LC/MS Biomarker Assay Validation Strategies Using Surrogate Matrix and Surrogate Analyte Approaches

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Advion

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Outline

- How to deal with target analyte in control matrix?
  - Surrogate Matrix – Parallelism and Dilutional Linearity
    - Case Studies
  - Surrogate Analyte – Response Factor and Parallelism
    - Case Studies
- Biomarker Validations
  - What QC samples to use?
  - Endogenous QC
  - Fit-for-Purpose (FFP)
  - Biomarker Validation SOP and the Validation Plan
    - Which experiments are to be performed?
    - What are the acceptance criteria?

Biomarker Methods: Biological Control Matrix Contains the Target Analyte

Two Main Approaches

- Surrogate Matrix
  - Authentic analyte
  - Calibration standards in an analyte-free diluent
  - Must demonstrate parallelism between matrices
- Surrogate Analyte
  - Stable-Isotope Labeled (SIL) Analyte as Calibration Standard*
  - Unique to LC-MS assays
  - Must evaluate response factor between labeled and unlabeled analyte analytical standards
  - Must demonstrate parallelism between analytes

*W. Li et. al., Analytical Chemistry, Volume 75, No 21, 2003, 5854-5859.
M. Jemal et. al., Rapid Communications in Mass Spectrometry, Volume 17, 2003, 1723-1734

Parallelism

Standards

- Prepare duplicate standard curves in both surrogate matrix and biological matrix

Dilutional Linearity

- Biological matrix pool [to measure the endogenous level of the analyte(s)]
- Endogenous level in biological matrix diluted with surrogate matrix

Spike Recovery

- Low QC in biological matrix
- Mid-range QC in biological matrix
- Upper-quartile QC in biological matrix
- Dilution QC

•Theoretical Concentration = -x intercept from standard addition (endogenous) + spiked concentration

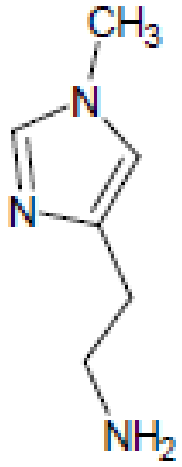
•%Relative Error of these QCs is the determining factor in parallelism

Surrogate Matrix Example: Histamine Metabolites in Human Plasma

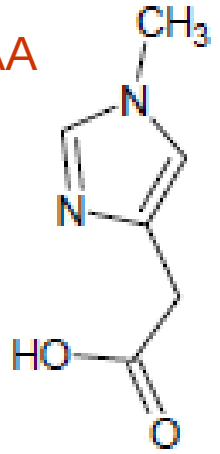
Establish a definitive quantitation method for two histamine metabolites

- Tele-methylhistamine (TMH)
- Tele-methylimidazoleacetic acid (TMIAA)
- Two stable isotope-labeled standards for each analyte were not available so a surrogate matrix was necessary – BSA in PBS

