Q² Solutions

a Quintiles Quest Joint Venture

Applying the lessons learned from small molecule biomarker analysis to method development for xenobiotics

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Outline

- Project Brief
- Method Development & Challenges
- Use of alternative approaches
- Assay Validation
- Clinical Sample Analysis
- Summary

Project Brief

- Development of assay requested for caffeine & paraxanthine
- Requested LLOQ of 20.0 ng/mL for both analytes
- Validation of assay to support clinical drug-drug interaction study with investigative drug

Extraction Development

- Preferential approach to use protein precipitation or liquid/liquid extraction
- Solid-phase extraction held in reserve if problems observed with simpler approaches
- Protein precipitation assessed with acetonitrile, 0.1% formic acid in acetonitrile, 50:50 acetonitrile/methanol and methanol.
- Liquid-liquid extraction tested with heptane, MTBE & ethyl acetate
- Sensitivity (recovery) reduced with liquid-liquid extraction
- Methanol best protein precipitation solvent in terms of overall signal

Troubleshooting Issues

- Erratic internal standard response –changing precipitation solvent to 50:50 methanol:acetonitrile improved the reproducibility

- Theophylline (formed by caffeine metabolism in the liver) interference on paraxanthine – shallow gradient employed to resolve the peaks

- Overriding problem –ubiquity of caffeine & metabolites.
  - Observed at low levels in purified water
  - Massive potential for interference in plasma

Issues with Control Plasma

- Typical method development & sample analysis for available drugs includes matrix pre-screening to eliminate plasma lots with any analyte response

- 39 plasma lots from supplier screened
  - Observed caffeine concentrations range from 20% to 22,000% of LLOQ
  - Observed paraxanthine concentrations range from 30% to 11,000% of LLOQ
  - One lot was clean

- Alternative approach – plasma from in-house volunteers instructed to avoid caffeine

Screening Control Plasma

- Based on literature information on caffeine metabolism, the 12 in-house volunteers were instructed to eliminate all sources of caffeine (medicine, chocolate, tea, coffee) for 48 hours before blood draw
- Only one lot was clean
- Observed responses for caffeine in other lots ranging from 50% to 18,000% of LLOQ

- Second draw scheduled with 96 hours caffeine avoidance
- Two lots were clean for both analytes but from different volunteers to the first draw
- Previously clean volunteer showed caffeine levels at 50% of LLOQ and paraxanthine levels at 137% of LLOQ

- Showed that we could not control amount of caffeine in control matrix.

