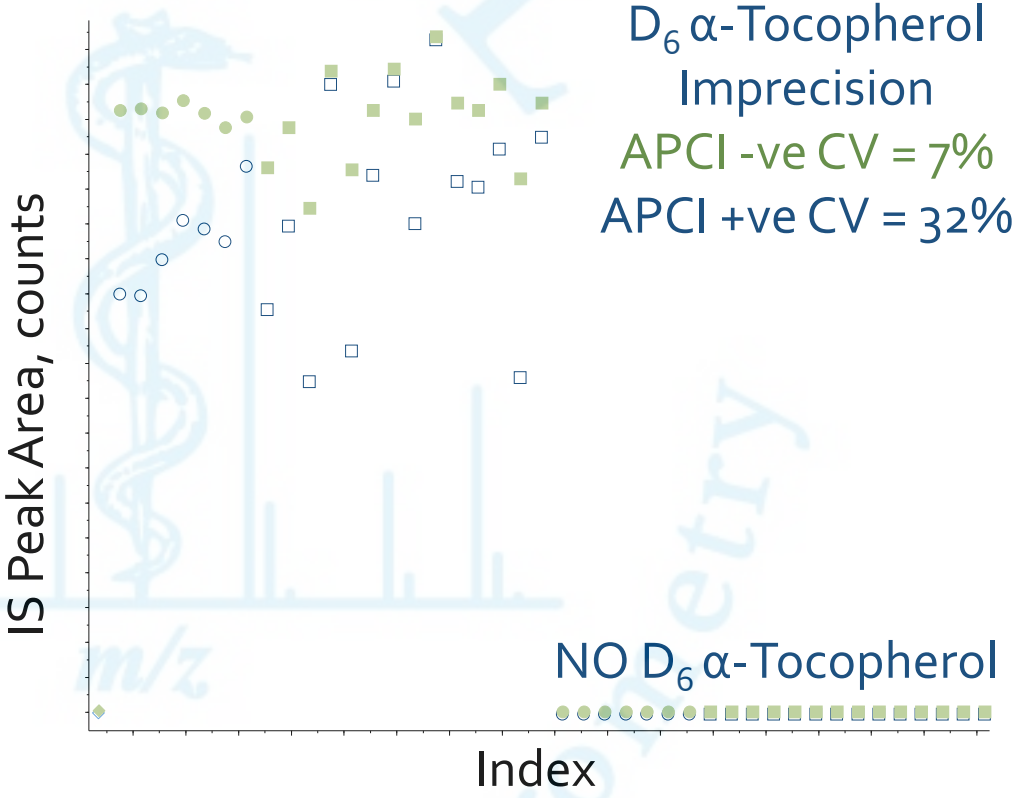
Ion Sources do weird things



Q1 Scan APCI +ve ion



Q1 Scan APCI -ve ion



D_6 α-Tocopherol Imprecision

APCI -ve CV = 7%

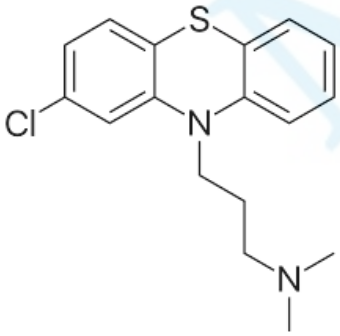
APCI +ve CV = 32%

NO D_6 α-Tocopherol

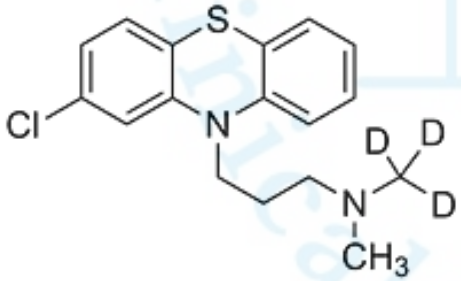
Radical Cation and isotopic variance leads to measurement variance

IS imprecision in calibrators and samples should guide Ion Source conditions

Curves Diverge?

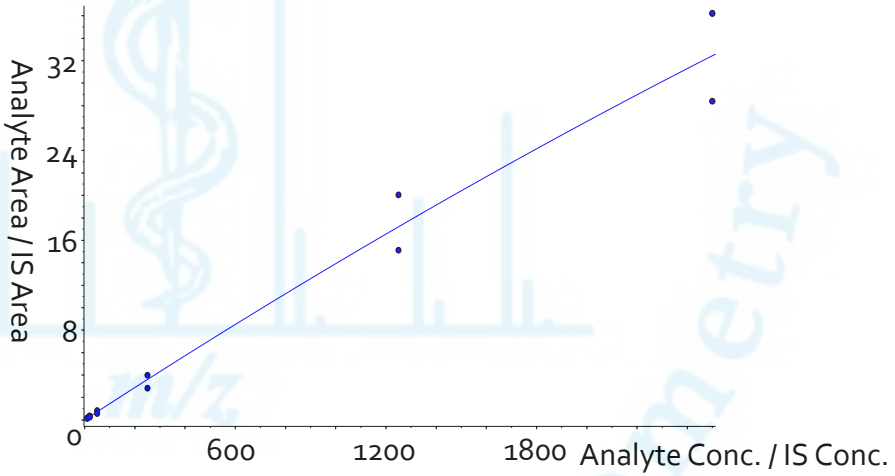


Chlorpromazine
m/z 319-86

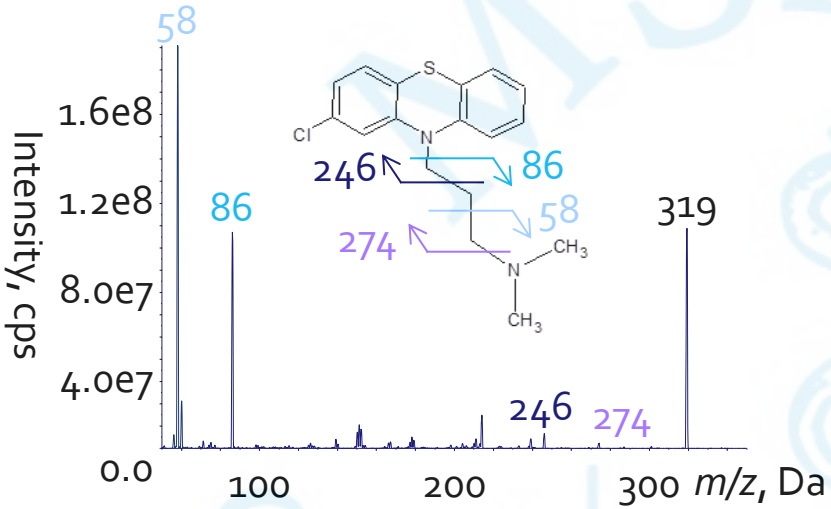


D3-Chlorpromazine
m/z 322-89

1st (top) verses 8th (bottom) divergence using "matched" transitions

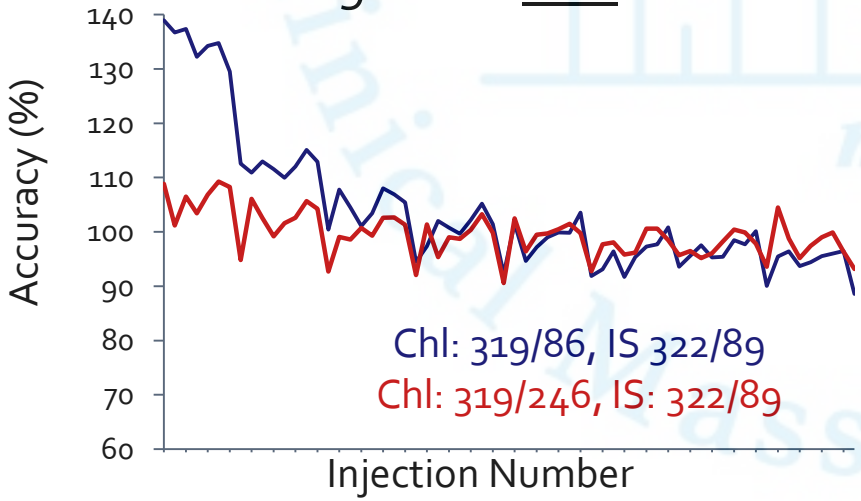


Facile Fragmentation

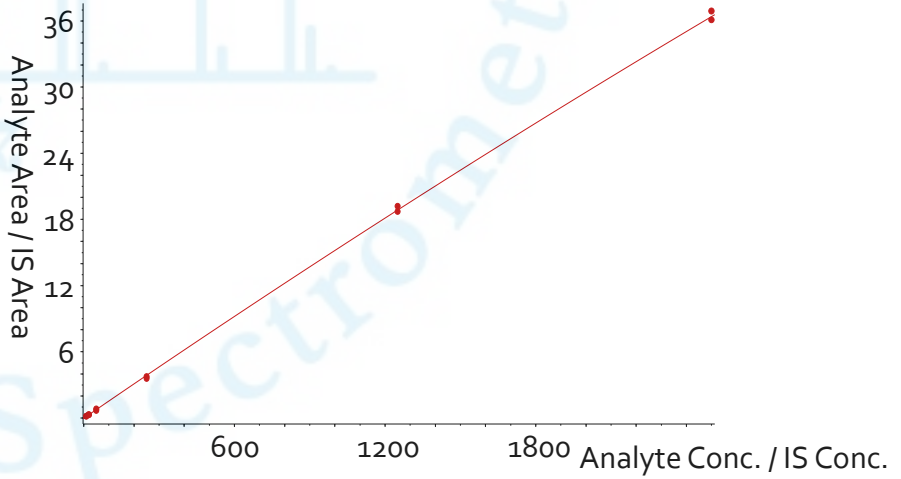


Product Ion retains N-dimethyl functionality (Deuterons)

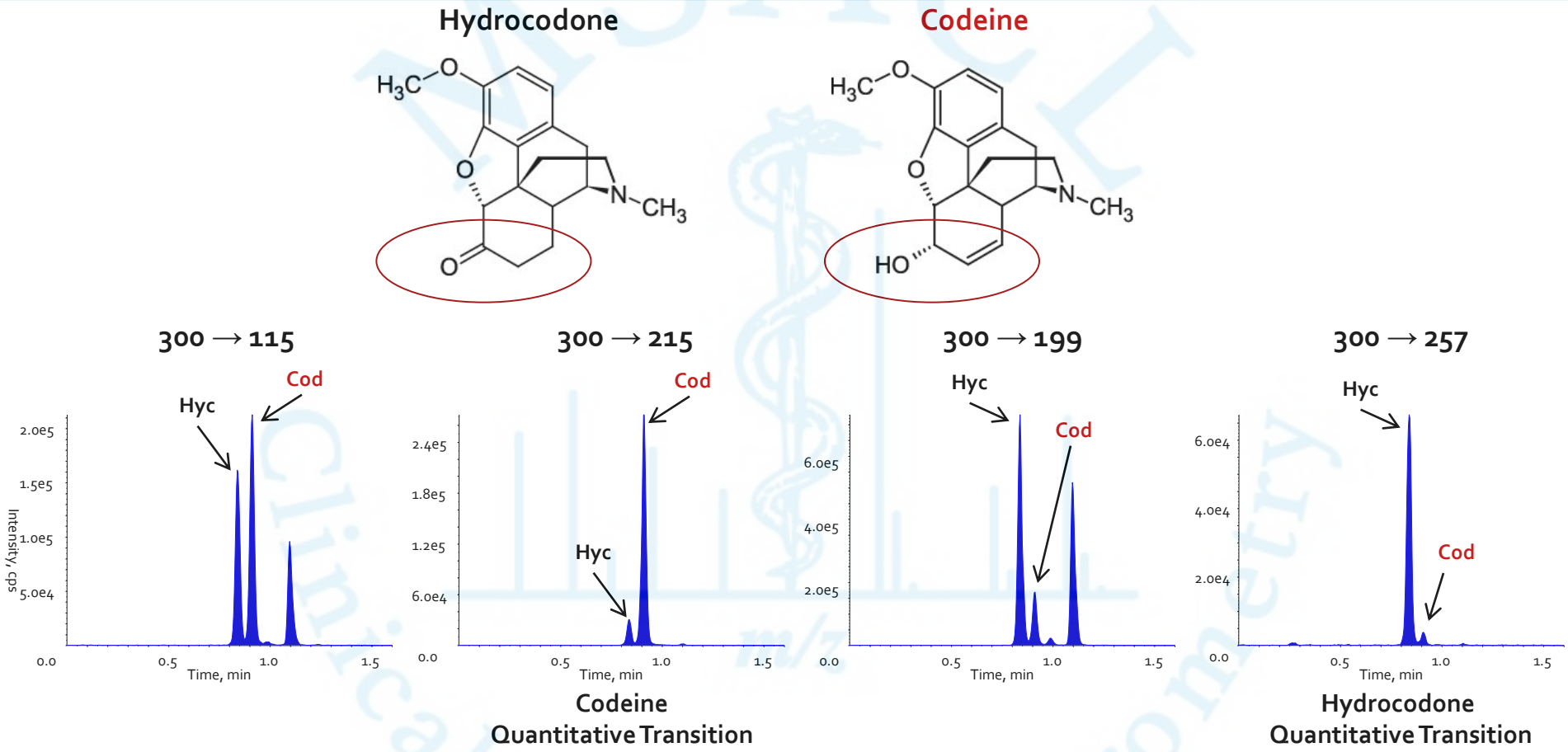
Repeat injection of a Known sample Starting with a Cold ion source



1st verses 8th Curves using "mis-matched" transitions



Transition Selection to minimize Isobaric Contribution



>20 transitions screened per isobaric pair in matrix samples, not just neats

Most sensitive ≠ best transition to use

More Isobaric (and Isotopic) Hindrances – selectivity!