TMH



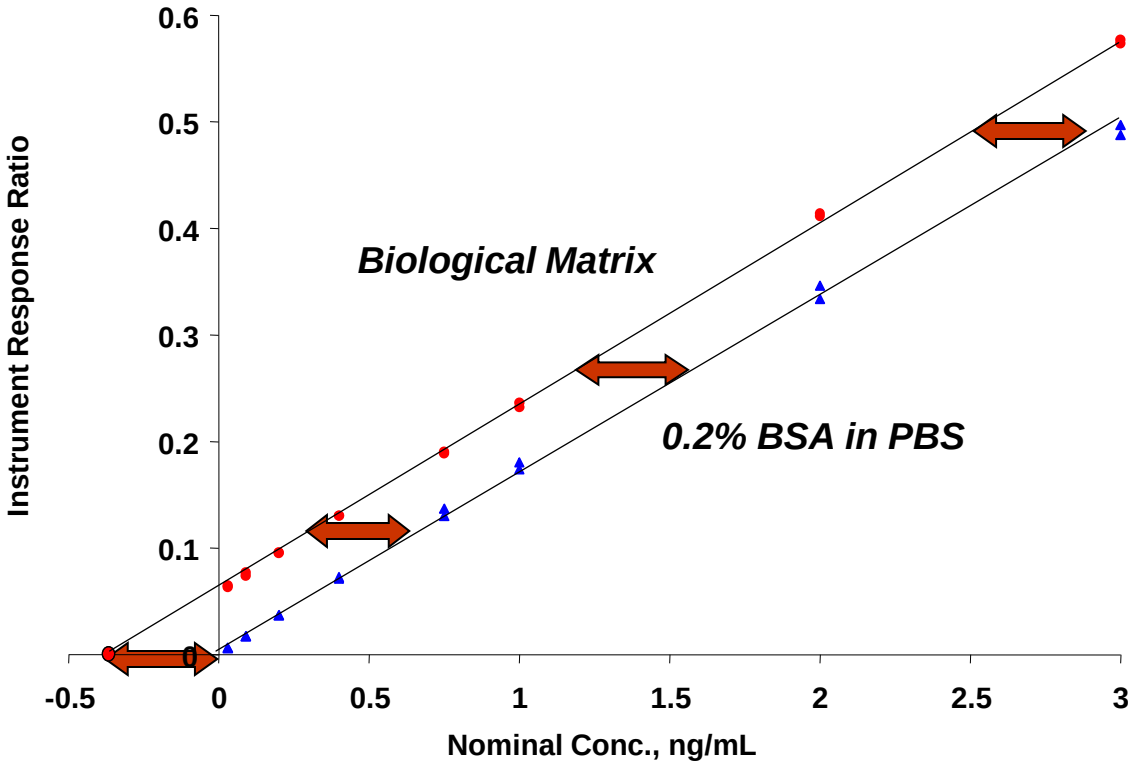
TMIAA



TMH Parallelism

TMH Biological Matrix Curve

Slope:	**0.17446**
Intercept:	**0.05937**
Negative x:	**0.340 ng/mL**



	Dilutional Linearity			Spiked Plasma			
	Plasma pool 5x	Plasma pool 2.5x	Plasma pool 1.5x	Plasma pool Low	Plasma pool Mid	Plasma pool High	Plasma pool 5xDilQC
Mean Calc., ng/mL	0.330	0.336	0.334	0.459	1.952	2.827	13.612
Theoretical, ng/mL	0.340	0.340	0.340	0.460	1.840	2.840	12.840
n	6	6	6	6	6	6	6
%RSD	3.0	1.4	3.1	3.4	1.4	0.7	1.5
% RE	-3.0	-1.4	-1.8	-0.3	6.1	-0.5	6.0

%RE = 100 x (Calculated Conc. – Theoretical Conc.)/Theoretical Conc.

