HOW TO ACHIEVE LOWER QUANTIFICATION LIMITS:

PART 1: FOUNDATIONS…for consideration

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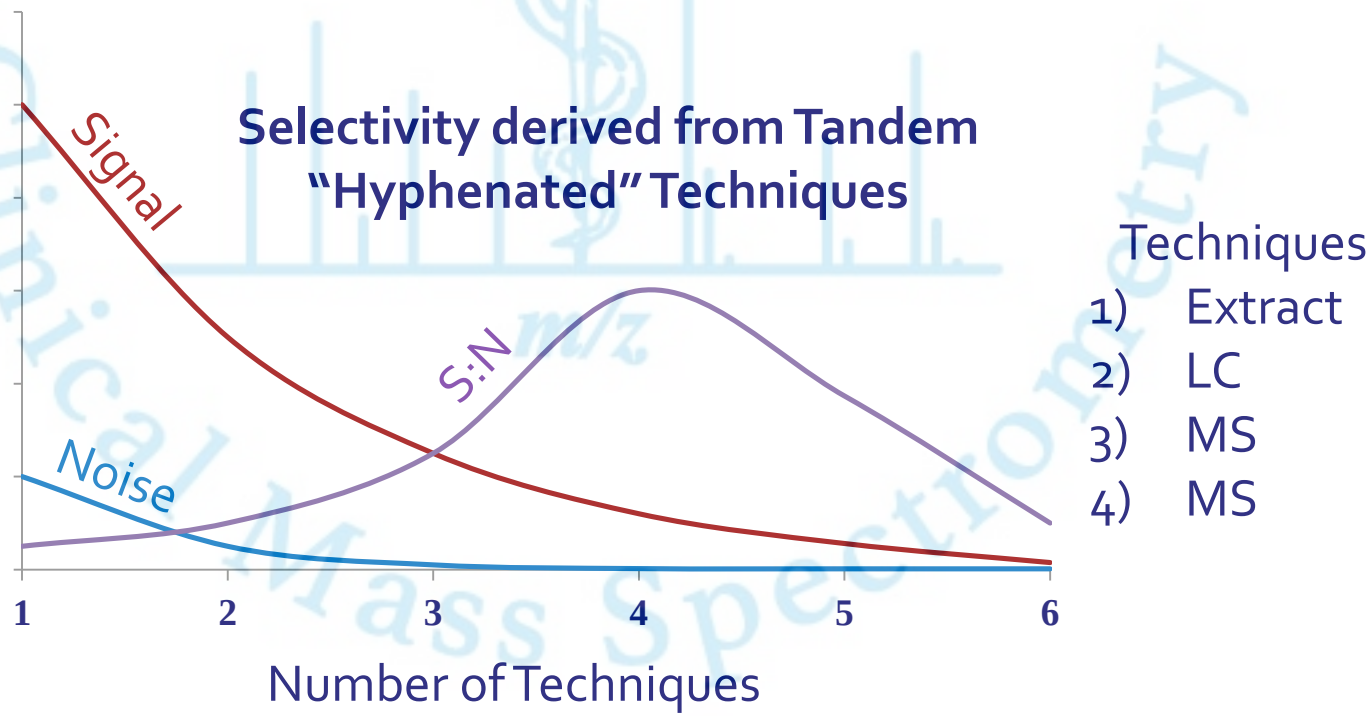
LC-MS/MS Principles of Analytical Measurement

Complexity reduction of sample – *Isolation*
Ideally selective for analyte(s) – *Sum of Complimentary techniques*
Ideally simple – *balancing the 4 S's*
Concentration of analyte(s) versus concentration of interferents
External Calibration with internal standardization (IS) – *Unique for MS (ECIDMS)*
Reproducible before Quantitative Recovery (*for Isotope Dilution, add IS first*)

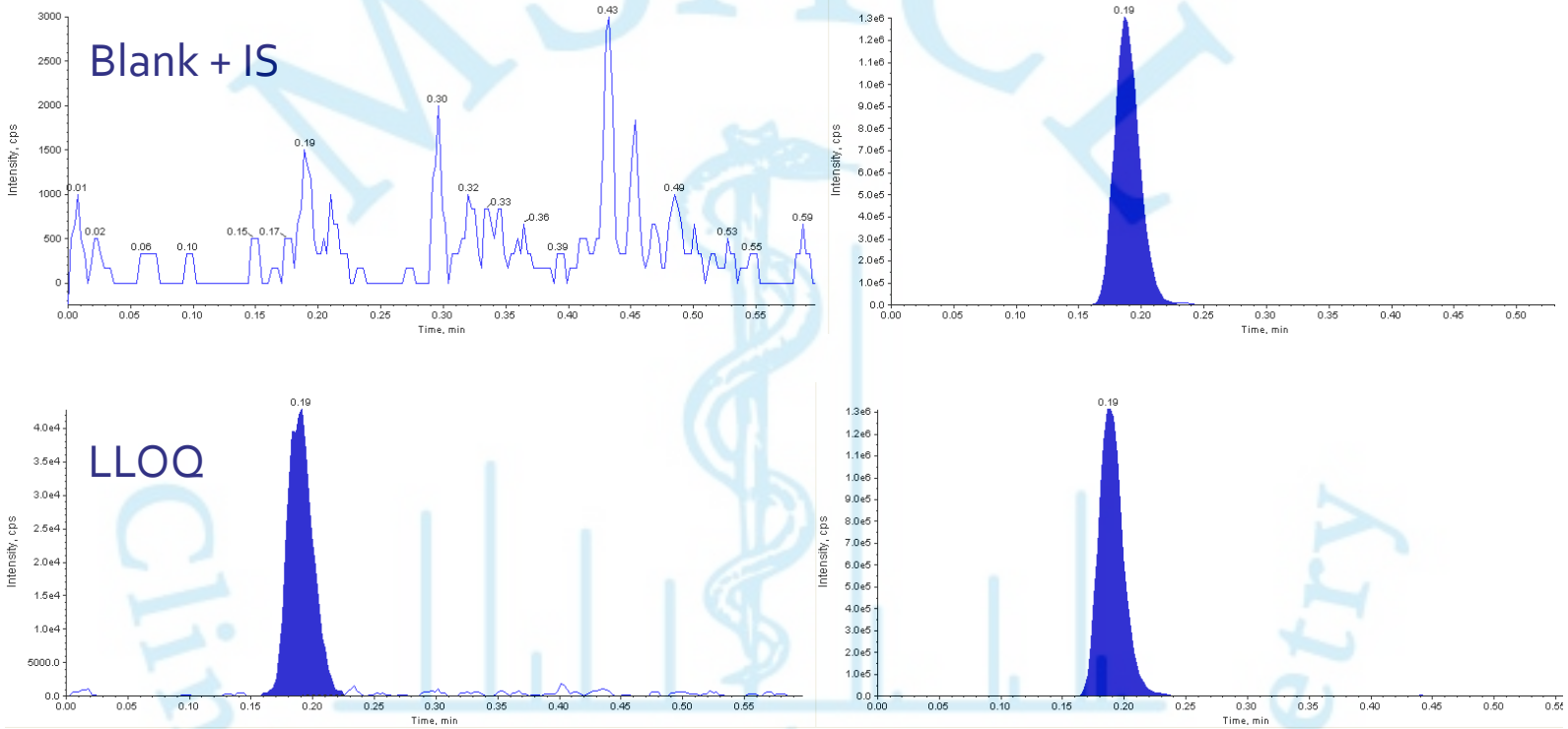


Modified from Cooks, R.G. et al, J. Chem Edu, 59, 926-933 (1982) and Rick Yost (TAMS 2016)

"The LC-MS/MS Experiment"

Ensure Homogeneity	**Thaw and mix Calibrators/QC's/Specimens**
Recovery/Sample variance Correction	**Addition of Internal Standard**
Reduce Complexity, remove interferences	**Extraction/Purification**
Reduce Complexity, remove interferences	**Liquid Chromatography**
Gas phase ion cluster generation	**Interface**
Tandem MS Selectivity step 1	**Precursor Ion Selection**
Reproducible fragmentation to generate structurally unique motif	**Fragmentation**
Tandem MS Selectivity step 2	**Product Ion Selection**
Ion counting/signal amplification	**Detection**

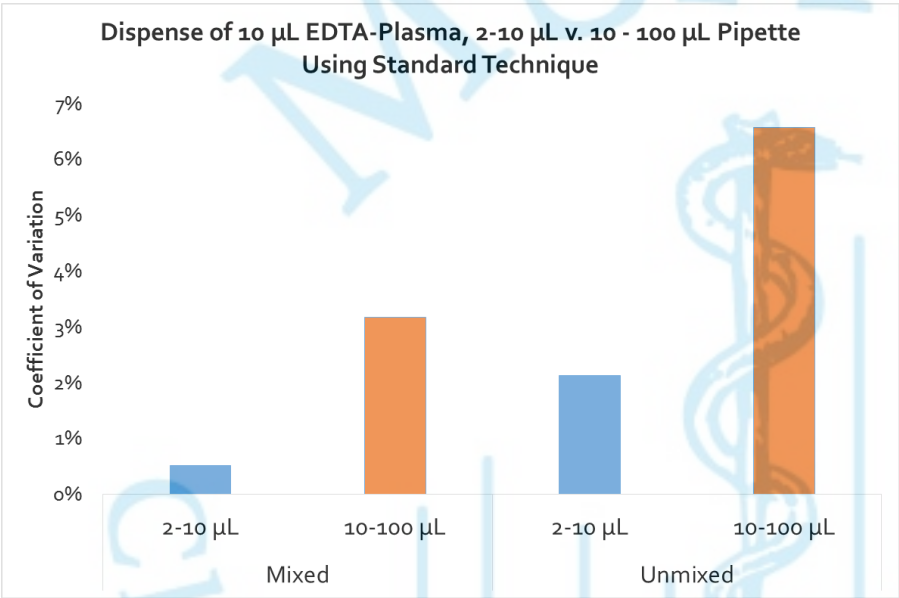
What defines the LLOQ? Bias and CV <20% ?



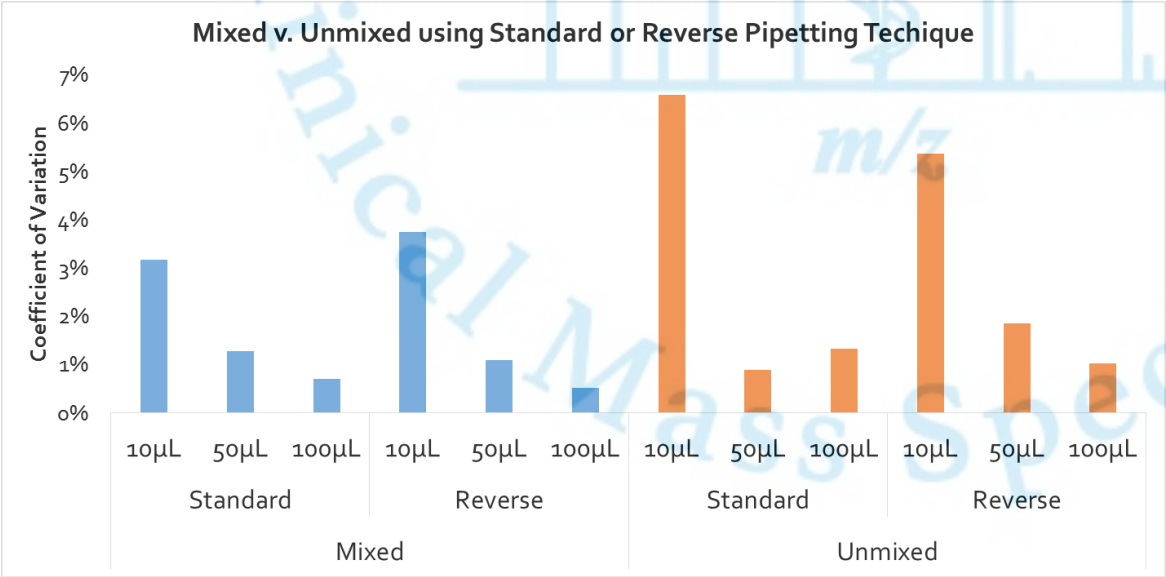
Accuracy (Bias): Metrology of Calibration, Lossless (corrected in assay process), Assay Selectivity, Purity of materials

Precision (CV): Signal : Noise?
Literally EVERYTHING involved in the assay and instrument (and all of the above plus repeatability over time)

Pipetting precision (Contact dispense, Balance and EDTA plasma)



Mix the sample first
Select the correct pipet and tip for desired volume (10µL shown)

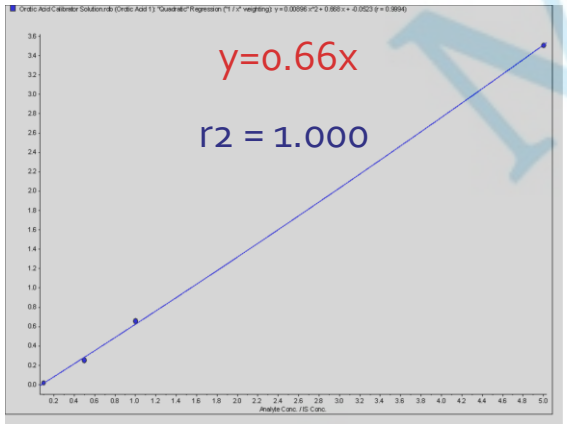


Know the imprecision of your pipetting system (100µL pipet shown) <u>and</u> technique

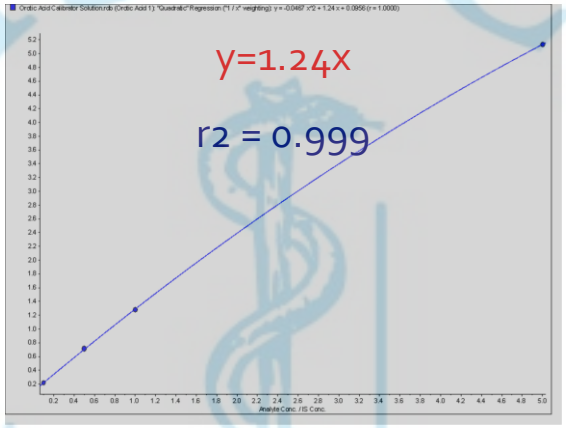
Calibration slope: Solubility/Adsorptive Loss Trigger

The "10 touch" experiment

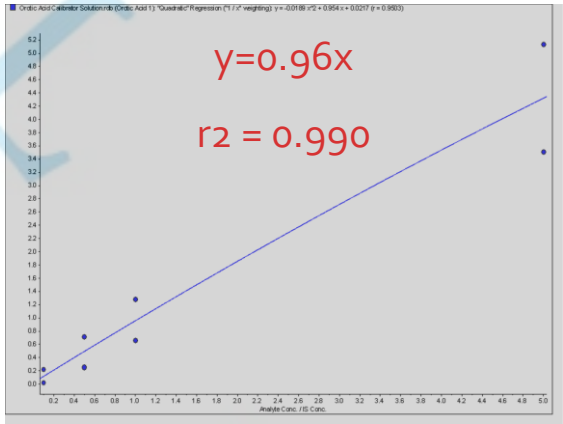
Orotic Acid MeOH calibrator



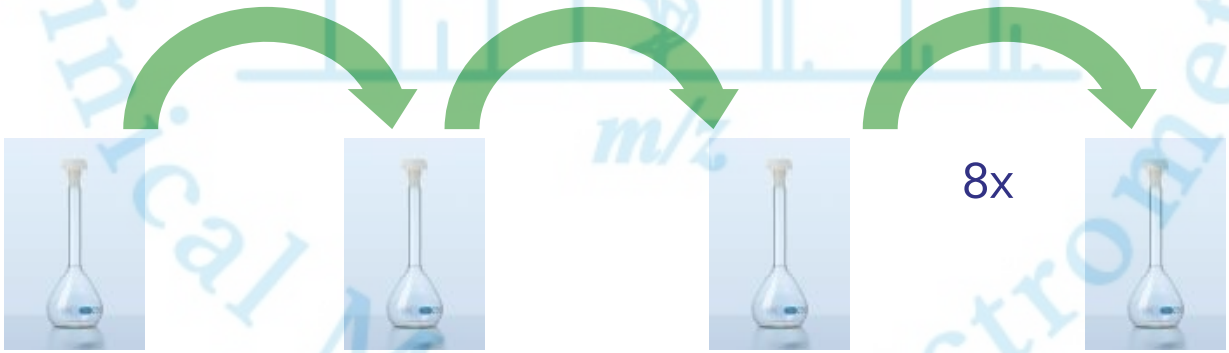
Orotic Acid 0.1% NaOH calibrator



Combined



Every surface (pipettes/glassware/plastics) for Stock and Calibrator manufacturing with the solute condition in consideration



Baseline (1 flask contact)

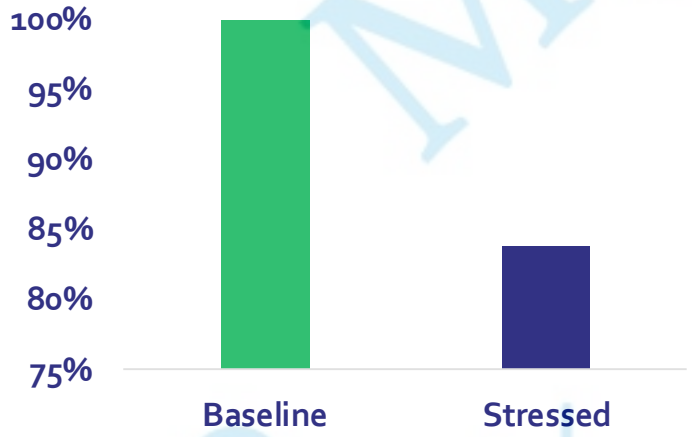
Pour over to fresh container

Stressed – Compare to Baseline

Consider "concentration range" and ameliorate with chemistry and materials

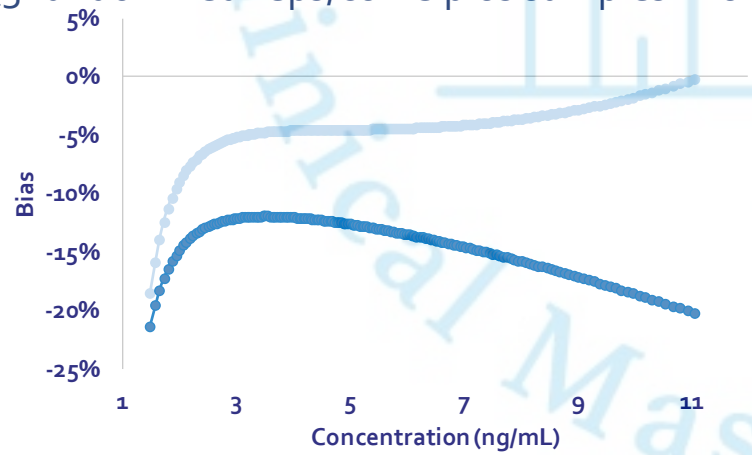
Addition to plates/tips of Calibrator Prior to IS addition

Gluten peptide in PBS, pH 7.4, 0.5% BSA

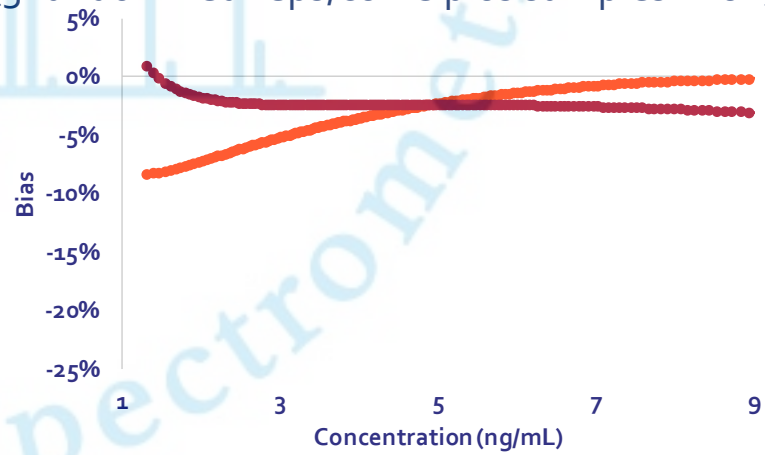


Gluten peptide in Tris, pH 8, 3% BSA

Stressed Curves 2 and 3 vs stressed Curve **1**
(3 randomized reps/curve plus samples 1 run)