- Next steps were to raise the LLOQ or treat caffeine as an “endogenous” analyte

Measurement of Endogenous Analytes

- 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

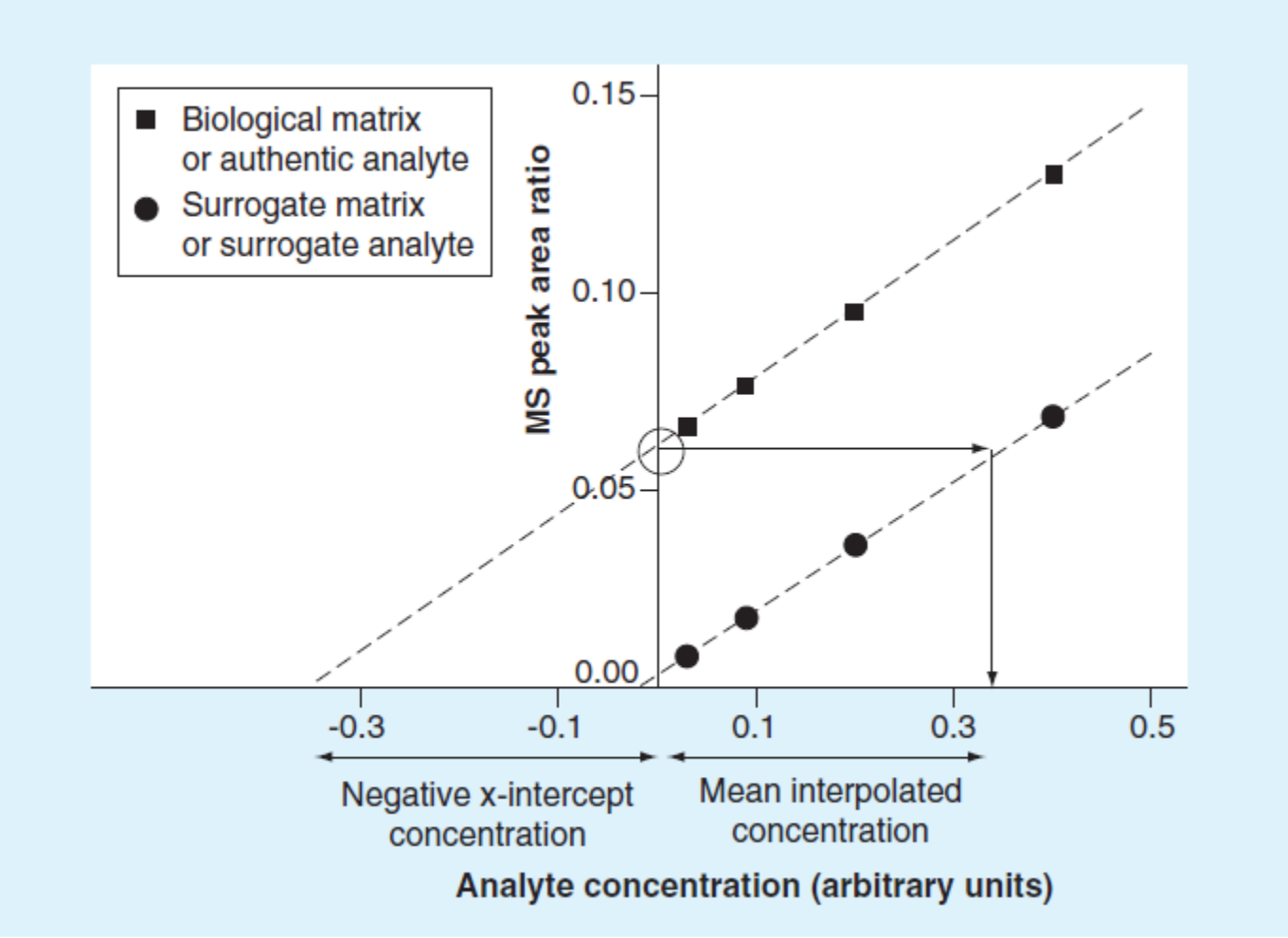
W. Li et. al., Analytical Chemistry, Volume 75, No 21, 2003, 5854-5859.
M. Jemal et. al., Rapid Communications in Mass Spectrometry, Volume 17, 2003, 1723-1734

Measurement of Endogenous Analytes

- Surrogate matrix options
  - Animal plasma where controlled diet does not include caffeine
    - Rat, dog & minipig plasma all clean
  - 5% (w/v) Bovine Serum Albumin in 10 mM Phosphate Buffered Saline – any interference would come from purified water