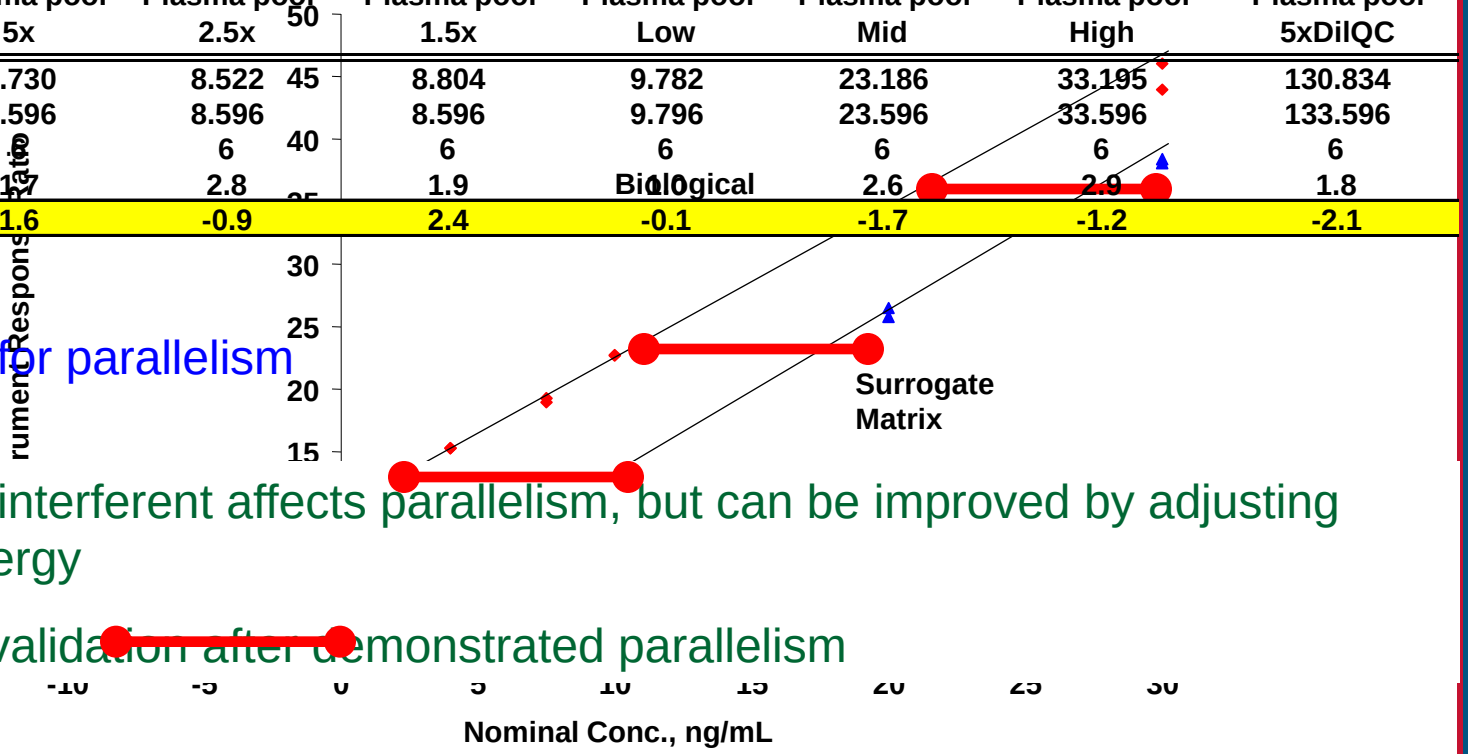
TMIAA Parallelism in Human Plasma

Optimized for maximum SRM intensity

CE= 35eV	Plasma pool 5x	Plasma pool 2.5x	Plasma pool 1.5x	Plasma pool Low	Plasma pool Mid	Plasma pool High	Plasma pool 5xDilQC
Mean Calc., ng/mL	7.550	7.709	7.600	8.596	21.852	29.509	124.513
Theoretical, ng/mL	8.448	8.448	8.448	9.648	23.448	33.448	133.448
n	6	6	6	6	6	6	6
%RSD	1.5	1.1	1.4	2.4	1.4	2.1	1.4
% RE	-10.6	-8.7	-10.0	-10.9	-6.8	-11.8	-6.7

CE= 17eV	Plasma pool 5x	Plasma pool 2.5x	Plasma pool 1.5x	Plasma pool Low	Plasma pool Mid	Plasma pool High	Plasma pool 5xDilQC
Mean Calc., ng/mL	8.730	8.522	8.804	9.782	23.186	33.195	130.834
Theoretical, ng/mL	8.596	8.596	8.596	9.796	23.596	33.596	133.596
n	6	6	6	6	6	6	6
RSD (%)	1.7	2.8	1.9	1.0	2.6	2.9	1.8
RE (%)	1.6	-0.9	2.4	-0.1	-1.7	-1.2	-2.1

Optimized for parallelism



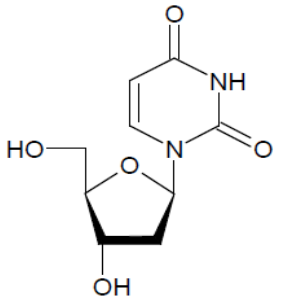
- Co-eluting interferent affects parallelism, but can be improved by adjusting Collision Energy
- Ready for validation after demonstrated parallelism

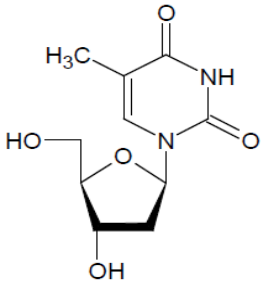
Surrogate Matrix Example: Thymidine and Deoxyuridine in Mouse Plasma

- Surrogate matrix – BSA in PBS
- Unacceptable parallelism with simple protein precipitation

Protein Precipitation Alone, ULOQs = 50µg/mL

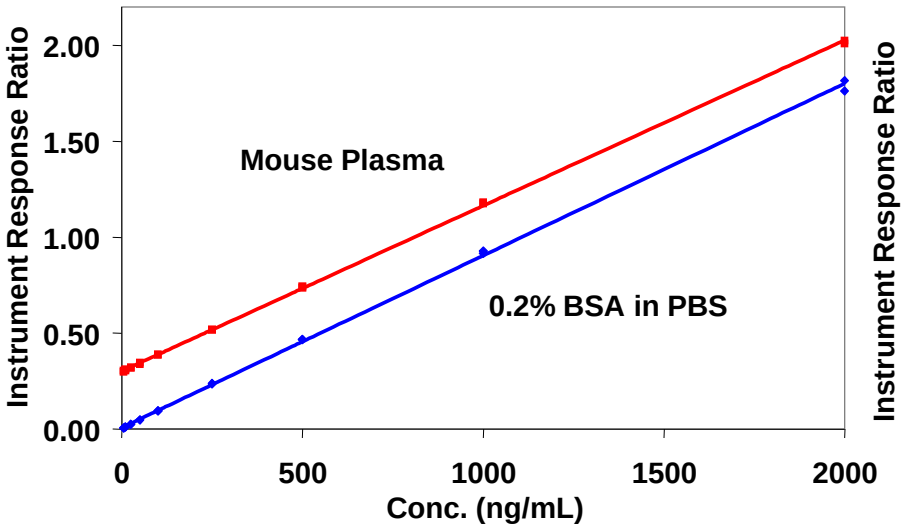
		% Difference
Endogenous Thymidine Concentration		
From negative x-intercept of plasma calibration curve:	279.4 ng/mL	**-17.6%**
Calculated concentration of mouse plasma from surrogate matrix curve:	230.3 ng/mL	
Endogenous Deoxyuridine Concentration		
From negative x-intercept of plasma calibration curve:	502.5 ng/mL	**-18.2%**
Calculated concentration of mouse plasma from surrogate matrix curve:	411.0 ng/mL	