Hydrocodone

m/z = 300.2→257.2

Codeine

m/z = 300.2→215.2

Hydromorphone

m/z = 286.2→185.2

Morphine

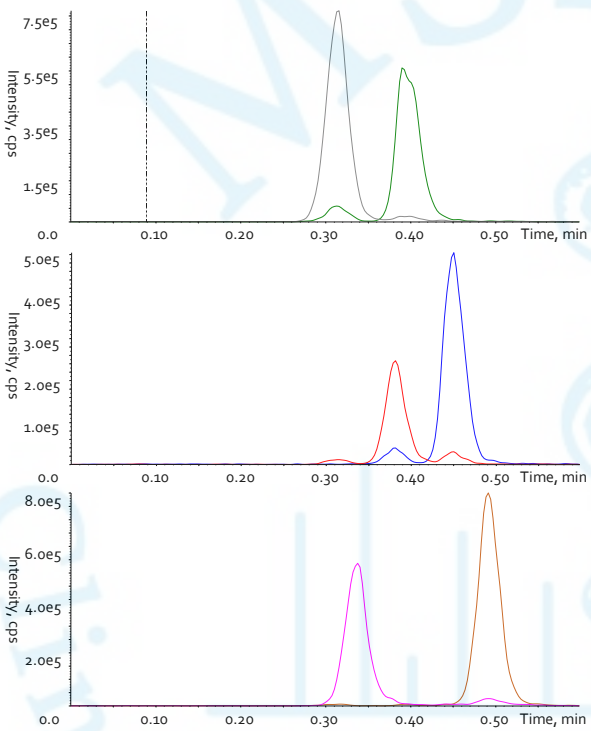
m/z = 286.2→155.2

Oxymorphone

m/z = 302.2→242.2

Dihydrocodeine

m/z = 302.2→245.2



Hydrocodone-d3

m/z = 303.2→241.2

Codeine-d6

m/z = 306.2→115.2

Hydromorphone-d6

m/z = 292.2→185.2

Morphine-d3

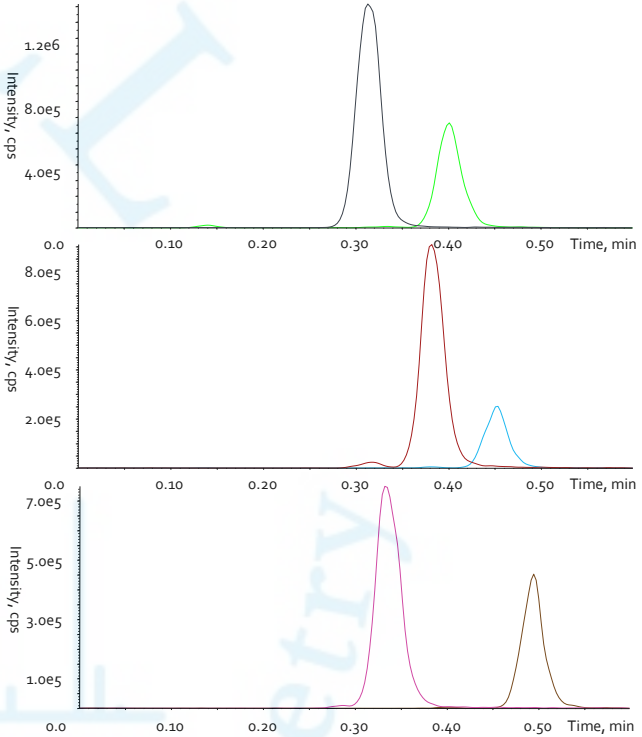
m/z = 289.2→152.2

Oxymorphone-d3

m/z = 305.2→230.2

Dihydrocodeine-d6

m/z = 308.2→230.2



4 analytes and 4 IS's in 8 mass unit range

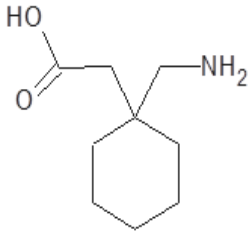
D_3 or D_6 IS's in isobaric pairs for automated peak selection
Mismatched IS transitions for peak purity

D_6- Codeine because of ^{13}C contribution of Oxym/DHC

Too many Deuterons can hurt you

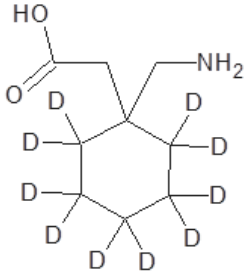
Gabapentin

172.2 - 137.1

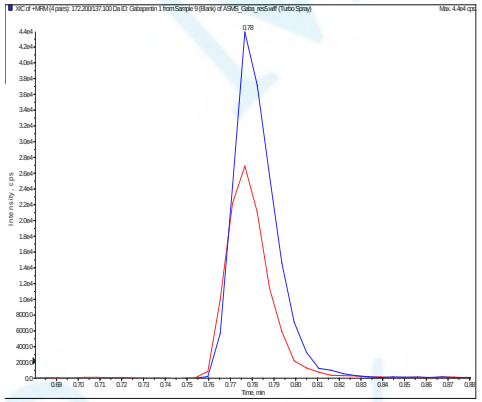


D10-Gabapentin

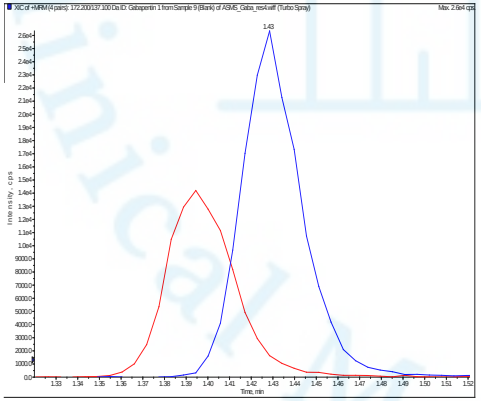
182.2 -147.1



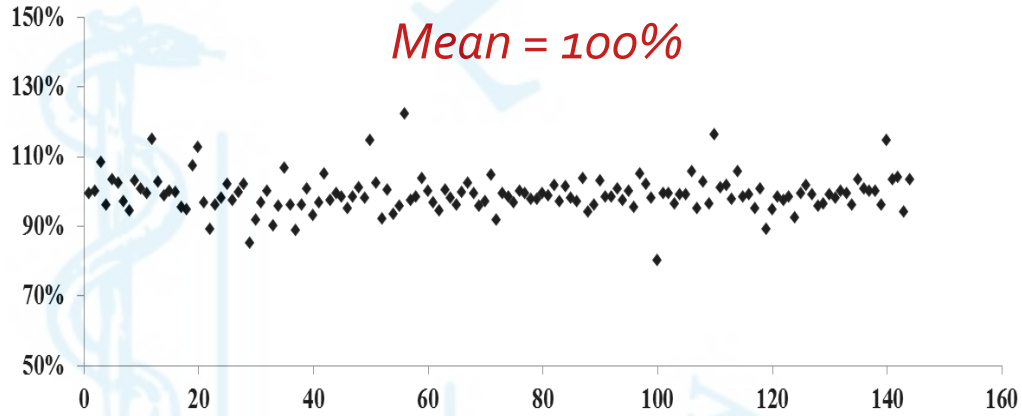
Fast LC: Co-elution



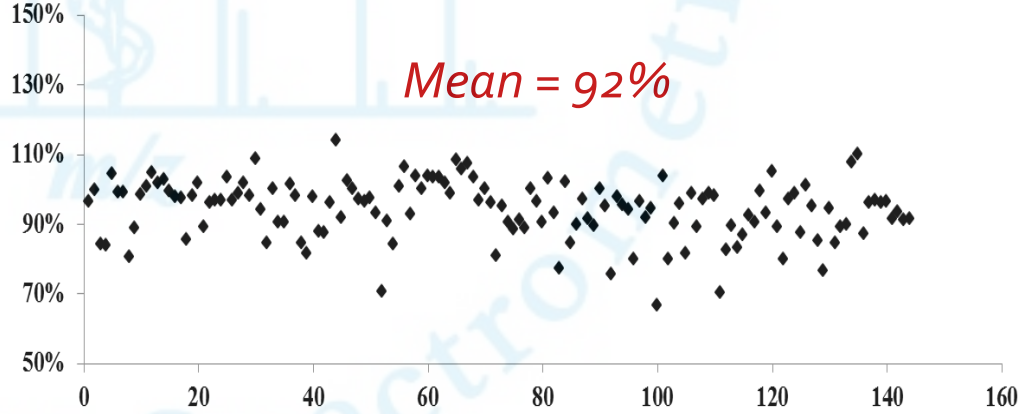
Slow LC: Separation



1:1 Gabapentin:D10 IS in Clean Urine



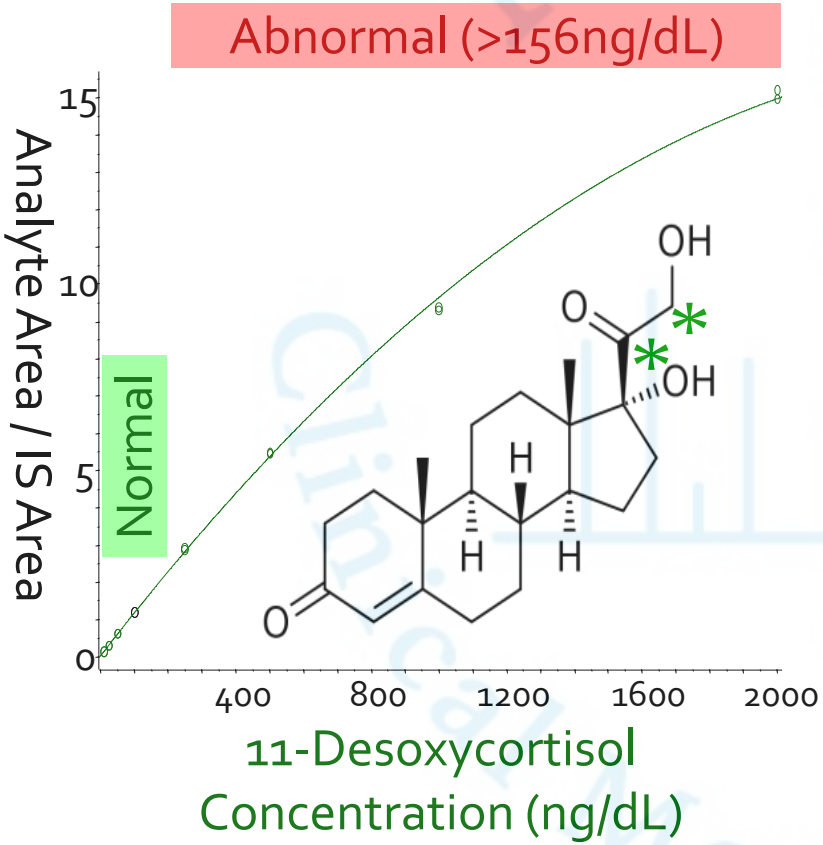
D10-Gabapentin IS not Co-eluting



Co-elution is <u>VERY</u> important

Courtesy of Brian Rappold, ASMS poster 2012 – Deception in the Deuteriums

Too few labels can hurt you also



Calibration curve non-linear 3-log range for 11-Desoxycortisol

$^{13}C_2$ labelled IS only (*)

Analyte isotopically contributes to IS

Solutions:

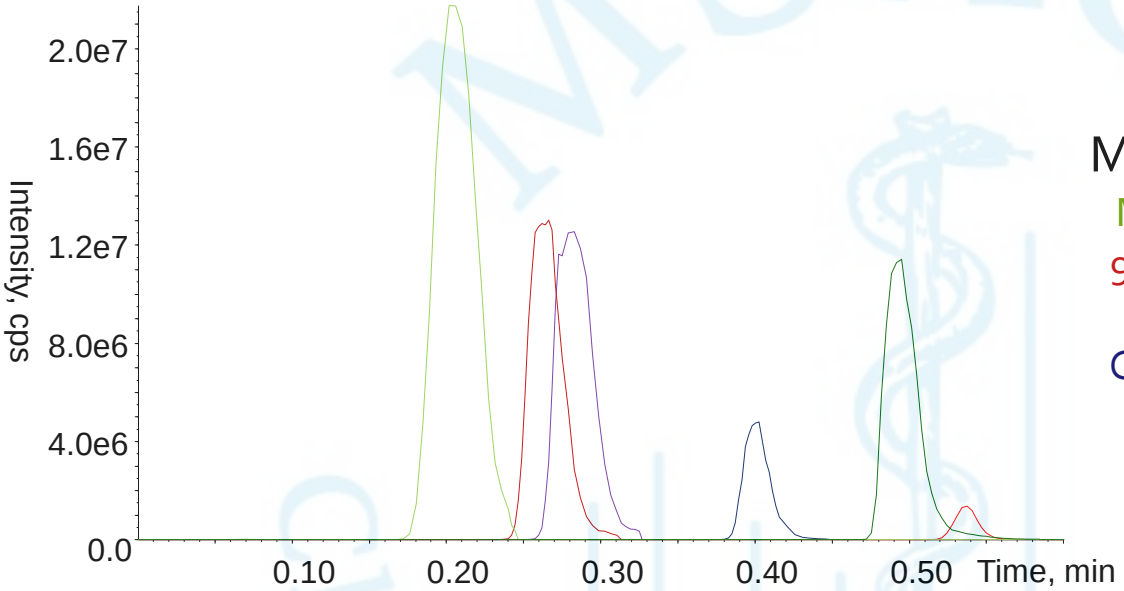
Truncate to linear range assay neat and pre-dilute samples (<u>two analysis</u>!),

Repeat on dilution > mid point

Increase concentration/alternate IS

<u>Clinically acceptable as is</u>

IS response decreases in Calibrators + Quadratic Curve



Multiplex assay 25000-fold range

Methylphenidate (1-250 ng/mL, D_9 IS),

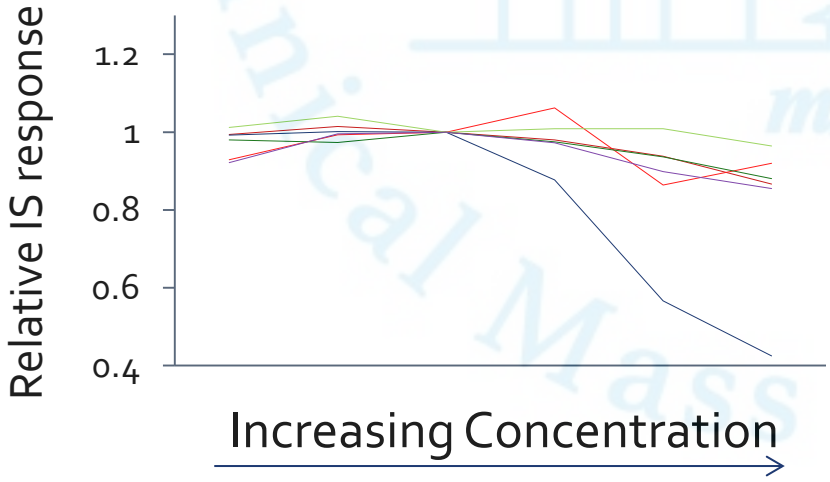
9-OH Risperidone (1-250 ng/mL, D_4 IS),

Risperidone (1-250 ng/mL, D_4 IS),

Chlorpromazine (10-2500 ng/mL, D_3 IS)