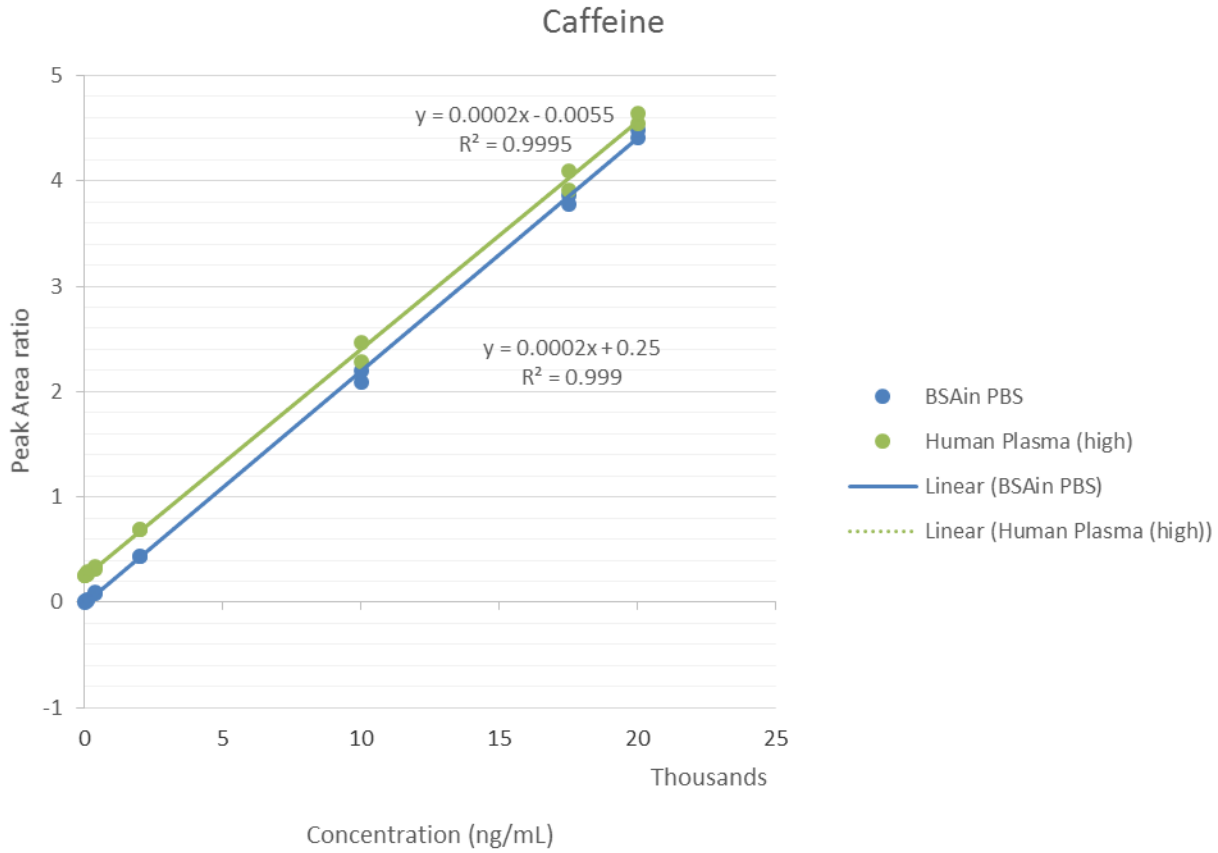
- 5% (w/v) Bovine Serum Albumin in 10 mM Phosphate Buffered Saline chosen matrix due to ready availability

Parallelism



B.R. Jones, G.A. Schultz, J.A. Eckstein, B.L. Ackermann, "Surrogate Matrix and Surrogate Analyte Approaches for Definitive Quantitation of Endogenous Biomolecules", *Bioanalysis* **2012**; 4(19):2343 - 2356.

Parallelism - Caffeine



Extrapolated

Negative X calc.

negative X = intercept / slope (-X = b/m)