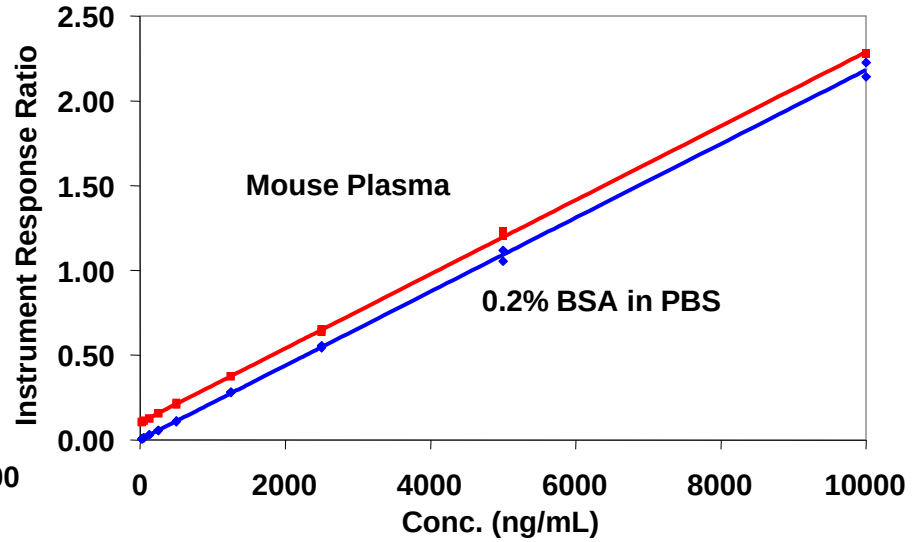


Improved Parallelism: Reduce linear range and include SPE cleanup

Thymidine



Deoxyuridine



		% Difference
Endogenous Thymidine Concentration		
From negative x-intercept of plasma calibration curve:	349.9 ng/mL	-9.6%
Calculated concentration of mouse plasma from surrogate matrix curve:	316.5 ng/mL	
Endogenous Deoxyuridine Concentration		
From negative x-intercept of plasma calibration curve:	456.8 ng/mL	-3.5%
Calculated concentration of mouse plasma from surrogate matrix curve:	440.6 ng/mL	

Thymidine and Deoxyuridine in Mouse Plasma

- Protein Precipitation Followed by SPE, Truncated Curve Ranges

- RE (%) Extrapolated = negative X intercept used as endogenous value
- RE (%) Interpolated = interpolation from surrogate matrix curve used as endogenous value

Thymidine	Plasma pool 15x	Plasma pool 2.5x	Plasma Pool Low	Plasma Pool Mid	Plasma Pool High	Plasma Pool 5X Dilution
Mean Calc., ng/mL	335.9	324.8	506.1	1251.7	1784.4	7915.5
Theoretical, ng/mL	349.9	349.9	549.9	1349.9	1949.9	8349.9
n	6	6	6	6	6	6
RSD (%)	2.1	1.8	0.7	1.0	0.8	0.7
RE (%) Extrapolated	-4.0	-7.2	-8.0	-7.3	-8.5	-5.2
RE (%) Interpolated	6.1	2.6	-2.0	-4.9	-6.9	-4.8

Deoxyuridine	Plasma pool 15x	Plasma pool 2.5x	Plasma Pool Low	Plasma Pool Mid	Plasma Pool High	Plasma Pool 5X Dilution
Mean Calc., ng/mL	470.8	458.8	1459.1	5297.9	8023.5	39631.5
Theoretical, ng/mL	456.8	456.8	1456.8	5456.8	8456.8	40456.8
n	6	6	6	6	6	6
RSD (%)	7.8	2.9	2.1	3.3	2.7	1.2
RE (%) Extrapolated	3.1	0.4	0.2	-2.9	-5.1	-2.0
RE (%) Interpolated	6.8	4.1	1.3	-2.6	-4.9	-2.0