Stressed Curves **2** and **3** vs stressed Curve **1**
(3 randomized reps/curve plus samples 1 run)



Even 15% Adsorptive loss = Calibration imprecision that IS <u>cannot</u> correct for

See : Where Did My Analyte Go? – Coping with Poor Solubility and Non-Specific Binding, Catarina Horro Pita

Addition of IS in serial mode (4 dispenses per aspiration)

Bias and Imprecision

1st vs 4th Delivery
8-tip liquid handler
No "prewetting"

Baseline

	Replicate/Tip	Addition Step 1	Addition Step 4	Bias	Addition Step 1	Addition Step 4	Bias	Addition Step 1	Addition Step 4	Bias	Including all data points Average	S.D.	CV (%)
IS Peak Area (counts)	1	989299.1	927191.9		1049259.5	946906.7		1030562.8	911475.1				
	2	1029303.6	942528.5		1065516.4	991245.5		997552.9	899031.4				
	3	1061115.4	948176.9		1060470.1	989430.2		988456.5	884794.9				
	4	1060079.4	952630.4		1020440.0	966136.4		980805.5	906279.3				
	5	1051722.1	934064.9		1042351.4	961229.2		1016556.3	900131.1				
	6	1080978.6	954270.0		1060356.0	979481.4		988227.7	916055.2				
	7	998548.0	956196.8		1012357.3	985305.3		1007473.7	961248.3				
	8	1026631.9	941873.8	Step 4 to 1	1077069.3	921012.7	Step 4 to 1	974348.4	928087.2	Step 4 to 1	984880.5	53348	
Mean		1037209.8	944616.6		1048477.5	967593.4		997998.0	913387.8				
S.D.		32038.8	10219.2	**-8.9%**	22429.7	24294.7	**-7.7%**	19034.7	23199.1	**-8.5%**			**5.41**
CV (%)		3.1	1.1		2.1	2.5		1.9	2.5				

1X "Pre-wetted"

1X Pre-wet

	Replicate/Tip	Addition Step 1	Addition Step 4	Bias	Addition Step 1	Addition Step 4	Bias	Addition Step 1	Addition Step 4	Bias	Including all data points Average	S.D.	CV (%)
IS Peak Area (counts)	1	400675.1	390357.2		395417.9	394114.1		395814.7	402188.3				
	2	416891.7	397422.9		398281.4	395746.3		396414.7	404584.2				
	3	403699.8	392128.0		402128.5	395993.3		408201.3	402101.2				
	4	403017.0	394389.2		396687.1	397949.4		406706.0	393925.7				
	5	408418.1	397334.9		405532.6	398622.3		409820.6	401478.8				
	6	399635.7	392591.3		404128.7	399847.5		404494.2	399721.2				
	7	401346.2	393197.3		403289.1	402496.8		402245.8	398567.5				
	8	404320.0	392820.4	Step 4 to 1	399895.9	401725.6	Step 4 to 1	401544.8	389046.8	Step 4 to 1	399936.6	5443.9	
Mean		404750.5	393780.1		400670.2	398311.9		403155.2	398951.7				
S.D.		5599.9	2489.9	**-2.7%**	3671.2	2960.0	**-0.6%**	5169.0	5107.6	**-1.0%**			**1.36**
CV (%)		1.4	0.6		0.9	0.7		1.3	1.3				

3X "Pre-wetted"

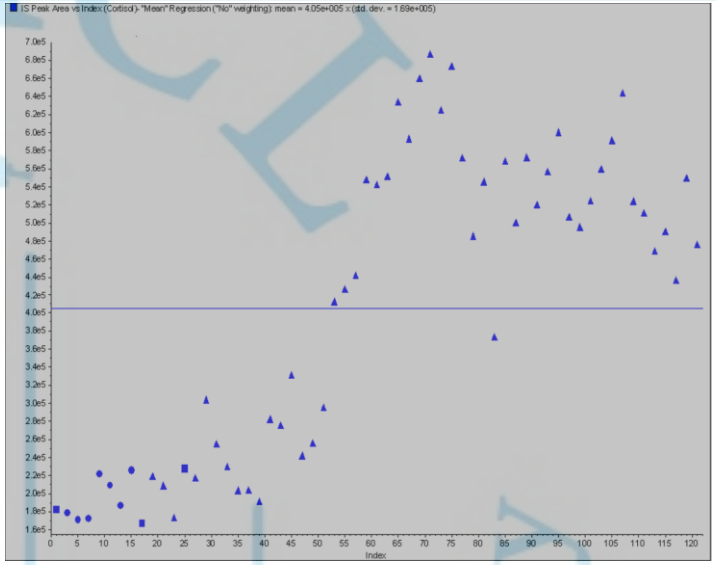
3X Pre-wet

	Replicate/Tip	Addition Step 1	Addition Step 4	Bias	Addition Step 1	Addition Step 4	Bias	Addition Step 1	Addition Step 4	Bias	Including all data points Average	S.D.	CV (%)
IS Peak Area (counts)	1	392787.7	388537.1		393217.6	385940.9		382757.2	389361.9				
	2	400111.2	389610.5		389785.9	384788.7		384557.5	382013.7				
	3	394277.8	390411.6		388066.6	385958.7		389315.3	380779.6				
	4	397538.3	392501.1		392502.8	388224.3		380559.2	381812.1				
	5	387718.3	388289.5		389380.7	391244.4		382829.8	381799.5				
	6	396628.3	388850.7		388969.9	392289.7		383727.3	377978.7				
	7	388807.5	391158.5		383562.6	378048.8		384367.2	389546.0				
	8	399712.6	385670.3	Step 4 to 1	388177.1	379897.1	Step 4 to 1	387594.3	390233.7	Step 4 to 1	1387956.2	5202.7	
Mean		394697.7	389378.7		389207.9	385799.1		384463.5	384190.7				
S.D.		4682.5	2068.9	**-1.3%**	2966.2	4980.4	**-0.9%**	2796.1	4755.1	**-0.1%**			**1.34**
CV (%)		1.2	0.5		0.8	1.3		0.7	1.2				

Pre-condition tips, Automation tips ≠ Manual pipet tips

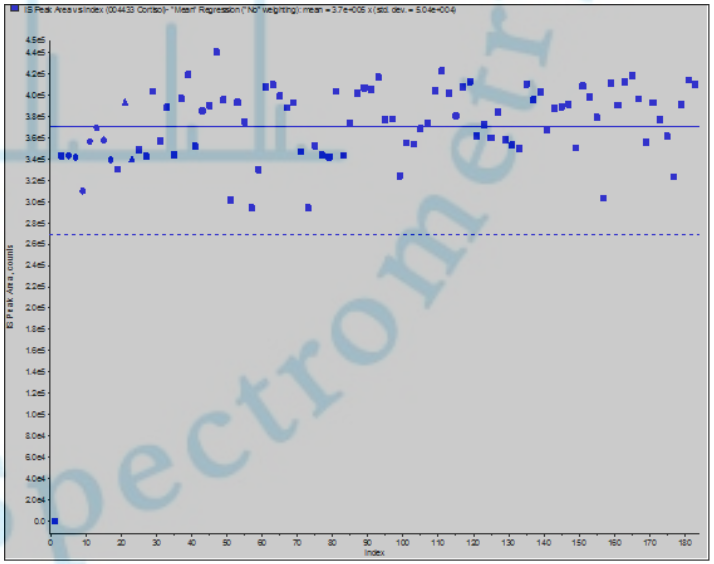
Internal Standard addition – its not just about weight

8-tip liquid handler IS addition using same 8 tips



Pre-wet tips (x3) with IS solution
Single dispense
D_4-Cortisol IS includes 0.1% BSA

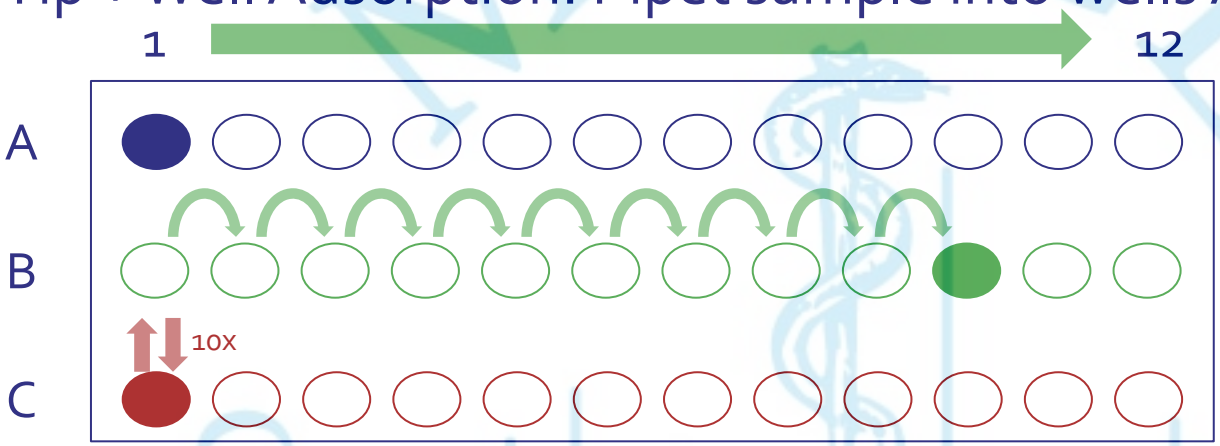
CV = 8.6%



Use reagent additives to reduce adsorption, IS precision enables outlier detection

Elucidating Adsorptive Post IS

Tip + Well Adsorption: Pipet sample into wells A1, B1 and C1

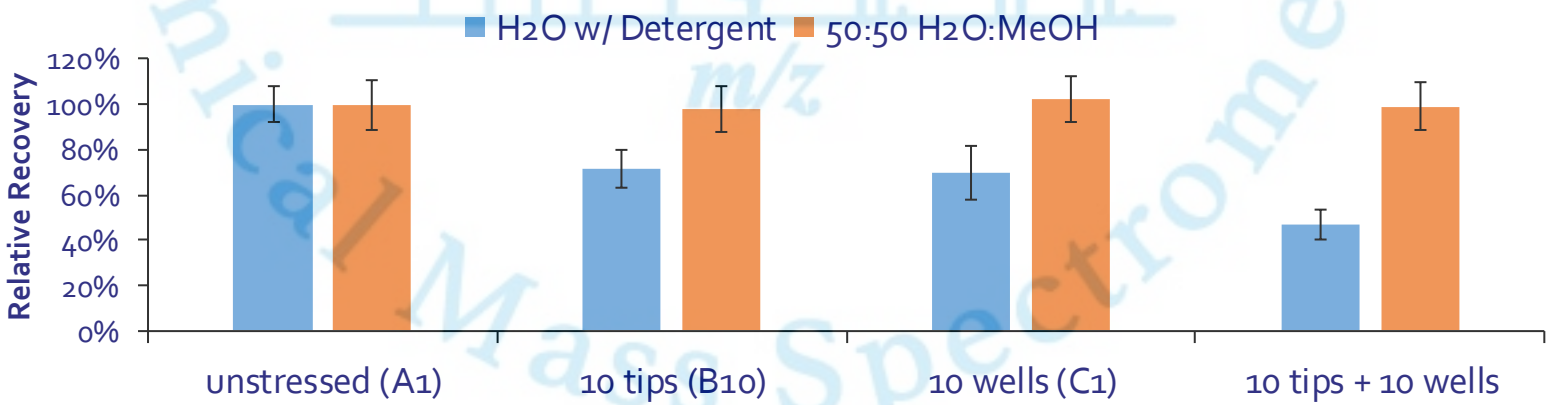


A1: 1 tip 1 well

B10: 1 tip transfer all sample over 10 wells

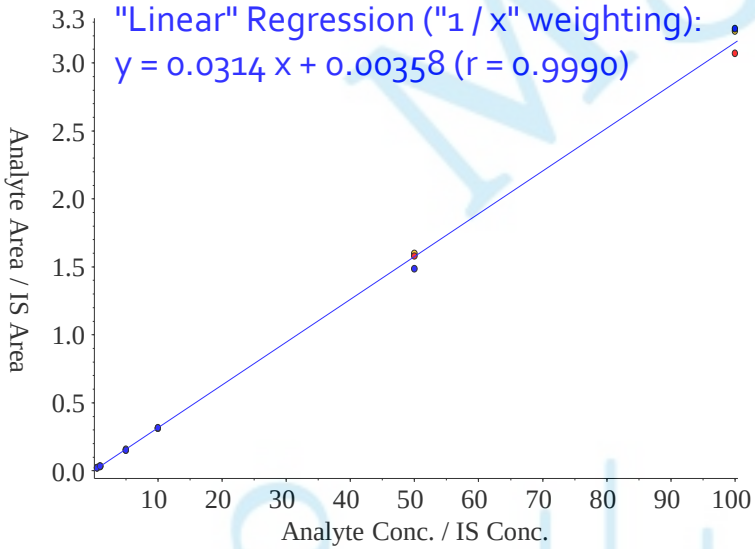
C1: 10 fresh tips aspirate and dispense into 1 well

B10 vs A1 adsorption to wells, C1 versus A1 - adsorption to tips

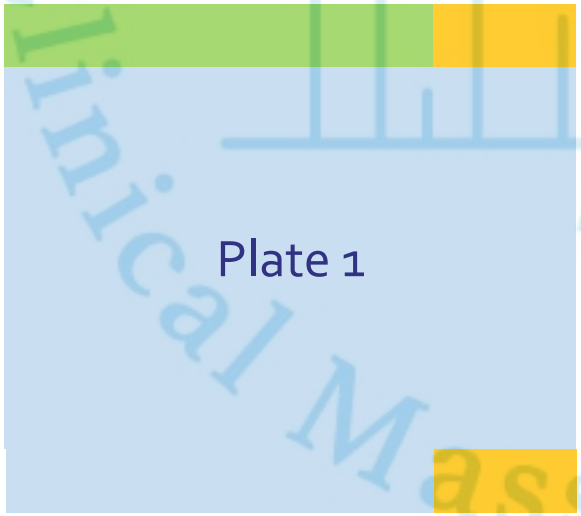


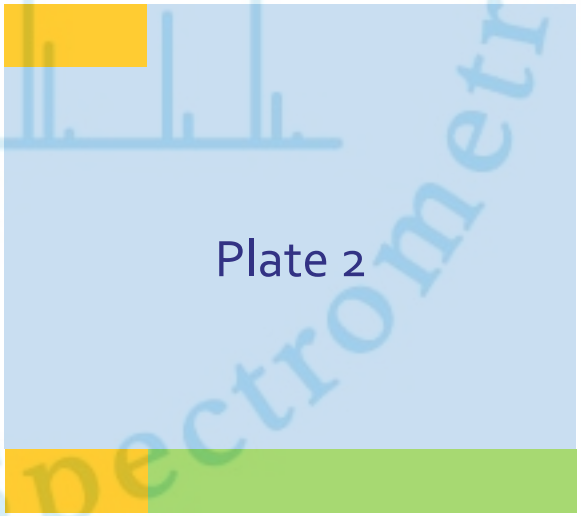
Consider every step and ameliorate with chemistry and materials

Precision and "Maximum batch size" – Influence of Time



Concentration Target (ng/mL)	Back-Fit Accuracy (%)		
	Curve 1	Curve 2	Curve 1 re-inject
0.5	113.7	111.4	112.8
1	90.3	88.3	93.6
5	95.0	96.7	94.0
10	99.3	96.7	99.1
50	102.8	98.7	94.2
100	99.0	101.2	103.3





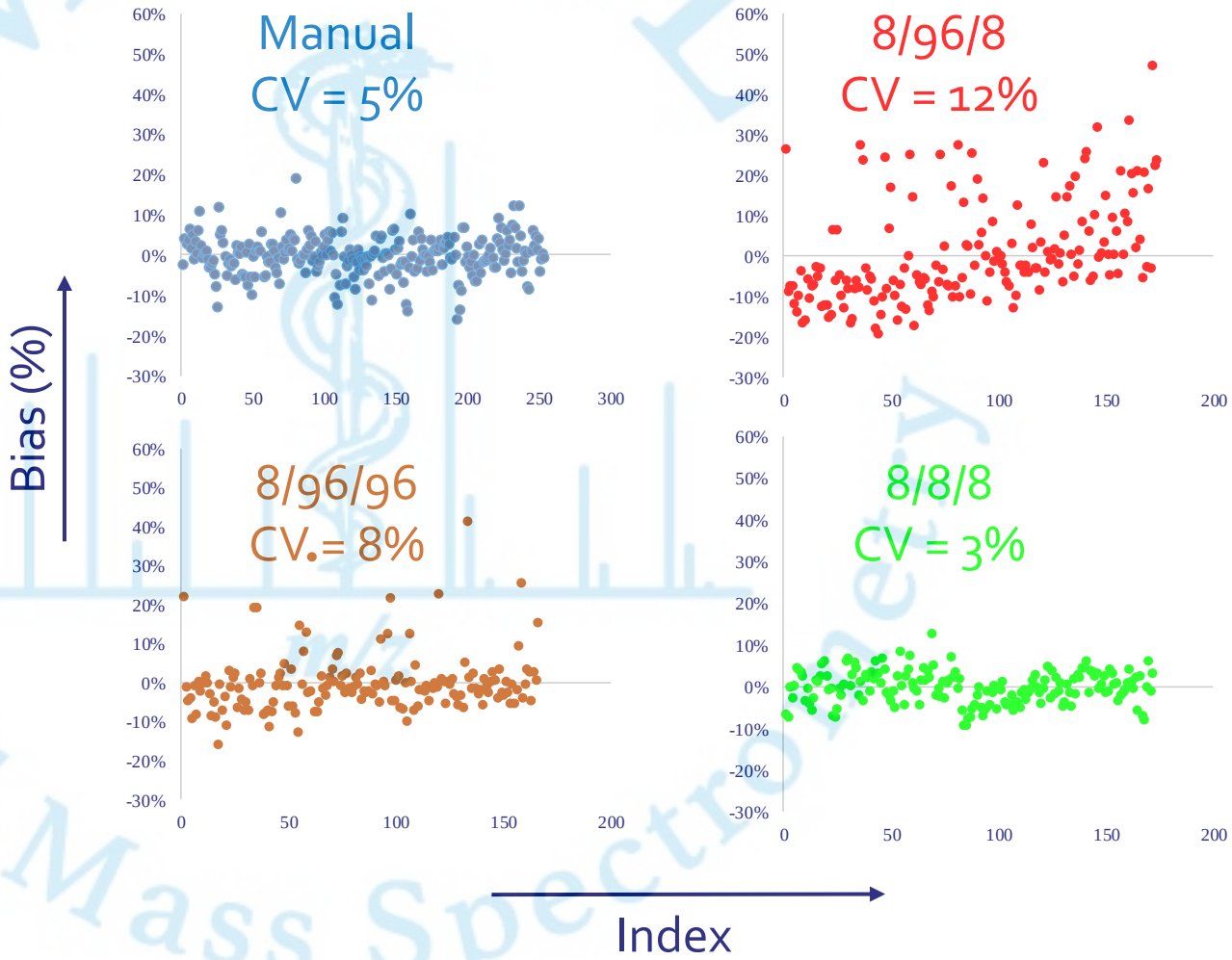
Two 96-well plates: Blanks and Calibrators, QC's, **Sample Pool**

Which pipetting technique to use (8 or 96-tip)?

