EBF Open Symposium 2025

Comparative Evaluation of Bioanalytical Techniques for Lipid Extraction from Lipid Nanoparticles in Human Plasma Using LC-MS/MS

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Disclosure

- I am an employee and stockholder of Moderna, Inc
- Opinions expressed are solely my own and do not express the views or opinions of my employer

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Road Map

- Intro to mRNA platform and lipids for assessment
- Sample Extraction for LC-MS
- Lipids 1 - 3
- Next Steps / Conclusions
- Acknowledgments

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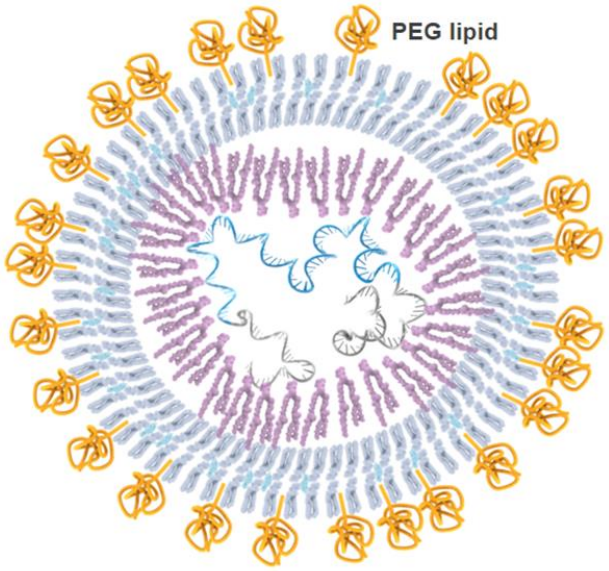
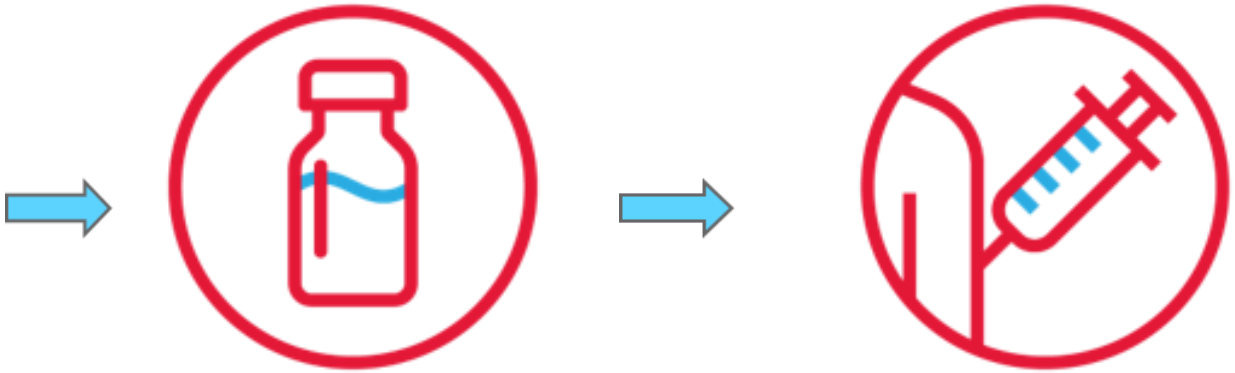
Intro into mRNA Platform





mRNA is packaged into lipid nanoparticles (LNP) to enter cells

mRNA + LNP results in a mRNA therapy or drug product that target the disease

After administration (SC, IV, IM, Intra-tumoural), mRNA is translated into proteins

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Sample Extraction for LC-MS

Technique	Pros	Cons
Manual Protein Precipitation	- Simple and low cost - Fast sample prep - No special equipment needed	- Low selectivity - Risk of inconsistent results (operator variability) - Poor removal of phospholipids
Automated Protein Precipitation	- High throughput - Reproducible and consistent - Reduced hands-on time	- Requires automation equipment - Still low selectivity - Phospholipids and other interferences may persist
Phospholipid Removal	- Reduces matrix effects in LC-MS - Better reproducibility - Cleaner extracts	- Additional cost - May require optimization for different matrices
Liquid–Liquid Extraction (LLE)	- Good selectivity for non-polar analytes - Removes many interferences - Widely used	- Labor-intensive - Emulsion formation risk - Solvent-intensive - Limited automation
Solid-supported Liquid Extraction (SLE)	- Cleaner than LLE - Easier workflow (less emulsion risk) - Semi-automatable	- Limited to certain analyte chemistries - Cartridge cost - Less selective than SPE
Solid Phase Extraction (SPE)	- Highly selective - Very clean extracts - Broad application range - Automatable	- More complex method development - Higher cost (cartridges + time) - Can be slower

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Lipid 1

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Project Requirements

- Method supplied with an LLOQ of 0.5 ng/mL using Protein precipitation
- To transfer/develop a method with an objective of achieving as low an LLOQ as possible
- Method to support upcoming clinical trial to assess clearance
- Method to be used as a platform assay for additional clinical studies
- Assess potential of developing multi-lipid analyte method

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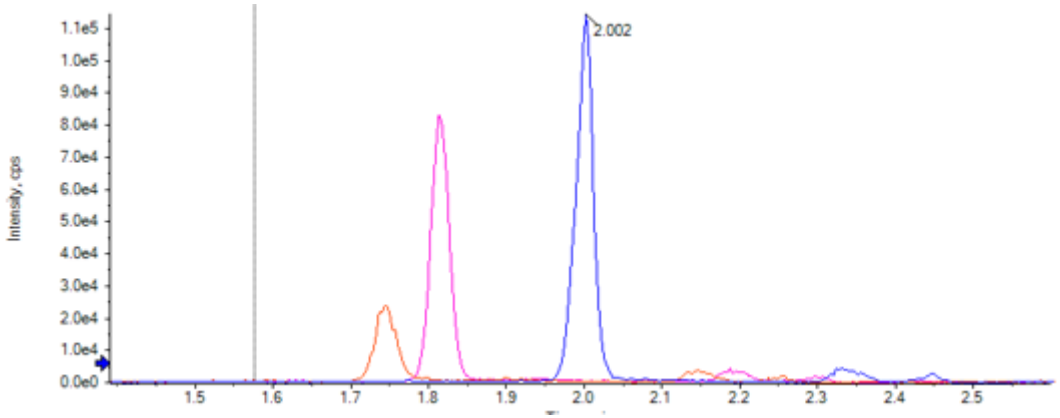
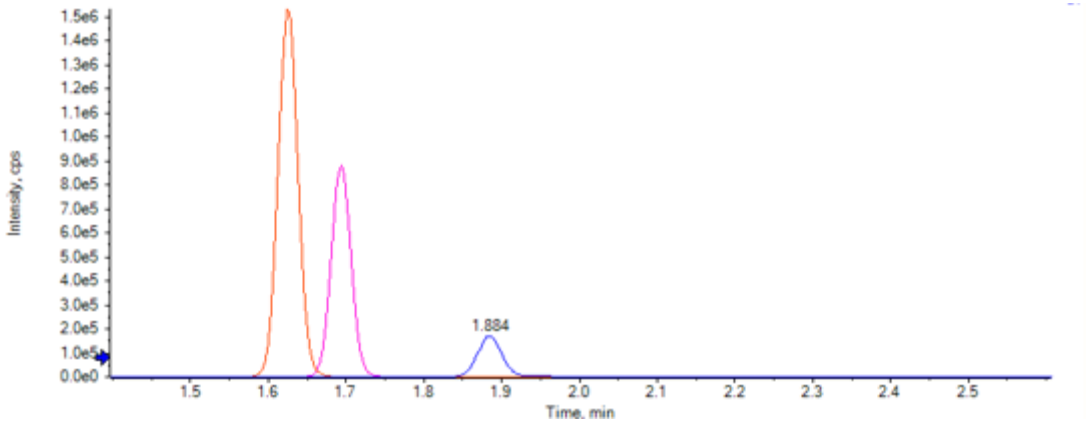
Protein precipitation - Chromatography

Lipid 1



Lipid 2 IS



LC Gradient;
0.5 min hold
0.2 min hold
No hold

Evidence of Ion suppression

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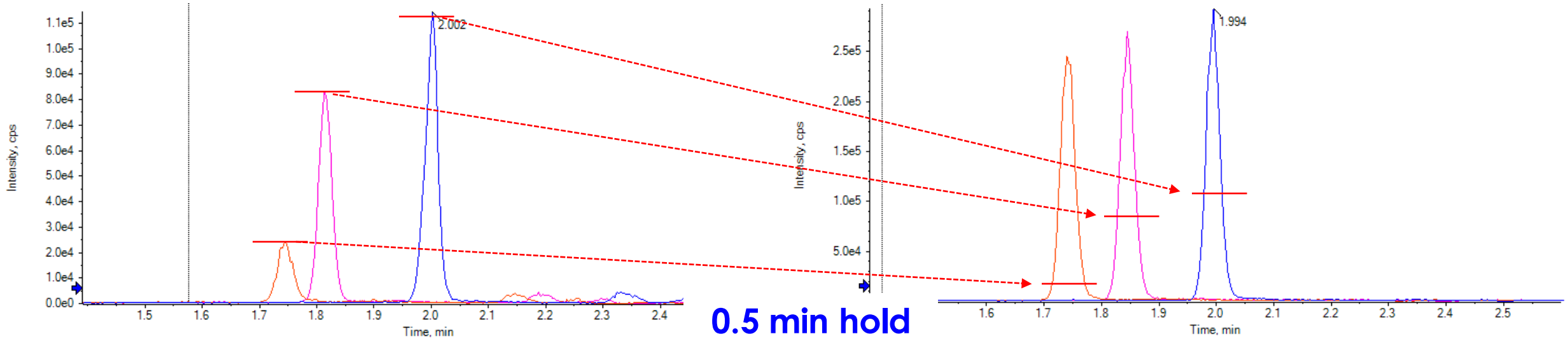
Protein crash