Surrogate Analyte – Response Factors

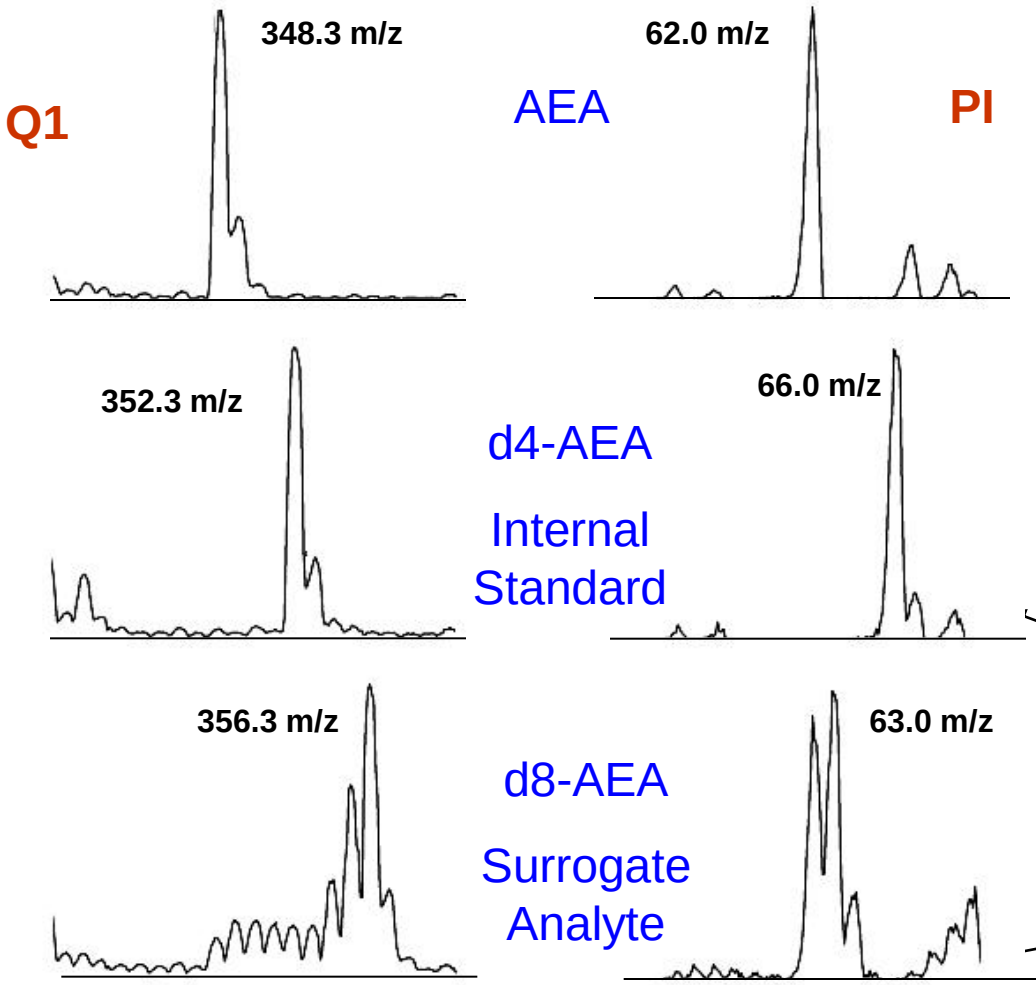
- The LC-MS response of the SIL must be shown to be equivalent to the response of the authentic analyte
- Response factor experimentally determined and balanced by adjustment of calibrator concentrations or by SRM detuning
- Response balance demonstrated in a formal pre-validation experiment and verified as a suitability check before each batch
- Responses of labeled analytes must balance with unlabeled within acceptance criteria
- **Many factors affect response differences***

**R. MacNeill et al, Bioanalysis, Volume 2, No. 1, 2010, 69-80.*

Factors Affecting Response Balance

- Ionization differences
- Gas-phase deuterium exchange
- Inaccuracies of purity characterization
- Kinetic Isotope Effect
- Compression of isotope distribution for the SIL compared to the native compound – favors a higher response of the labeled analyte

Arachidonoyl Ethanolamide (AEA)



Sources of Imbalance

- Impure standards
- Deuterium Scrambling

Deuteriums on ethanolamide less prone to scrambling than those on chain*

**G. Schultz et al, ASMS 2009 poster.*

Balancing the Responses

- d4-AEA used as internal standard (no d0)
- d8-AEA used as calibrator
- Large response factors – AEA = 6.2 (unlabeled response/labeled response for equal nominal concentration)