1: Add Sample

2: Add Generation buffer

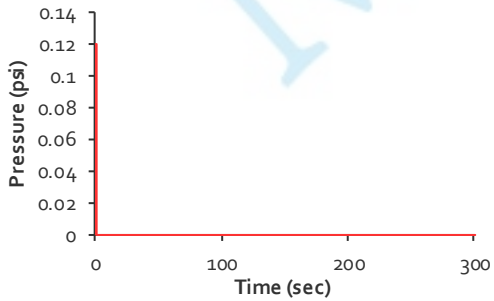
3: Add IS (after generation)



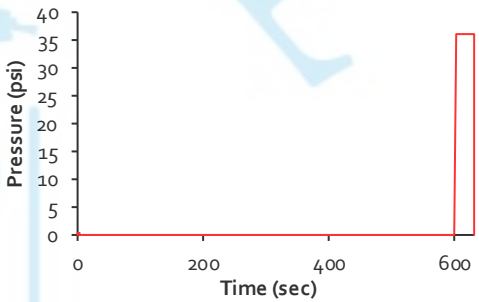
Let repeatability of a pool guide automation technique

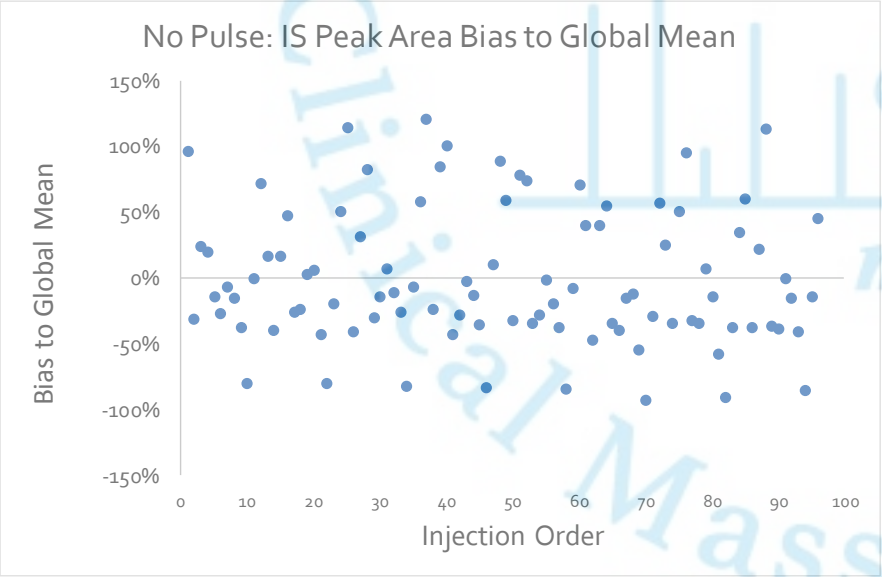
SLE precision of elution volume

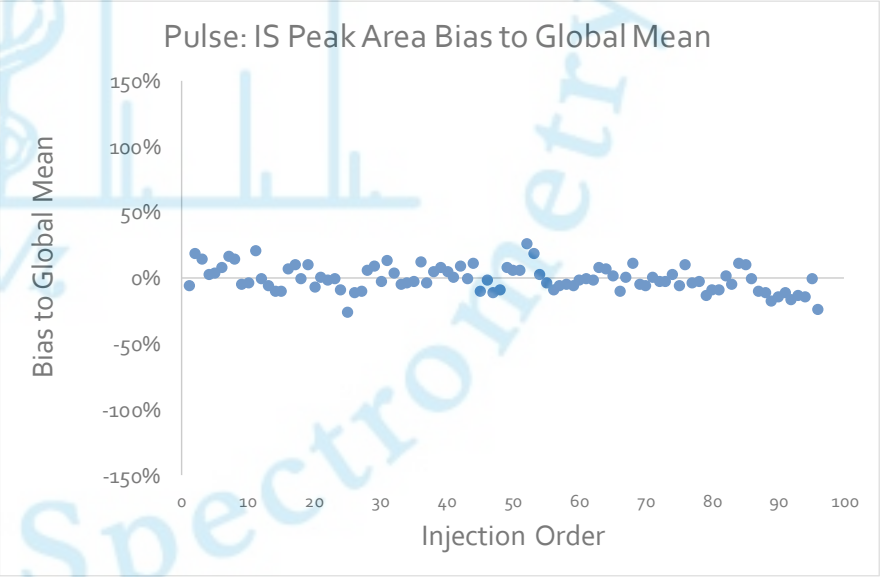
LOAD: PRE-MIXED SAMPLE + IS
(5 SEC 0.12PSI, HOLD 5 MIN)



ADD SOLVENT AND ELUTE
(5 SEC 0.12PSI, HOLD 10 MIN, 30 SEC 36PSI)

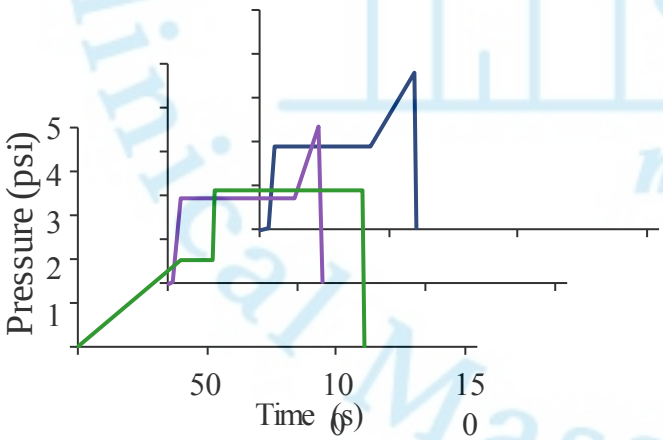




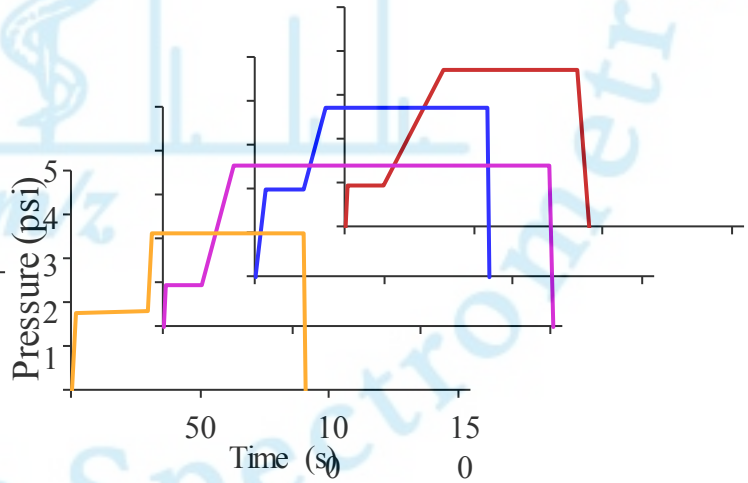


Multi-step SPE for Reverse T3: Very Complicated

Step	Solvent	Volume (mL)	Function
1	CH_3OH	1	Clean 96-well plate
2	H_2O	1	Pre-condition plate
3	Sample	0.4	Load 1:1 diluted sample containing IS
4	5% NH_4OH:CH_3CN 1:1	1	Elute soluble acids, proteins and neutrals
5	5% NH_4OH	1	Elute aqueous soluble acids, proteins and neutrals
6	CH_3OH	1	Elute acids and neutrals
7	C_3H_7OH	1	Elute lipids and polar analytes
8	2% HCOOH in CH_2Cl_2	1	Elute lipids and polar analytes
9	5% HCOOH in CH_3OH	1	Elute anionic moieties
10	Evaporate 40°C, 45 minutes, reconstitute, seal plate, mix, centrifuge and inject		



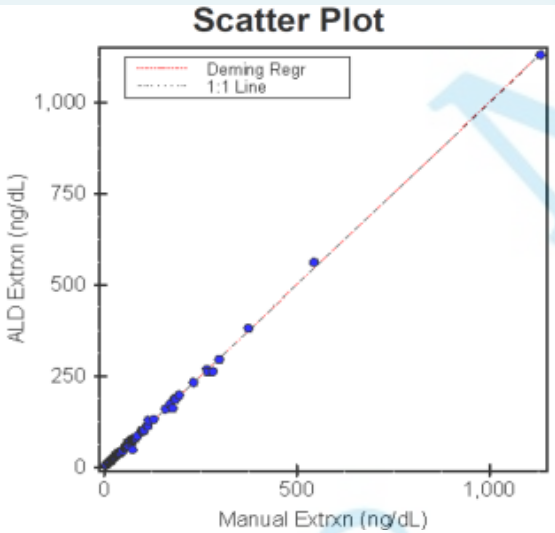
SPE Plate Condition and Loading

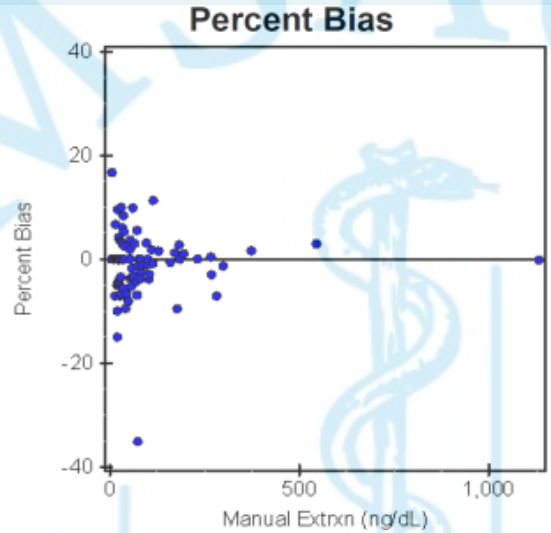


SPE Plate Washing and Eluting

Precision of technique is critical, particularly in complicated workflows

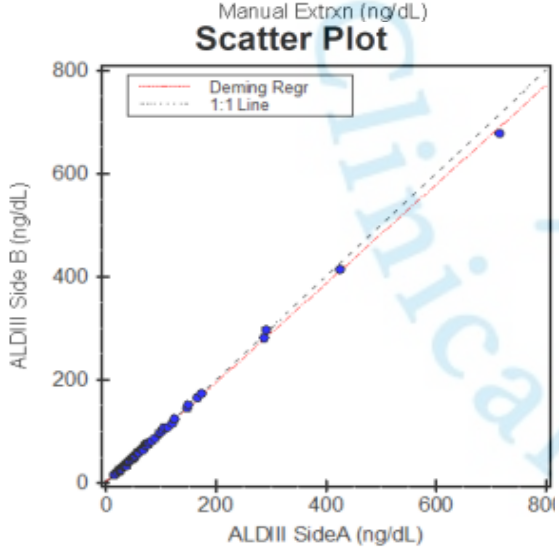
Using duplicate single plates – Curve and identical distributed samples on each plate (Note Percent Bias Scale)

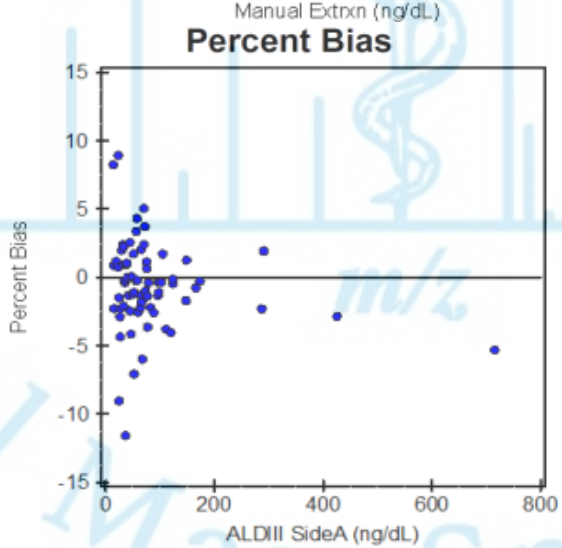




Manual versus Automated

Deming Slope	1.002
r	0.9994
Bias (%)	-0.700
IS Precision (%)	**16%** vs **7%**





ALDIII Left versus Right

Deming Slope	0.961
r	0.9995
Bias (%)	-1.345
IS Precision (%)	**6%** vs **8%**

Use automation and perform triplicate runs for method comparison (reduce influence of single batch variance on comparative slope bias)

Part 1 summary..what we learned

You must know and improve the imprecision of pipetting techniques

Control of adsorptive losses is critical
(concentration/solute/solvent/surface dependent)

Bias = imprecision over time

Internal standard response and single pool imprecision are go to tools

HOW TO ACHIEVE LOWER QUANTIFICATION LIMITS:

PART 2: FORMULATION…of an assay and system

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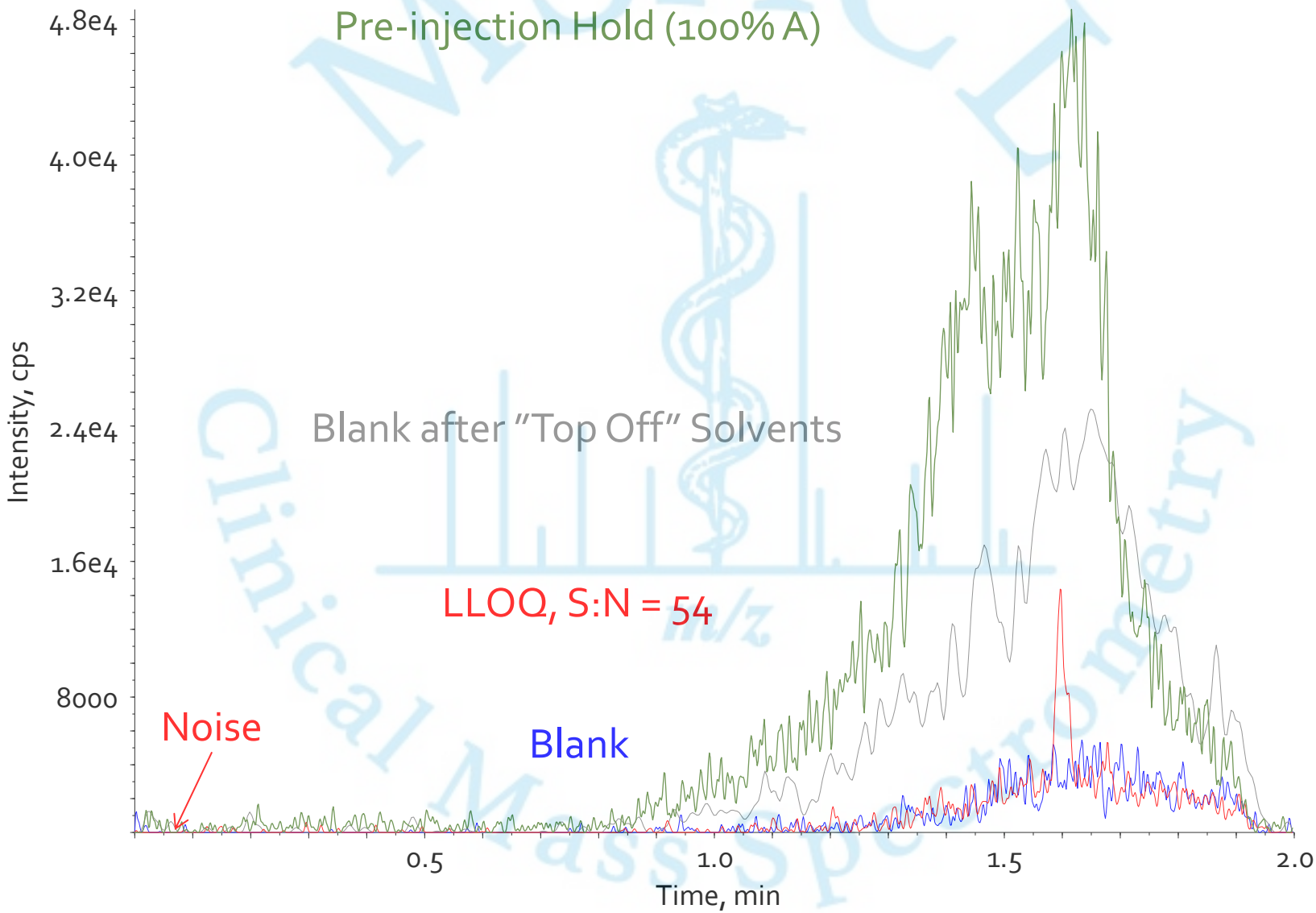
Cleanliness of System

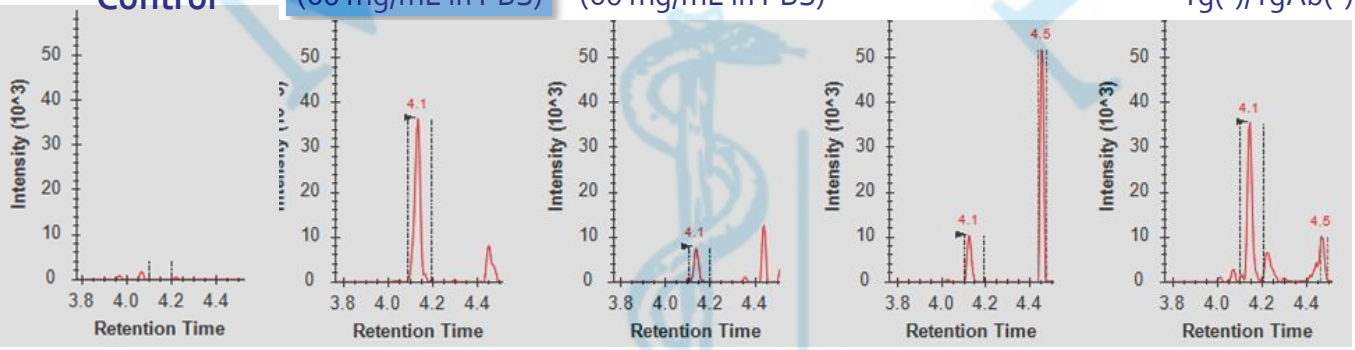


"Absence" of signal and statistics required to define LLOQ, not S:N

More "sensitivity" = More interferences

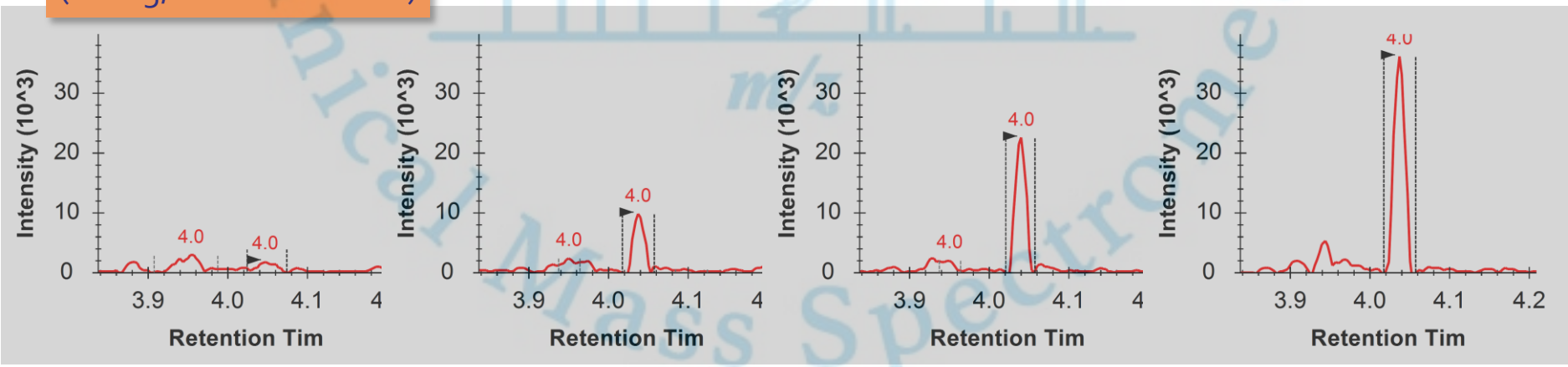
LabCorp Surrogate Matrix

Elution Control | 96% HSA (60 mg/mL in PBS) | 99% HSA (60 mg/mL in PBS) | Chicken Serum | Human Serum "Tg(-)/TgAb(-)"



Final µLC-MS/MS Surrogate Matrix

SigMatrix (20 mg/mL rHSA in PBS) | +0.02 ng/mL | +0.04 ng/mL | +0.08 ng/mL



See: How Low Can You Go? Using Flow to Measure Zero, Chris Shuford

Precision of Recovery and Volumes (Organic Precipitant to Sample Ratio's)

2mL Pooled Serum + 3:1, 2:1, 1:1 Acetonitrile or Methanol
Mix 5 minutes, Centrifuge at 2500g for 10 minutes

Acetonitrile Methanol

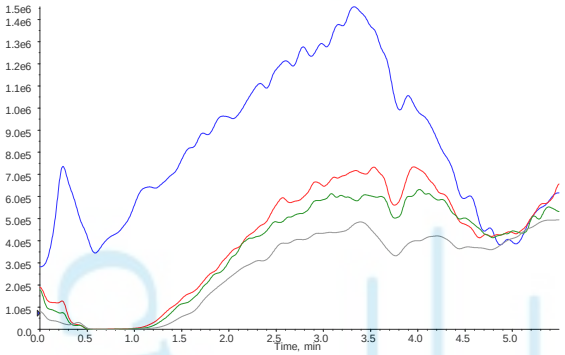
3:1 2:1 1:1 3:1 2:1 1:1

Acetonitrile - smaller floccule, different "texture", less precipitation than Methanol?
Floccule size 3:1 > 2:1 > 1:1 – Subtle, precision of supernatant removal?
Turbidity 1:1 >2:1 > 3:1

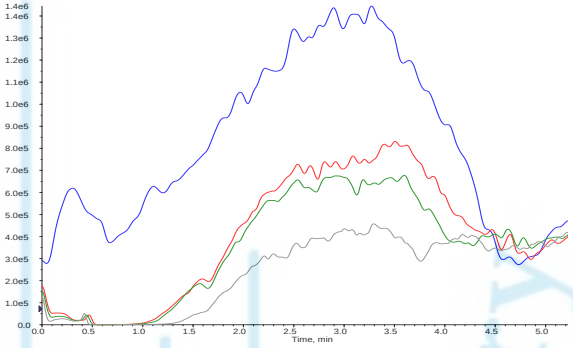
Polson et al, Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry, J Chromatogr B Analyt Technol Biomed Life Sci. 2003 Mar 5;785(2):263-75.