Haloperidol (1-250 ng/mL, D_4 IS),

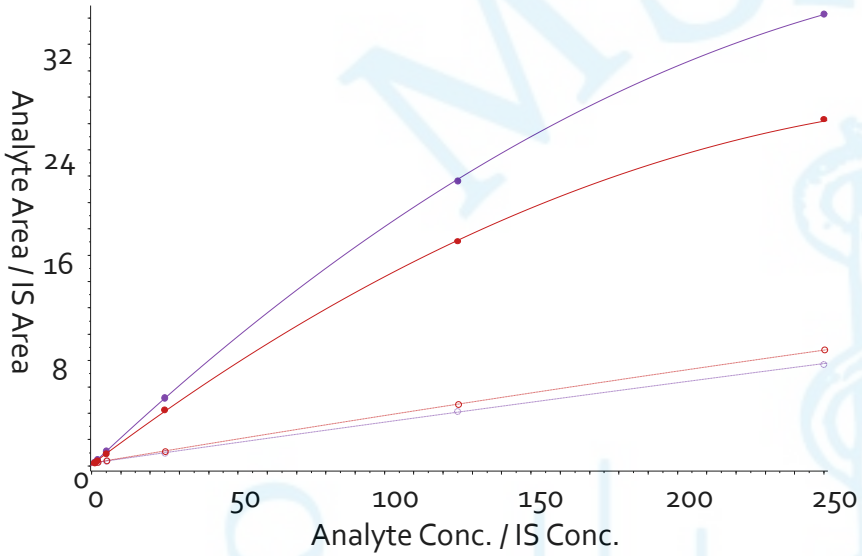
Fluphenazine (0.1-25 ng/mL, D_8 IS*),



IS response versus 3rd Calibrator with increased analyte concentrations

Most show some reduction in signal

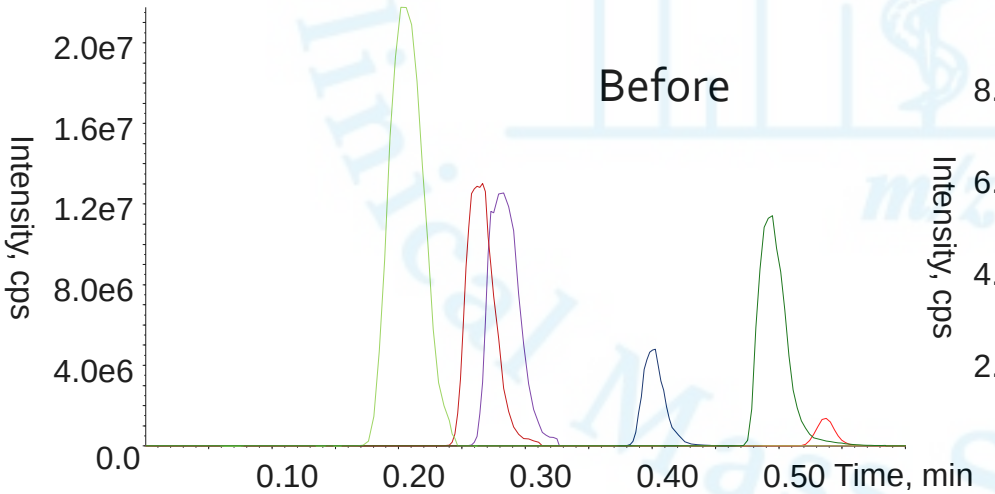
Does it result in error?

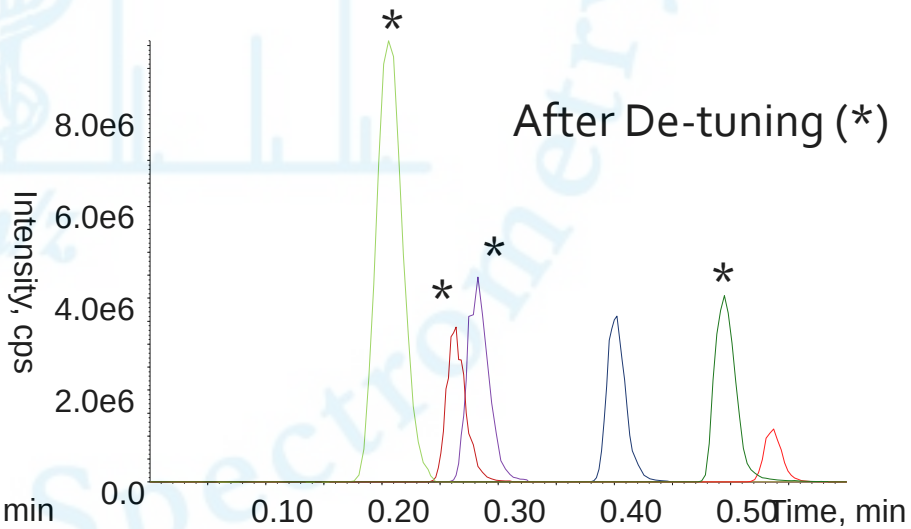


Risperidone and 9-OH Risperidone
IS response reduction and quadratic curve

Detuned MASS Spectrometer Transition efficiency for Analyte Transitions ONLY

Culprit was Multiplier Blinding as source suppression affects BOTH Analyte and IS

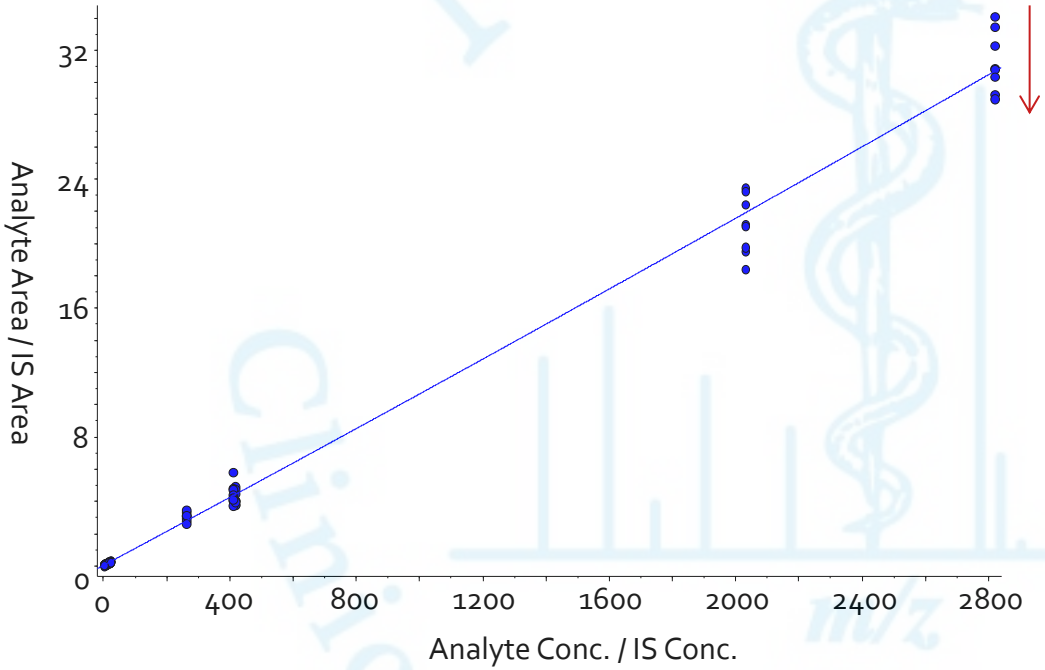




Are you observing it co-suppression or detector blinding

Dynamic Extraction with PPT plates

Testosterone + $^{13}C_3$ Testosterone in Methanol
Add IS to plate, then add Serum, mix 5 min, vacuum

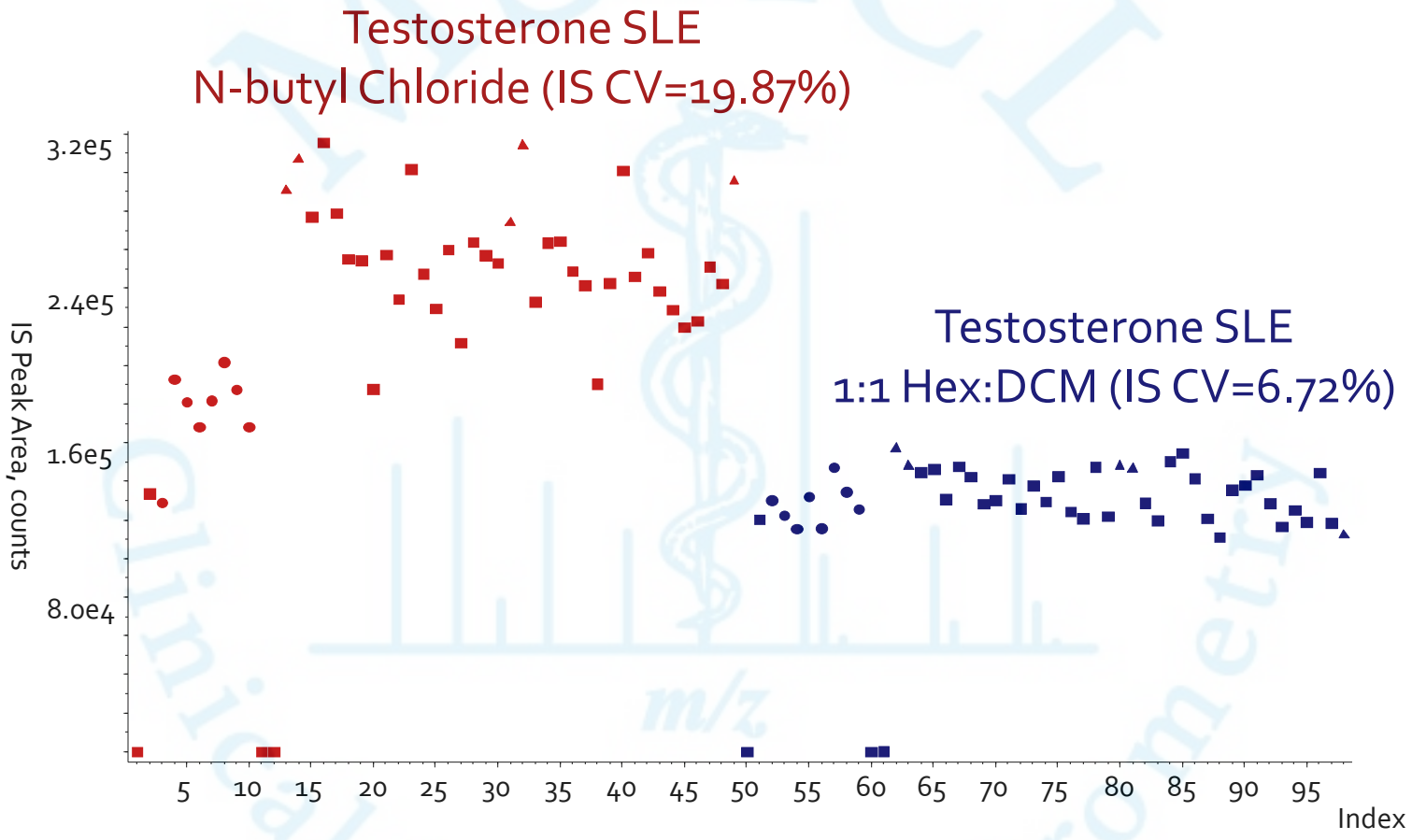


Curve 1 - 8

Dynamic (non-equilibrated) extraction obvious in charcoal stripped serum calibration matrix

Off line mixing, centrifugation then addition to PPT plate OK (ish)

Extraction: Increased Recovery or reduced Imprecision?



Go for reduced imprecision of IS every time

Enables outlier detection!

Estrone and Estradiol using Supported Liquid Extraction

Back-fit Bias compared to 5 minute mixing

	Temperature	5min	15min	30min	60min
Estrone Calibrator Mean Bias (%)	RT	NA	4.6%	5.3%	8.1%
Estrone QC Mean Bias (%)	RT	NA	2.1%	-1.7%	-1.7%
Estrone Samples Mean Bias (%)	RT	NA	-3.3%	-4.9%	-3.7%
Estradiol Calibrator Mean Bias (%)	RT	NA	16.3%	20.5%	21.0%
Estradiol QC Mean Bias (%)	RT	NA	5.2%	15.7%	17.2%
Estradiol Samples Mean Bias (%)	RT	NA	9.8%	16.8%	15.1%

Estradiol bias for Calibrators, QC pools/Samples when mixing prior to SLE

Do we believe the MS results? YES

Consistent across matrix types? YES

Timing of the experiment (temperature of samples/pipetting consistency?) YES

High quality IS? $^{13}C_6$ for BOTH

Observed in predicate LLE assay? NO..it was a 90 minute mixing step

Solution: *Evaluate Equilibration of Analyte and IS*

Temperature affects binding kinetics..obviously..

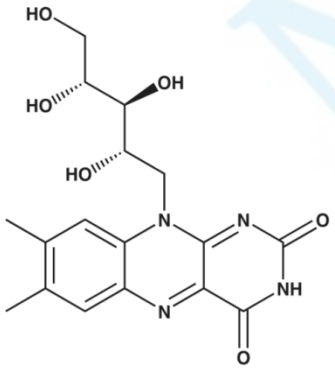
Back-fit Bias compared to 5 minute mixing

	Temperature	5min	15min	30min	60min
Estrone Calibrator Mean Bias (%)	RT	NA	4.6%	5.3%	8.1%
Estrone QC Mean Bias (%)	RT	NA	2.1%	-1.7%	-1.7%
Estrone Samples Mean Bias (%)	RT	NA	-3.3%	-4.9%	-3.7%
Estrone Calibrator Mean Bias (%)	37C	NA	0.3%	-0.9%	0.9%
Estrone QC Mean Bias (%)	37C	NA	-2.5%	-0.3%	-2.7%
Estrone Samples Mean Bias (%)	37C	NA	1.4%	1.1%	1.6%
Estradiol Calibrator Mean Bias (%)	RT	NA	16.3%	20.5%	21.0%
Estradiol QC Mean Bias (%)	RT	NA	5.2%	15.7%	17.2%
Estradiol Samples Mean Bias (%)	RT	NA	9.8%	16.8%	15.1%
Estradiol Calibrator Mean Bias (%)	37C	NA	0.5%	5.6%	3.1%
Estradiol QC Mean Bias (%)	37C	NA	1.4%	-2.8%	3.8%
Estradiol Samples Mean Bias (%)	37C	NA	3.1%	1.6%	2.4%

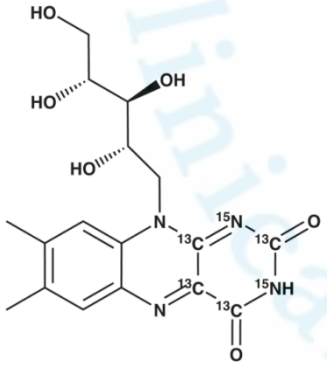
Mixing at 37C – equivalent accuracy (analyte/IS) recovery over time