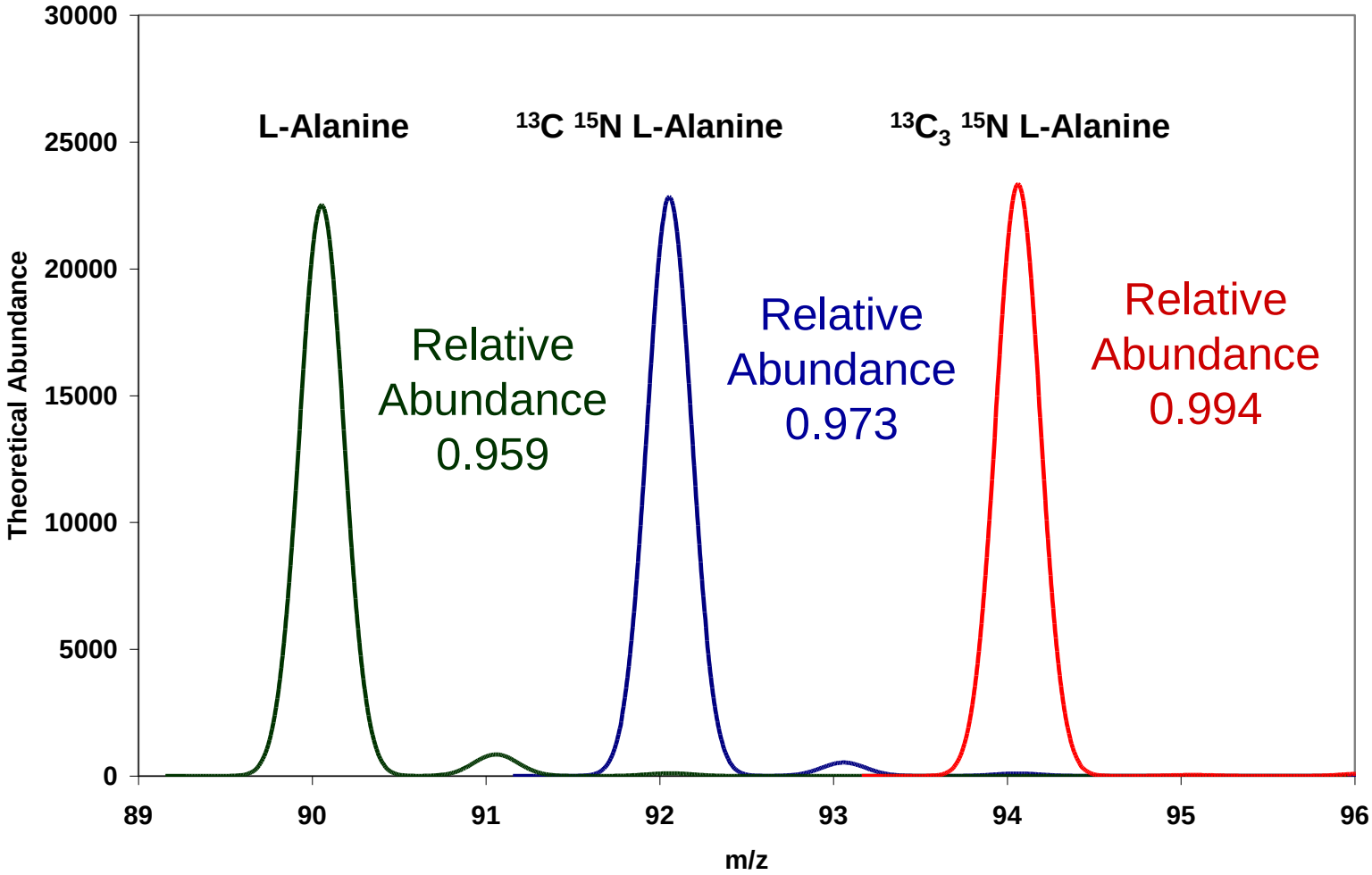
AEA Response Balance

	Peak Area Ratio	
	d8 AEA	AEA
Collision Energy	40	34
Rep 1	0.811665	0.816656
Rep 2	0.801124	0.824462
Rep 3	0.807438	0.812055
Mean	0.806742	0.817724
RSD (%)	0.7	0.8

% Difference = 1.3%

% Difference = 100*(Mean AEA response ratio – Mean d8-AEA response ratio)/Mean AEA response ratio