Neg X = 1161.3 ng/mL

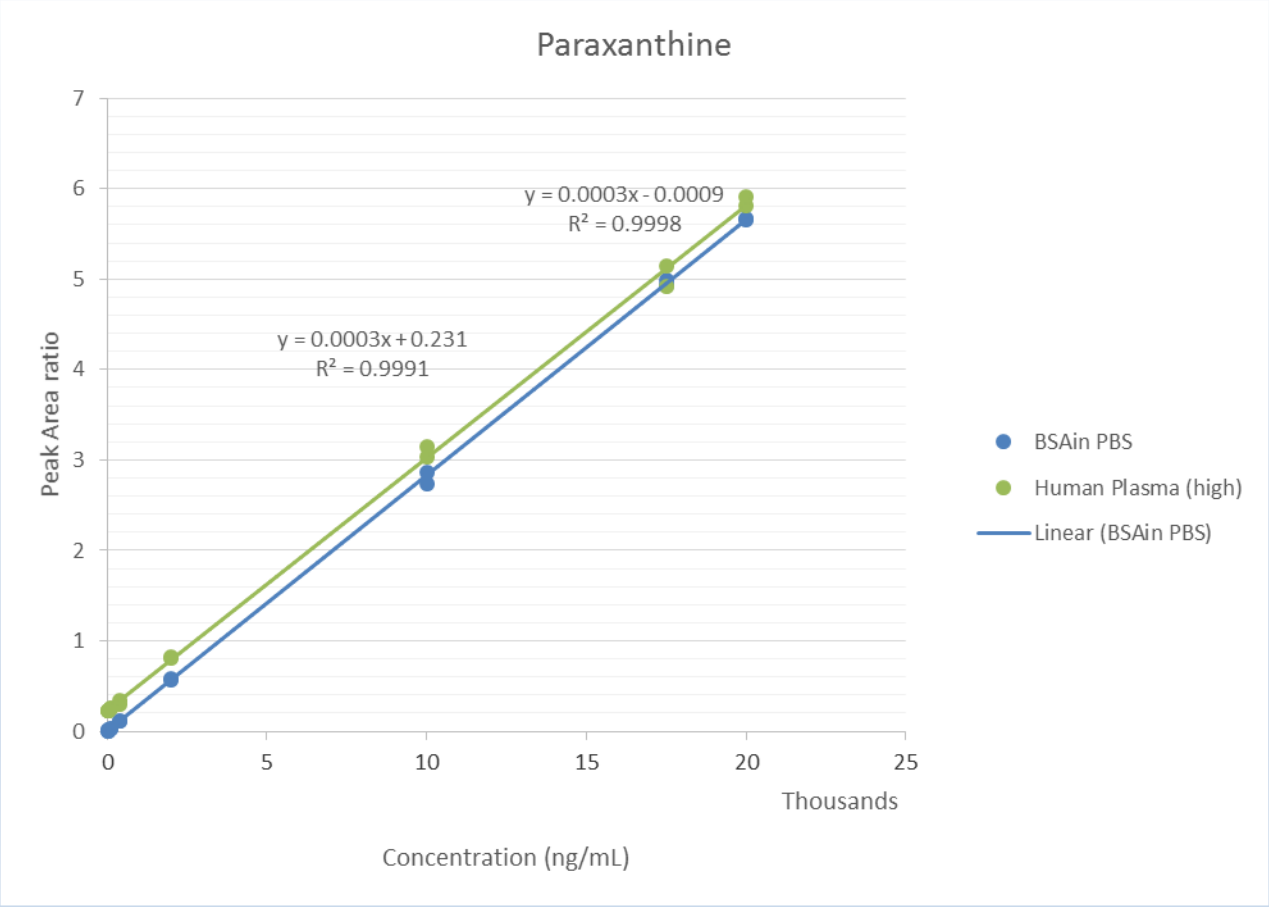
Interpolated

Mean calculated concentration of 6 replicates of unfortified plasma, (interpolated from the surrogate matrix curve)

Mean = 1129.5 ng/mL

%Difference = -2.8%

Parallelism - Paraxanthine



Extrapolated

Negative X calc.

negative X = intercept / slope

(-X = b/m)

Neg X = 787.8 ng/mL

Interpolated

Mean calculated concentration of 6 replicates of unfortified plasma, (interpolated from the surrogate matrix curve)