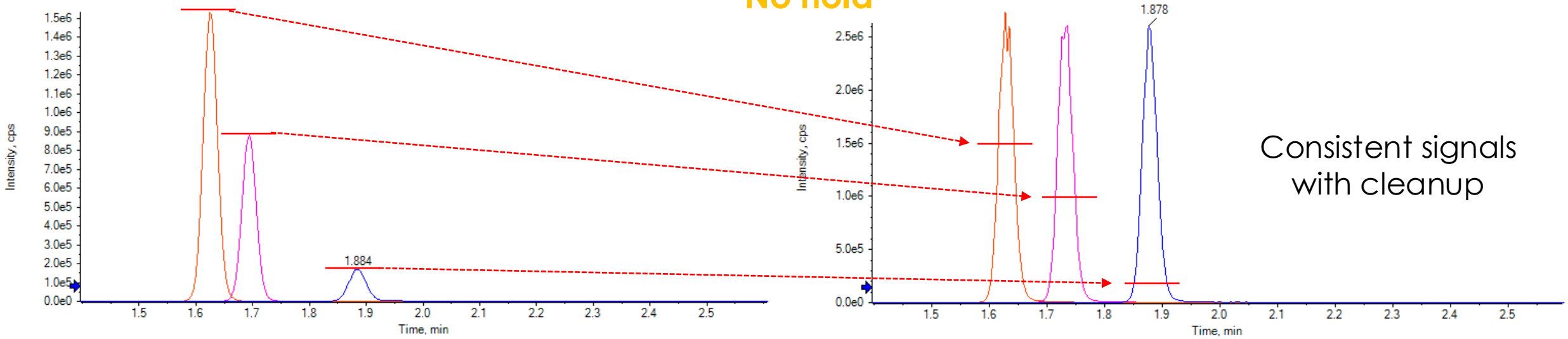
PL removal plate



0.5 min hold

0.2 min hold

No hold



Consistent signals with cleanup

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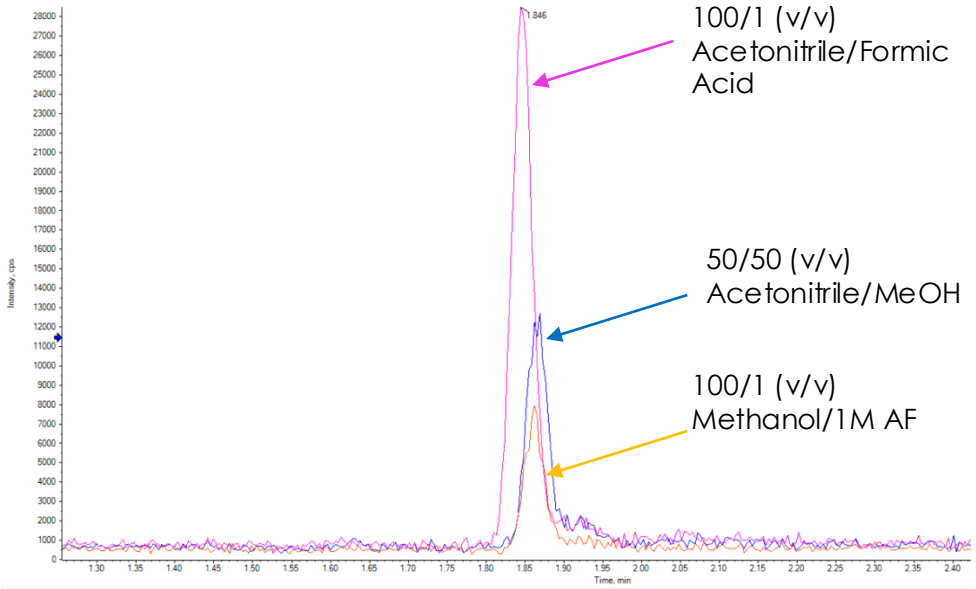
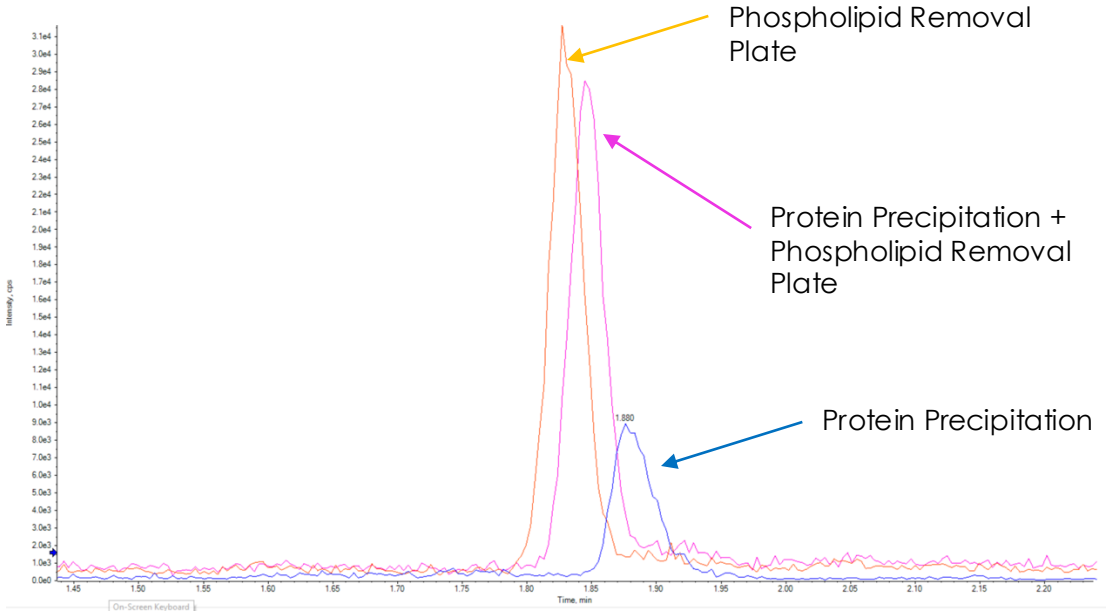
Extraction Procedure Adapted and Optimised

Acetonitrile/Formic Acid (100/1) has greatest response





- Acidified acetonitrile has the greatest peak response of Lipid 1.
- Protein Precipitation showcases a 5-fold reduction in peak response compared to the other two methods, indicating lost recovery or potential suppression caused by a protein precipitation method.

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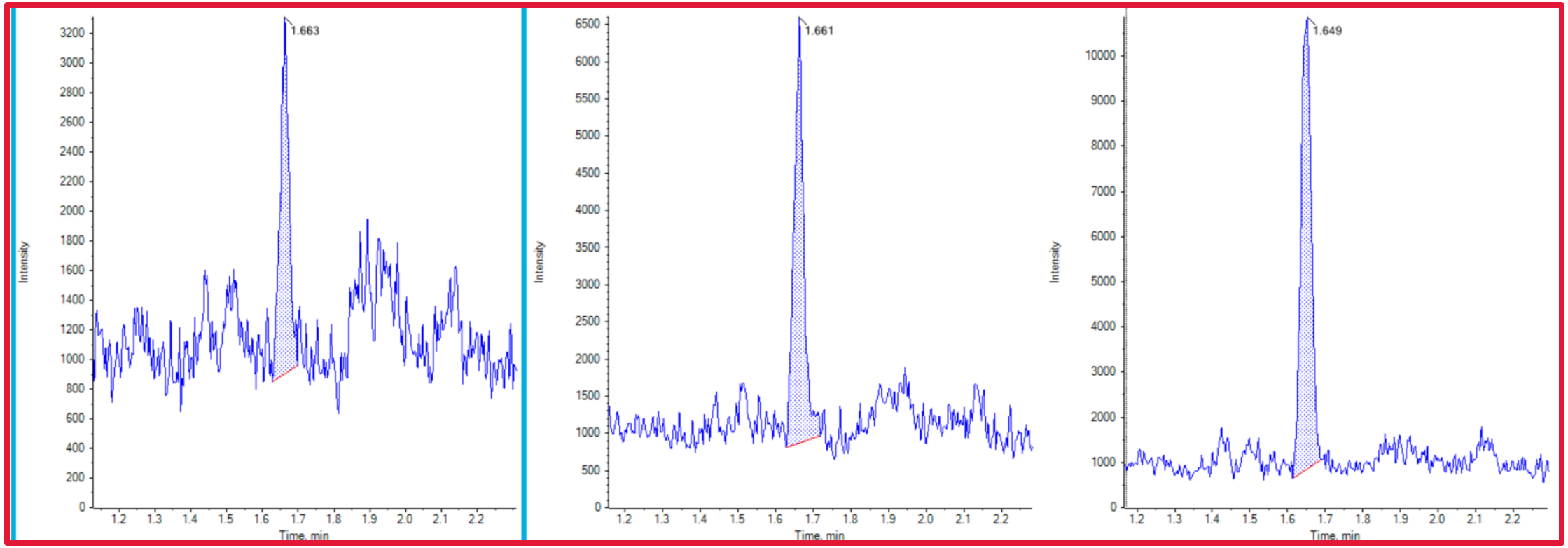
LLOQ Too Ambitious – Comparison between Peaks

1 , 2.5 and 5 pg/mL Peak Comparison



1 pg/mL | 2.5 pg/mL | 5 pg/mL

- Signal-to-noise of 1 pg/mL is not quite sufficient and high risk for day-to-day sensitivity changes. A challenge of this development was reaching as low a LLOQ as possible with only 25 µL of the plasma sample, 5 pg/mL is the confirmed choice at this point in method development.

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Precision & Accuracy Results (1pg/mL – 1000 pg/mL)

Run Statistics out – LLOQ raised to STD2 due to insufficient S/N

- STD1 (1 pg/mL) deactivated from calibration curve as S/N < 5:1

Actual Concentration (pg/mL)	%CV	%Bias	n
1	N/A	N/A	0/2
2.5	N/A	-5.25	1/2
5	3.05	-4.11	2/2
10	8.13	-5.5	2/2
50	0.68	12.3	2/2
200	8.20	0.79	2/2
500	N/A	-0.69	1/2
1000	12.97	-0.52	2/2

Sample ID	Actual Concentration (pg/mL)	%CV	%Bias	n
QCLLOQ	1	N/A	N/A	0/6
QCLOW	3	>15	>15	6/6
QCMID	300	8.34	4.11	6/6
QCHIGH	800	2.53	2.39	6/6

- QC-LOW outside acceptance criteria – potentially due to the limited data at the low limit of the $\frac{1}{x^2}$ weighted calibration curve.
- IS metrics show peak are consistent, so the errors point towards potential aliquoting and/or mixing issues prior to the plate elution.

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Precision & Accuracy Results (5pg/mL to 1000 pg/mL)

Actual Concentration (pg/mL)	%CV	%Bias	n
5	5.64	4.03	2/2
10	2.80	-7.26	2/2
25	13.38	-1.16	2/2
100	1.58	-4.38	2/2
250	6.73	1.82	2/2
500	5.14	-8.29	2/2
750	0.44	11.56	2/2
1000	N/A	7.38	1/2

Sample ID	Actual Concentration (pg/mL)	%CV	%Bias	n
QCLLOQ	5	10.89	-6.52	6/6
QCLOW	15	11.65	1.76	6/6
QCMID	300	6.04	7.83	6/6
QCHIGH	800	5.73	9.11	6/6

- Further assessments using different lots of plasma resulted in QC samples being outside acceptance criteria – potentially due to matrix effects.