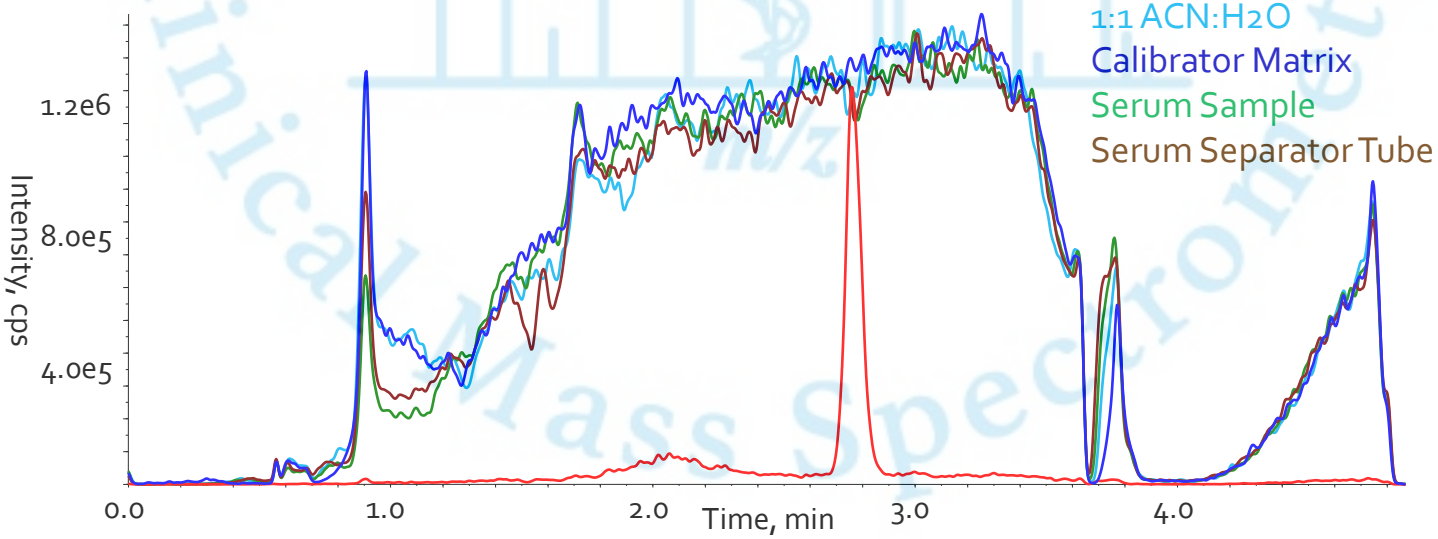
Post Column Infusion of Extracts to define LC needs

Acetonitrile PPT (10µL injected)
5 min Gradient 0 – 100% MeOH
50x2.1mm, 5µm XDB-C18 1mL/min
Neat, 3:1, 2:1, 1:1



Methanol PPT (10µL injected)
5 min Gradient 0 – 100% MeOH
50x2.1mm, 5µm XDB-C18 1mL/min
Neat, 3:1, 2:1, 1:1

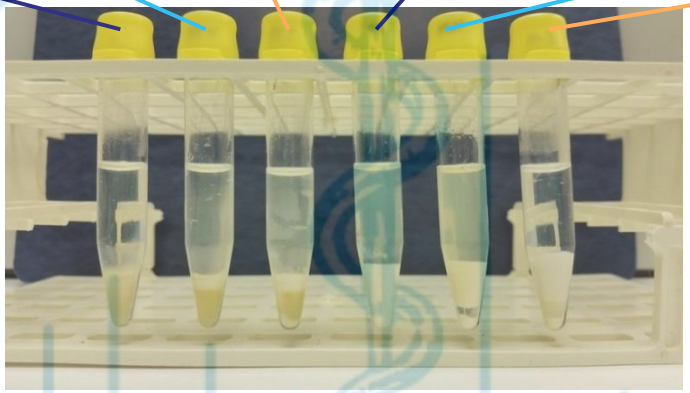




Reducing dilution in Protein Precipitation

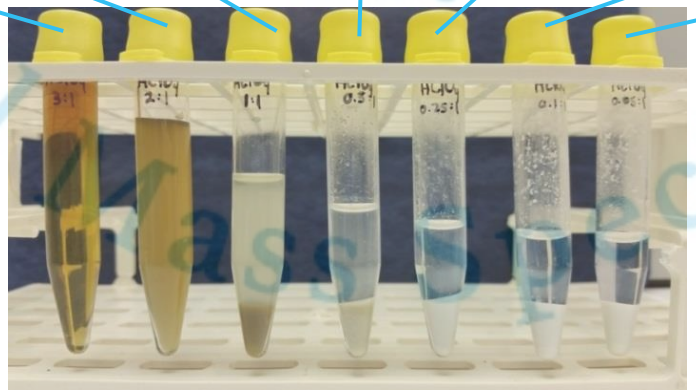
Solvent + Saturated Salt (90:10 aqueous) at 1:1 PPT ratio

90:10 ACN:H_2O +$(NH_4)_2SO_4$ +$ZnSO_4$ 90:10 MeOH:H_2O +$(NH_4)_2SO_4$ +$ZnSO_4$

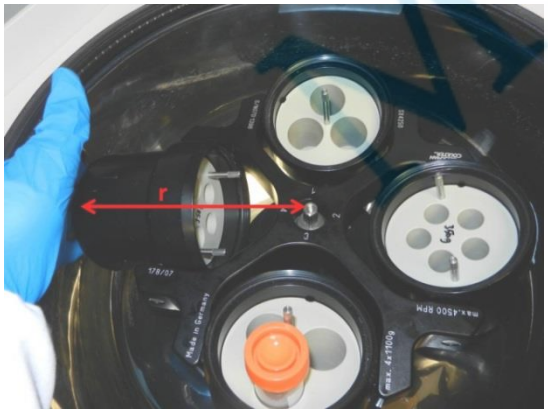


70% Perchloric Acid at various Acid:Sample PPT ratios

3:1 2:1 1:1 0.5:1 0.25:1 0.1:1 0.05:1



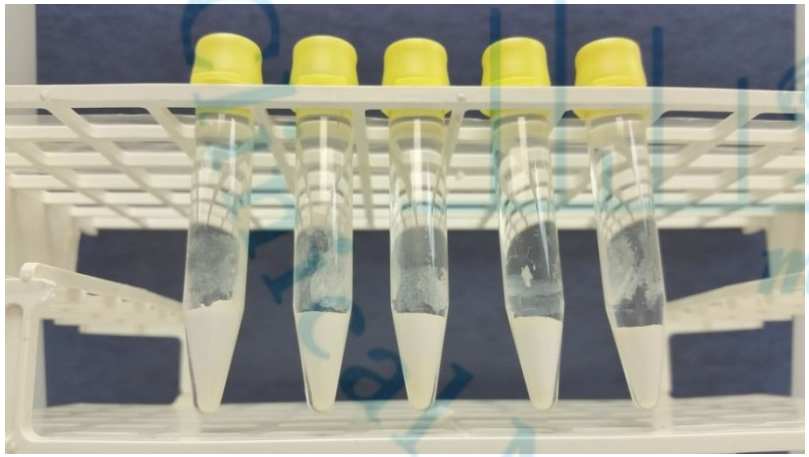
Centrifugal Force (not RPM) – Higher and longer is better



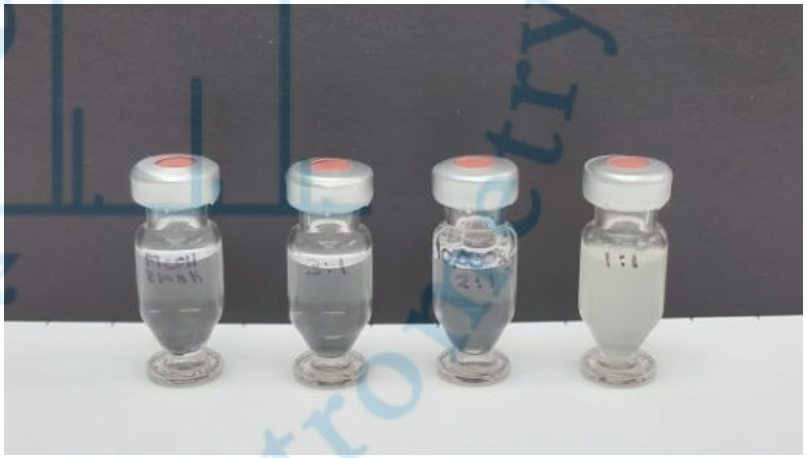
$$G = (1.118 \times 10^{-5}) \times r \times (RPM)^2$$

G = RCF (relative centrifugal force)
r = rotational radius (centimeter, cm)
RPM = evolutions per minute

Database for centrifuge conversions: http://www.endmemo.com/bio/grpm.php



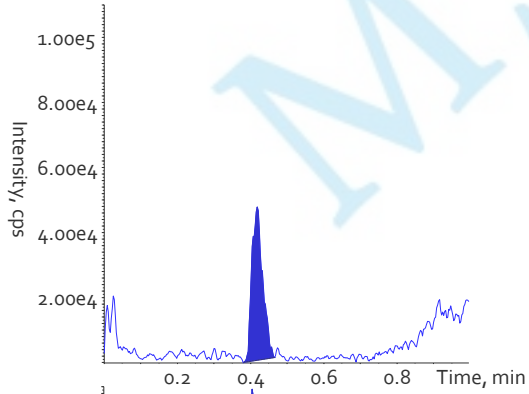
500 1000 1500 2000 2500g
Acetonitrile PPT 3:1



Blank 3:1 2:1 1:1
Methanol PPT, 10,000g, 24h @10C

*96-well plates Centrifuge at ~3600g **

Control your LC system dead volume

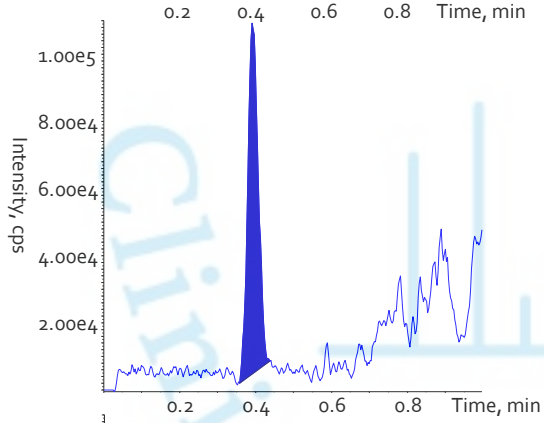


40µL injection into a 100µL Loop

Analyte Area CV = 29%

IS Area CV = 21%

Ratio CV = 13%

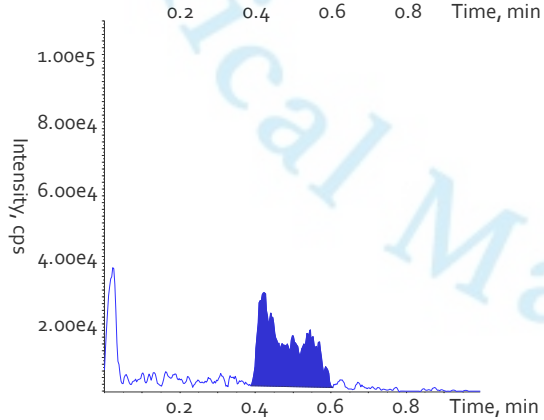


40µL injection into a 20µL Loop

Analyte Area CV = 12%

IS Area CV = 12%

Ratio CV = 5%



Post-column "Orange versus Red peak"

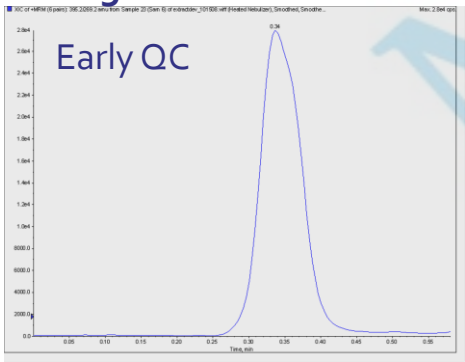
Analyte Area CV = 74%

IS Area CV = 71%

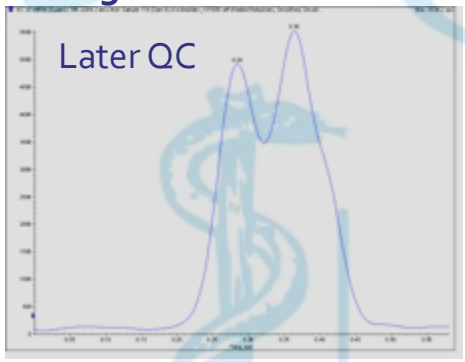
Ratio CV = 40%

Why Isocratic LC eventually fails you

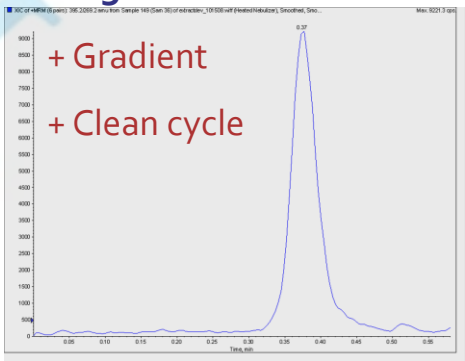
25OH Vitamin D2



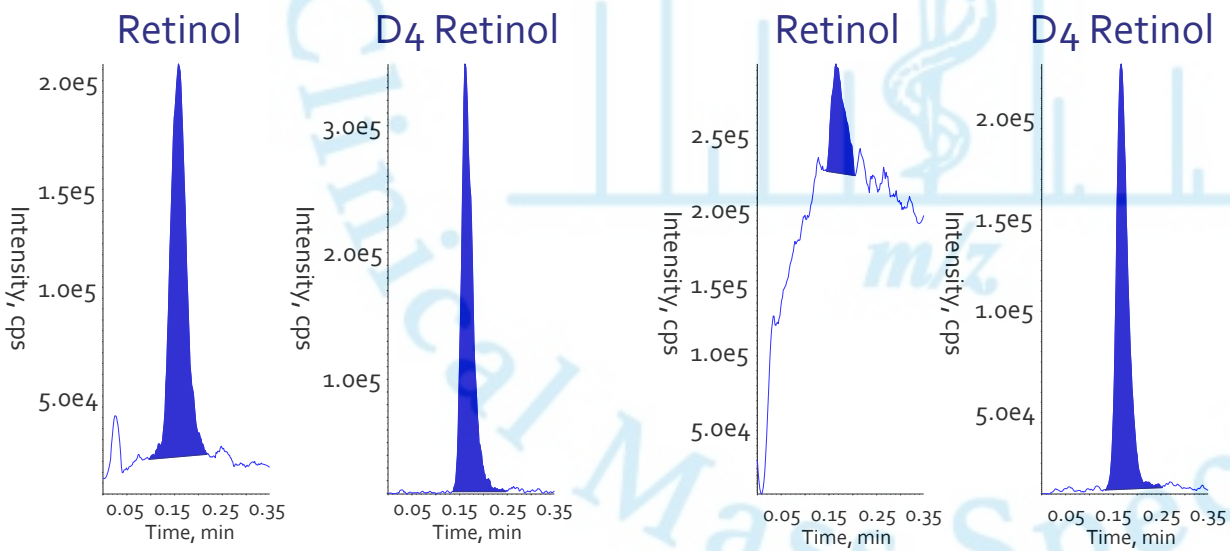
25OH Vitamin D2



25OH Vitamin D2



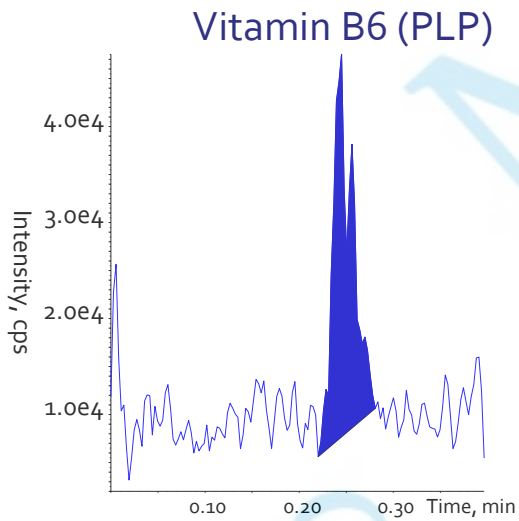
MeOH PPT, Isocratic MeOH:H_2O

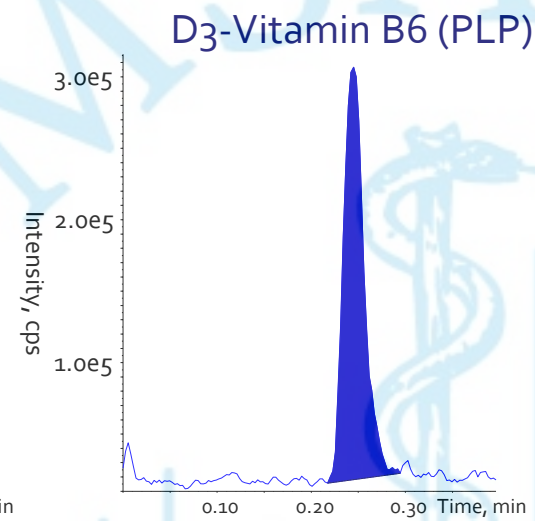


IPA PPT,
Gradient ACN:H_2O
LLOQ injection
1st v 2nd curve
Bias ~90%
+ MeOH wash
Bias <7%, CV< 5%

Use gradients, better prep/LC and elutropic wash for "reproducible integration"
See: Pragmatic HILIC for the Clinic by Brian Rappold