Mean = 769.8 ng/mL

%Difference = -2.3%

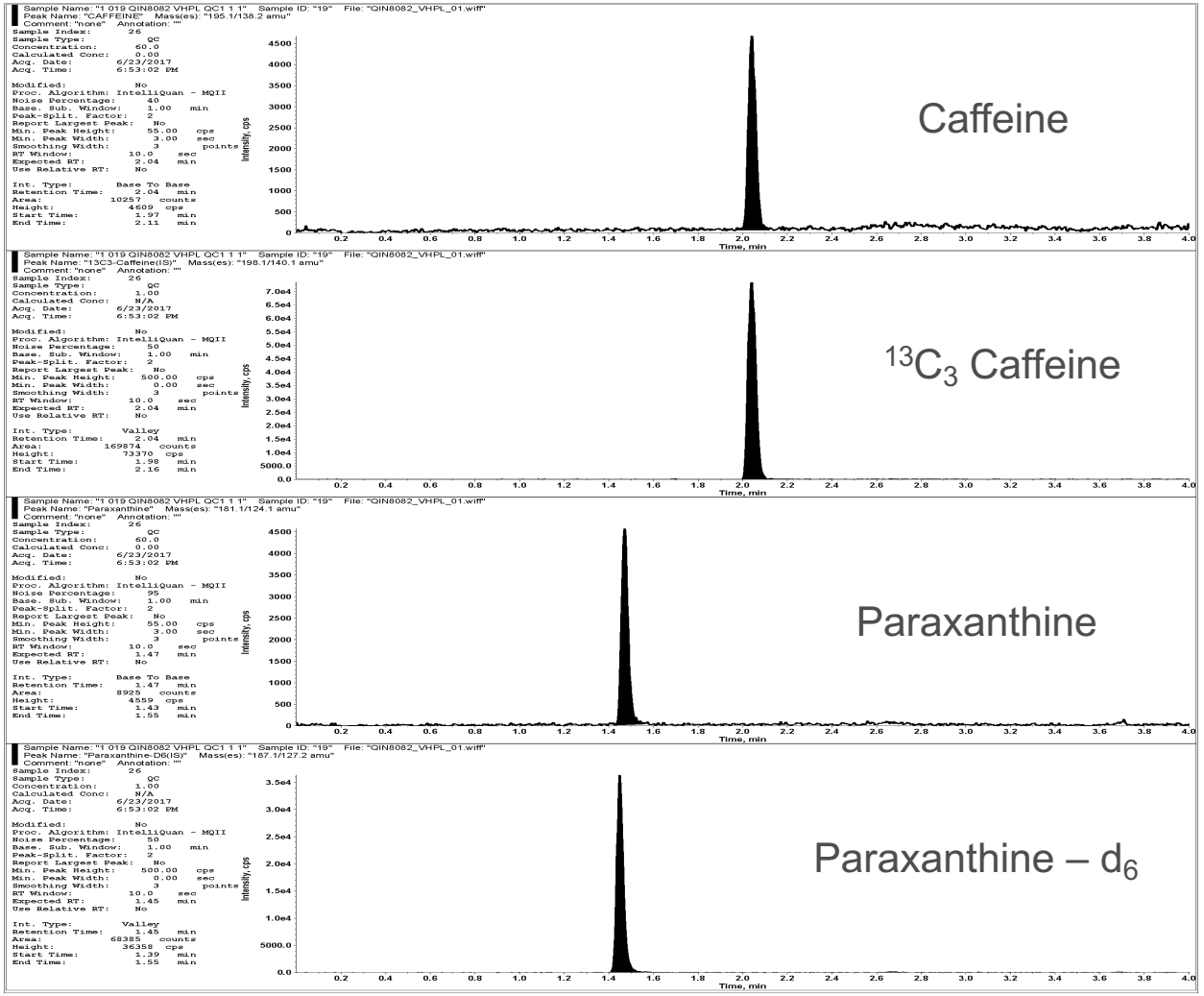
Sample Clean-up

- Calibration Standards & Low QC sample – 100 μL aliquot of 5% (w/v) Bovine Serum Albumin in 10 mM Phosphate Buffered Saline
- Quality Controls & samples – 100 μL human plasma with K_2EDTA anti-coagulant
- Dilution QCs diluted 1:10 with 5% (w/v) Bovine Serum Albumin in 10 mM Phosphate Buffered Saline
- Addition of 20 μL methanol (blanks) or methanolic solution of stable-labeled internal standards
- Precipitation with 500 μL 50:50 methanol/acetonitrile.
- Reconstitution in 150 μL 10:90 methanol/water