Note: This informs how you perform Spike and Recovery in validation

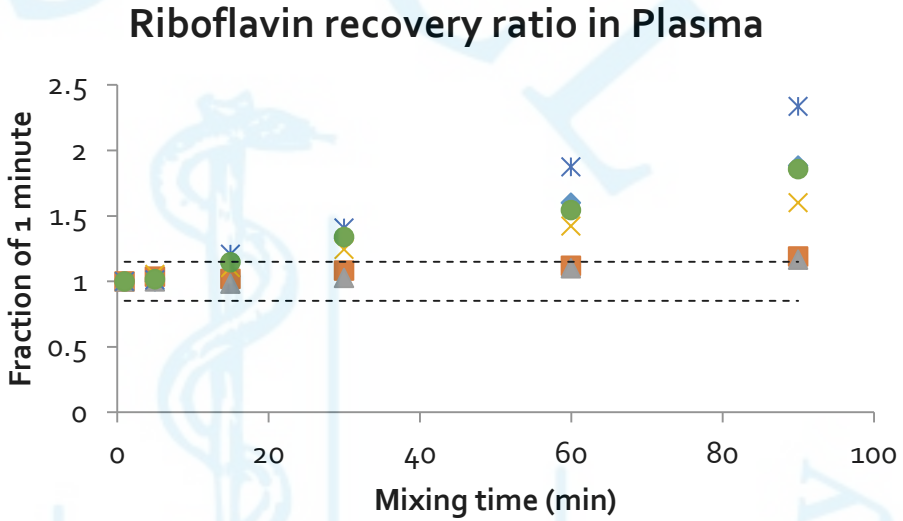
Perfectly labelled Internal standard added in Methanol (PPT/Mix)

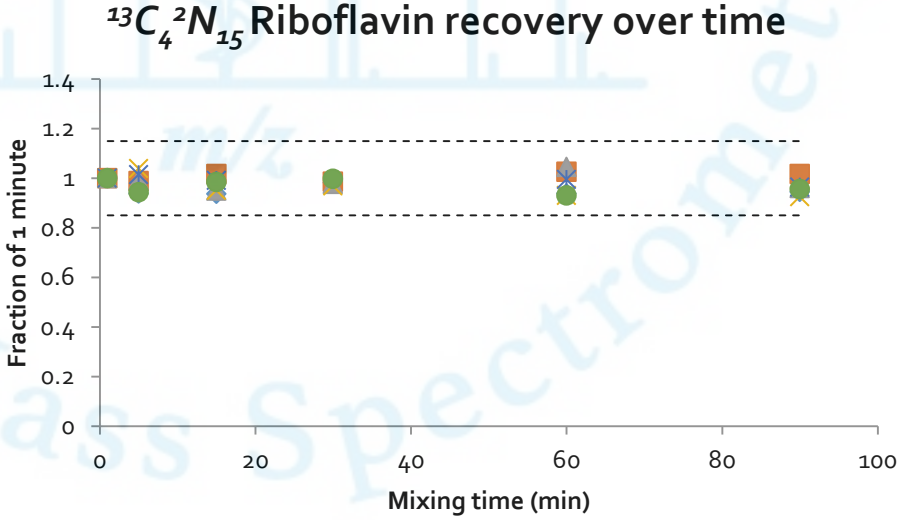


Riboflavin



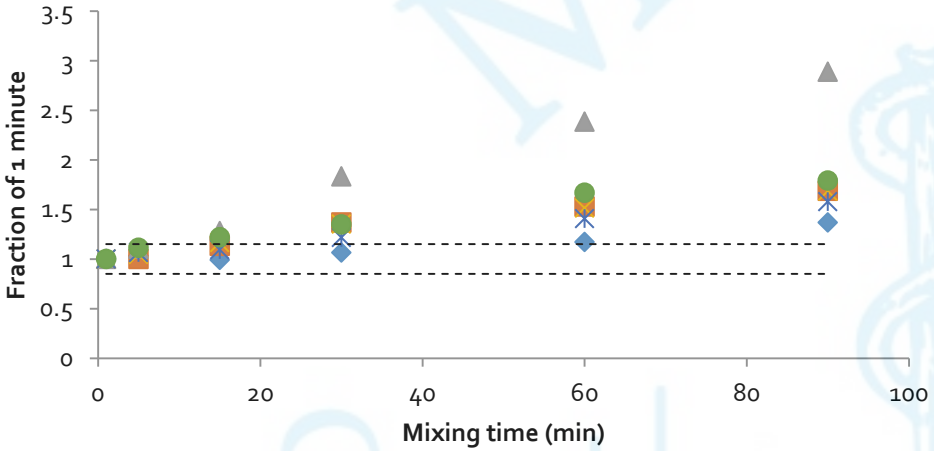
$^{13}C_4{}^2N_{15}$ Riboflavin



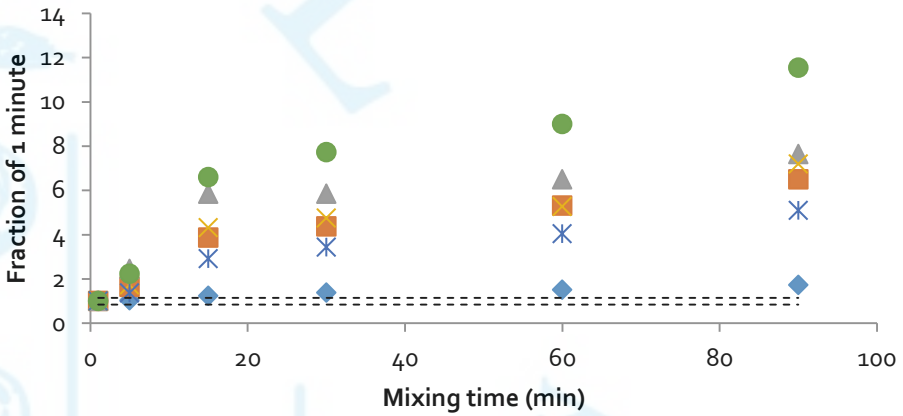


Reached equilibrium yet? Incomplete recovery?

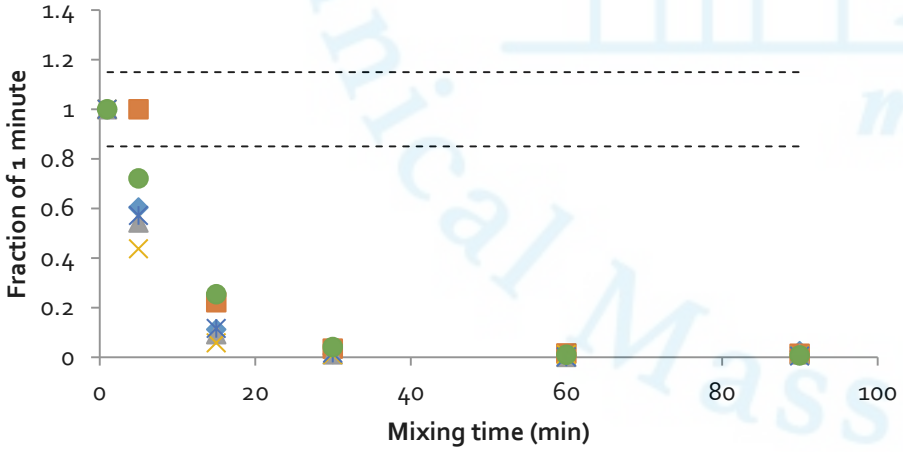
Riboflavin recovery ratio in Plasma 37 °C



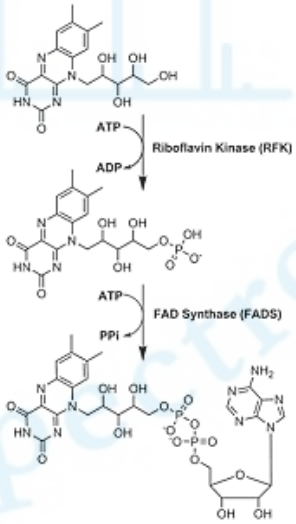
Riboflavin recovery ratio in Plasma + 0.1% Formic Acid



FAD-IS recovery in Plasma 37 °C



VitB2 RBC Biology



VitB2 Plasma Biology

Riboflavin

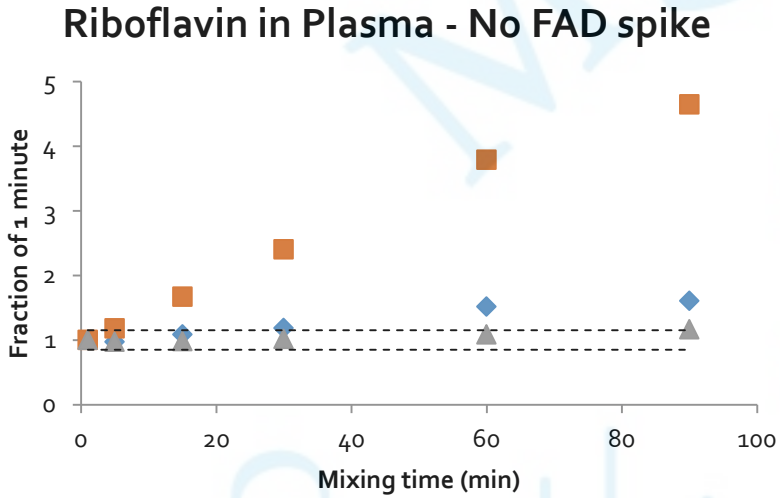
Slow in plasma

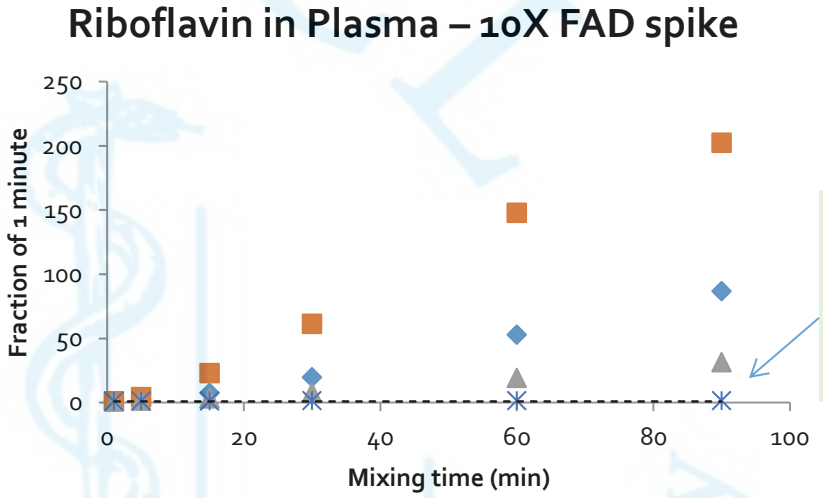
Flavin Mononucleotide

Fast in plasma

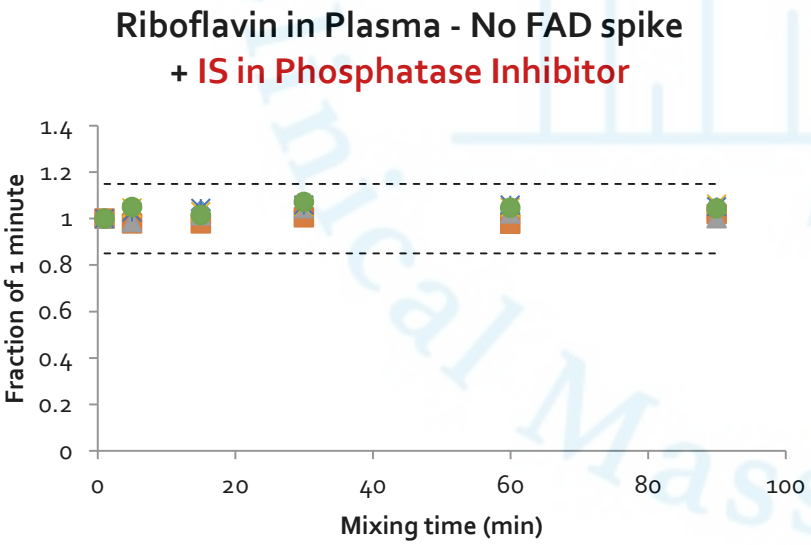
Flavin Adenine Dinucleotide

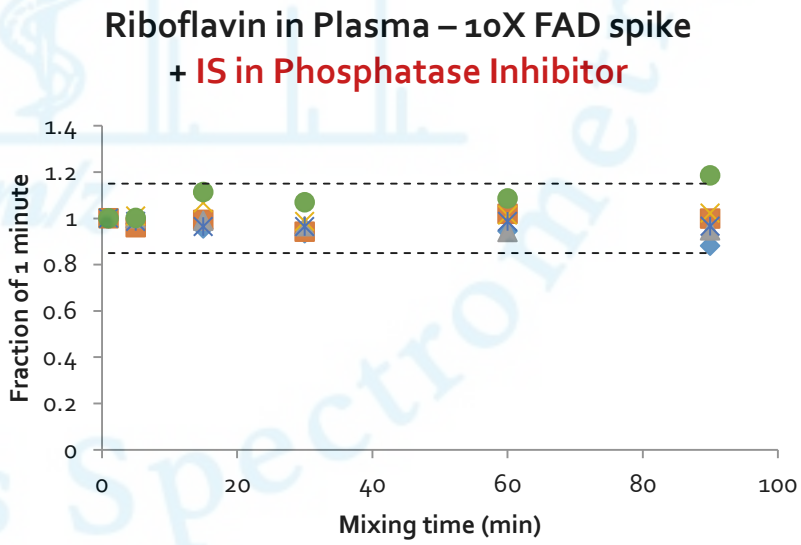
FAD overspike, Test and correct biological error





X = FAD Spiked into Water





Oddly…Riboflavin unchanged up to 4 hours following thaw in plasma samples

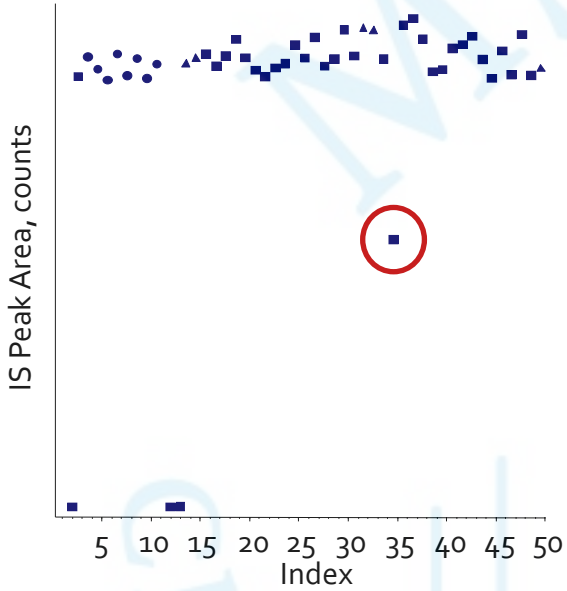
EVERYTHING YOU WANTED TO KNOW ABOUT INTERNAL STANDARDS, BUT WERE TOO AFRAID TO ASK

PART 3: UNIQUE CAPABILITIES

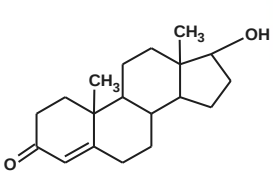
RUSSELL GRANT

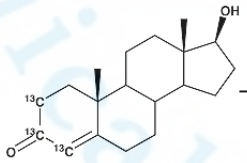
LABORATORY CORPORATION OF AMERICA® HOLDINGS, BURLINGTON, NC USA

Release? Reinject or Re-extract?