Importance of maintenance

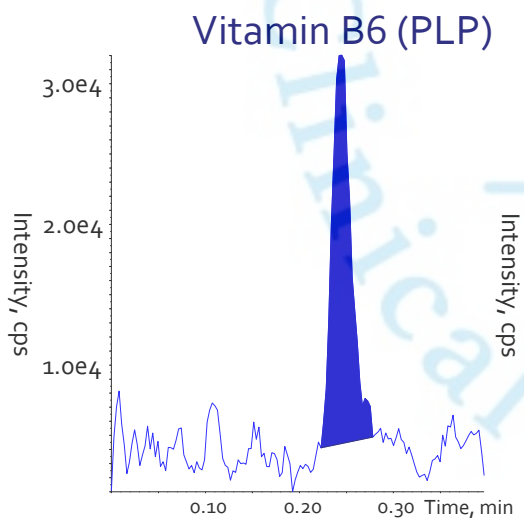


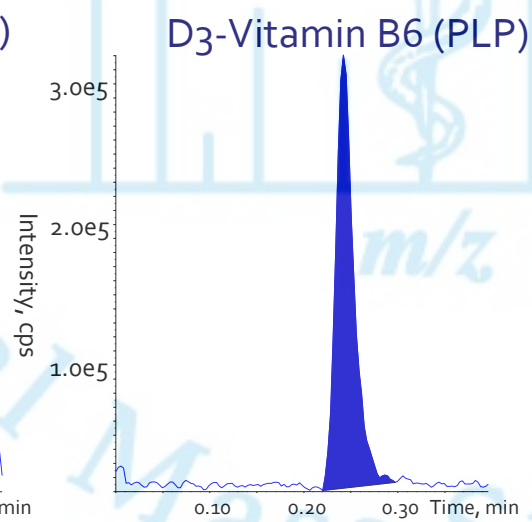


Ion sources are an electrochemical cell

System operates in Negative ion mode

Mean Bias = 4.4%
CV = 27.7%



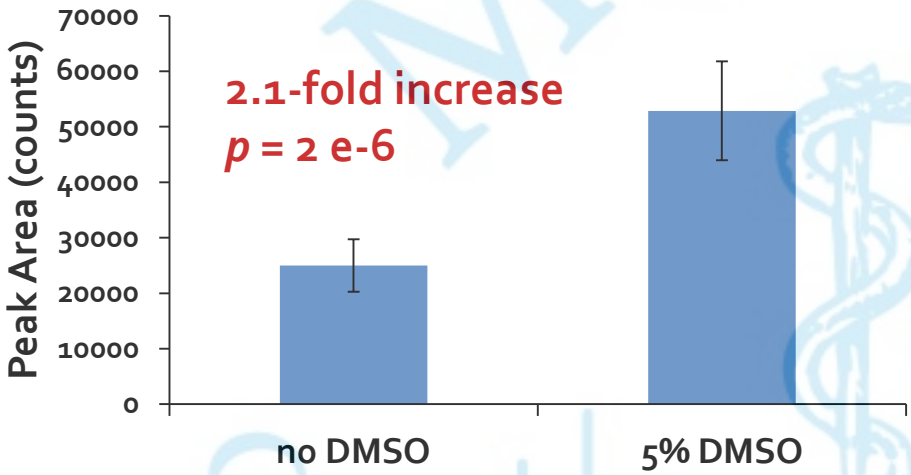


New emitter/source clean

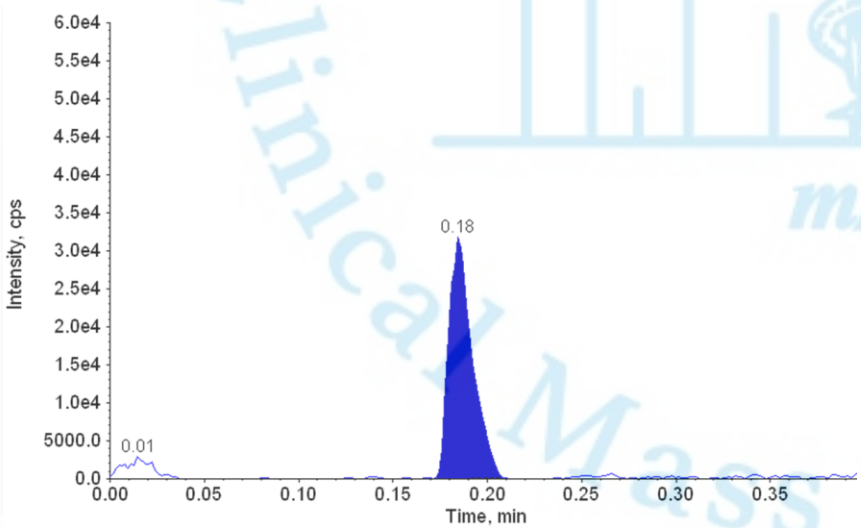
Mean Bias = 3.2%
CV = 10.9%

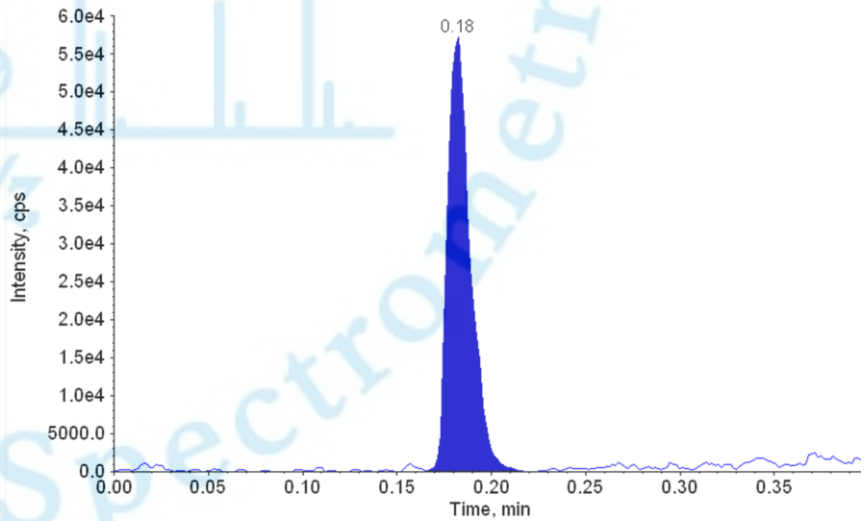
Learn to interpret <u>Noise</u> around and superimposed on your analyte peak

Solvent (distillation) chemistry



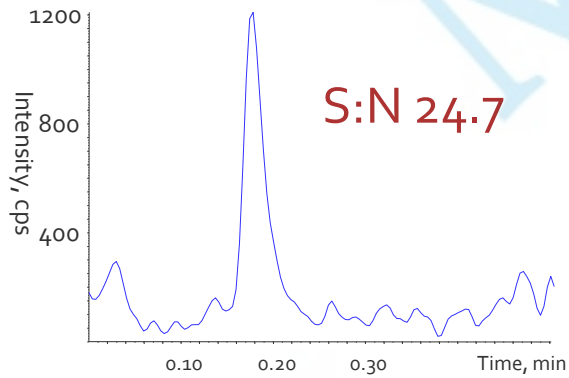
Alternating injections for A peptide (Load/Elute/Load/Elute…N=8)





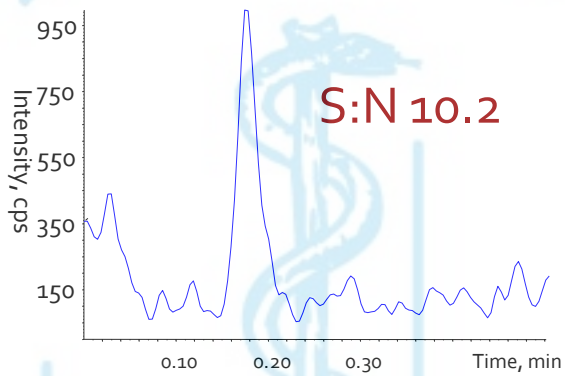
Transition summing: Discovered by accident in 2003

Norfentanyl 200pg/mL
233.20→84.0



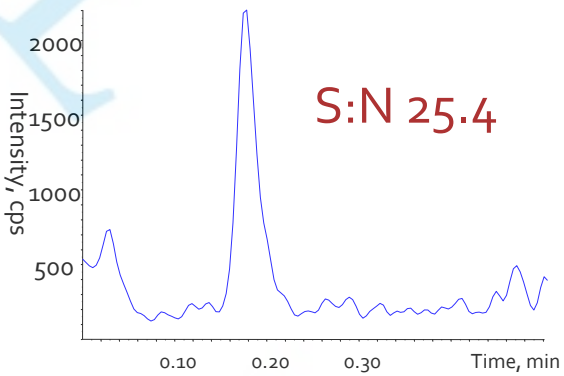
CV: **28.89%**
Bias: -5.60%

Norfentanyl 200pg/mL
233.21→84.0



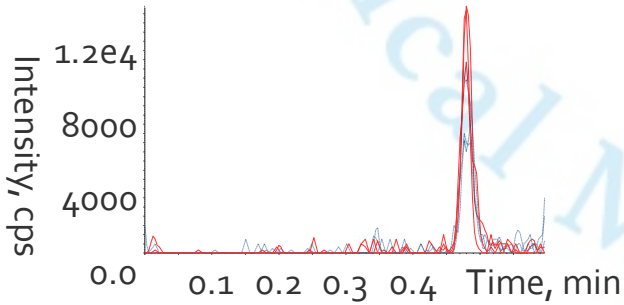
CV: 12.97%
Bias: **18.55%**

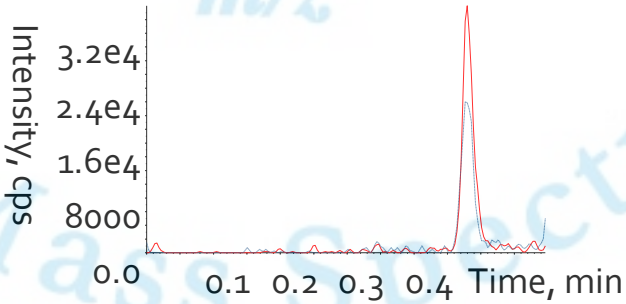
Norfentanyl 200pg/mL
Transition Sum



CV: **9.63%**
Bias: **6.50%**

6 Fluphenazine transitions (10ms, 3 for QN and QL), product ions offset by 0.01amu



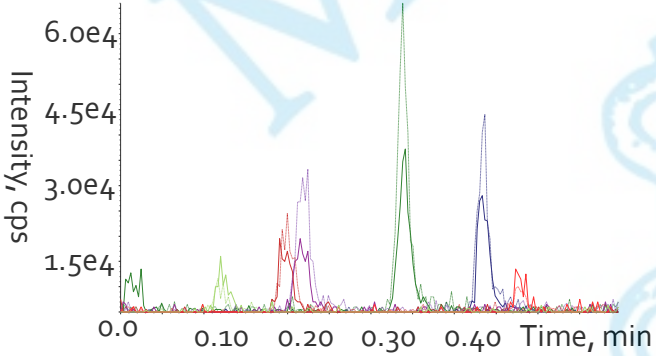


	Single (30ms)	Summed (3*10mS)
S:N	16 - 40	50
Bias (%)	-10.0	1.2
CV (%)	16.4	4.5

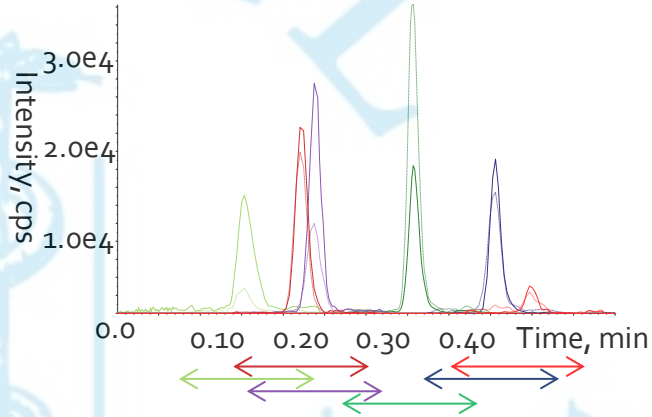
Quite simply you should do this…it literally feels like cheating

Scheduled MRM to solve noisy peaks from summation and ensure appropriate peak sampling, plus signal averaging

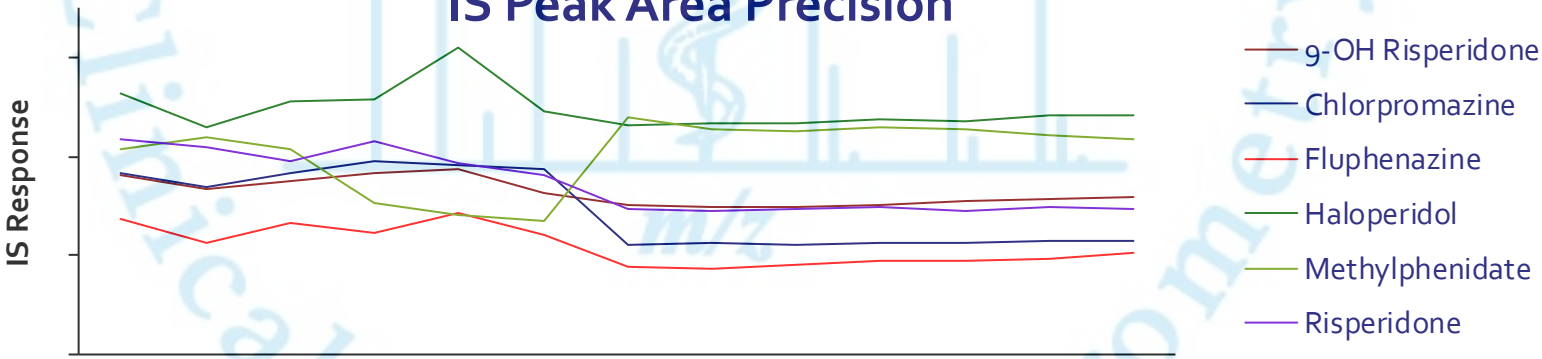
SRM
24 transitions, 12 points/peak



Scheduled MRM
24 transitions, 12 points/peak



IS Peak Area Precision



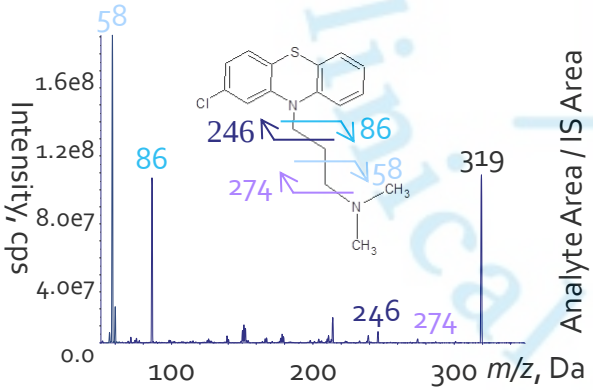
SRM
2-5mS per transition
9.7% CV

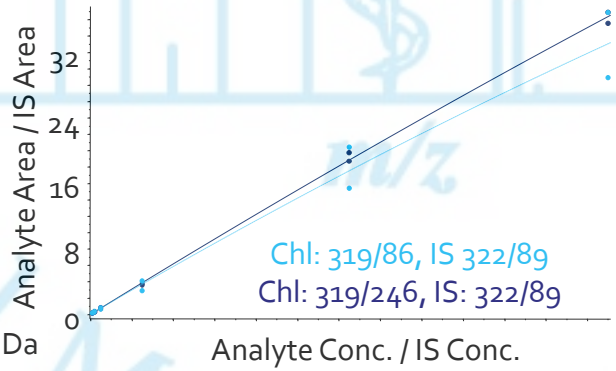
Scheduled MRM
11 – 55 mS per transition,
2.4% CV

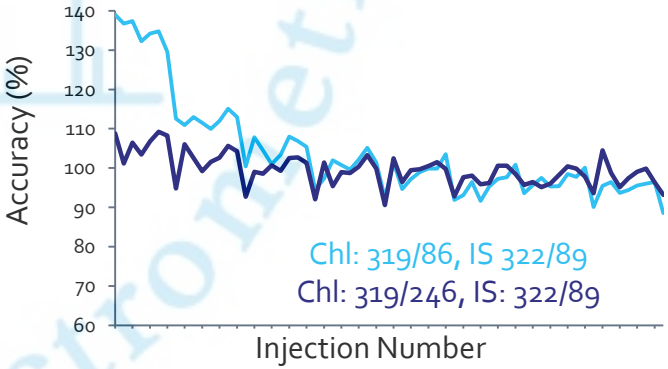
Peak sampling, Noise and Scheduled MRM: Impact on transition ratio precision

	Selected Reaction Monitoring			Scheduled MRM		
	Dwell (mS)	Ion Ratio	CV (%)	Dwell (mS)	Ion Ratio	CV (%)
Methylphenidate	2 – 5	0.44	21.6	11 – 31	0.44	5.2
9-OH Risperidone	2 – 5	0.74	18.5	11 – 25	0.82	5.1
Risperidone	2 – 5	0.52	11.1	11 – 25	0.51	6.2
Haloperidol	2 – 5	2.07	6.6	25 – 55	2.07	3.0
Chlorpromazine	2 – 5	1.32	7.2	13	0.97	13.5*
Fluphenazine	2 – 5	0.81	7.9	13	0.85	9.9

*** Differential Fragmentation over time for Chlorpromazine**





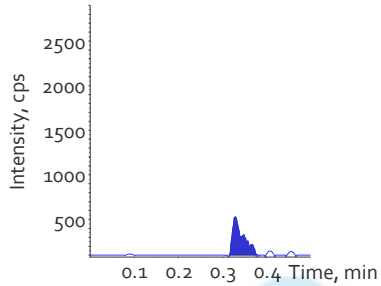


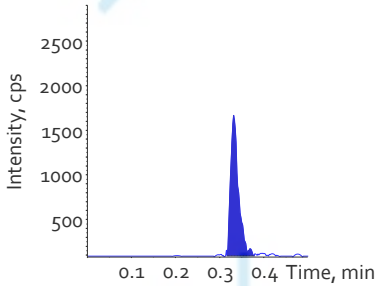
Short dwell times for peak sampling = Noisy data

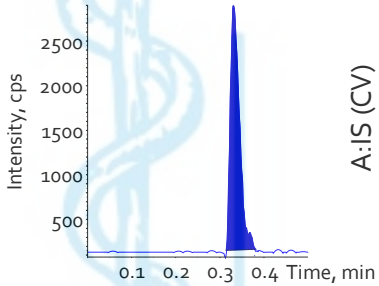
Scheduled MRM increases dwell time per transition = Signal averaging

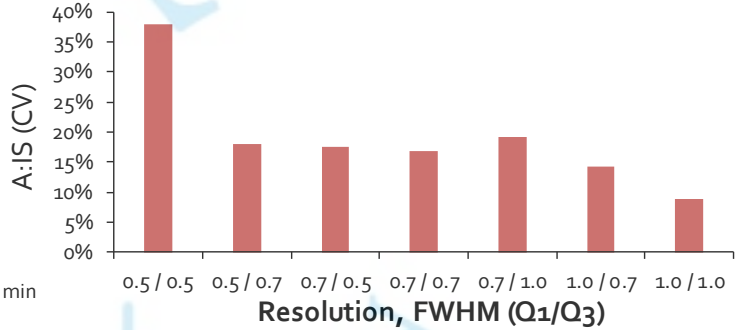
Modifying Quadrupole Resolution (Q1 and Q3)
..in the absence and presence of noise

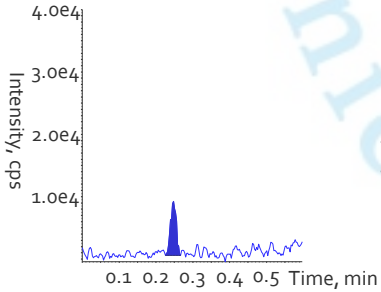
"**High**" Resolution (0.5 amu, FWHM) | **Unit** Resolution (0.7 amu, FWHM) | "**Low**" Resolution (1.0 amu, FWHM) | Analyte to IS Precision

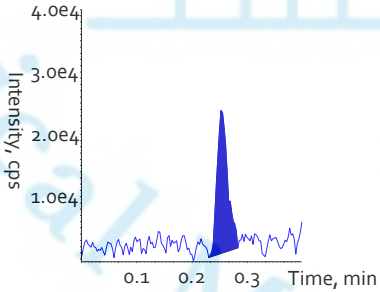


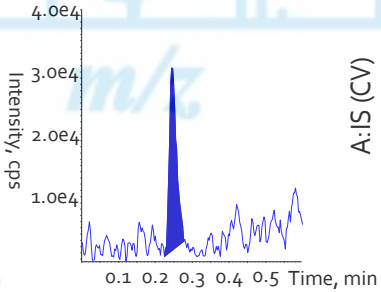


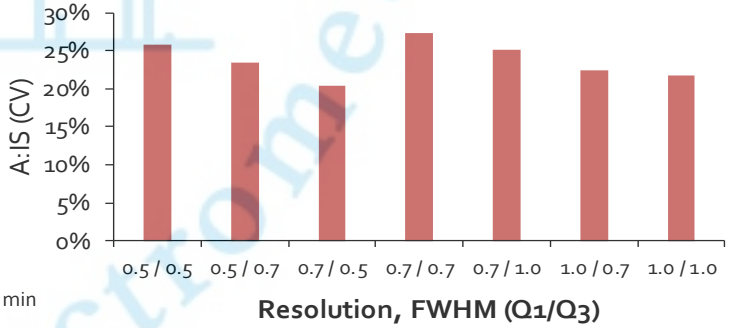












Absence of noise = More "signal"
Presence of noise (biological and/or chemical) use ***caution***

Part 2 summary…what we learned

You must have a "clean system and matrix blank"

Calculation of signal:noise is somewhat irrelevant (can we agree?)