Isotope Distribution Compression*



Theoretical Alanine Spectra

**B. Jones et al, ASMS 2011 poster.*

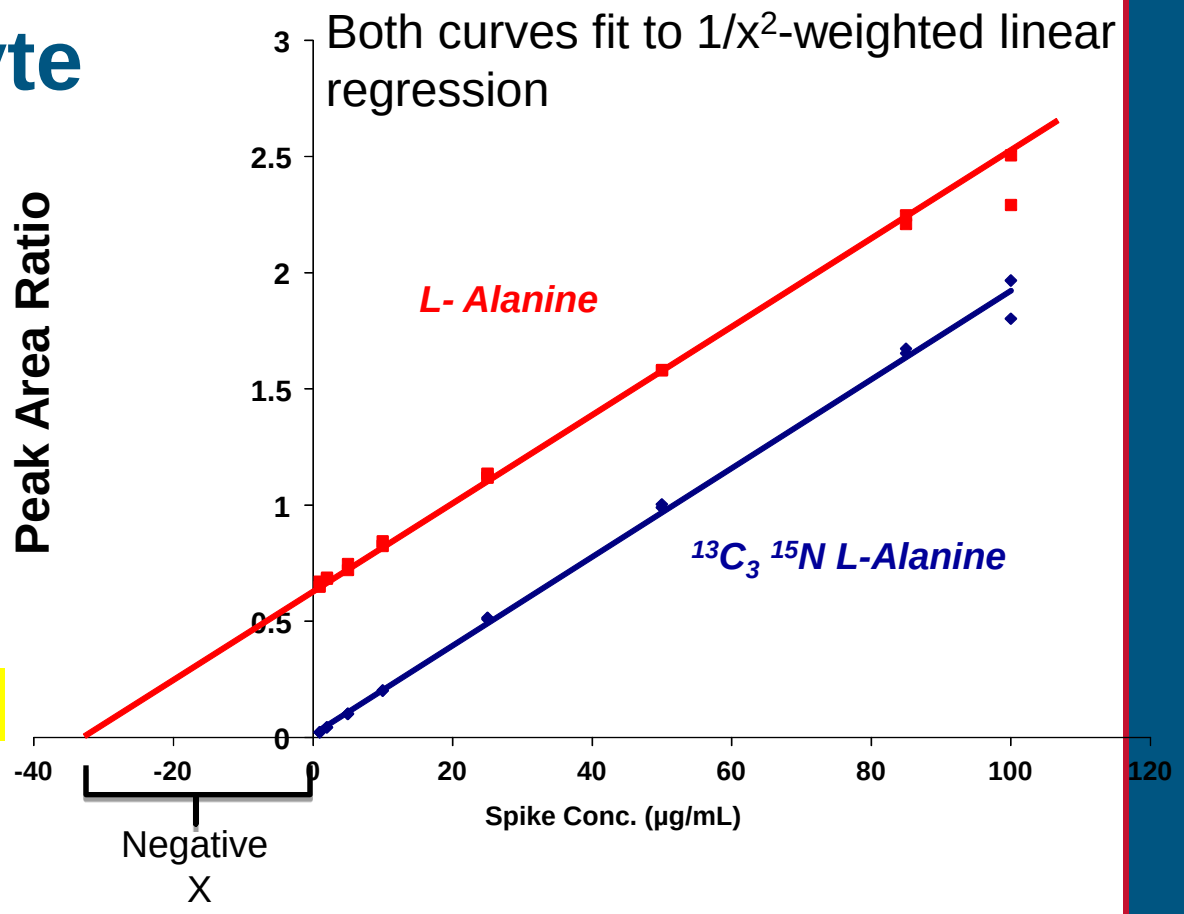
SIL vs Authentic Analyte Imbalance – Not Easily Assigned

- The measured % differences are greater than predicted
- Necessary to detune the more responsive transition to achieve balance

Analyte	Mean Theoretical % Difference	Mean Observed % Difference	% Diff after adjustment of Declustering potential
L-Alanine vs [$^{13}C_1$, $^{15}N_1$] L-Alanine	1.4%	9.0%	-2.0%
L-Alanine vs [$^{13}C_3$, $^{15}N_1$] L-Alanine	3.6%	16.1%	1.5%

Surrogate Analyte Parallelism Alanine

Alanine Native	
Slope =	0.018910
y intercept =	0.641074
$^{13}C_3{}^{15}N$ L-Alanine	
Slope =	0.019731
y intercept =	0.001789
-x intercept =	33.9 µg/mL
From surrogate curve =	31.3 µg/mL
% Difference of Slopes =	2.9%



Spiked Plasma

Spike Conc.	0 ng/mL	1 µg/mL	3 µg/mL	50 µg/mL	80 µg/mL	100 µg/mL
Calc. Conc (µg/mL)	31.3	33.3	35.0	80.3	109	125
Theo. Conc. (µg/mL)	33.9	34.9	36.9	83.9	114	134
%RSD	3.3%	1.7%	1.7%	1.0%	1.0%	0.3%
%RE Extrapolated	-7.8%	-4.5%	-5.0%	-4.3%	-4.6%	-6.5%
%RE Interpolated	0.0%	3.1%	-2.9%	-1.2%	-2.1%	-4.8%

What types of QCs are needed for the Validation?