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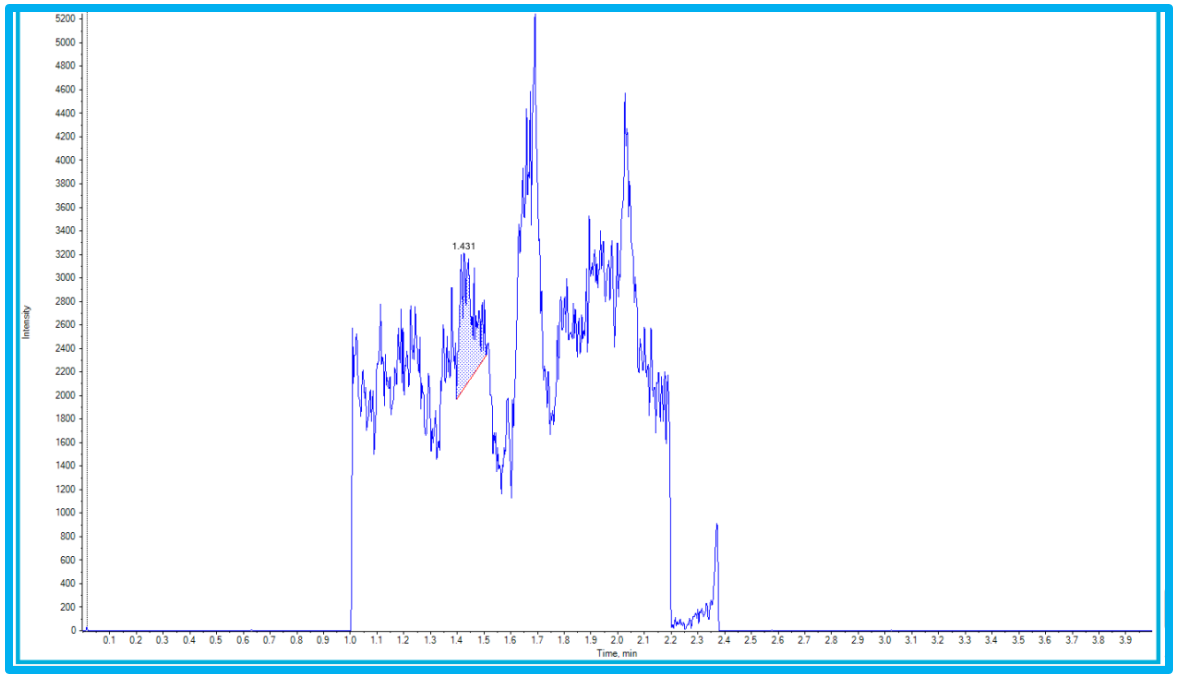
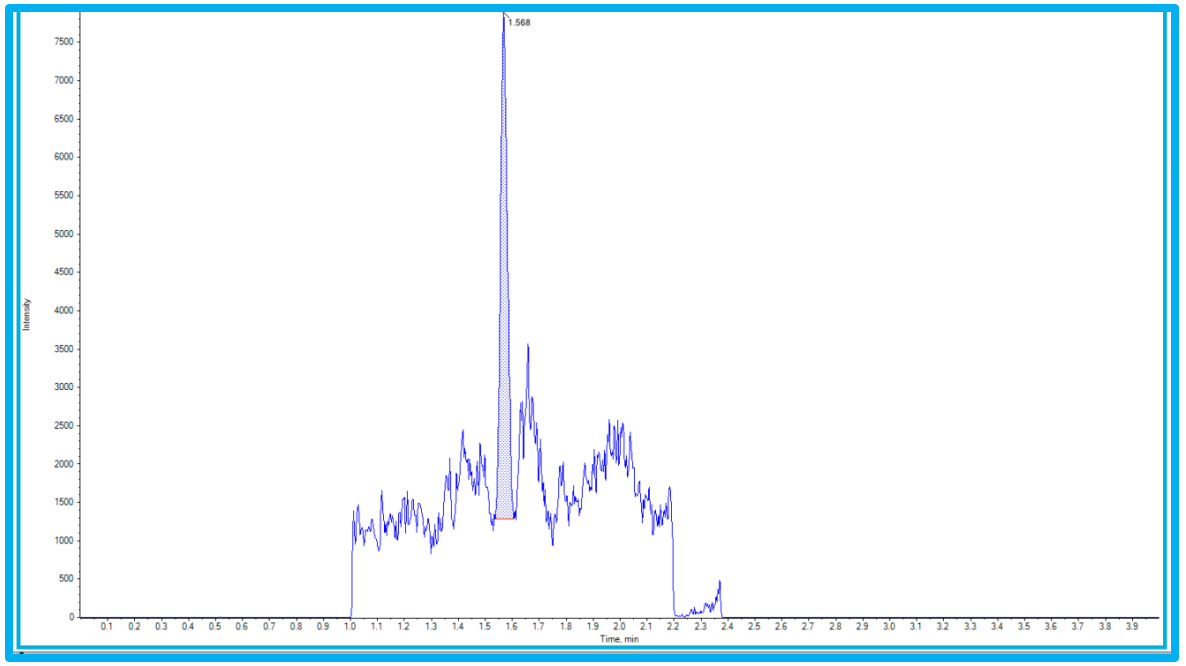
Chromatography Problem – Baseline is NOT consistent or flat

Peak integration within a valley may cause run-to-run problems at the LLOQ



Matrix Blank



QC – LLOQ (5 pg/mL)

- Not only is baseline relatively high – the analyte RT is eluting between two endogenous peaks. This is high-risk chromatography.
- Baseline is not consistent– varies on matrix blank injections.
- Method potentially not suitable for Validation

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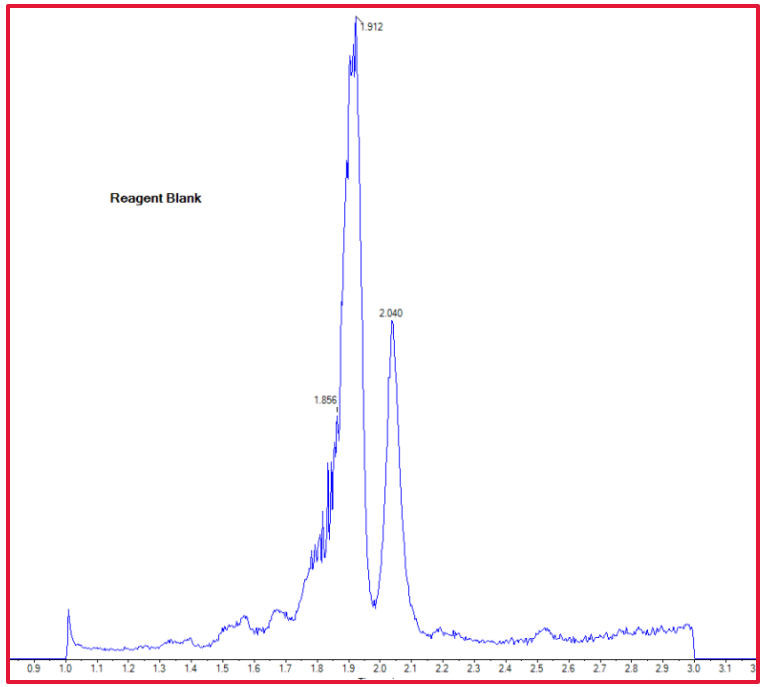
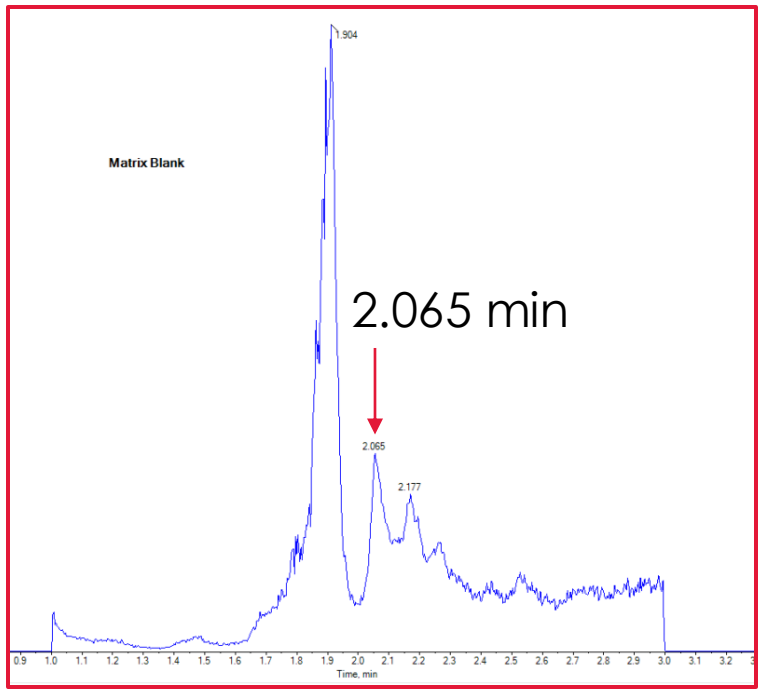
Liquid-Liquid Extraction Assessment

MTBE causing potential plastic degradation to occur

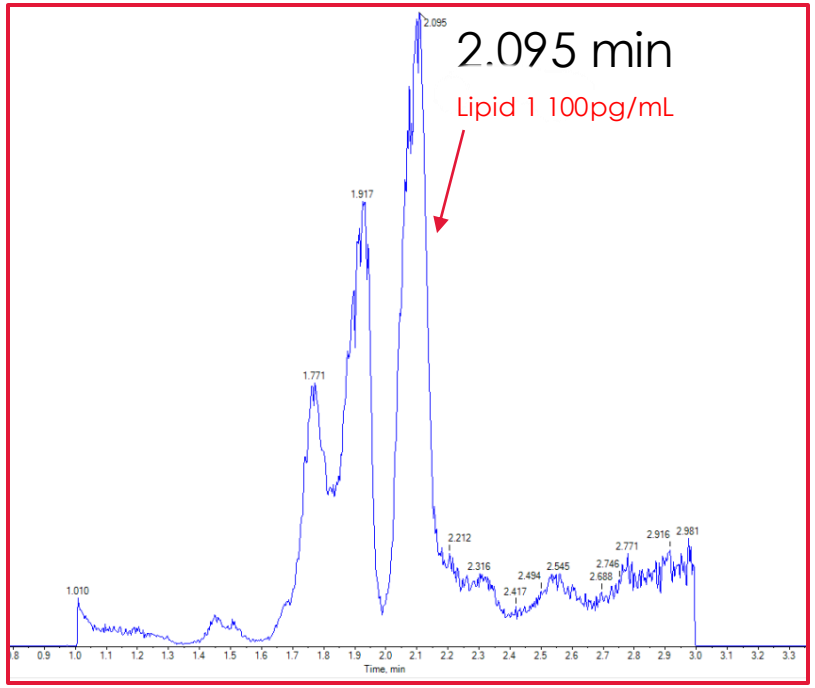
- Endogenous levels co-eluting with Lipid 1. Potentially a polypropylene glycol from the extraction plates – no HRMS to confirm.



Reagent Blank



Matrix Blank



Lipid 1-100 pg/mL

- Peak response from the 100 pg/mL sample proving Lipid 1 extraction from the organic layer during the LLE means the methodology is possible.