Sample preparation should enable precision of aspiration (volume/centrifugation)

Sample preparation ideally be non-dilutionary and must resolve matrix effects

LC system dead-volume must be minimized and use gradients

Maintain instrument source/optic cleanliness (plus use a bypass valve)

Ion source solvent chemistry is a key variable to enhance ionization efficiency

Use signal summing always and scheduled MRM for large panels

Be careful with quadrupole de-resolution (monitor ion ratios in many samples)

HOW TO ACHIEVE LOWER QUANTIFICATION LIMITS:

PART 3: FINESSE…tying the pieces together

RUSSELL P. GRANT

LABORATORY CORPORATION OF AMERICA® HOLDINGS, BURLINGTON, NC, USA

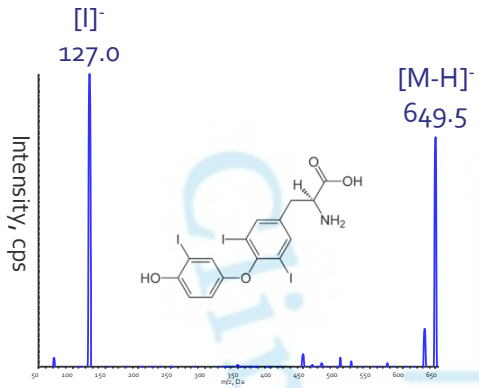
Free T3 and T4 in 2003 ...*adsorption!*

- **Challenges**
  - Sensitivity - 1pg/mL LLOQ from 200µL sample (<200 fg on column)
  - Adsorptive losses (>99%) in aqueous solutions
  - Over-dilution in equilibrium dialysis (disrupting free/bound)
  - Internal standardization (and calibration) <u>after</u> dialysis
  - T3 and rT3 are isomer
  - Fragmentation of T4 produces T3 and rT3 – "in-source" isobar (resolution if assaying together)

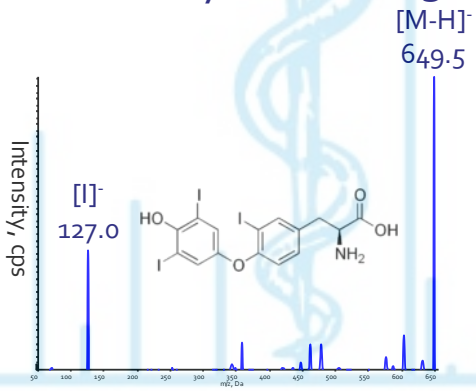
Thyroid Metabolites – Signal gain and Ionization

- **Good News:**
  - 3-4 common product ions from 1 precursor, yet rare product ion in biochemistry
  - Negative ion mode (less noise/interferences)
  - Uncommon mass range for Small Molecules

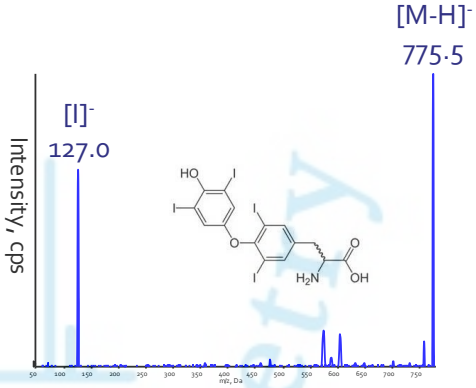
Triiodothyronine (T3)

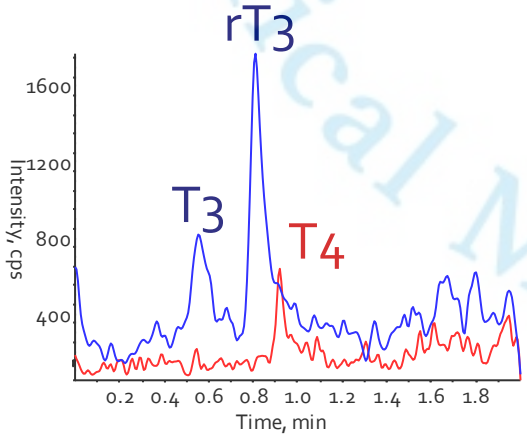


Reverse Triiodothyronine (rT3)



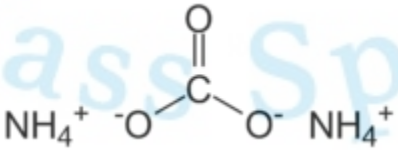
Thyroxine (T4)

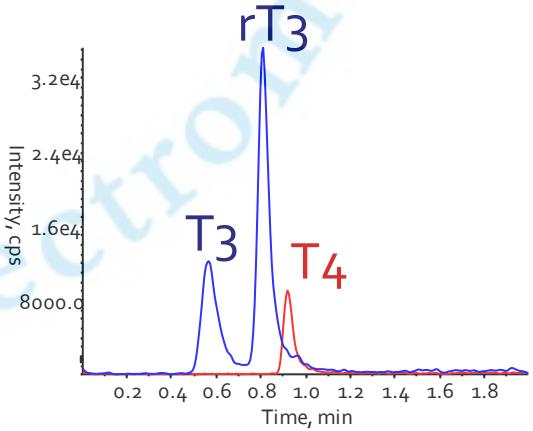




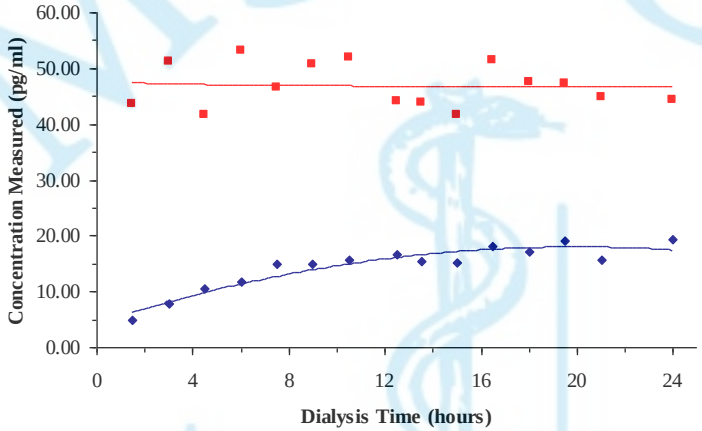
Late elution (MeOH)

Post Colum Addition





Free Thyroxine (T4) – 96-well Equilibrium Dialysis



T4 Dialysis Losses absent following addition in dialysate on BOTH sides of membrane

Time-course of Free T4 with Equilibrium Dialysis at 37C

Sample: 200µL sample and dialysate volume – mitigate dilutionary disruption

Sample: Dextran Blue (2 MDa) – Well malfunction (T4 99.9% Bound) Carry-over mitigation (do not inject)

Dialysate: Isotonic physiological - non disruptive to free fraction

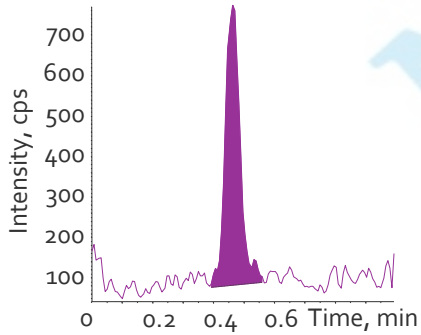
Dialysate: Heat denatured protein added – non disruptive and mitigate adsorption

Post dialysis: Pre-wet & pre-fill IS in MeOH into aspiration tip – mitigate adsorption

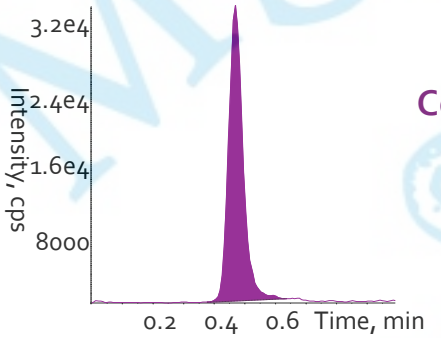
Calibration: Dialysate matrix plus T1 (iodothyronine)* - mitigate adsorption

** Van Houcke et al.: Free thyroxine conventional reference procedure, Clin Chem Lab Med 2011;49(8):Ad1–Ad5*

Free Thyroxine (T4) Assay Data



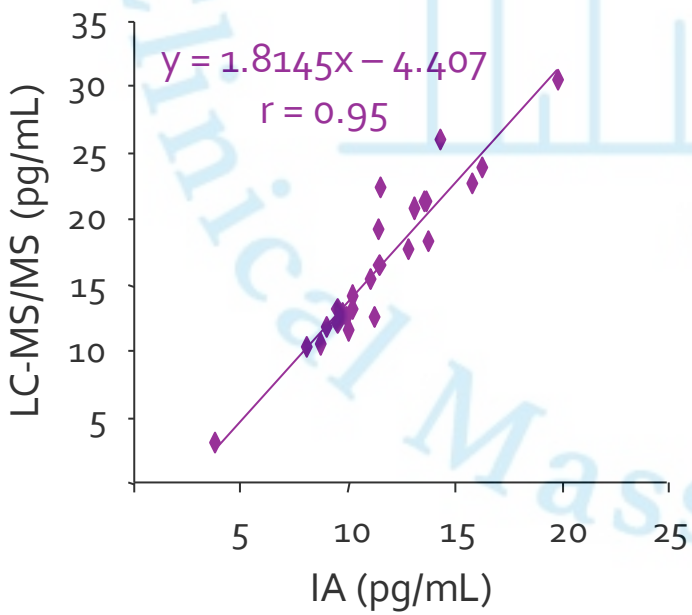
LLOQ 2 pg/mL
(2.5pmol/L, 50fg on column)

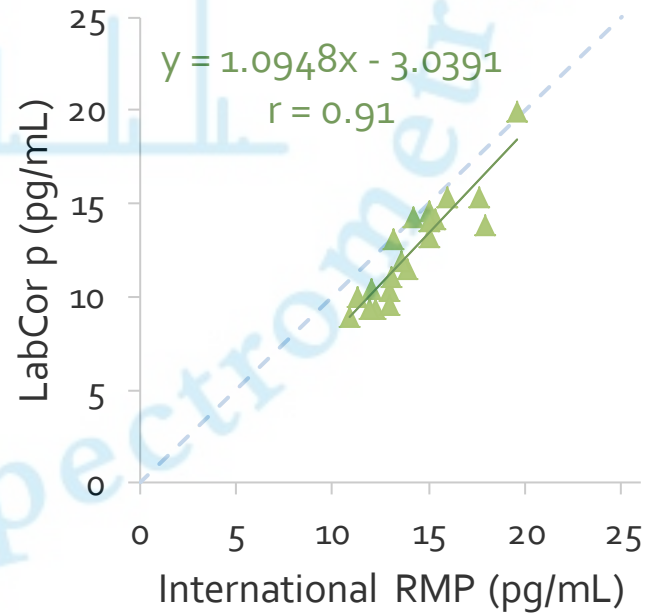


ULOQ 100 pg/mL
(128.7pmol/L)

Free Thyroxine

	Accuracy (%)			Precision (%)		
Conc.(pg/mL)	2	50	100	2	50	100
Intra - 1	-3.4	-7.0	3.0	5.0	6.2	5.5
Intra - 2	0.1	-1.2	-0.3	5.1	5.2	2.2
Intra - 3	-0.4	0.6	3.7	4.4	4.1	5.5
Inter	-1.3	-2.5	2.1	4.8	6.0	4.8



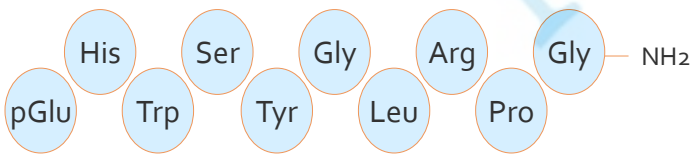


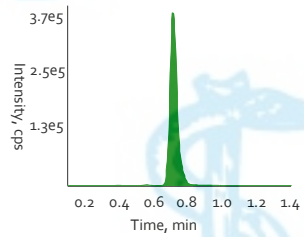
GnRH…*decoupling selectivity and sensitivity*

- **Challenges**
  - Sensitivity - 10pg/mL LLOQ from 50µL sample (<200fg on column)
  - Adsorptive losses (>90%) in aqueous solutions
  - First quantitative peptide assay for clinical diagnostics (2005)
  - Unstable in specimens
  - Isotopic separation of IS from analyte?
- **Good news**
  - Comparator immunoassay
  - Its good to try to be first
  - "labeled peptide" synthesis capability (malibu!)

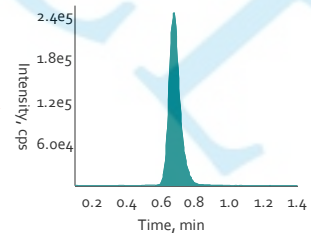
Decoupling Selectivity and Sensitivity with 2D LC

GnRH

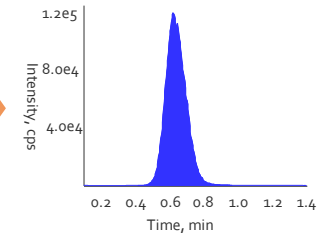




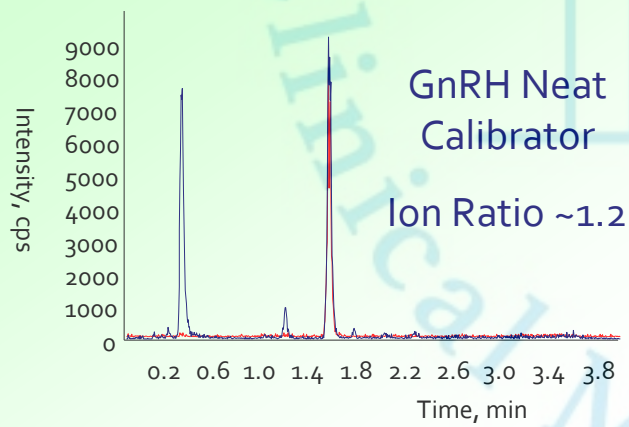
1st Injection
3.7e5



32nd Injection
2.4e5

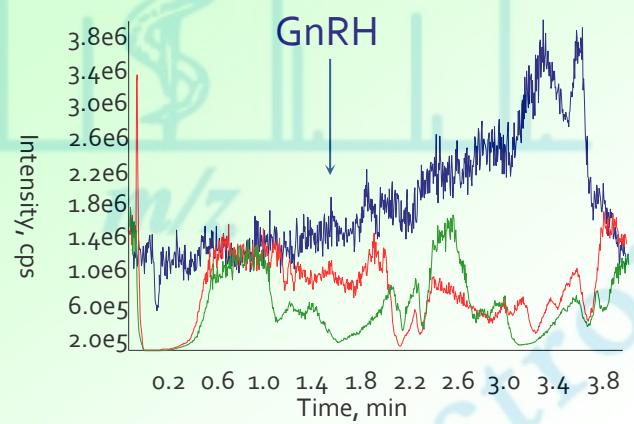


79th Injection
1.2e5



GnRH Neat Calibrator

Ion Ratio ~1.2



Post Column Infusion

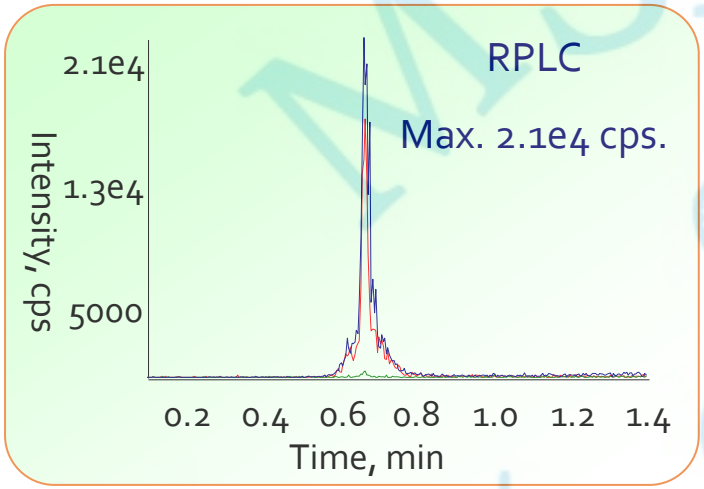
Water Injection

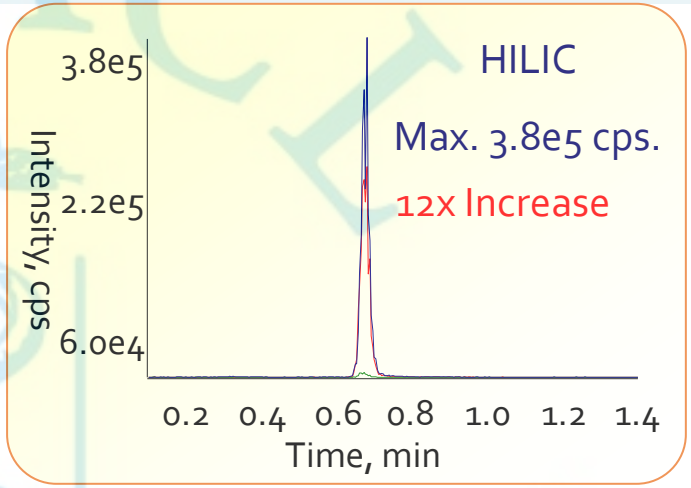
ACN Plasma PPT

MeOH Plasma PPT

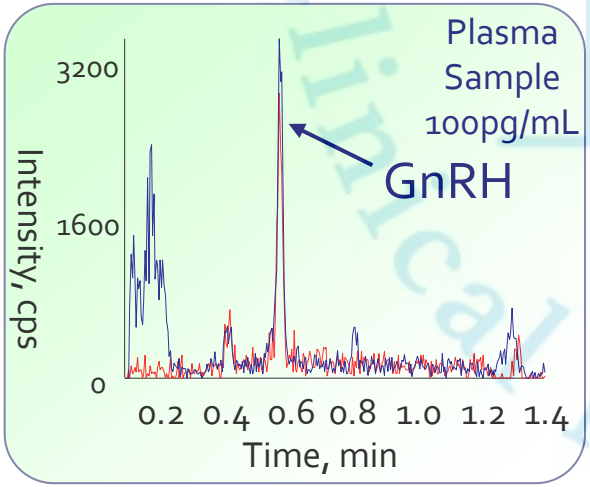
Ref: Wagner A, Grant R , Poster, ASMS, 2007

Decoupling Selectivity and Sensitivity

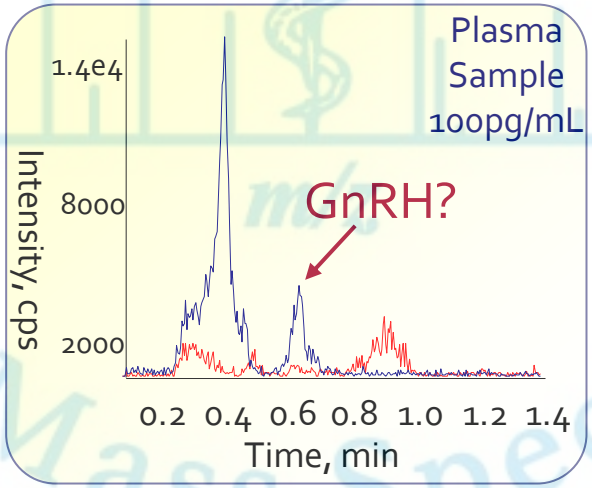




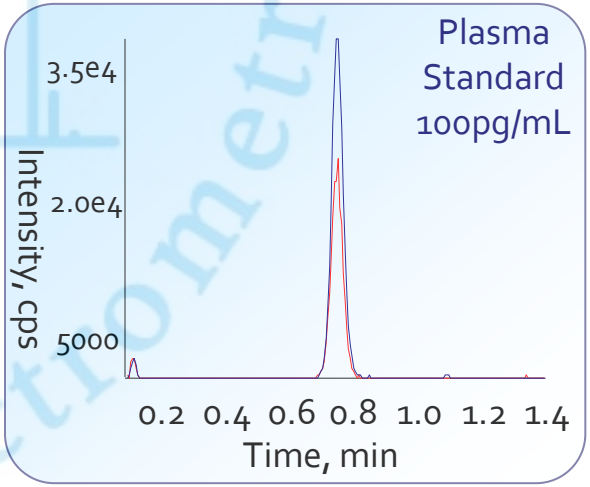
GnRH Elutes at <5% Organic in RPLC but 95% Organic in HILIC mode