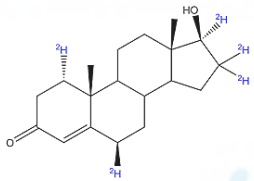


What is the IS correcting for
Recovery
Transfer
Injection
Ionization?





Mix, Equilibrate,
SLE, Evaporate



Mix, Transfer,
LC-MS/MS

Testosterone
m/z 289 – 97, 109

$^{3}C_{13}$ Testosterone
m/z 292 – 100, 112

Recon with different IS
D_5-Testosterone
m/z 294 – 100, 111

Two IS's is better than one

	1	2	3	4	5	6	7	8
Volume Added to SLE (%)	100	75	50	25	100	100	100	100
Volume Injected (%)	100	100	100	100	100	75	50	25

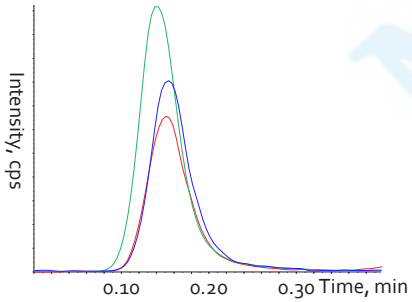
Testosterone

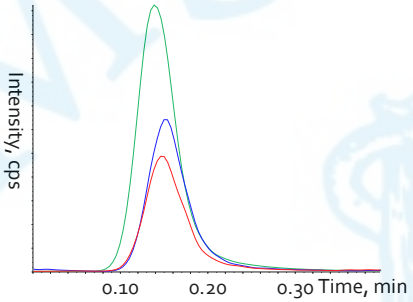
$^{3}C_{13}$ Testosterone

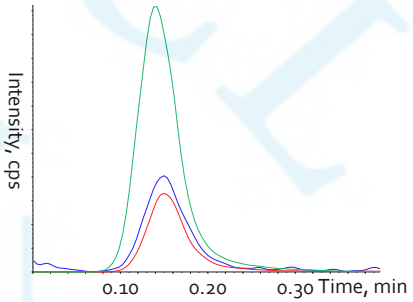
D_5-Testosterone

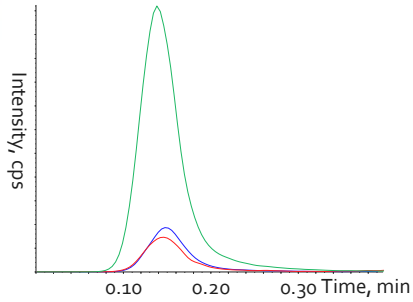
$^{3}C_{13}$ Testosterone

And when tested experimentally….



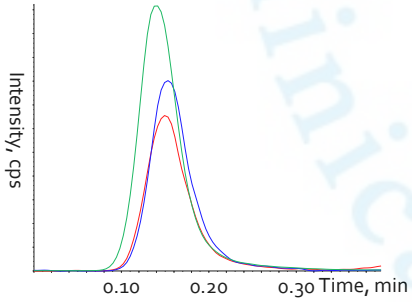


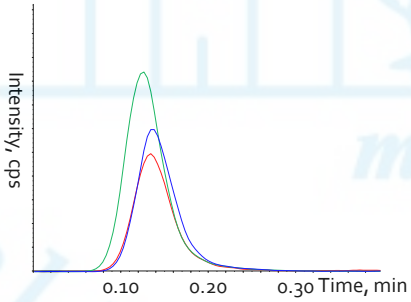


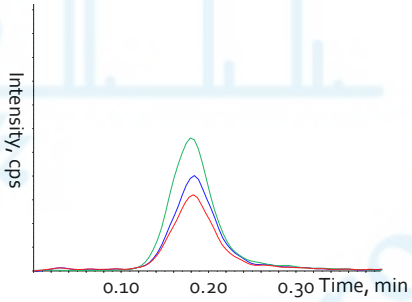


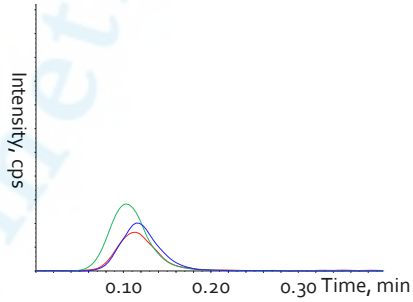
Testosterone/$^{13}C_3$ Testosterone Ratio	1.2	1.2	1.1	1.1
D_5 Testosterone/$^{13}C_3$ Testosterone Ratio	1.7	2.1	3.3	6.8

SLE Under delivery: Unless you REALLY know of volumetric errors – Re-Extract









Testosterone/$^{13}C_3$ Testosterone Ratio	1.2	1.2	1.1	1.1
D_5 Testosterone/$^{13}C_3$ Testosterone Ratio	1.7	1.7	1.6	1.5

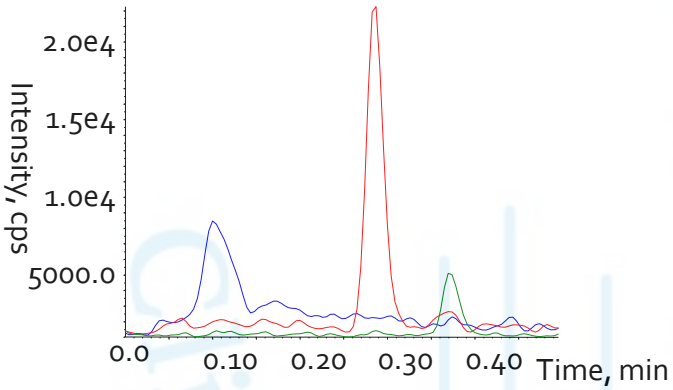
Injection/Ionization error: Release if responses acceptable or re-inject

Calibration conundrum

Using "bracketing" calibration curves…you are averaging drift across a run

Using a single curve…you are performing Historical Calibration

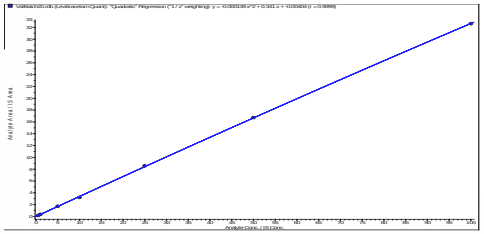
…….because the IS enables you to do so



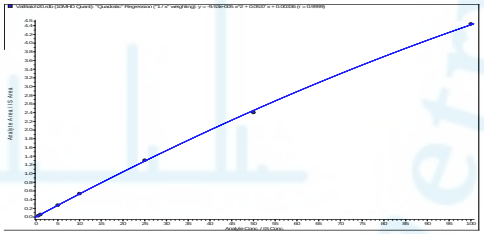
LLOQ 0.5µg/mL

Levetiracetam, Lacosamide,

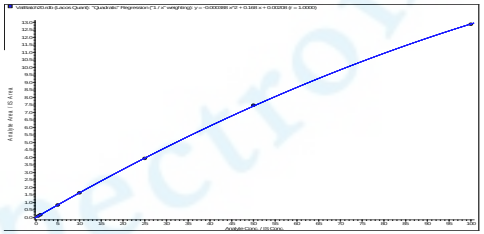
Oxcarbazepine (metabolite)



Levetiracetam, 0.5 – 100µg/mL, r = 0.9999



Oxcarbazepine, 0.5 – 100µg/mL, r = 0.9999



Lacosamide, 0.5 – 100µg/mL, r = 1.0000

Figures of merit for Inter-assay (n=20 at 6 levels)

Levetiracetam: Imprecision < 10.07%, Bias < 6.35%

Oxcarbazepine: Imprecision < 6.03%, Bias < 9.98%

Lacosamide: Imprecision < 8.58%, Bias < 6.03%

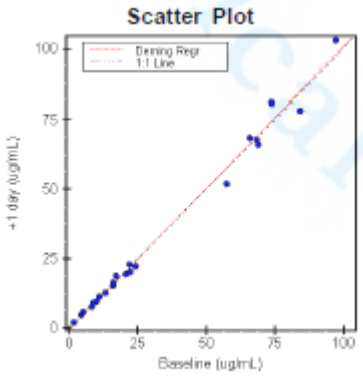
The IS actually enables Historical calibration

Freeze separate aliquots, thaw a single aliquot and assay (Day zero)
Store working IS solution in stable conditions (solution, container, temperature)

24 hours later..thaw another aliquot and use stored IS –
No calibrators – measure Analyte/IS ratio against day zero curve
Compare Day zero (x) to + 24 hours (y)

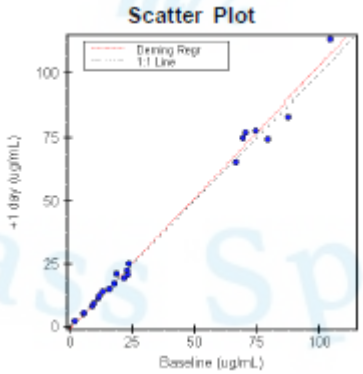
Levetiracetam + 24 hours

Deming Slope = 1.016
Intercept = -0.7749
Corr Coef, r = 0.9955



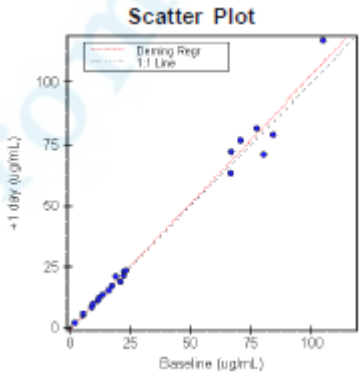
Oxcarbazepine + 24 hours

Deming Slope = 1.039
Intercept = -0.8327
Corr Coef, r = 0.9956

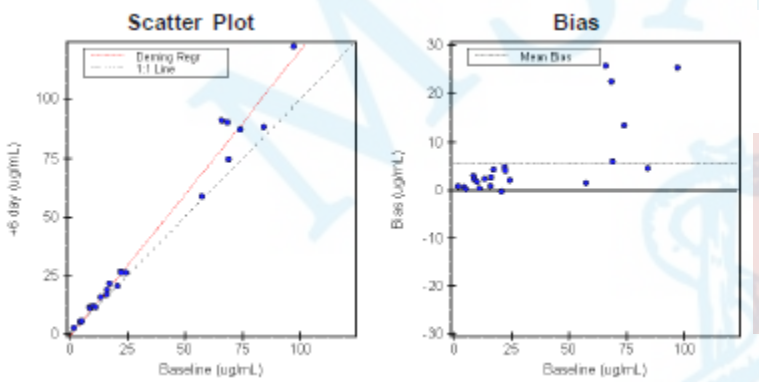


Lacosamide + 24 hours

Deming Slope = 1.039
Intercept = -0.9056
Corr Coef, r = 0.9927

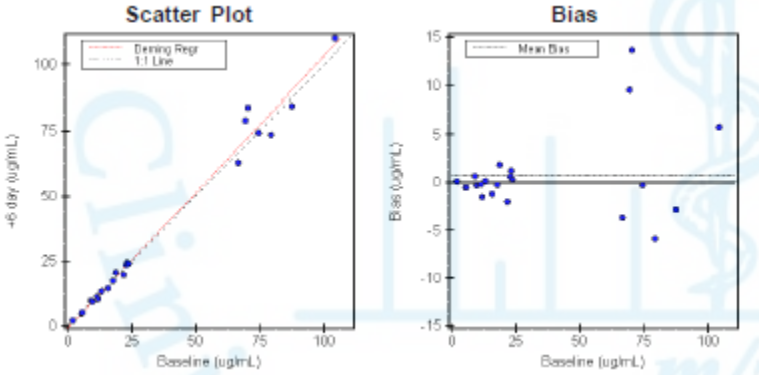


7 days later



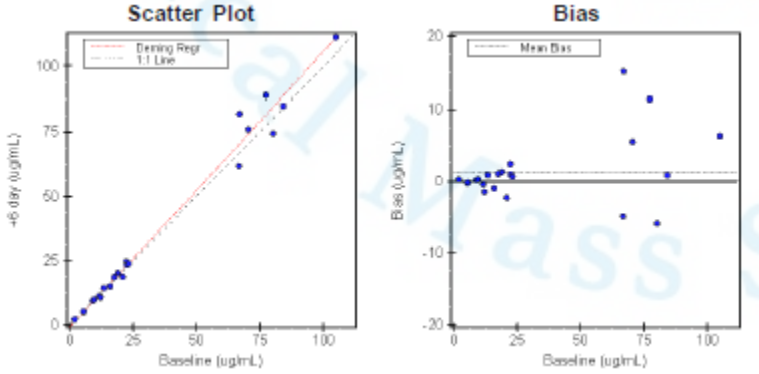
Levetiracetam + 144 hours

Deming Slope = 1.217
IS Degraded - only good for 3 days
Intercept = -1.1966
Corr Coef, r = 0.9892



Oxcarbazepine + 144 hours

Deming Slope = 1.041
Intercept = -0.7982
Corr Coef, r = 0.9927

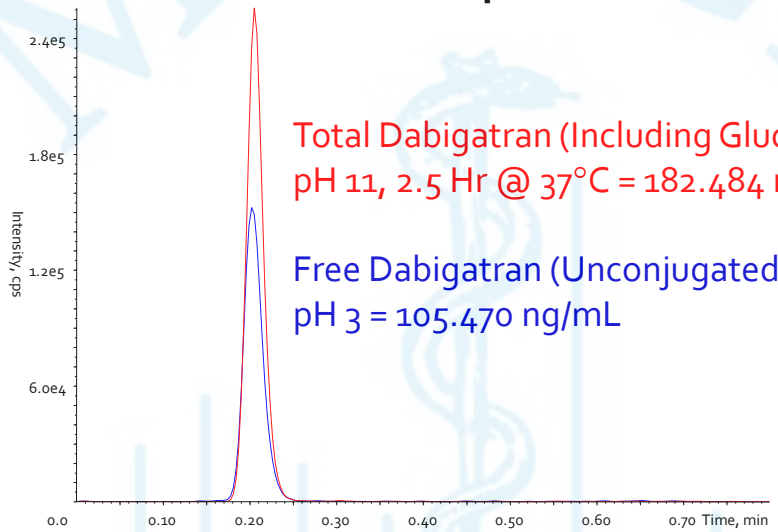


Lacosamide + 144 hours

Deming Slope = 1.065
Intercept = -0.8589
Corr Coef, r = 0.9920

When sample preparation is painful

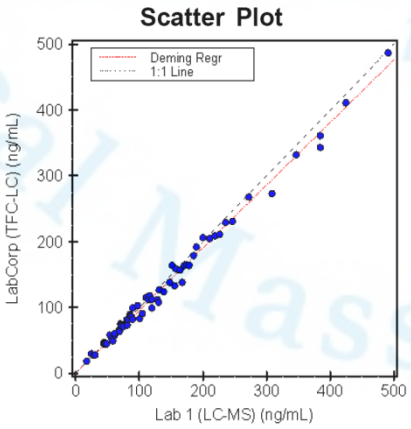
TFC-LC-MS/MS – pH Modification required



Total Dabigatran (Including Glucoronide)
pH 11, 2.5 Hr @ 37°C = 182.484 ng/mL

Free Dabigatran (Unconjugated)
pH 3 = 105.470 ng/mL

LC-MS/MS (FDA Trial samples) versus TFC-LC-MS/MS

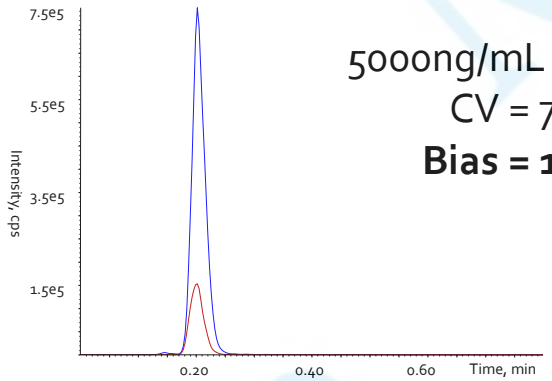


	Deming
Slope:	0.951 (0.930 to 0.973)
Intercept:	0.6182 (-3.0005 to 4.2369)
Std Err Est:	8.7582