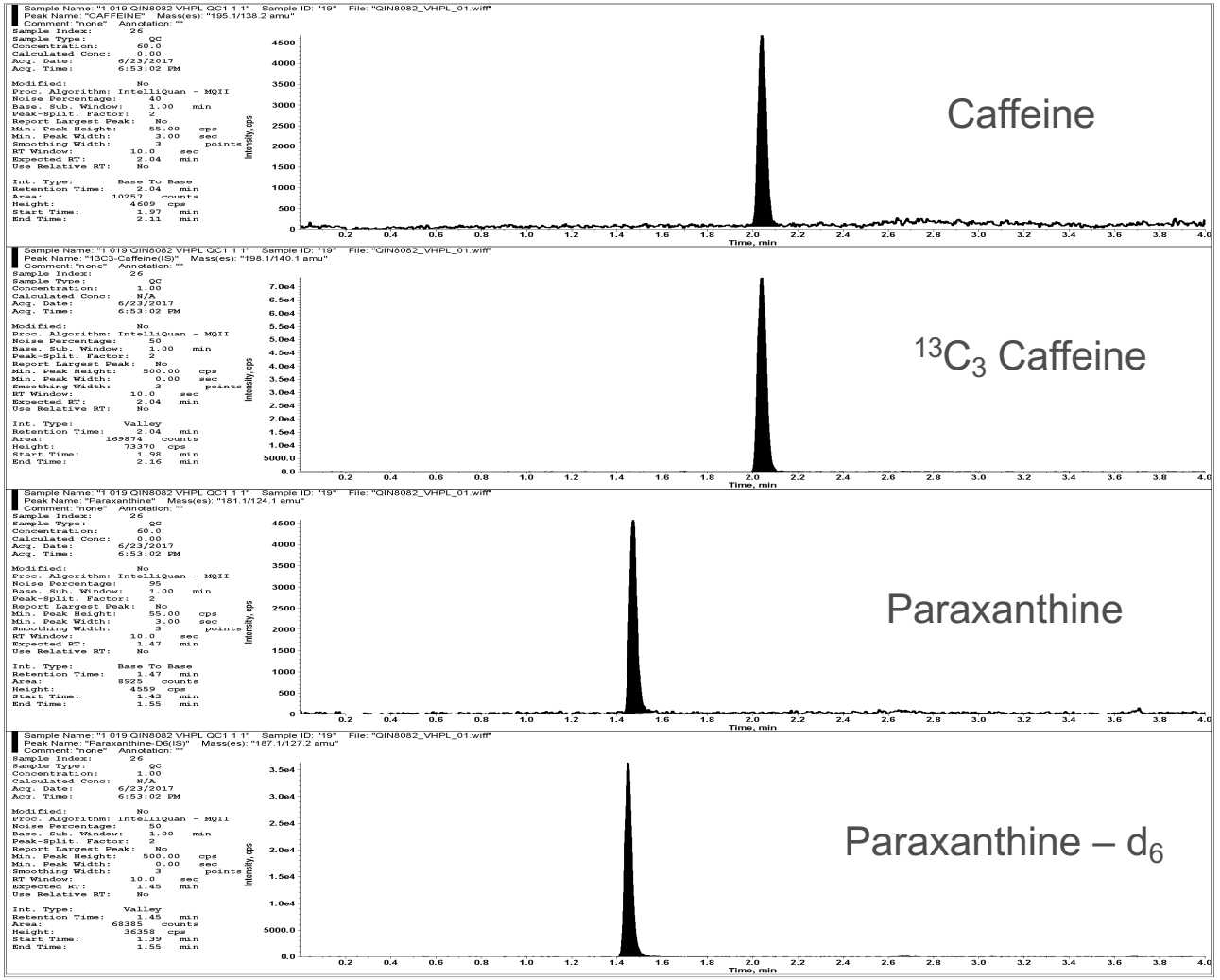
LC/MS/MS Conditions

- LC System – Shimadzu Nexera UHPLC consisting of two LC-30AD pumps with Shimadzu SIL-30AC autosampler
- HPLC column – Acquity UPLC HSS T3, 1.8 μm, 2.1 x 50 mm
- Mobile Phase – Gradient of 1000:1 water/formic acid and methanol.
  - Flow 600 μL/min
  - 2 μL injection per sample
- Mass Spectrometer – Sciex API-4000
  - TurboIonSpray™ at 500 °C
  - Positive Ionization
  - Cycle Time 4.8 minutes