RP Only



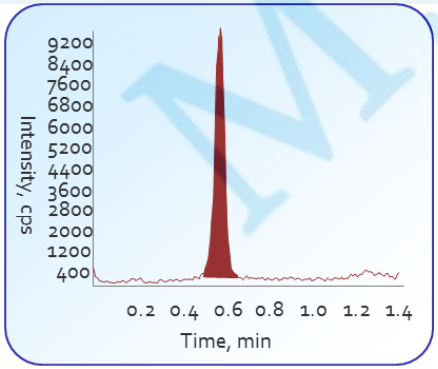
HILIC Only



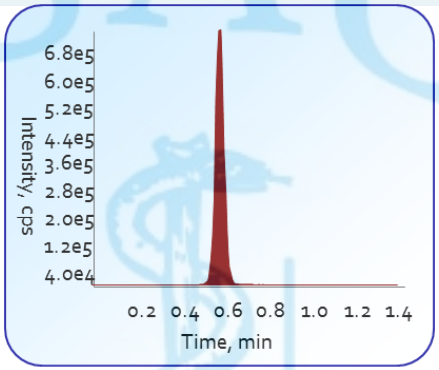
2D RP - HILIC

Selectivity derived in 1st Dimension, Sensitivity generated in 2nd dimension

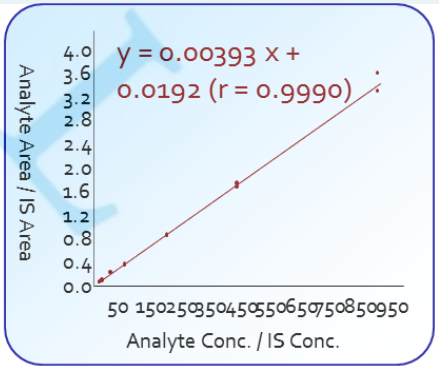
FIGURES OF MERIT: 2D-LC FOR GNRH



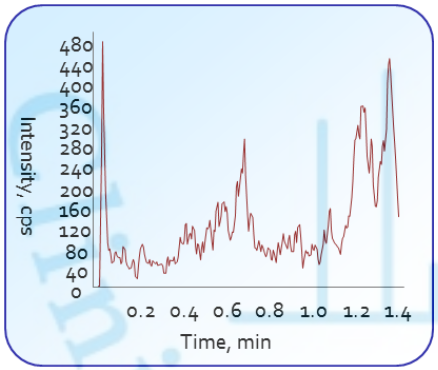
LLOQ (10pg/mL)



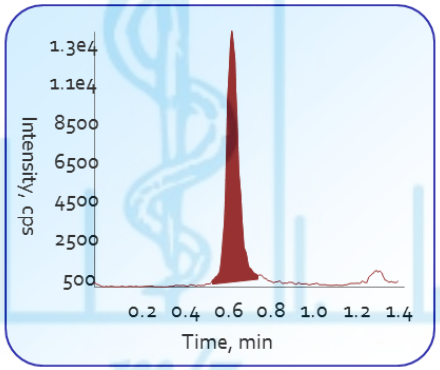
ULOQ (1000pg/mL)



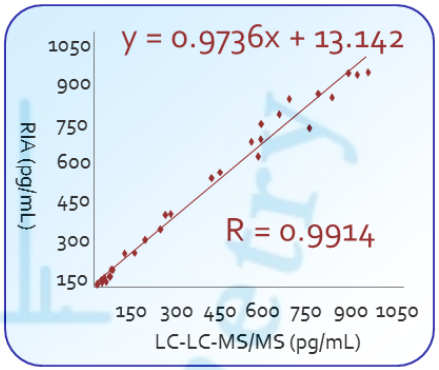
Calibration Curve



Carryover Blank



Patient Sample



Cross-Validation
N= 30, Avg Bias = 1.08%

	Accuracy (%)			Precision (%)			
Conc. (pg/mL)	10	500	1000	30	100	400	800
Intra - 1	-4.9	0.7	-1.4	3.5	1.7	1.5	1.9
Intra - 2	0.5	-1.1	-1.1	3.5	3.3	2.8	2.9
Intra - 3	-8.3	-1.0	-1.7	6.4	3.1	3.2	1.8
Inter	-4.2	-0.5	-1.4	4.6	2.8	2.6	2.2

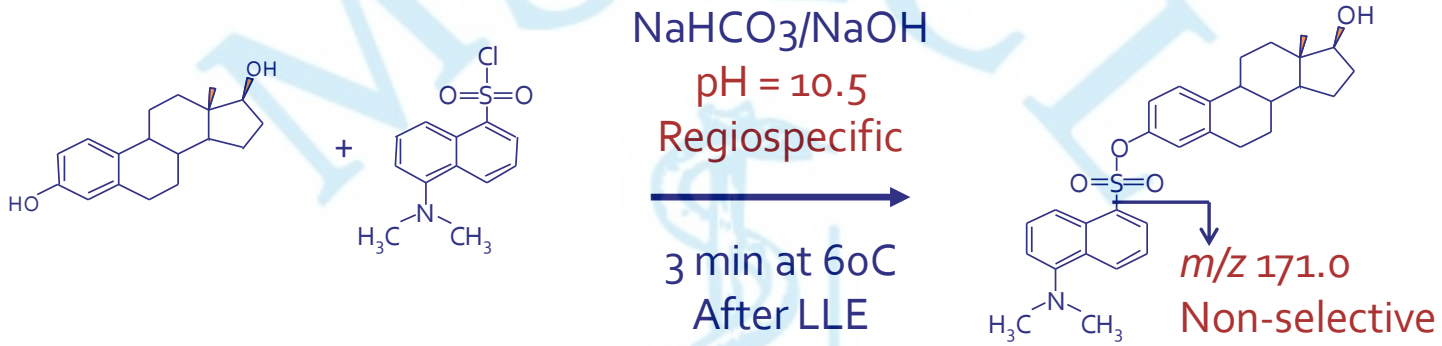
Validation	Bias (%)
Plasma	2.5 – 3.7
Lipemia	6.6
Whole Blood	6.2
Recovery	97.9 – 106.8

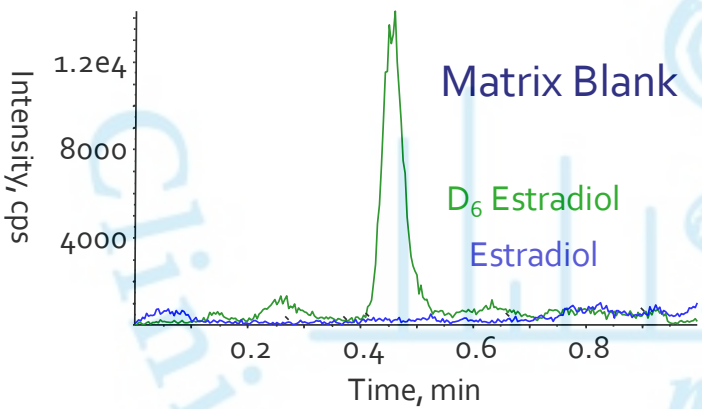
Ref: Wagner A, Grant R , ASMS, 2007

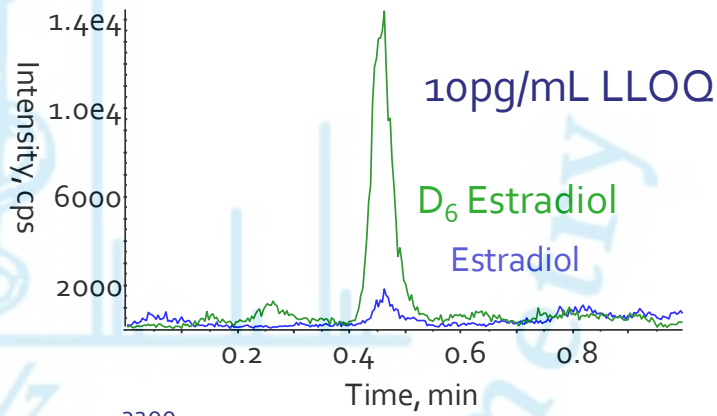
Estradiol…*additional orthogonality*

- **What is special about <1pg/mL (<4pmol/L)?**
  - Estrogen suppression "controlled" (Often < LLOQ)
  - Pre-pubertal onset
  - Free Estradiol
  - Salivary Estradiol?
- **Challenges**
  - Sensitivity - 1pg/mL LLOQ every day
  - Neutral molecule
  - Very common mass range for Small Molecules
- **Good News:**
  - Extensively studied and many tools to explore
  - 14 years working on it (gen 3)

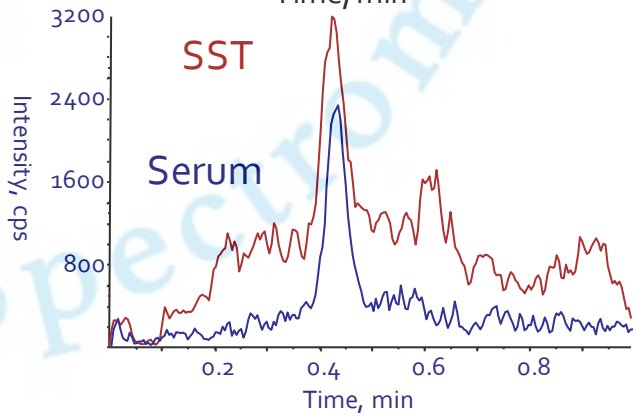
In 2003 on an API4000







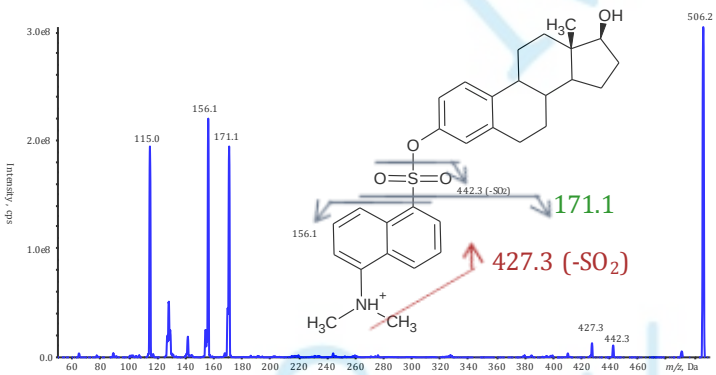
Serum Separator Tube

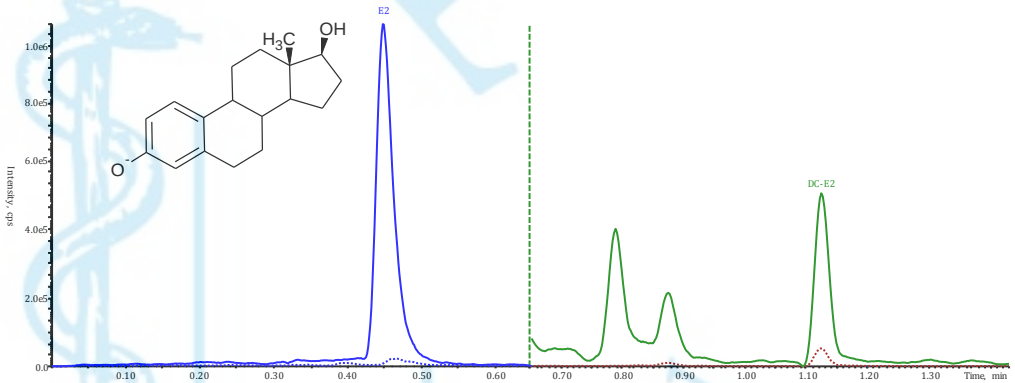


Derivatization, Yield and Suppression - zero sum game?

Estradiol (substrate) to Dansyl Derivative (product) with LC + polarity switch

Determine disappearance/appearance using "disease and dirty samples"

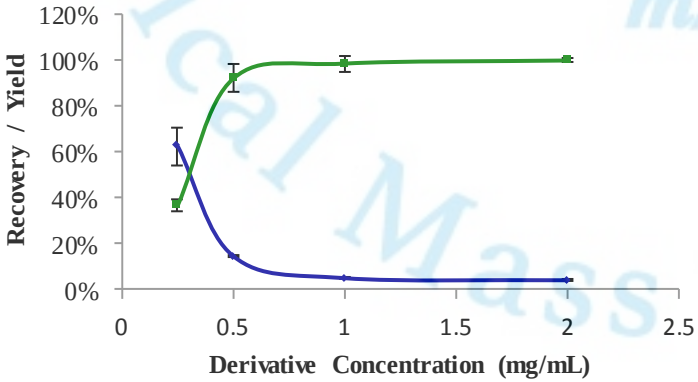


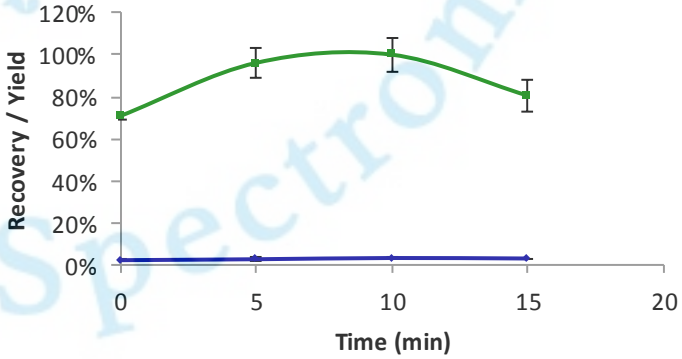


Efficiency sensitivity) Loss = 50% (95%)

Matrix effects ~85%

Add neat and derivatized IS post extraction – Recovery yield + Matrix Effects

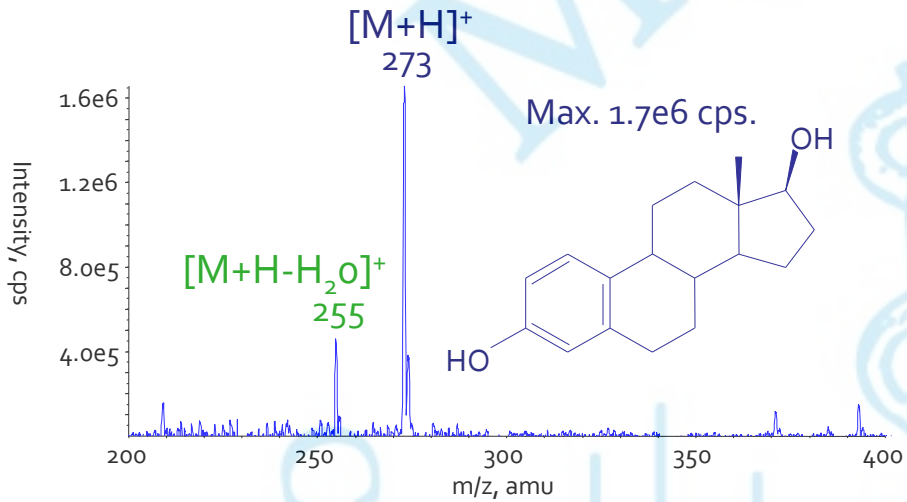




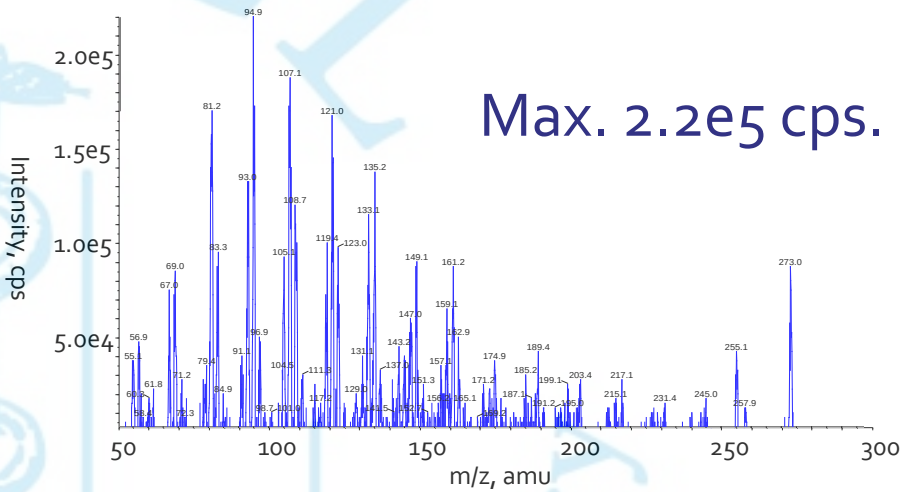
Measurement of Low Level Endogenous Biomarkers for Use in Clinical Diagnostics, *Dee et al, ASMS 2014, Poster*

Increasing MSMS transmission efficiency API5000

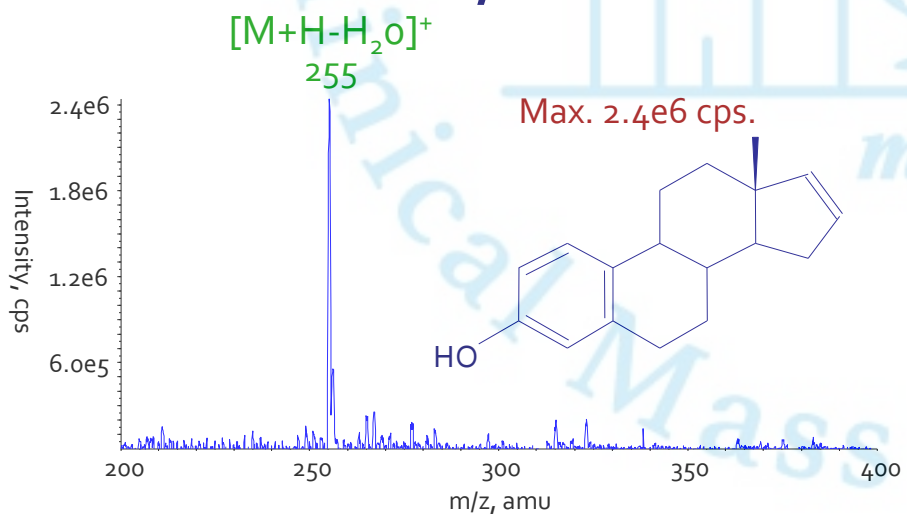
Estradiol APCI 400C, Precursor Ion scan



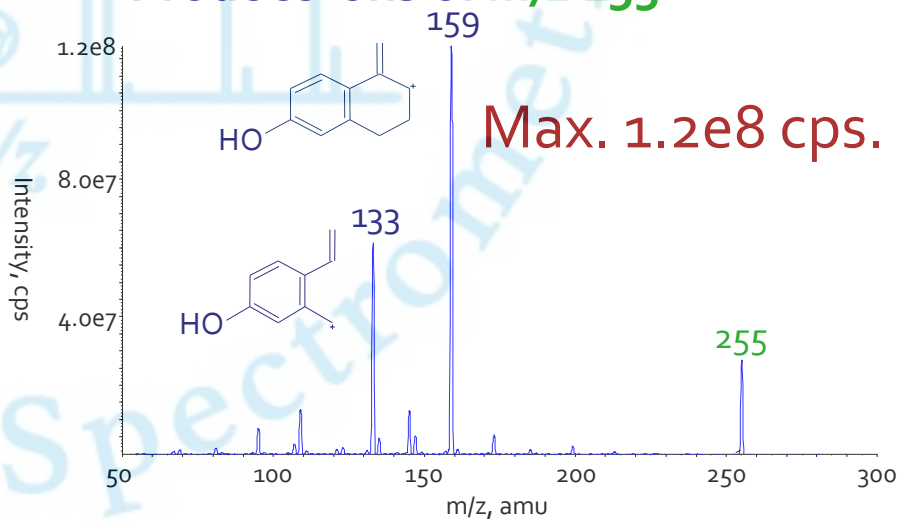
Product Ions of *m/z* 273



Estradiol APCI 600C, Precursor Ion scan



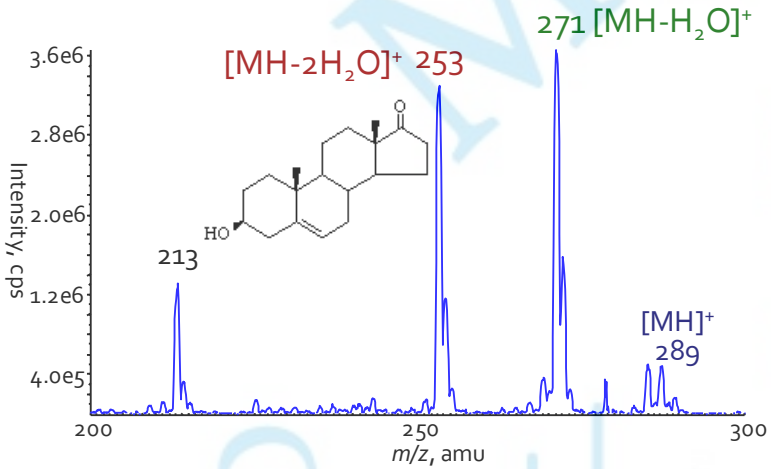
Product Ions of *m/z* 255



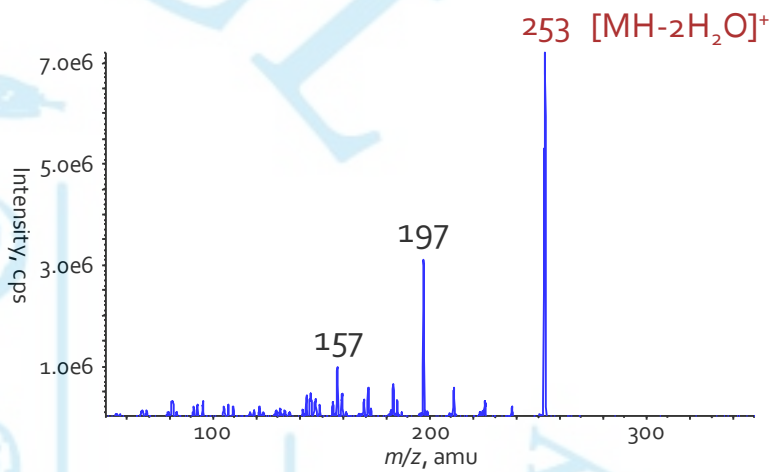
> 2 orders of magnitude increase in MS/MS transmission efficiency