Corr Coef (R):	0.9959
Bias:	-6.0322
X Mean ± SD:	136.5904 ± 100.7016
Y Mean ± SD:	130.5582 ± 95.8182
Std Dev Diffs:	10.1615

For samples > ULOQ (Calibrated Analyte peak area)

Dilute then add IS



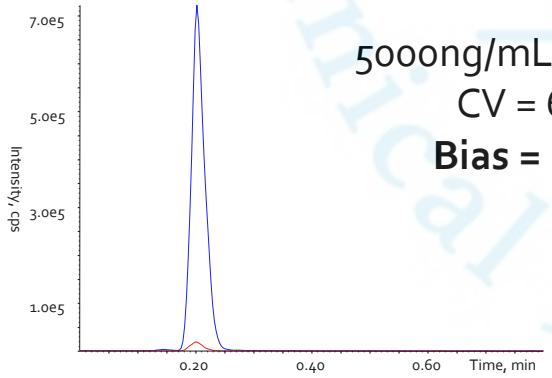
5000ng/mL (5 x ULOQ)
CV = 7.52%
Bias = 12.11%

Analyte peak area (Ar)
IS peak Area (Ir)

BUT…Look closely at the Axis

Analyte concentration (Ac)
Internal standard concentration (Ic)

Dilute 10x in well, re-inject or inject less



5000ng/mL (5 x ULOQ)
CV = 6.57%
Bias = -2.20%

For Linear-fit calibration curves: (y=mX-b)

Dilute "in-well" and re-inject or inject less so that

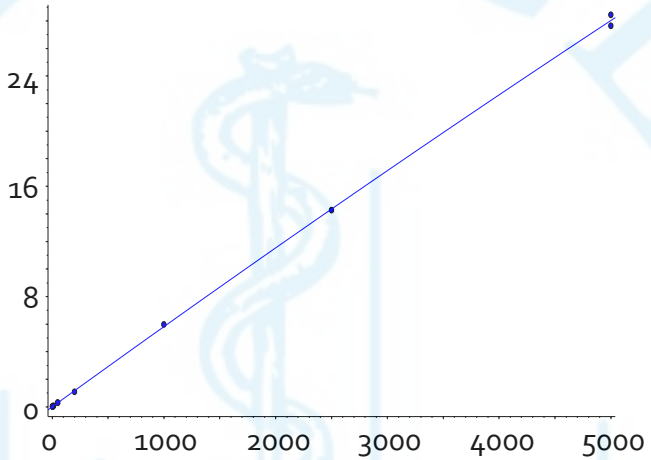
Analyte peak area response ≤ ULOQ (linear range)
IS peak area response ≥ Analyte LLOQ (ideally 10x)

Linear calibration fit = linear response of analyte - applies to good IS's, even lower "amounts"
Reduced bias observed as only 1 step pre IS not 4

Now you are thinking about calibration differently…RMP's

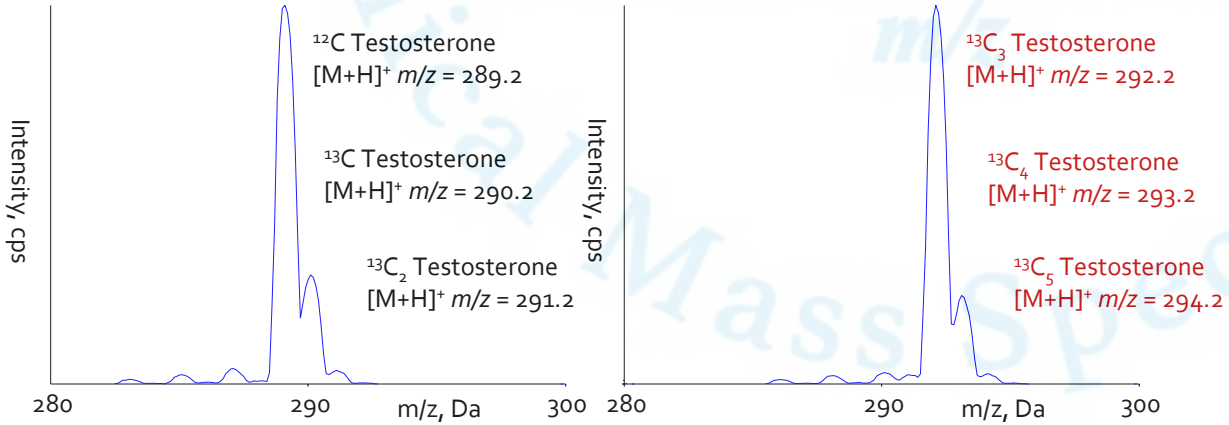
Step 1: Analyze samples using external calibration and isotope dilution

Analyte peak area response (Ar)
IS peak Area response (Ir)



Analyte concentration (Ac) / Internal standard concentration (Ic)

Step 2: Determine Equimolar relationship between Analyte and IS responses



1.1% Carbon is in the form of ^{13}C, Per NIST:

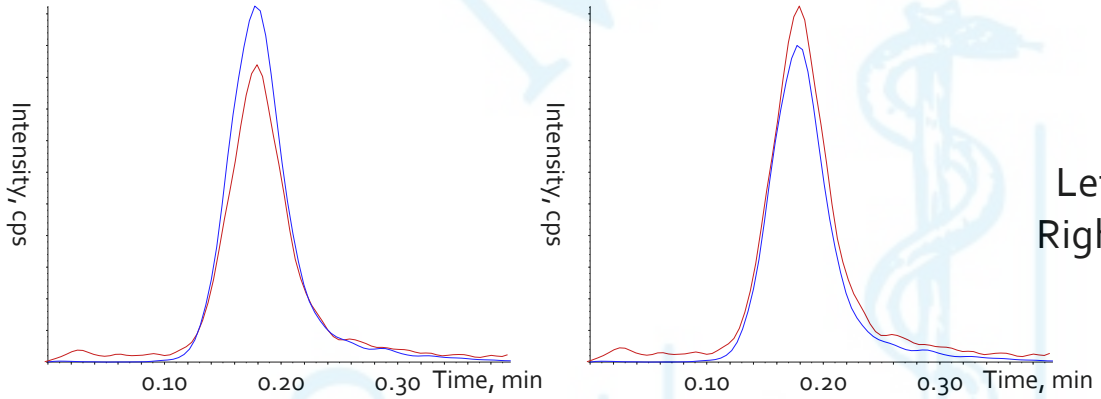
Testosterone ($C_{19}H_{28}O_2$) isotope ratio's
77%, 21%, 2%

$^{13}C_3$ Testosterone ($^{13}C_3{}^{12}C_{16}H_{28}O_2$) isotope ratio's
80%, 18%, 2%

Difference in Isotopic Contribution = 3%
100µM $^{13}C_3$ Testosterone = 103µM Testosterone

Bracketing or Absolute Matching with IS

Step 3: Analyze using "corrected" response function with Bracketing IS concentration



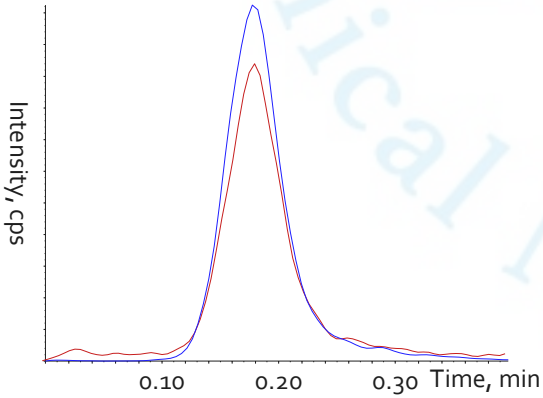
$$[\text{Analyte}] = \frac{\text{Analyte Area}}{\text{IS Area}} * [\text{IS}]$$

Left: 100000/90000 * 500 ng/dL = 555 ng/dL
Right: 100000/115000 * 600 ng/dL = 521 ng/dL

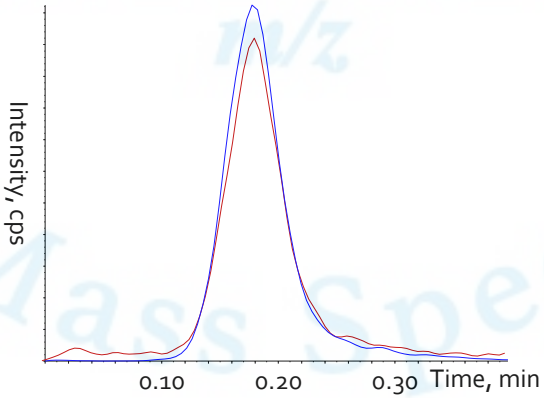
Average = 538 ng/dL

Or repeat analysis with IS modification until signal responses match [A] = [IS]

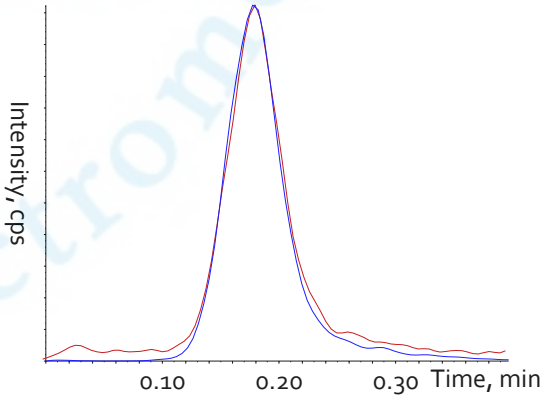
[IS] = 520 ng/dL < [A]



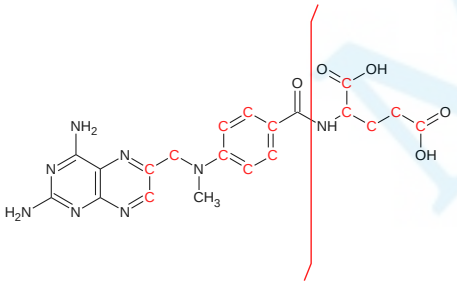
[IS] = 530 ng/dL < [A]



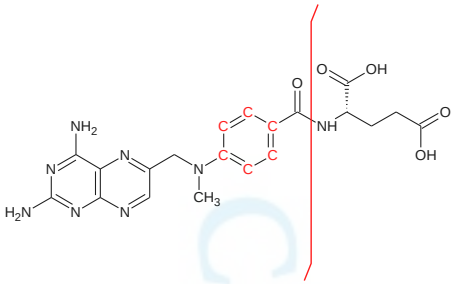
[IS] = 540 ng/dL = [A]



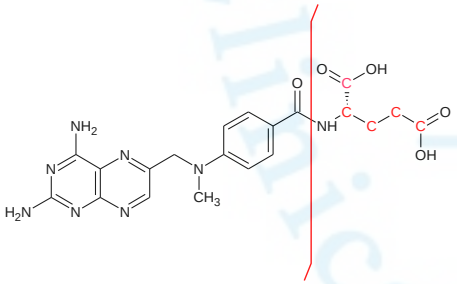
Now you are really thinking about calibration differently.. How about no external calibration whatsoever? Instrinsix ®



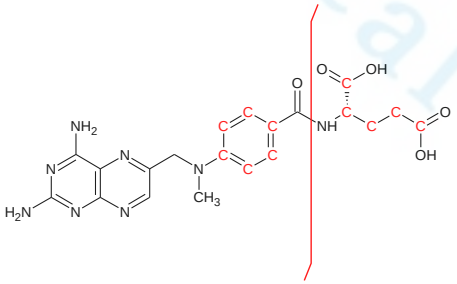
Methotrexate Calibrator 4
$C_6{}^{13}C_{14}H_{22}N_8O_5$
(10 µM)



Methotrexate Calibrator 3
$C_{14}{}^{13}C_6H_{22}N_8O_5$
(1.0 µM)



Methotrexate Calibrator 2
$C_{15}{}^{13}C_5H_{22}N_8O_5$
(0.1 µM)



Methotrexate Calibrator 1
$C_9{}^{13}C_{11}H_{22}N_8O_5$
(0.025 µM)