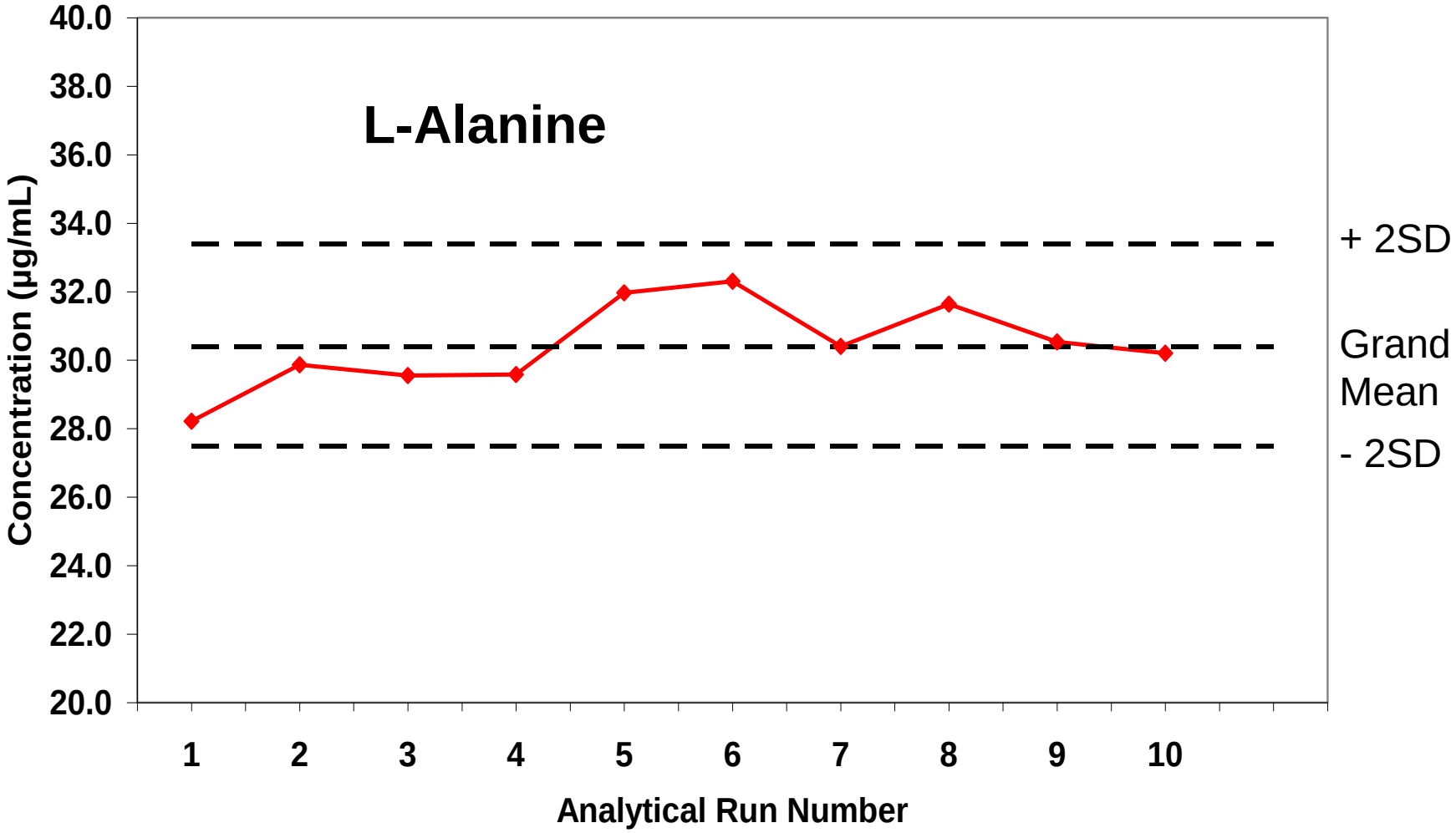
Surrogate Matrix Validation Samples

- LLOQ in surrogate matrix
- Diluted Endogenous Pool
- Endogenous Pool
- Low (Biological Matrix)
- Mid (Biological Matrix)
- High (Biological Matrix)
- ULOQ in surrogate matrix
- Dilution QC (if needed)

Surrogate Analyte Validation Samples

- LLOQ
- Low
- Mid
- High
- ULOQ
- Dilution QC (if needed)
- “Endogenous QC”

Endogenous QC tracking for Study Samples



LC/MS/MS Method Validation of Endogenous Compounds

Currently:

- No formal guidance exists for LC-MS/MS method validation for endogenous biochemicals (Biomarkers)
- Anticipated that the next release of FDA BMV guidance will include endogenous compound assays
- Fit-for-Purpose (FFP) approach recommended by J.W. Lee paper*
- Many follow modified version of FDA BMV guidance

**J. W. Lee et al, Pharmaceutical Research, Volume 23, No. 2, 2006, 312 – 328*

Fit for Purpose Validations

- SOP that describes each experiment that may be needed for a validation
- Validation plan that selects the needed experiments and assigns acceptance criteria
- Criteria may depend on the intended use of generated data, the limitations of the analytical assay, the fold change of the marker, etc

- Question: When is it permissible to relax criteria?

Conclusions

- Surrogate Matrix: Important to demonstrate dilutional linearity and parallelism of analyte between surrogate and biological matrices
- Surrogate Analyte: Important to balance responses and demonstrate parallelism between surrogate and authentic analytes
- Many factors affect the response of a stable-isotope labeled analog compared to the authentic analyte
- Can have prescribed rigor yet accommodate fit for purpose using a Validation SOP plus Validation Plan

Acknowledgements

Advion LC/MS

Biomarker Team

- John E. Buckholz
- Kathlyn M. Porter
- Kristen M. Bearup
- Lian Shan
- Johnson Zhang
- Danielle R. Strong
- Kathleen A. Cormack