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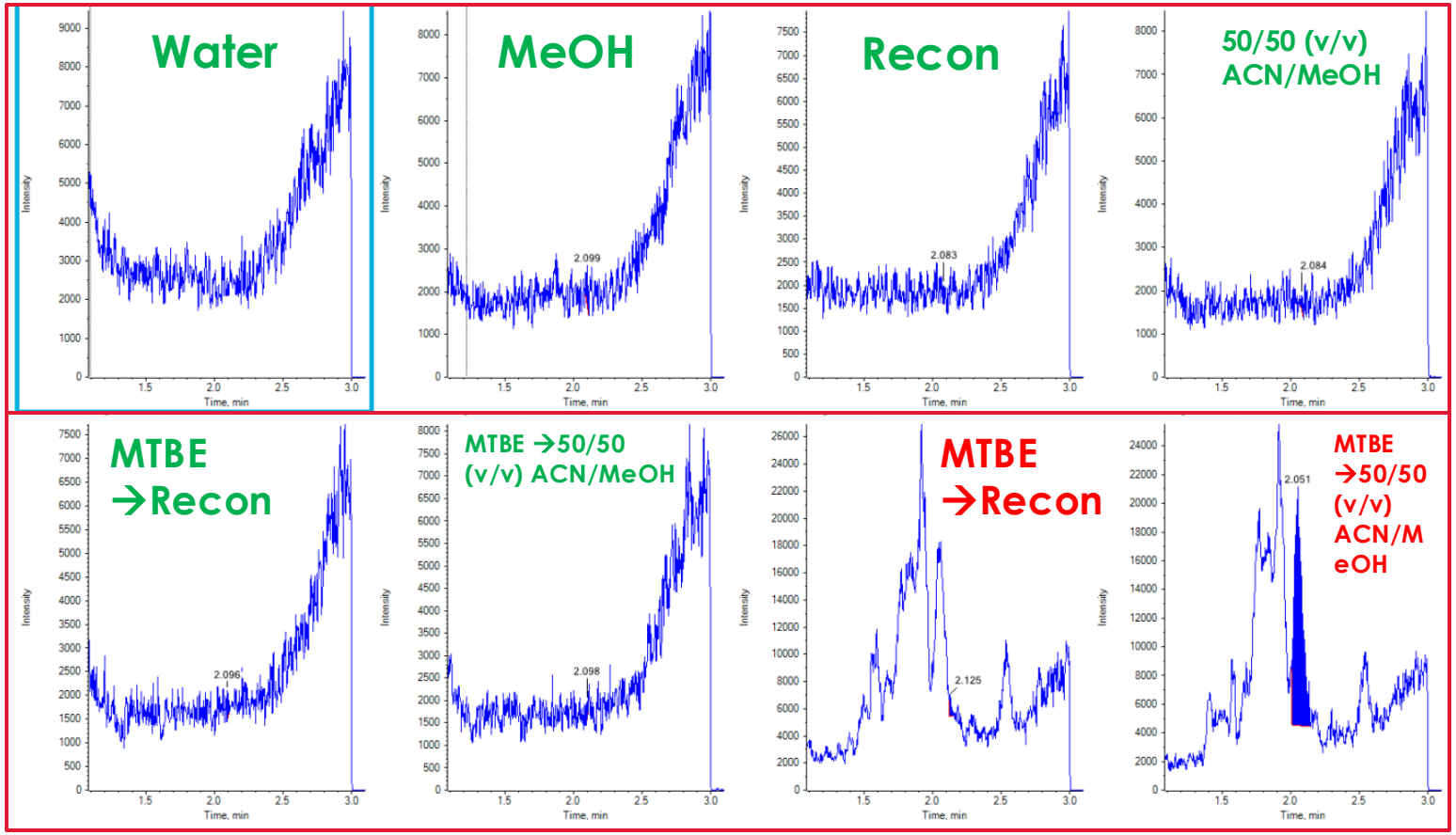
Concerns with LLE - Reagent contamination or container issue?

Isolating the source of the peaks to the plate materials



Injection from Glass Vials

Injection from PP Matrix Tubes

- Polypropylene based plates or matrix tubes seem to be interacting with MTBE and eluting with the current chromatography at Lipid 1 RT.
- Extraction within glass vials is a possibility as well as trying alternative organic solvents for the upper phase of LLE.

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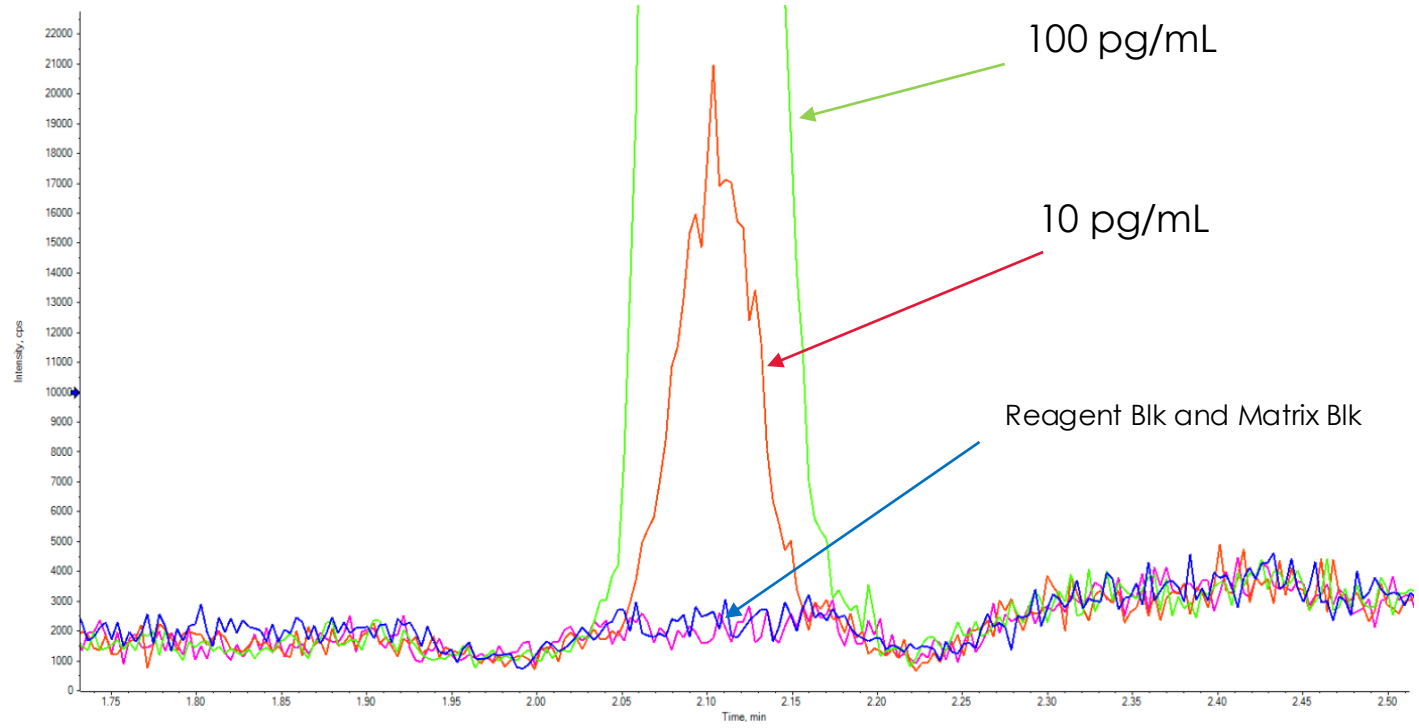
Liquid-Liquid Extraction Assessment

Achievable low pg/mL level LLOQ's with LLE using glass inserts

Extraction procedure in glass vials successful



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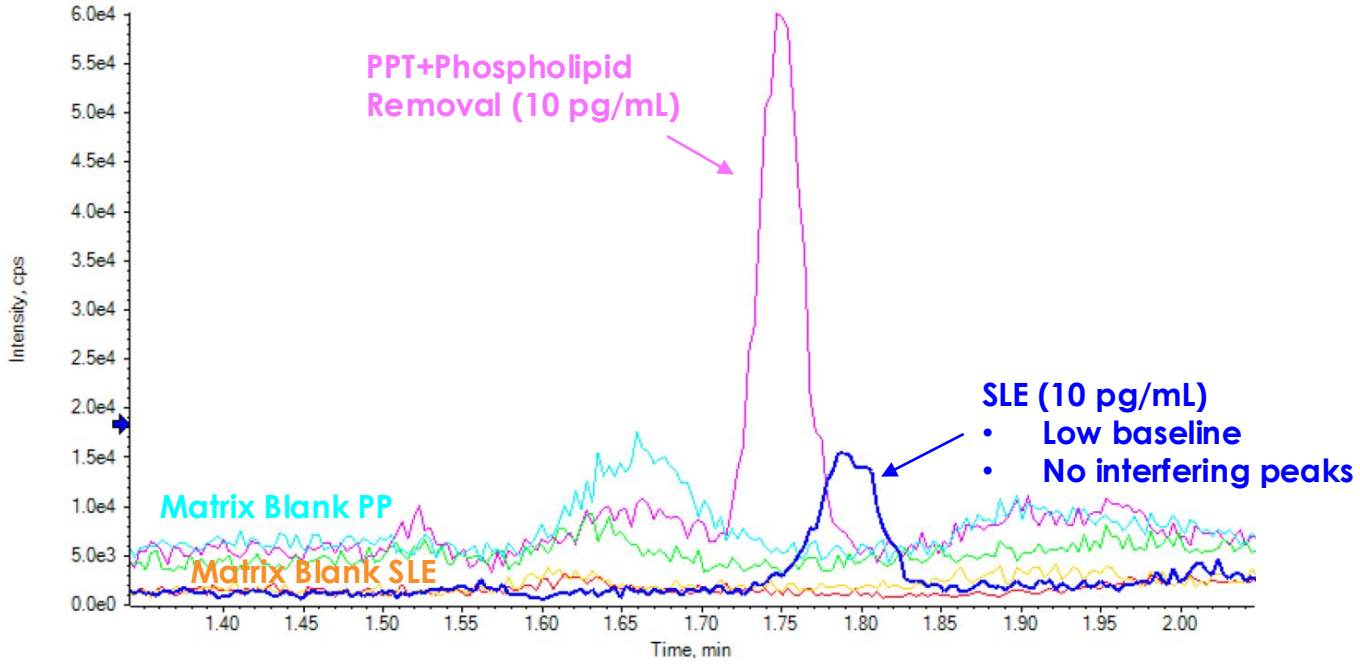
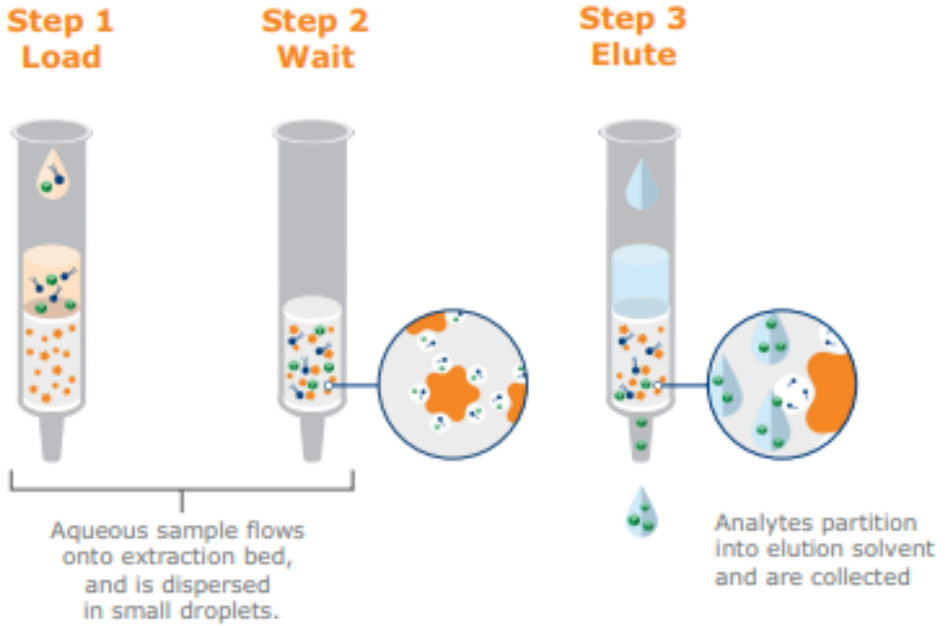
SLE (Supported Liquid Extraction)