Protein precipitation workflow

Addition of differentially labelled C_{13} Methotrexate Calibrators

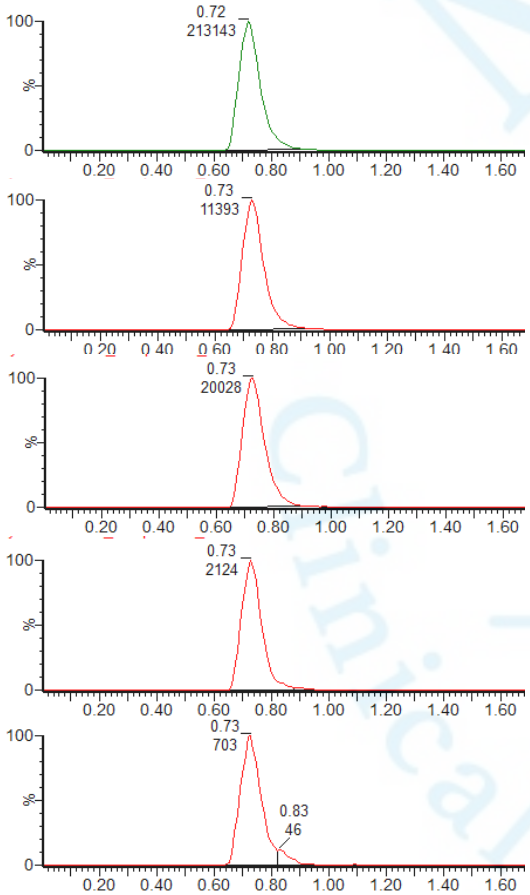
Known amounts added

No contribution between or to Methotrexate

^{13}C has no effect on retention time

Courtesy of Don Cooper and Don Mason, Waters Corporation

Intrinsix ® = Internal Calibration with an IS curve per sample



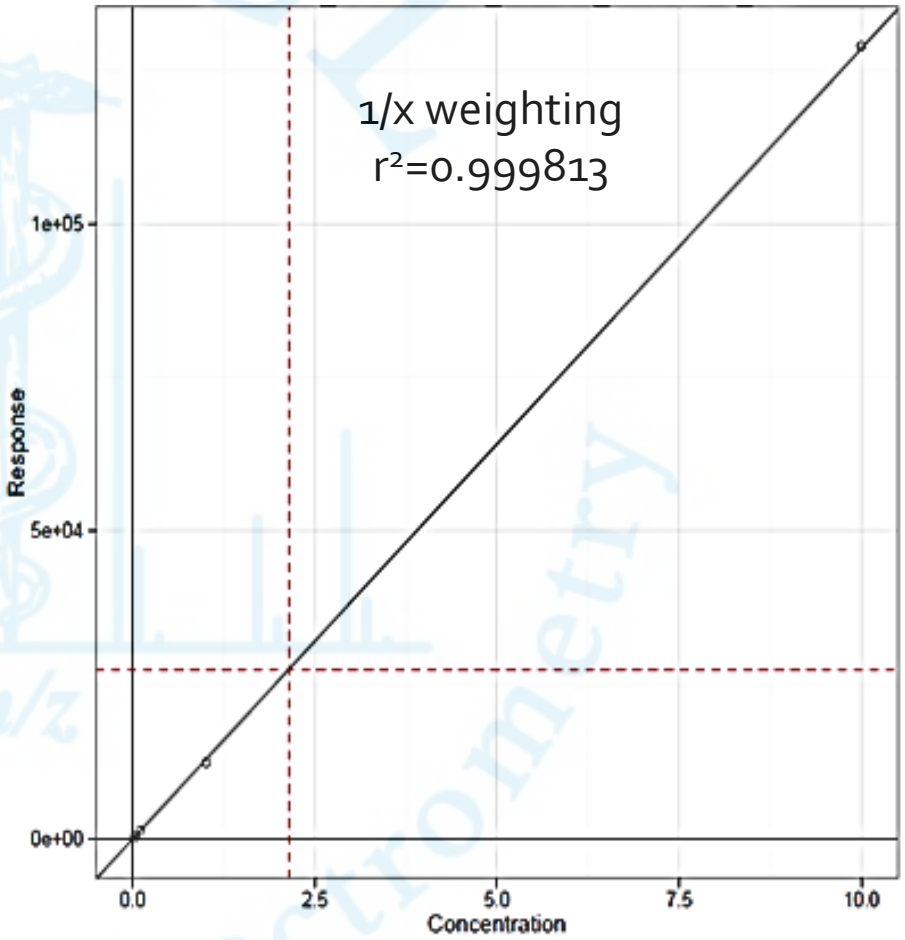
Calibrator 4
$C_6{}^{13}C_{14}H_{22}N_8O_5$
(10 µM)

Methotrexate
$C_{21}H_{22}N_8O_5$
?

Calibrator 3
$C_{14}{}^{13}C_6H_{22}N_8O_5$
(1.0 µM)

Calibrator 2
$C_{15}{}^{13}C_5H_{22}N_8O_5$
(0.1 µM)

Calibrator 1
$C_9{}^{13}C_{11}H_{22}N_8O_5$
(0.025 µM)



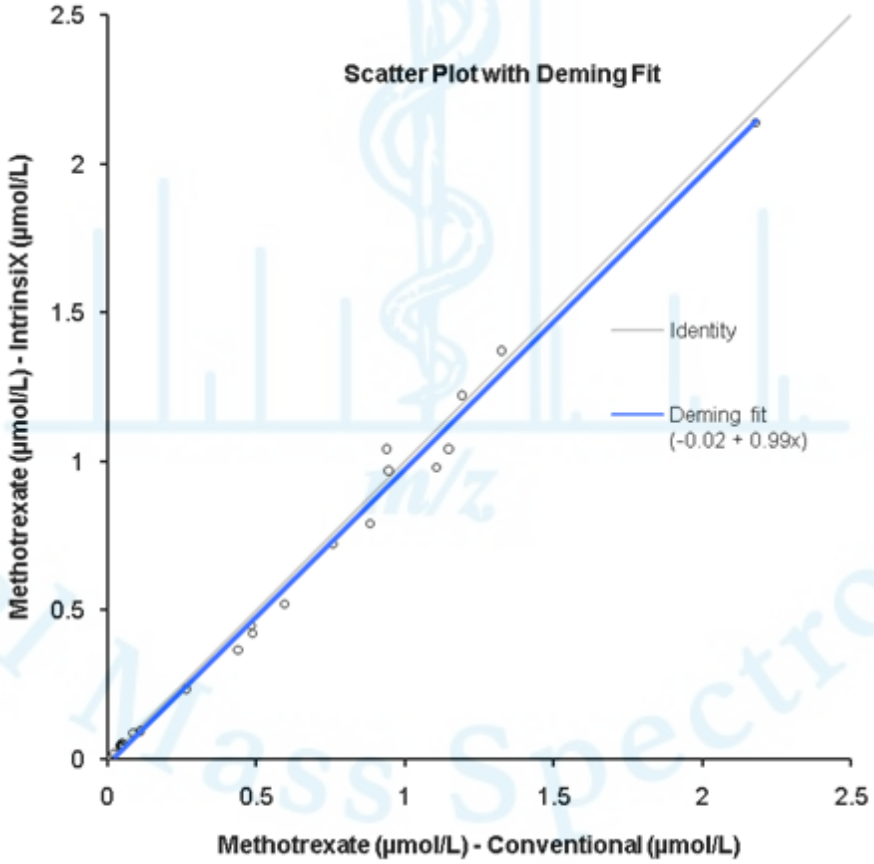
Measured = 2.14 µM (bias < 7.5%)

Courtesy of Don Cooper and Don Mason, Waters Corporation

Method Performance: EQA Analysis

QA materials from UK NEQAS (pilot scheme) and WEQAS

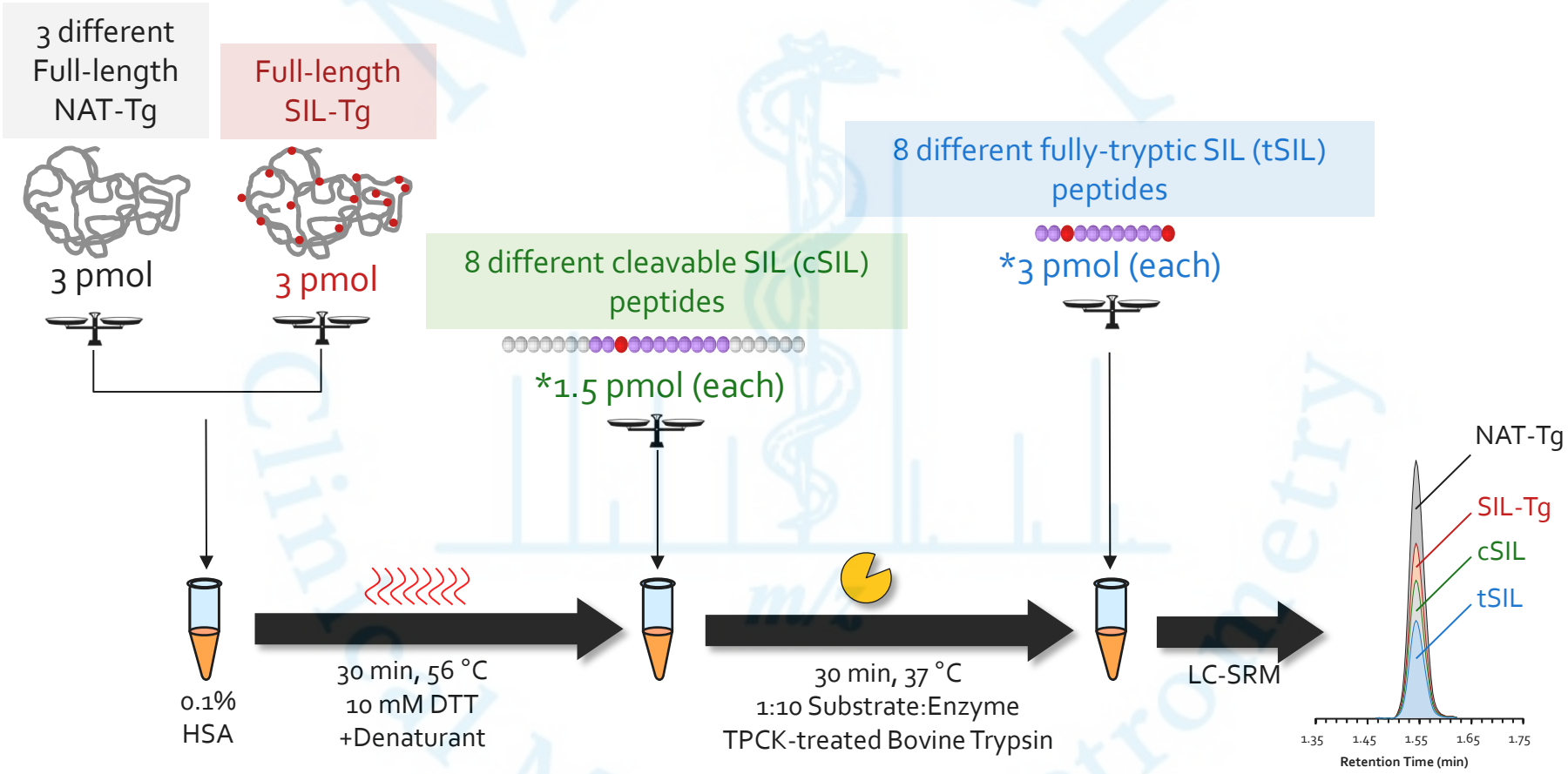
Correlation between IntrinsiX and conventional UPLC-MS/MS analysis described by Deming equation y=-0.99x-0.02 (n=23, range 0.025-2.18 µmol/L)



Courtesy of Don Cooper and Don Mason, Waters Corporation

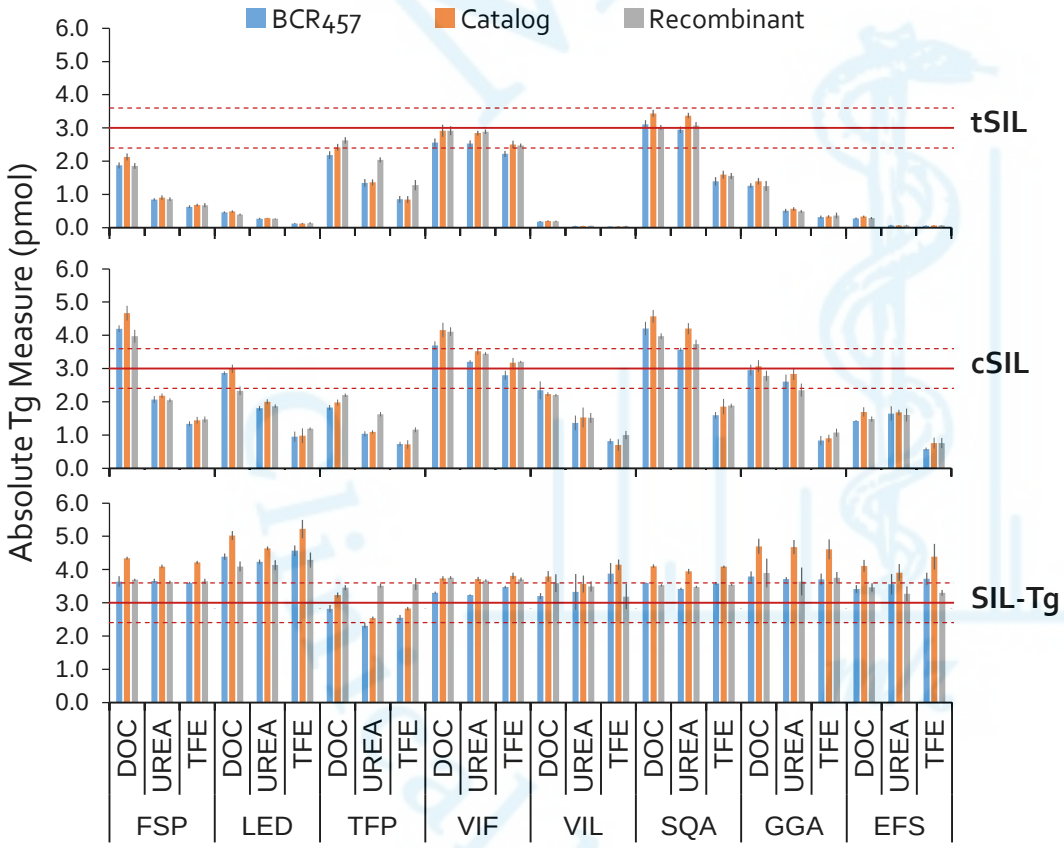
Go Big or Go Home

Q: Internal Calibration versus External calibration and Internal Standardization?



C.M. Shuford & co-workers, Anal. Chem. ***2017****, 89 (14), 7406–7415.*

Internal Calibration

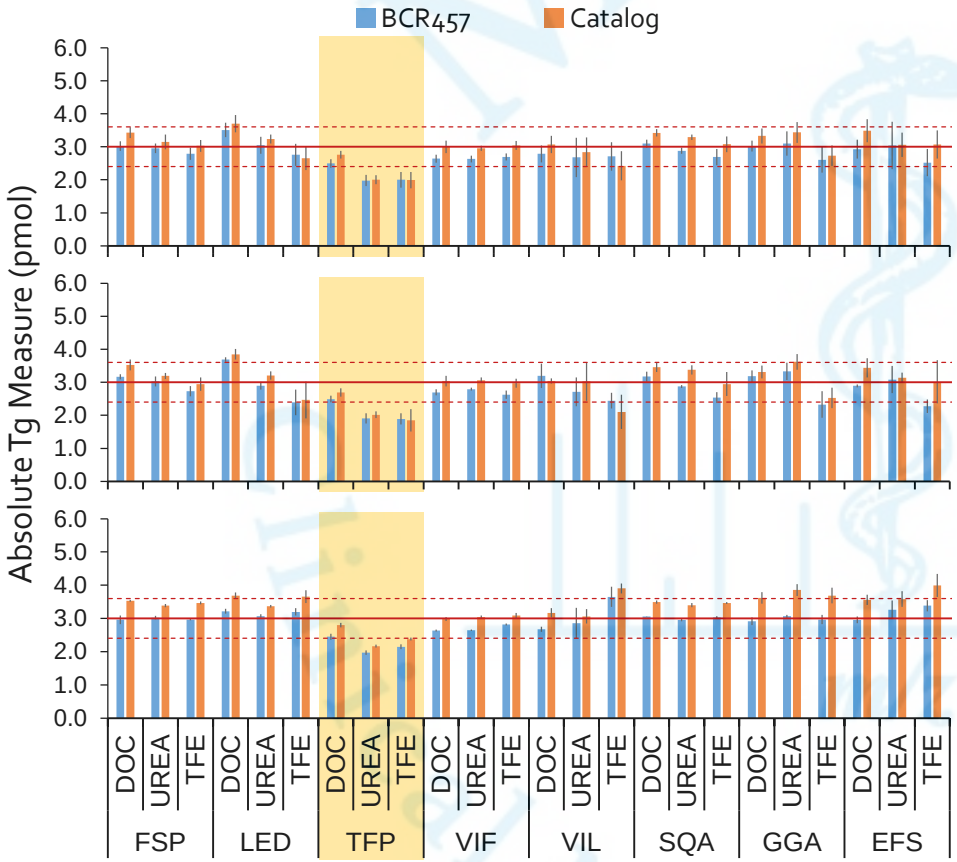


Under-recovery
Digestion Efficiency of SIL > Analyte

Over-recovery
Digestion Efficiency of SIL < Analyte

Isotope Effect?