Heat induced stress…*for m/z 255 - 159*

Precursor Ion Scan of DHEA

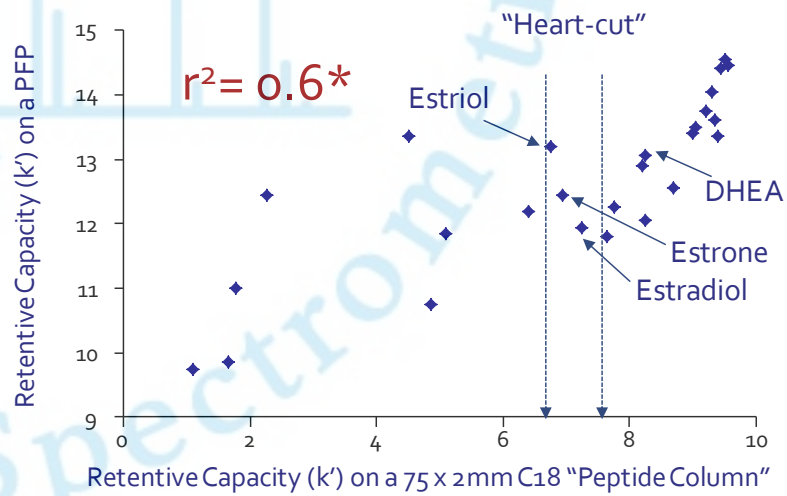


Product Ion Scan of dehydrated DHEA



$^{2}C_{13}$ DHEA has a *m/z* 255 – 159 transition, circulates at 300 – 1500 x Estradiol

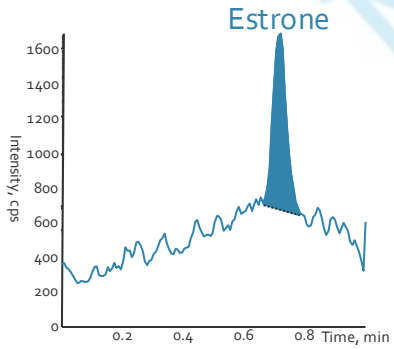
Estradiol : 24 interferences (*m/z* 255 → 159/133)
Estrone : 16 interferences (*m/z* 273 → 159/133)



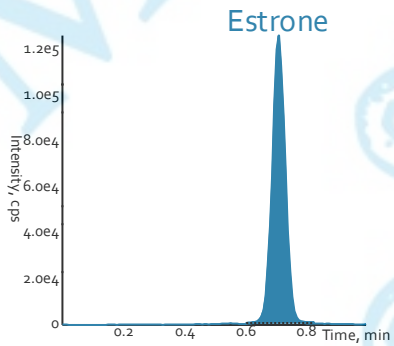
** Development and Application of 2D-LC-MS/MS in Clinical Diagnostics, Grant et al, ASMS 2007, Poster*

Underivatized estrogens (LLE-LC-LC-MS/MS)

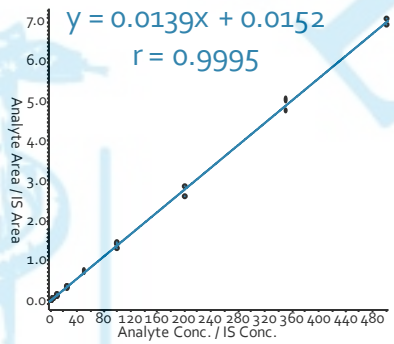
LLE (0.5mL), 2D-LC (5 min cycle time), Heat assisted APCI, 350 – 850fg on column



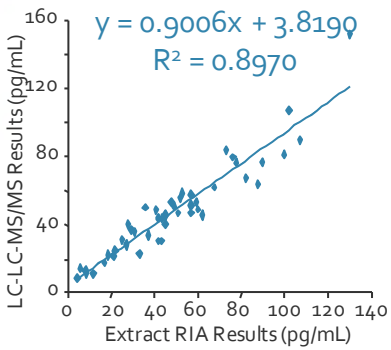
LOQ (2.5 pg/mL)



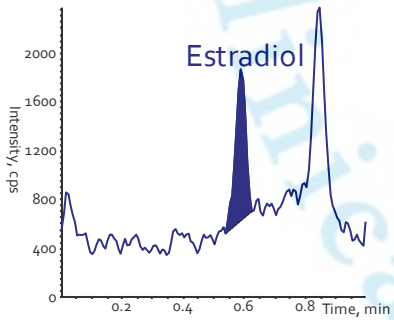
ULOQ (500 pg/mL)



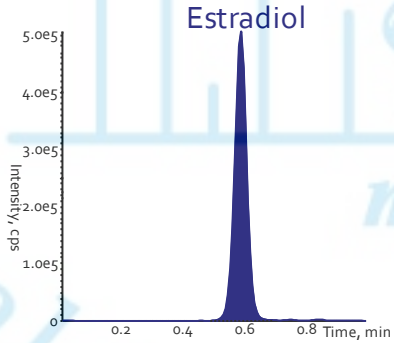
Calibration Curve



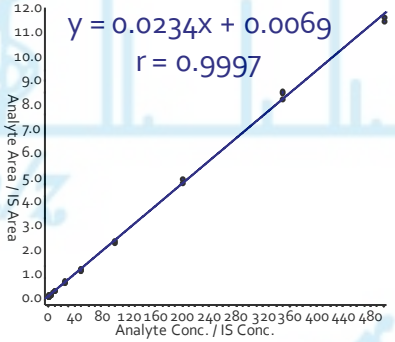
Inter-assay comparison (with extraction/RIA)



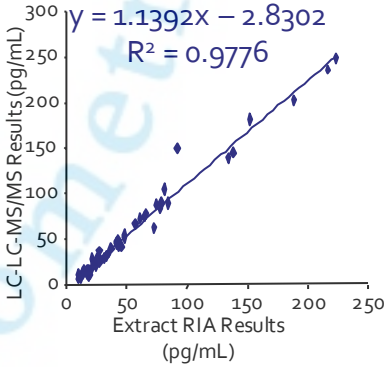
LOQ (1 pg/mL)



ULOQ (500 pg/dL)



Calibration Curve

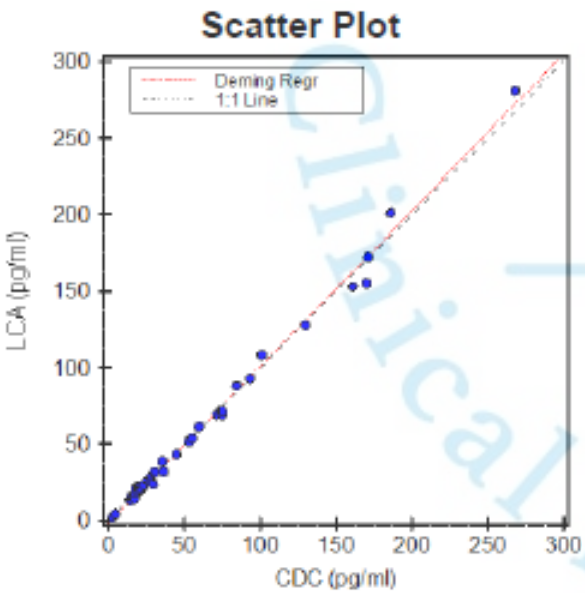


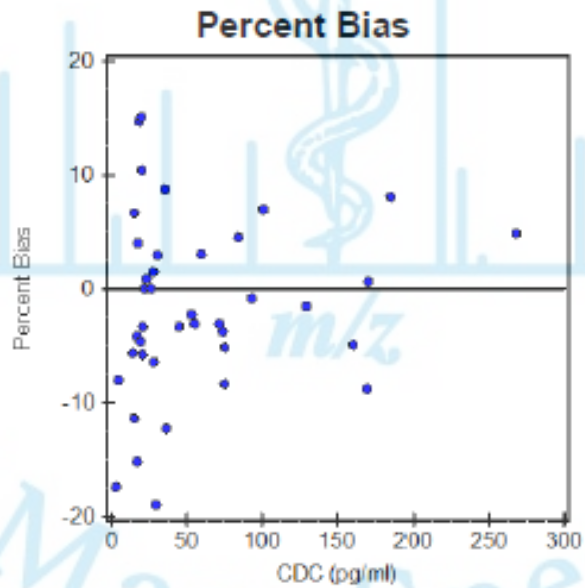
Inter-assay comparison (with extraction RIA)

Quantitative LC-MS/MS Analysis of Endogenous Biomarkers for Clinical Diagnosis in Endocrinology, Morr et al, Poster ASMS 2006

Validation Statistics (2006) and CDC Standardization Phase 1 (Aug 2014)

	Estrone						Estradiol							
	Accuracy (%)			Precision (%)			Accuracy (%)				Precision (%)			
Conc.(pg/mL)	2.5	250	500	0.1	250	500	1.0	2.5	250	500	1.0	2.5	250	500
Intra - 1	12.4	1.1	-1.4	2.9	5.5	3.3	-7.1	1.1	-1.1	0.4	4.7	4.8	2.8	1.2
Intra - 2	-0.3	9.2	4.0	7.3	2.8	2.4	-7.4	1.1	6.4	6.5	4.4	6.3	1.6	4.3
Intra - 3	3.6	6.3	5.1	6.3	3.8	1.6	4.7	5.6	0.6	1.4	4.7	5.6	2.6	3.5
Inter	4.8	5.5	2.6	7.4	5.1	3.7	-3.3	2.7	1.9	2.8	7.4	5.2	3.9	4.1

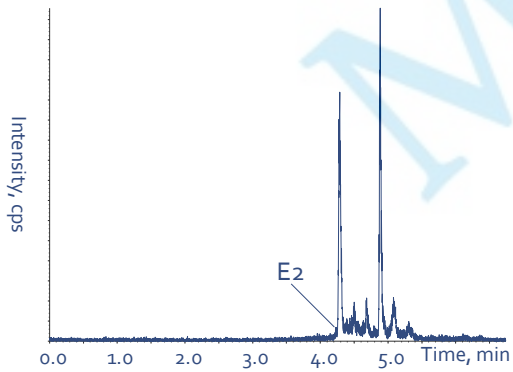




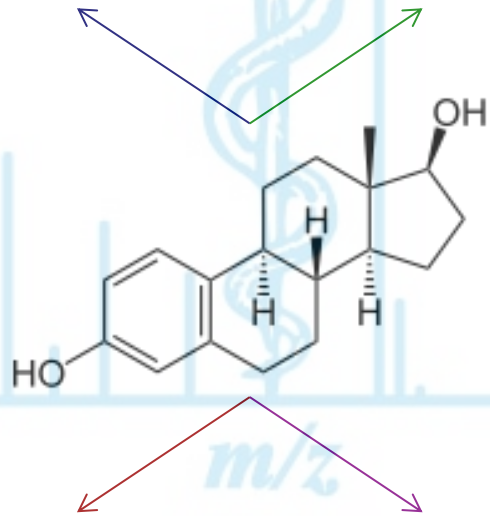
LabCorp vs CDC (phase 1)
Slope = 1.021
R= 0.9970
Bias = -0.469%

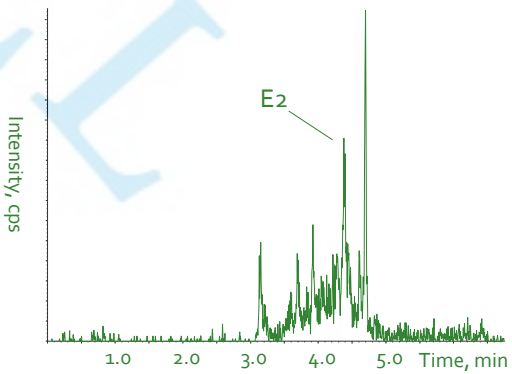
Vesper et al., Steroids 82 (2014) 7–13, 11 by IA, 6 by MS, Sample at 14.1 pg/ml – results from 9.4 – 64.8 pg/mL

Since 2014..API5500..Estrogen suppression samples (~1pg on column)



APCI +ve; $-H_2O$





ESI –ve; Na quench

Combinations of

SLE plates

TICE plates

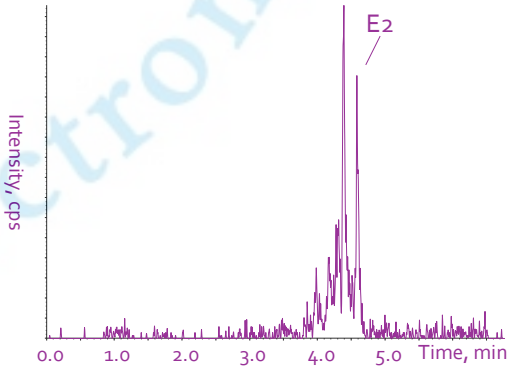
Phospholipid depletion plates

6 Derivatization schemes

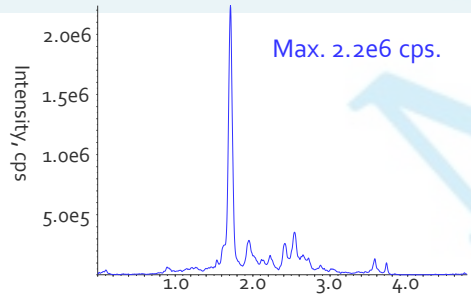
2D-LC

DMS (+ dopants)

ESI +ve; PSE2 Derivative

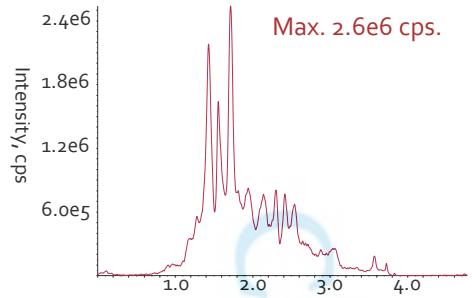


Selectivity with 7 degrees of Orthogonality
Dansylated Estradiol Red top serum vs Serum Separator Tube



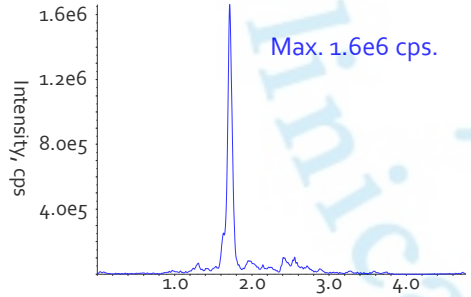
SLE

1D-LC



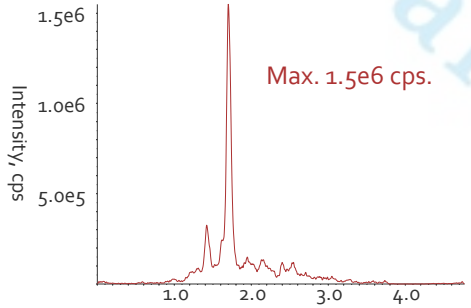
CV 40%

Bias 500%



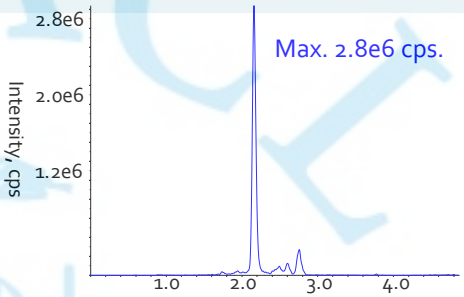
SLE / +Ab Enrich
60% "Efficiency"

1D-LC



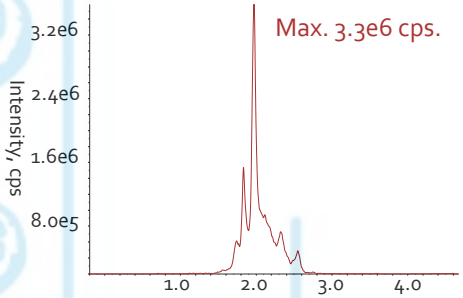
CV 15%

Bias 40%



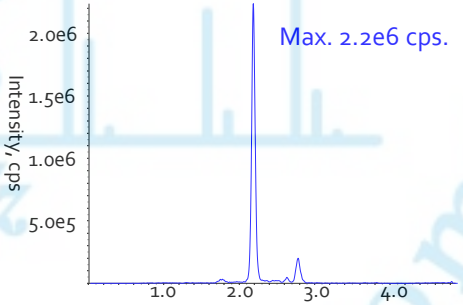
SLE

2D-LC



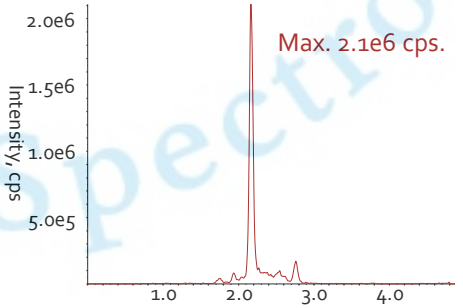
CV 20%

Bias 200%



SLE / +Ab Enrich
60% "Efficiency"

2D-LC



CV 7%

Bias 5%

Part 3 summary…what we learned

"On-column" amount is important , not LLOQ (yoctogram/attoliter)

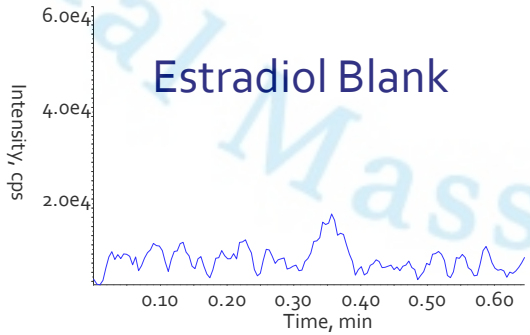
Adsorption must be controlled at each step of the assay

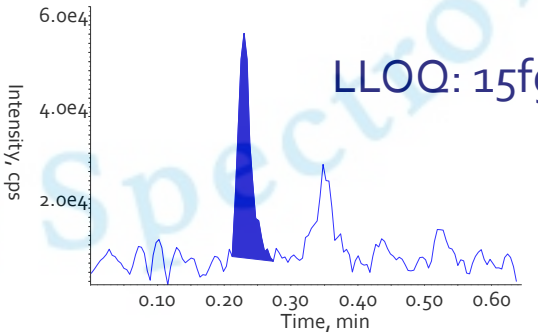
Anchor to reference methods if at all possible

Use 2D-LC to decouple selectivity and sensitivity variables

Establish selectivity and stoichiometry of reagents in worst case specimens

Improved LLOQ often requires "recovery losses"
or
While counter-intuitive, more degrees of selectivity may be optimal





Thank You!

Pat Holland
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Andrew Wagner
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William Slade, Ph.D.
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