First try using SLE+ 200uL plates





Step 1: load 1:1 sample: aqueous buffer

Step 2: Sample Soak

Step 3: Elute with MTBE, dry down and recon

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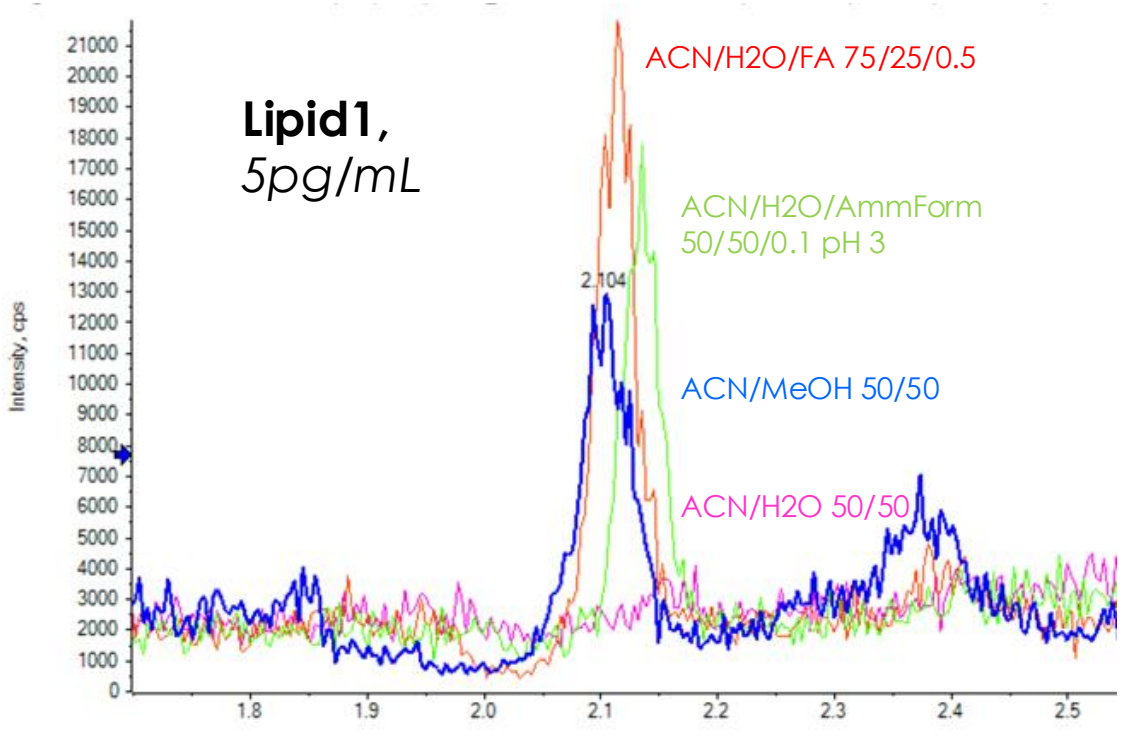
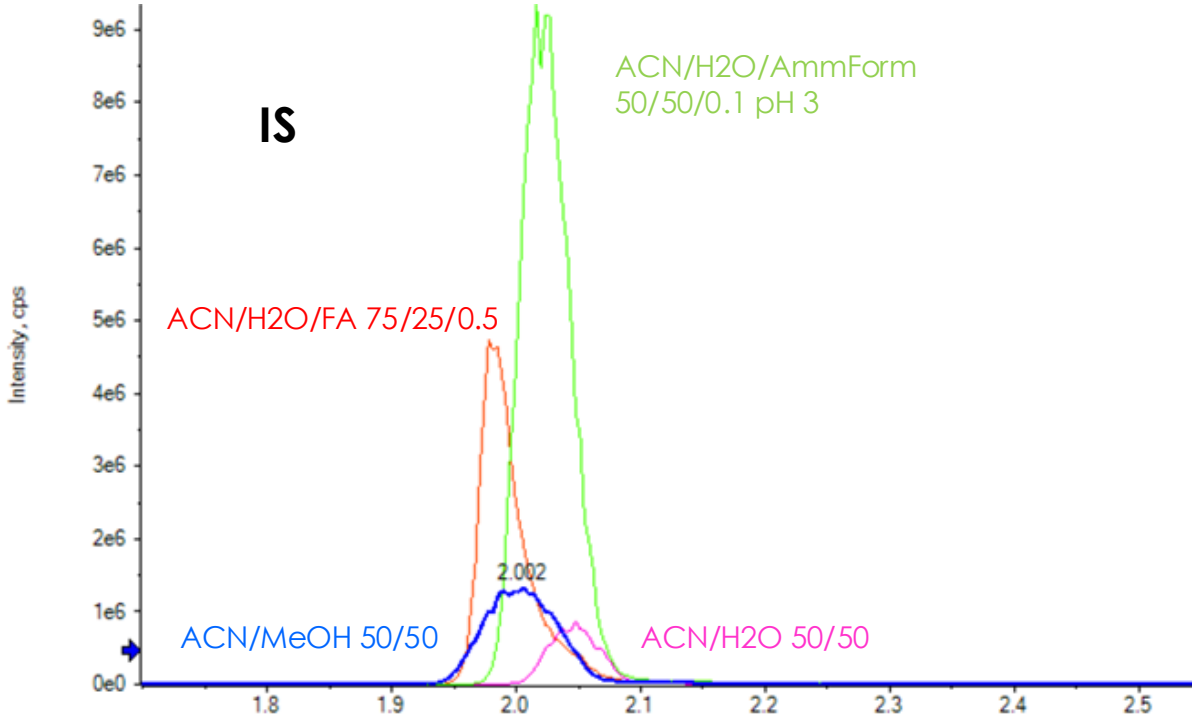
SLE – recon solution testing

With Alkaline Mobile phases





- **Higher sensitivity from acidic recon solution, especially in IS**

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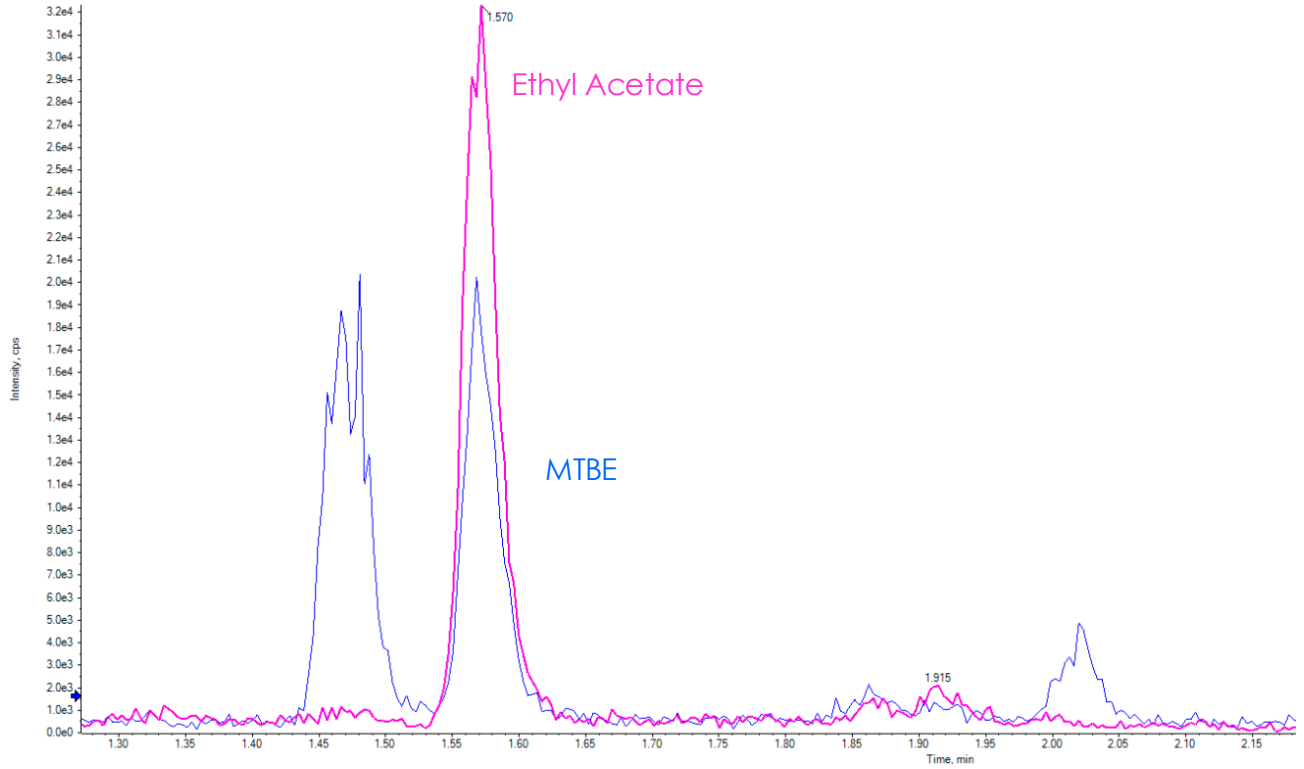
SLE – MTBE vs Ethyl Acetate

Eluent for SLE



Lipid 1 (25 pg/mL)

- **Higher sensitivity using Ethyl Acetate**
- **Cleaner baseline when Ethyl Acetate used as eluent**