External Calibration with Recombinant Protein and Internal standardization



TFP peptide generation in Recombinant Tg > Human Tg

Absolute Protein Quantification, Not as Simple as Advertised, C.M. Shuford & co-workers, Anal. Chem. ***2017****, 89 (14), 7406–7415.*

Acknowledgements and Additional Literature

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Meghan Bradley
Brian Rappold

Waters Corporation
Don Mason
Don Cooper

MilliporeSigma Corporation*
Mitzi Rettinger
Uma Sreenivasan
Jim Walters
Kevin Ray

Cerilliant Corp is a subsidiary of MilliporeSigma (formerly Sigma-Aldrich)/Merck KGaA)

Internal Calibration and Drift

Improving quantitative precision and throughput by reducing calibrator use in liquid chromatography-tandem mass spectrometry.
Rule GS, *et al*, Anal Chim Acta. 2016 May 5;919:55-61. doi: 10.1016/j.aca.2016.03.020. Epub 2016 Mar 19.

Alternative calibration strategies for the clinical laboratory: application to nortriptyline therapeutic drug monitoring.
Olson MT et al., Clin Chem. 2013 Jun;59(6):920-7. doi: 10.1373/clinchem.2012.194639. Epub 2013 Feb 20.

Correcting Sample Degradation for Glutathione (GSH GSSG)

Molecular speciated isotope dilution mass spectrometric methods for accurate, reproducible and direct quantification of reduced, oxidized and total glutathione in biological samples.
Fahrenholz T *et al*, Anal Chem. 2015 Jan 20;87(2):1232-40. doi: 10.1021/ac503933t. Epub 2015 Jan 8.

I hope I passed the audition…Questions?

Slide Notes

Slide 1: Hello
Slide 2: No disclosures, except I do like Guinness…particularly if you are buying
Slide 3: External calibrators are used to generate a dose dependent response when used in the assay – i.e. generating a Calibration curve. Analyte recovery and matrix effects (total efficiency) must be identical between calibrators and samples for accurate measurement of analyte in unknown samples
Slide 4: In this example, hydrolysis of the glucoronide to the parent molecule is complete in calibrators but incomplete in an unknown samples resulting in a negative bias.
Slide 5: Perfect Internal Standards behave identically to the analyte – both physicochemically and analytically – Stable labeled forms are the go to choice!
Slide 6: Internal standards only correct steps following addition…if used properly!
Slide 7: Addition of a stable labelled version of the analyte – the IS - to calibrators and samples prior to extraction, separation and ionization. Differences in the analyte are exhibited by the IS, thus the IS serves corrects efficiency losses between the calibrators and samples, minimizing bias. A properly selected IS fixes a lot of problems and no other technology has this powerful tool.
Slide 8: IS added after hydrolysis can correct for differences in injection volume, chromatography retention time variance and ionization differences – but – does not correct for the preparation efficiency difference between calibrators and samples, Bias still present.
Slide 9: The Internal standard is added prior to hydrolysis for both calibrators and samples, and most importantly, contains the glucoronide. The efficiency of hydrolysis for analyte and IS in samples is incomplete compared to calibrators, but the ratio is identical (2:1), thus the IS corrects for the efficiency difference observed in sample preparation, together with injection, separation and ionization.
Slide 10: Physicochemically identical AND Analytically identical..that's a lot of deuterons that can scramble. Analyte should not contribute to IS and vice versa
Slide 11: Isotopes are a concern – some particularly likely candidates are Carbon and Sulphur. Analyte should not contribute to IS..when multiple Chlorines present..walk away to ensure contribution from analyte to the IS transition is non-existent BY DESIGN
Slide 12: Takes some consideration and even when planned perfectly..there can still be under-incorporation and/or loss..Analyte should not contribute to IS if at ALL possible
Slide 13: Analysis of small molecule panels such as amino acids is challenged with many analytes across a narrow mass range. While Methionine generates a major product ion at mass-to-charge 104, a contribution to the D3-Lysine transition of 150 to 87 is observed. .. Making life even more complicated, The D5-Glutamuic acid internal standard contributes to the D3-Methionine transition. Addition of D3-Proline with a transition from mass-to-charge 119 – 73 is not selectively measured by the mass spectrometer as the carbon 13 isotope of Valine also contributes to the transition. There are three solutions to this phenomenon. Select a different IS, or, add a lot of IS for proline and lysine to minimize the contribution (not ideal), or – resolve chromatographically – which is the correct solution.

Slide Notes

Slide 14: Correctly selected IS defines analyte peak properties of retention time and shape
Slide 15: IS added after hydrolysis can correct for differences in injection volume, chromatography retention time variance and ionization differences – but – does not correct for the preparation efficiency difference between calibrators and samples, Bias still present.
Slide 16: *QC level 1 injection 12 versus injection 94…*D4 IS tells you – where analyte elutes and the shape of the eluted peak, so LC was working, IS response is identical between injection #12 and #94, so interface and mass spectrometer were working, but, the analyte transition shows elevated baseline NOT seen in the IS transition…shows contamination of LC-MS/MS system over time that is either carry-over from previous high level analyte sample (NOT the case) or another contaminant extracted from the specimen that shares the same transition and elutes later in the assay (YES). Solution, used a third washing solvent to clean off the column between each injection
Slide 17: There is always a sweet spot – if you think about it
Slide 18: Re-injection rules out preparation but imprecise preparation really makes the evaluation of drift impossible
Slide 19: Observation of IS peak area drift across a run using a liquid handler and the same 8-tips for IS addition. Reinjected the first part of the plate – same LOW IS response observed (LC-MS/MS system operating OK). Added a carrier to IS solution and pre-wetted the tip: aspirate/dispense to IS solution container 3 times prior to first aspiration to the 96-well plate
Slide 20: Speed isn't what you are looking for – it's control
Slide 21: And here's how you test for it
Slide 22: Look at both the ratio over time and the loss of the IS – to have confidence in your experimental conclusions
Slide 23: Should be pretty obvious…Carbon and Nitrogen isotopes are preferred. The Carbon – Deuteron bond is more acidic than the Carbon-proton bond..and that does matter…see later
Slide 24: It's the perfect correction tool and other technologies such as clinical autoanalyzers would love to have this capability
Slide 25: To correct for every step in the assay, the IS should be added immediately after mixing and pipetting calibrators, QC's and samples. The internal standard needs to experience the same environment as the analyte in calibrators and samples, thus, the goal of the IS is to be in the same equilibrium state as the analyte, free and bound to sample constituents such as proteins.
Slide 26: Oh yes… a lot of weird stuff happens…. O-o
Slide 27: Neat solution of D4-Dopamine was injected using an APCI source and a very large response for Dopamine was observed (>highest desired calibrator). After checking for contamination, purchasing new materials (and checking the label carefully), the position of deuteration drew our attention. It is in a very "active" region of the molecule and APCI involves proton donation through gas phase collisions. Potential solutions include reducing the amount of IS added, but we need to have 10 – 25x LLOQ so that a reproducibly measured response is observed in all samples. In this instance, 20 fold dopamine to D4-Dopamine response was seen – we cannot logically add IS < assay LLOQ and expect success. When we switched to the ESI source, we noted no contribution of the IS to analyte transition due to a fundamentally different ionization mechanism (solvent removal versus gas phase ionization). Labelling in the wrong place – last on – first off.

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Slide 28: Ionization mode provides multiple precursor ions in positive ion mode – contributing to response variance. You want low IS variance to spot outliers
Slide 29: Drift requires some experimentation, and cheaply made materials are not your friend
Slide 30: This isn't the only time we have observed this, Deuterated IS materials are really the least favorable label you can use
Slide 31: Biology meets chemistry and creates a mass spectrometry nightmare..keep watching
Slide 32: Told you….Oxym/Dhc could potentially have a +1 amu isotopic contribution to Cod-d3 therefore we choose the Cod-d6. However in doing so we also had to consider the m+2 contribution of Cod-d6 to Oxym-d6 which in fact has a selective transition and no contribution. Dhc-d3 is m-1 to Cod-d6 however we are both chromatographically separated and we have a selective transition. Hydrocodone-d6 is m+4 to Oxym but only m-2 from Oxym-d6 and we preferred the d3 as we choose Cod-d6. With the Hyc-d3 there's only +1 amu difference to Oxym but through judicious transition selection we have a selective transition with no contribution of Oxym to Hyc-d3.
Slide 33: Historically, GC-MS assays tended to use heavily deuterated IS materials, in this case, Gabapentin IS contains 10 deuterons and the carbon – deuteron bond is more acidic than the carbon – proton bond. This can result in the IS eluting earlier than the analyte in reverse phase LC. Addition of equal amounts of Gabapentin and D10-Gabapentin to 150 urine samples demonstrates the issue with this. When they co-elute, the recovery ratio is generally 100% (1:1). When a slower LC separation is used, the IS does not co-elute with the analyte, resulting in many samples recovering much lower than expected. The answer isn't fast LC – you lose selectivity, it's a better labelled IS, ideally with carbon-13 or nitrogen-15 isotopes.
Slide 34: dynamic range (3-logs) the analyte contributes to the IS transition, so non-linear calibration curve seen. There are many solutions to this – but, do you want to run each sample with and without dilution, or repeat on dilution, or add more IS material to minimize contribution from analyte (you must have no unlabelled analyte in the IS for this), or find an alternate IS..Clinically the normal range is unaffected by the contribution in the linear part of the calibration range…so..ask your medical director. If you see this – LOOK at lower yield (3-5 fold here) transitions and see if you observe same degree of non-linearity in their curves:
IF Yes: Isotopic contribution, Preparative error, source saturation affecting IS response
IF NO: Detector blinding Using High QC (~80% ULOQ), assay neat and on dilution into "linear" portion – determine bias (<15% OK). Add calibrator(s) to define non linearity…OR…Solution - truncate linear range, pre-dilute samples, modify collision energy…or ask yourself if it is Clinically acceptable
Slide 35: Mass spectrometers are mixture analysis tools – BUT response (as transitions) is very different and further exacerbated when measuring mixtures with different circulating concentrations
Slide 36: De-convoluting the observation measuring multiple analytes in a single run really needs solid foundations in analytical chemistry to know what your eyes are telling you isn't an issue…remember..Of the 5 senses, we trust our vision the most…but our eyes are the most easily fooled

Slide Notes

Slide 37: As stated before..fast isnt good, good is good and fast comes as a side benefit
Slide 38: While many would argue this point..and it is my humble opinion…you will see later why imprecision should always rule
Slide 39: Fast isnt better…see..told you…
Slide 40: But fast can be achieved if you work the problem some..
Slide 41: Why does biology make life so…blurry…?
Slide 42: Because…depending upon what you believe..Biology has had a 5 billion or 5000 year head-start on scientists like us
Slide 43: But chemical understanding to ameliorate biology is a unique trait in humans…right?
Slide 44: Open your mind…legally of course
Slide 45: So what do you do about low IS? Have a policy…go conservative and re-extract? what if you cant? Really not result out for a patient that really needs help?
Slide 46: Hope you like the colors…I'm a fan of purple..and chose the colors as the obvious mixture of the two of each pair..yep..I take this that seriously
Slide 47: Its Expensive..but sooo worth it (I use that line with my wife quite a lot…sadly she does the same to me..)
Slide 48: Calibration frequency is a pain when STAT analysis is needed and doesn't have to be done by regulations as frequently as you would think…see The March of The Masses..Grant RP, Clin Chem. 2013 Jun;59(6):871-3. doi: 10.1373/clinchem.2013.205435. Epub 2013 Apr 16.
Slide 49: Simple to validate and use QC's to monitor – the stability and storage of the IS..as a component of the entire protocol
Slide 50: Same principal applies…its worth it just to see the look on the face of the auditors .. ☺
Slide 51: Hmm…what to do what to do…when samples are >ULOQ…really thaw, dilute, transfer, mix, cook for 2.5 hours etc…or…read next page?
Slide 52: We do this A LOT! Honestly
Slide 53: An exemplar..not technically accurate but relatable..pretty much like teaching the plum pudding model of the atom to kids…not that you are kids or anything..that's merely an analogy of the concept of an analogy..as a teaching tool…erm…moving on…..
Slide 54: nd this can be VERY VERY accurate..the foundation of what I would call proper reference method procedures..thanks to all of our colleagues who do this for us..truly great of you
Slide 55: Now this is quite cool if I say so myself…
Slide 56: No external calibrators, each samples recovery an matrix effects are "intrinsically" corrected…see what I did there…yep..its cool
Slide 57: And works like a charm
Slide 58: This took 3 years..while conceptually obvious..the metrology required to prove this was very painstaking..thanks to many groups who helped on this one…

Slide Notes

Slide 59: Just look at the next slide and this one…accurate you say? When two peptides from the same protein give the same number from tryptic IS added as a "calibrator" they are CONCORDANT..not accurate..
Slide 60: Right..got off my soap box….materials can be used as internal standards though..just don't forget what you give up based on their form and where they are added…t make life more complicated…large protein IS materials are ANALOGS..because big proteins aren't an entity, theyre a family of things..and we all know what secrets families hide..right?…erm….just mine then?
Slide 61: If you're still reading…here's a joke…

"As a kid I was made to walk the plank. We couldn't afford a dog."

Okay here's one… "I was watching the London Marathon and saw one runner dressed as a chicken and another runner dressed as an egg. I thought: 'Ooh, this could be interesting".

Erm…"I'm sure wherever my dad is; he's looking down on me. He's not dead or tall, just very condescending."

☺ cheers, russ