Chromatography – LLOQ Calibration Standard in Surrogate Matrix



Chromatography – Endogenous QC Sample (K_2EDTA plasma)



Precision & Accuracy Data - Caffeine

	LLOQ QC	QC1	QC3	QC4	Dilution QC
	20.0	60.0	6250	15300	150000
Run 1	18.5	62.2	6320	15000	149000
	20.2	61.7	6280	15300	156000
	18.2	65	6400	15500	153000
	22.1	59	6140	16000	151000
	19.4	63.2	6060	15200	150000
	21.0	59.7	6310	15300	153000
Run 2	19.4	61	6310	15300	158000
	21.6	64.2	6090	15400	149000
	19.3	65.4	6260	14900	145000
	18.2	70.8	6110	14800	160000
	19.6	71.7	6340	15600	154000
	19.2	61.5	6410	15100	155000
Run 3	18.6	55.3	5710	13500	135000
	17.6	55.8	5680	13400	129000
	18.5	56.7	5800	13600	132000
	18.0	57.2	5590	13300	129000
	17.6	54.9	5510	12900	128000
	17.8	57.0	5590	13300	128000
Mean	19.2	61.2	6051	14633	145222
S.D.	1.34	4.93	315	991	11568
%RSD	6.99	8.05	5.20	6.77	7.97
%DEV	-4.22	2.06	-3.19	-4.36	-3.19

Endogenoous QC 2

Grand Mean over 3 runs

N= 30

Mean = 243 ng/mL

Precision & Accuracy Data - Paraxanthine

	LLOQ QC	QC1	QC3	QC4	Dilution QC
	20.0	60.0	6210	15200	150000
Run 1	20.1	62.8	5880	15600	151000
	19.4	59.5	6330	15300	146000
	19.4	61.2	6150	14600	150000
	20.8	59.9	6620	15900	145000
	19.3	63.7	5930	14900	152000
	19.5	57.1	6370	15000	146000
Run 2	19.4	61.8	6100	15700	154000
	19.9	58.7	6030	15500	153000
	19.0	56.2	5990	16000	152000
	20.2	63.4	5990	15500	144000
	21.2	65.0	5940	16600	157000
	18.4	60.9	6170	15000	154000
Run 3	17.9	53.0	5460	13000	132000
	18.8	56.0	5530	13700	128000
	18.2	58.0	5460	13000	128000
	17.7	55.3	5250	13800	131000
	17.7	55.0	5690	13700	127000
	18.8	55.1	5410	13900	128000
Mean	19.2	59.0	5906	14817	143222
S.D.	1.01	3.53	373	1068	10931
%RSD	5.25	5.98	6.32	7.21	7.63
%DEV	-3.97	-1.61	-4.90	-2.52	-4.52

Endogenous QC 2

Grand Mean over 3 runs

N= 30

Mean = 200 ng/mL

Sample Analysis

- Clinical study with 448 samples analyzed
- No >ULOQ repeats
- ISR performed on 50 samples
  - 100% passing rate for caffeine & paraxanthine

Conclusions

- Quantitative assay requested for caffeine & paraxanthine
- Method development on-routine due to issues with target compounds in control matrix
- Presence of caffeine in diet made it difficult to guarantee clean matrix from volunteers
- Treating the analytes as “endogenous” and following a similar approach to biomarker validation was successful
- Using a surrogate matrix approach gave a robust validated assay that was used to support a clinical study

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