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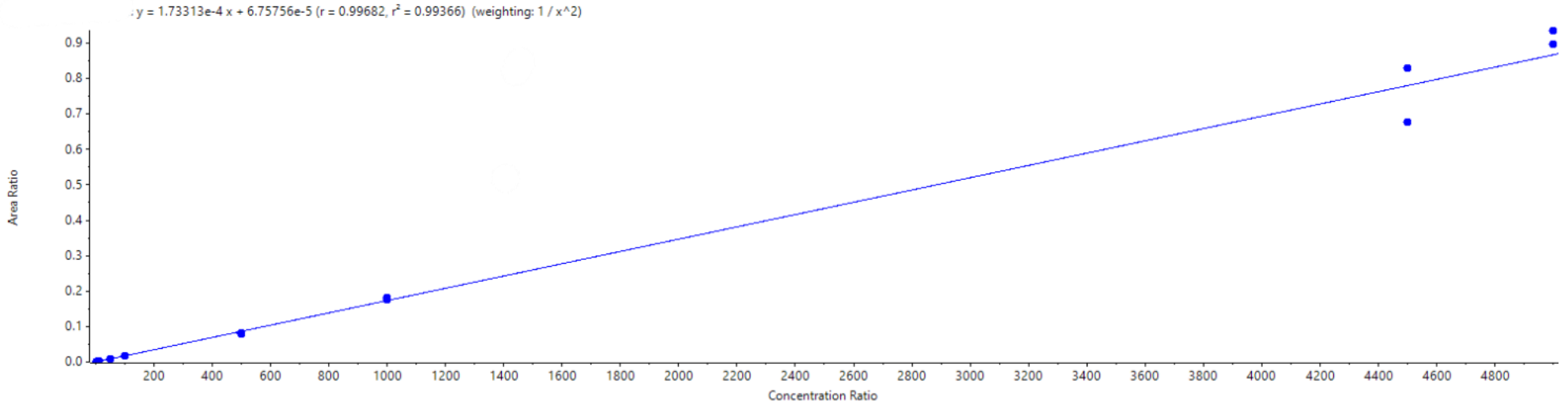
SLE– Initial assessment calibration line results (using Ethyl Acetate)

Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Average Accuracy across Replicates
5.00	1 of 2	4.705	N/A	N/A	94.10
10.00	2 of 2	10.590	0.514	4.85	105.90
50.00	2 of 2	50.910	5.912	11.61	101.82
100.00	2 of 2	97.469	2.944	3.02	97.47
500.00	2 of 2	463.011	21.452	4.63	92.60
1000.00	2 of 2	1028.075	17.112	1.66	102.81
4500.00	2 of 2	4348.730	615.225	14.15	96.64
5000.00	2 of 2	5285.459	152.925	2.89	105.71



To further optimise, use alternative column with suitable mobile phases (eg Premier version of the same column).

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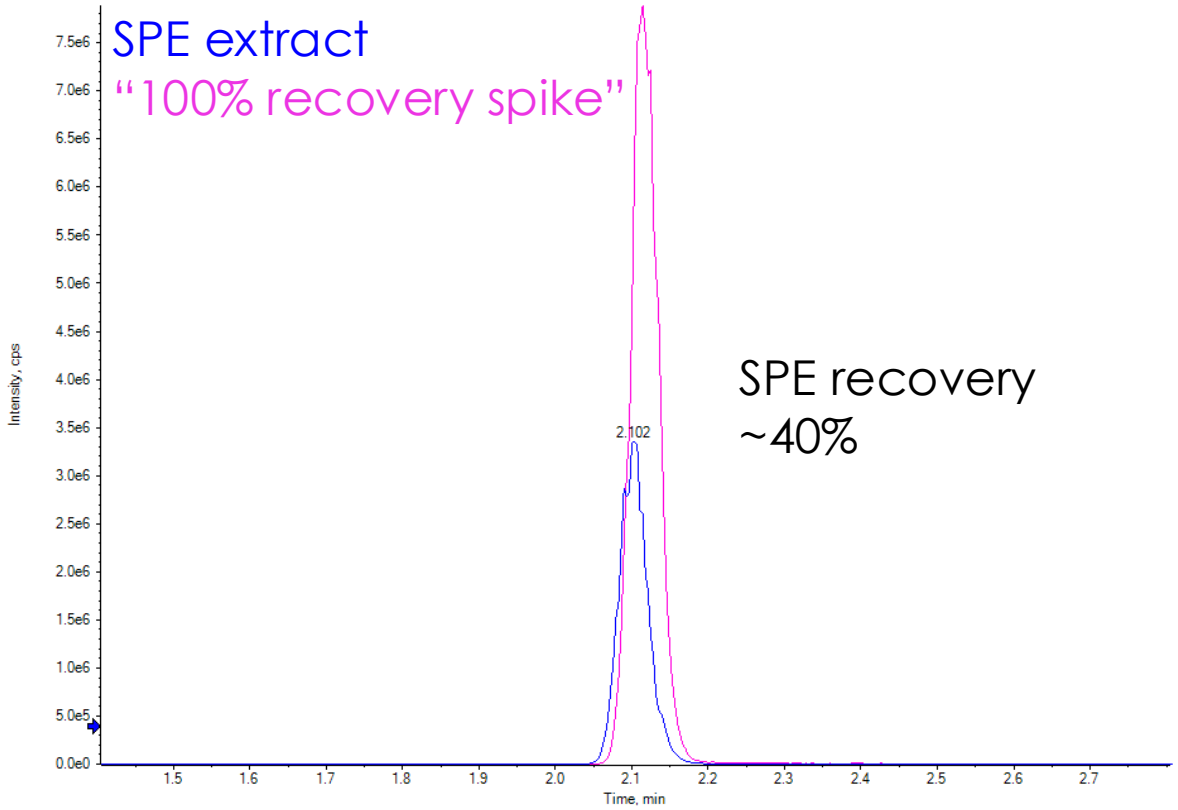
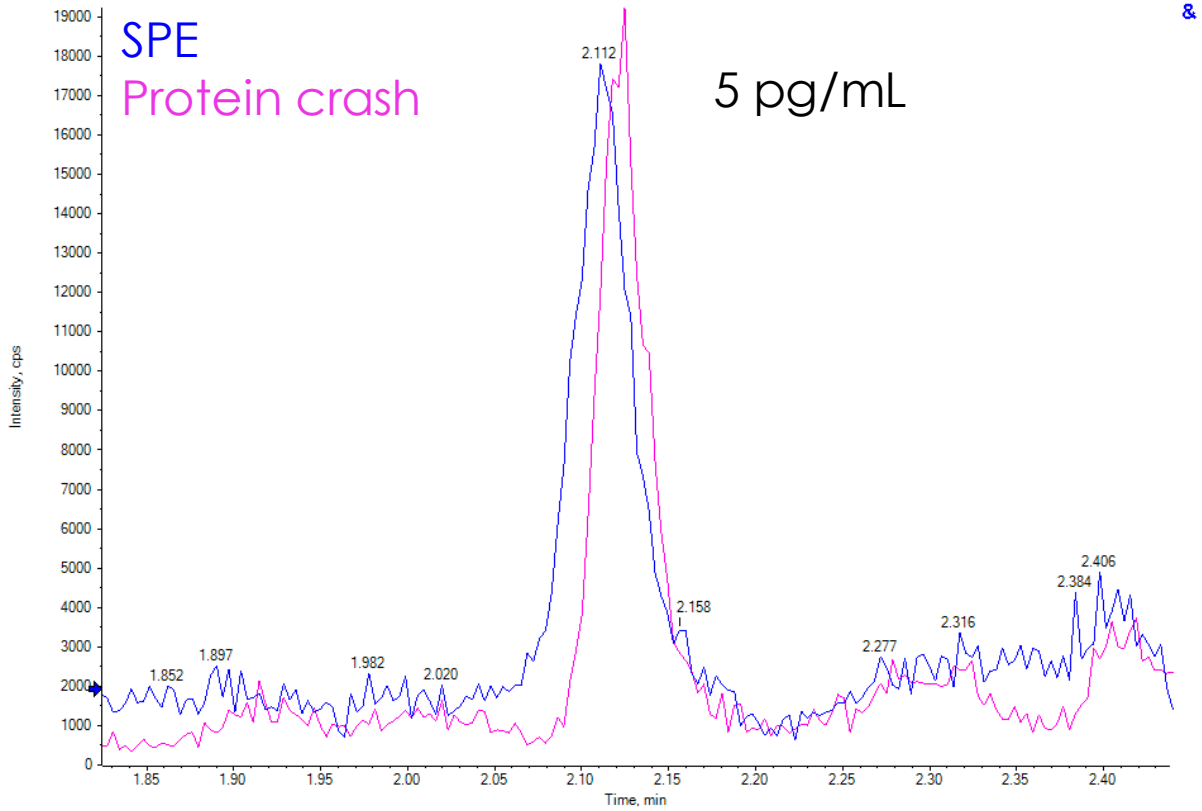
Solid Phase Extraction – Initial assessment (C18)





- Even at 40% recovery, SPE peak matches protein precipitation (reduced suppression)
- Further optimisation to increase signal possible

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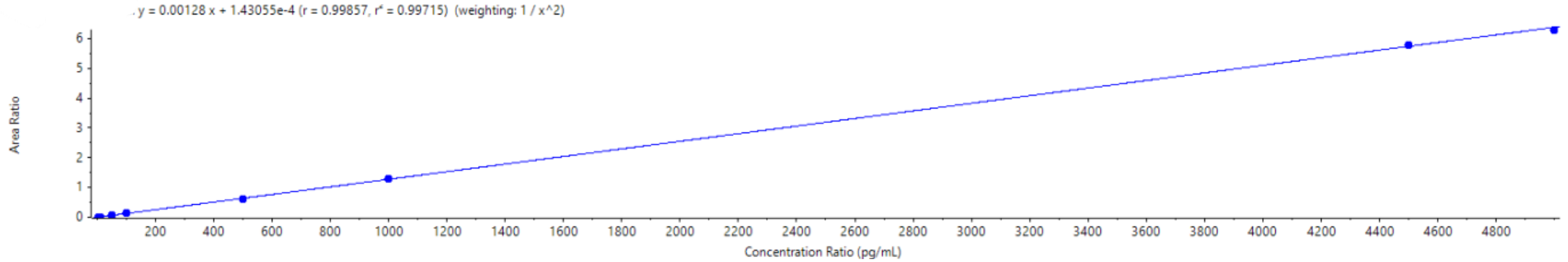
SPE C18 – Initial assessment calibration line results

Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Average Accuracy across Replicates	Value #1
5.00	1 of 1	5.099e0	N/A	N/A	101.98	5.099e0
10.00	1 of 1	9.586e0	N/A	N/A	95.86	9.586e0
50.00	1 of 1	4.800e1	N/A	N/A	95.99	4.800e1
100.00	1 of 1	1.106e2	N/A	N/A	110.59	1.106e2
500.00	1 of 1	4.812e2	N/A	N/A	96.24	4.812e2
1000.00	1 of 1	1.003e3	N/A	N/A	100.29	1.003e3
4500.00	1 of 1	4.523e3	N/A	N/A	100.52	4.523e3
5000.00	1 of 1	4.927e3	N/A	N/A	98.53	4.927e3



Further assessments were performed:
Chromatography issues still observed (using C8 column) resulting in failure of runs

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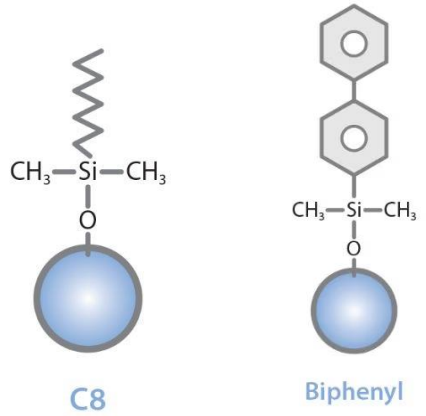
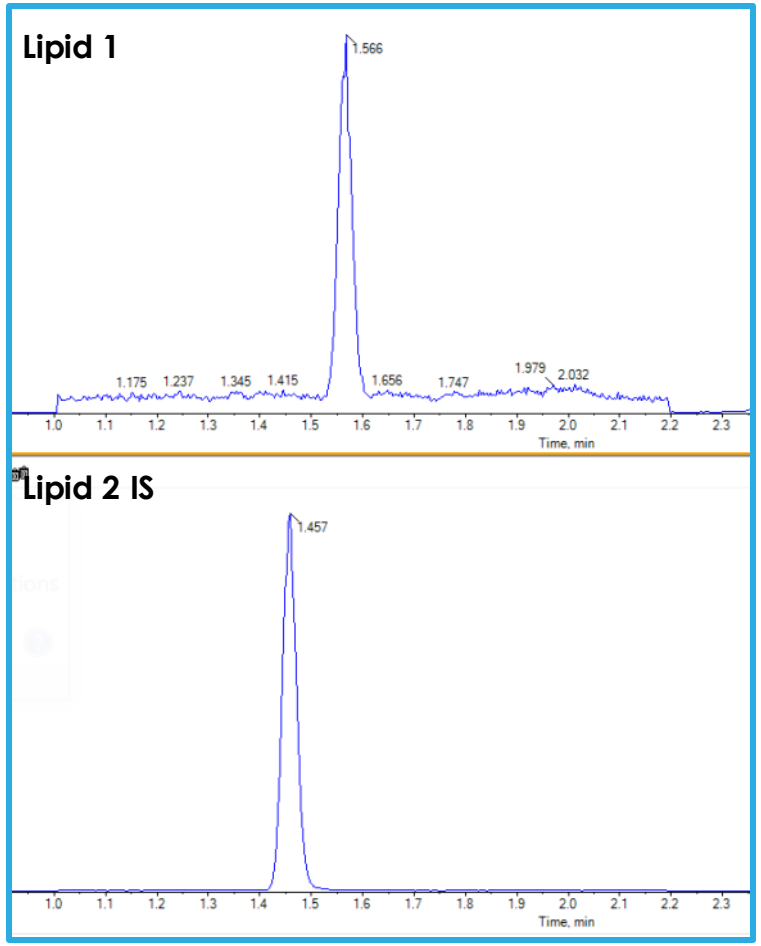
HPLC Column Change - Column Selectivity

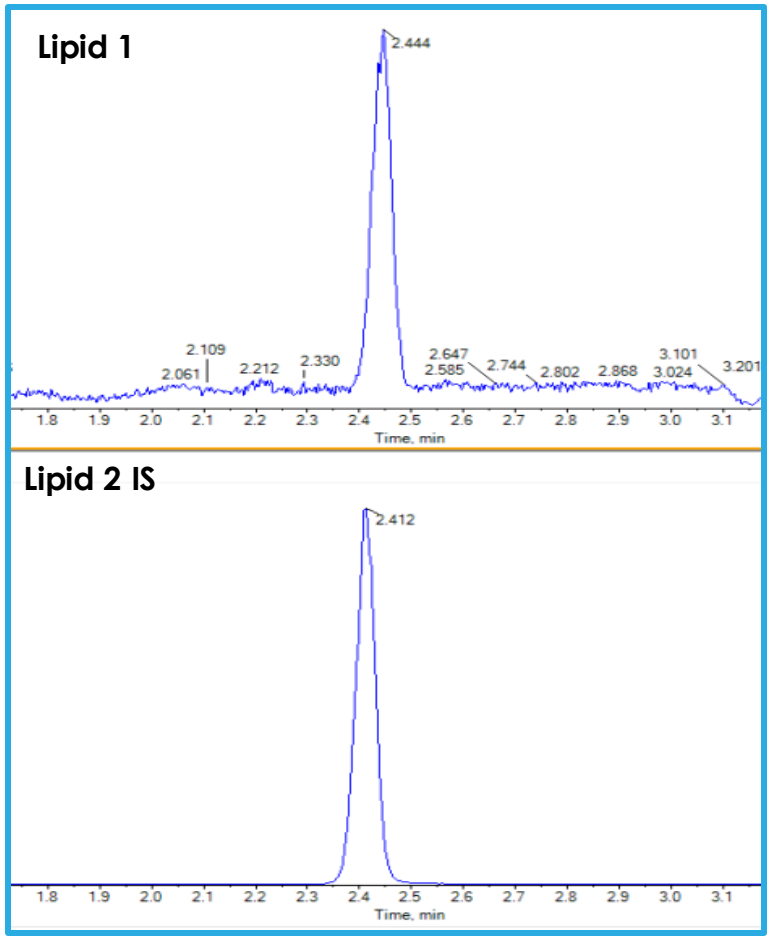
C8 vs Biphenyl



- C8 Column chemistry: separation of peaks is driven by hydrocarbon chain differences between Lipid 1 and Lipid 2
- Biphenyl column chemistry: difference in hydrophobic interactions less significant, therefore there is less peak separation.
- We are using Lipid 2 as internal standard. Need for separation is less significant. May be beneficial for the two analytes to co-elute and reach the ion source at the same time.



C8



Biphenyl

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Solid Phase Extraction (Cation exchange) - Developed Method

Dual protein precipitation/Solid phase extraction:

Protein precipitation:

- Add 200 µL methanol (with I.S) to 25 µL sample
- Centrifuge and remove 150 µL supernatant
- Dilute with 400 µL water + 2% formic acid

} Disrupt protein binding of lipids

Solid phase extraction:

- Condition SPE plate with 200 µL methanol + 200 µL water
- Add sample extracts to SPE plate + adsorb
- Wash with 200 µL methanol + 2% formic acid
- Elute with 75 µL methanol + 1% ammonia

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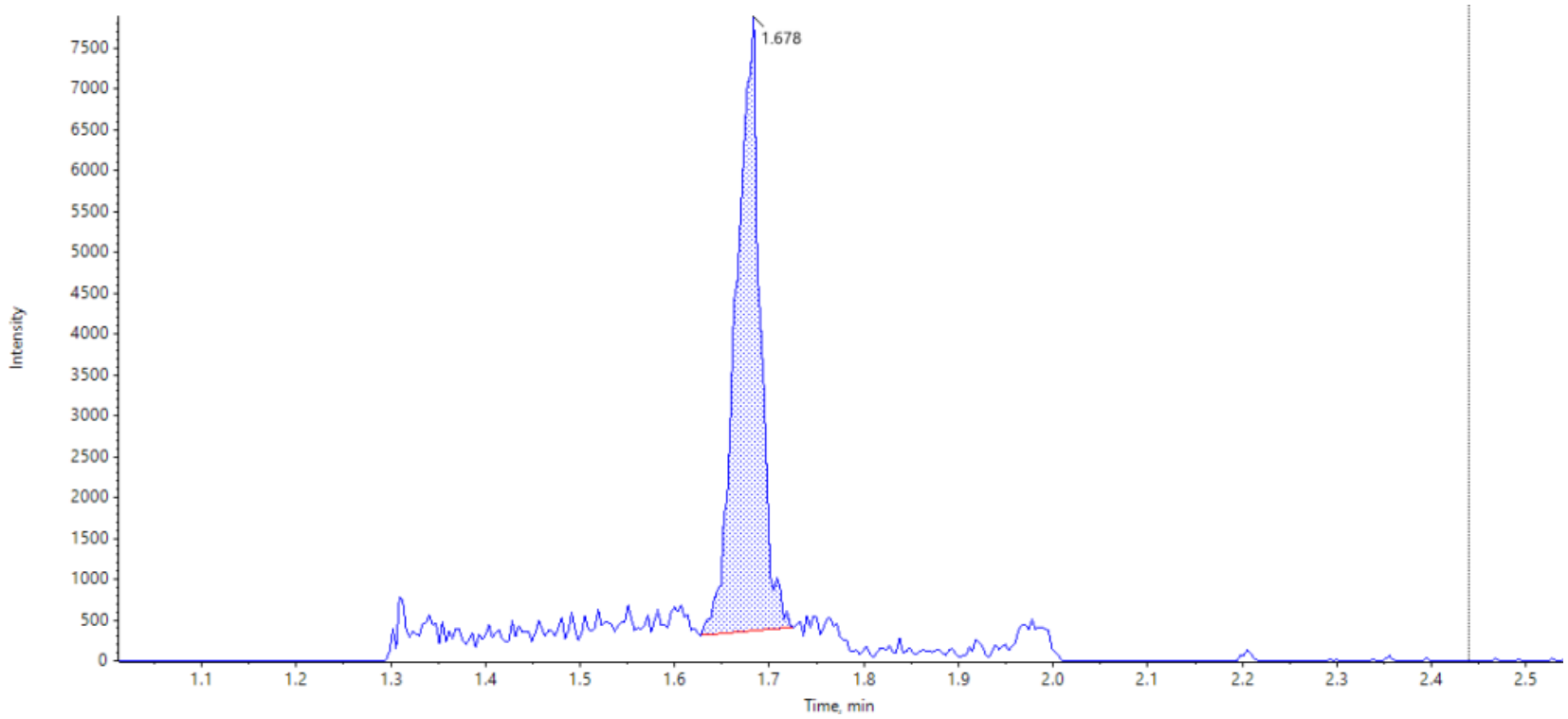
Solid Phase Extraction (Cation exchange) - Chromatography with biphenyl HPLC Column



LLOQ (5 pg/mL)

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Solid Phase Extraction (Cation exchange) - Precision & Accuracy (Run 1)

Calibration Standards

Sample ID	Actual Concentration (pg/mL)	SD	%CV	%Bias	n
STD1	5	0.25	5.08	-1.65	2
STD2	10	0.83	7.91	4.46	2
STD3	50	1.98	4.22	-6.01	2
STD4	100	0.6	0.59	0.84	2
STD5	500	14.64	3.01	-2.75	2
STD6	1000	24.21	2.39	1.18	2
STD7	4500	16.89	0.37	2.43	2
STD8	5000	8.65	0.17	1.5	2

Quality Controls

Sample ID	Actual Concentration (pg/mL)	SD	%CV	%Bias	n
QCLLOQ	5	0.15	2.97	2.49	6
QCLOW	15	0.77	4.81	6.16	6
QCMID	250	5.75	2.36	-2.68	6
QCHIGH	4000	88.8	2.31	-3.78	6

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Solid Phase Extraction (Cation exchange) - Precision & Accuracy (Run 2)

Calibration Standards

Sample ID	Actual Concentration (pg/mL)	SD	%CV	%Bias	n
STD1	5	0.41	8.2	0.82	2
STD2	10	0.57	5.71	-0.83	2
STD3	50	0.7	1.46	-4.59	2
STD4	100	3.28	3.24	1.19	2
STD5	500	3.53	0.72	-1.31	2
STD6	1000	N/A	N/A	3.26	1
STD7	4500	81.44	1.79	1.33	2
STD8	5000	135.14	2.66	1.76	2

Quality Controls

Sample ID	Actual Concentration (pg/mL)	SD	%CV	%Bias	n
QCLLOQ	5	0.48	9.25	3.65	6
QCLOW	15	0.44	2.92	1.60	6
QCMID	250	3.04	1.16	4.31	6
QCHIGH	4000	44.16	1.09	1.32	6

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Solid Phase Extraction (Cation exchange) - Matrix Effects (Run 3)

Calibration Standards

Sample ID	Actual Concentration (pg/mL)	SD	%CV	%Bias	n
STD1	5	0.17	3.4	2.3	2
STD2	10	N/A	N/A	-7.91	1
STD3	50	1.53	3.16	-3.31	2
STD4	100	1.49	1.48	0.87	2
STD5	500	14.96	3.19	-6.12	2
STD6	1000	32.29	3.09	4.44	2
STD7	4500	12.46	0.26	7.06	2
STD8	5000	109.81	2.22	-1.28	2

Quality Controls

Sample ID	Actual Concentration (pg/mL)	SD	%CV	%Bias	n
QCLOW	15	0.5	1.21	-10.13	2
QCMID	250	3.04	1.16	-2.68	2
QCHIGH	4000	44.16	1.09	-3.78	2

Matrix Effect

Donor ID	Actual Concentration (pg/mL)	SD	%CV	%Bias	n
A (Disease State Plasma)	15	0.08	0.56	-7.3	2
B (Disease State Plasma)	15	0.72	5.23	-8.6	3
C (Disease State Plasma)	15	0.3	2.04	-1.8	3
D	15	0.38	2.62	-4.3	3
E	15	0.79	5.41	-2.8	3
F	15	1.79	11.39	4.6	3

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Lipid 2

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Lipid 2 Results

Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy
5	4.748	94.96
4.5	4.224	93.87
1	0.935	93.49
0.5	0.492	98.45
0.1	0.103	103.18
0.05	0.045	90.98
0.01	0.009	90.6
0.005	0.005	95.43

Protein Precipitation

Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy
5	3.721	96.48
4.5	3.262	93.97
1	0.788	102.14
0.5	0.377	97.69
0.1	0.085	109.93
0.05	0.038	98.46
0.01	0.008	102.5
0.005	0.004	91.18

Sirocco Plate

Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy
5	4.526	90.51
4.5	4.101	91.14
1	1.006	100.56
0.5	0.493	98.55
0.1	0.104	104.36
0.05	0.049	98.58
0.01	0.01	103.44
0.005	0.005	95.02

PLR

Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy
5	4.789	95.78
4.5	3.834	85.2
1	0.624	62.35
0.5	0.556	111.18
0.1	0.114	114.25
0.05	0.05	99.3
0.01	0.011	108.54
0.005	0.005	95.16

PP+ PLR

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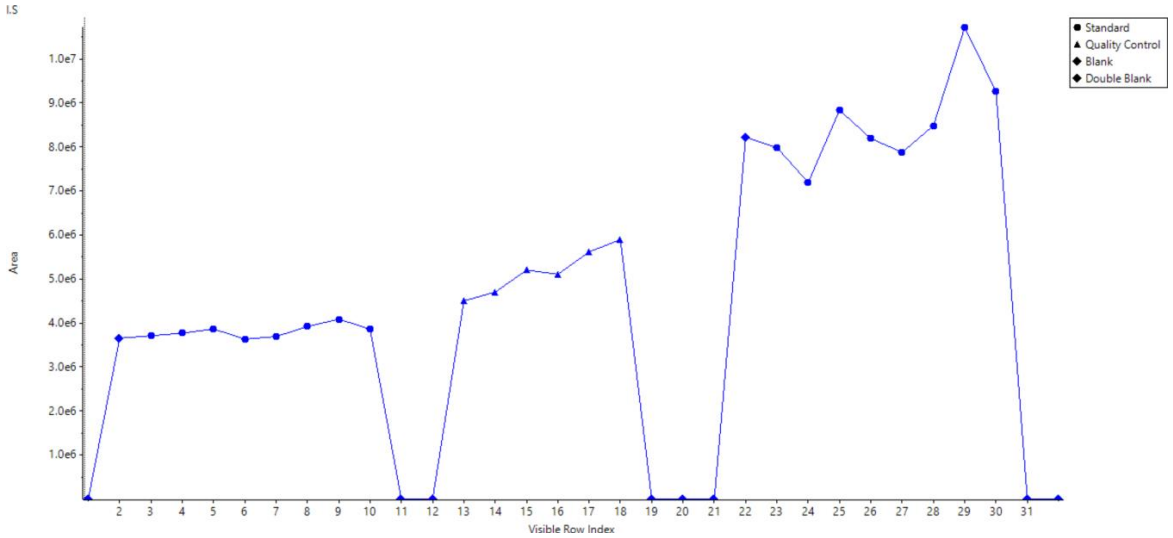
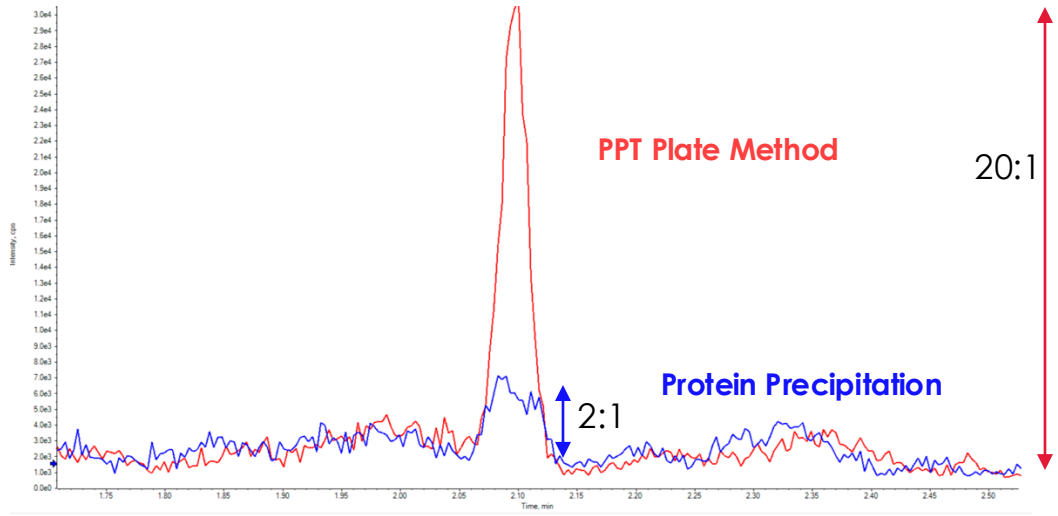
However…

The Lipid 2 internal standard and the analyte signal-to-noise ratio at LLOQ in the protein precipitation method **not acceptable.**



*I.S plot



*Lipid 2 analyte

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Lipid 2 SLE results

Actual Concentration (pg/mL)	Calculated Concentration (pg/mL)	Accuracy
5	4.978	99.55
10	7.962	79.62
50	49.347	98.69
100	112.675	112.67
500	494.928	98.99
1000	909.787	90.98
4500	4592.321	102.05
5000	4853.186	97.06

SLE using Ethyl Acetate

Actual Concentration (pg/mL)	Calculated Concentration (pg/mL)	Accuracy
5	5.636	112.72
10	7.481	74.81
50	45.704	91.41
100	112.943	112.94
500	556.022	111.2
1000	945.401	94.54
4500	4407.338	97.94
5000	5221.765	104.44

SLE using MTBE

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Lipid 2 SPE results

Actual Concentration (pg/mL)	Calculated Concentration (pg/mL)	Accuracy
5	4.27	85.45
10	8.64	86.41
50	45.97	91.94
100	99.65	99.65
500	479.16	95.83
1000	1052.86	105.29
4500	4398.12	97.74
5000	4824.02	96.48
5	5.73	114.7
10	11.49	114.85
50	49.15	98.31
100	104.88	104.88
500	493.14	98.63
1000	972.57	97.26
4500	4783.22	106.29
5000	5314.39	106.29

Cation exchange SPE using biphenyl chromatography

Actual Concentration (pg/mL)	Calculated Concentration (pg/mL)	Accuracy
5	4.402	88.04
10	9.122	91.22
50	46.104	92.21
100	103.278	103.28
500	492.997	98.6
1000	1052.2	105.22
4500	4284.93	95.22
5000	4874.961	97.5
5	5.908	118.16
10	9.667	96.67
50	50.997	101.99
100	105.946	105.95
500	488.606	97.72
1000	962.807	96.28
4500	4781.532	106.26
5000	5284.397	105.69

Cation exchange SPE using alkaline chromatography

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Lipid 3

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Lipid 3 Results

Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy
1	0.88	88.14
10	9.41	94.05
20	20.97	104.87
50	52.76	105.51
150	171.96	114.64
350	324.56	92.73
1000	1000.46	100.05

Protein Precipitation

Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy
1	0.99	98.89
2.5	2.53	101.26
10	10.01	100.05
20	22.49	112.45
50	51.01	102.02
150	140.93	93.95
350	319.82	91.38
1000	736.42	73.64

PPT Plate

Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy
1	0.96	96.26
2.5	2.91	116.37
10	8.05	80.54
20	18.24	91.21
50	32.49	64.98
150	204.6	136.4
350	394.78	112.79
1000	1014.5	101.45

PRL

Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy
1	0.79	79
2.5	4.48	179.34
10	3.26	32.61
20	6.63	33.17
50	21.58	43.16
150	196.24	130.83
350	675.9	193.11
1000	1087.79	108.78

PP+ PRL

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Next Steps / Conclusions

- Complete extraction assessments for Lipid 3 and further assessments on optimised extraction methods for Lipids 1 + 2
- When a High LLOQ level (i.e ng/mL) is required and/or if an analogue IS is available then protein precipitation/phospholipid removal is preferred
- When a low LLOQ level (i.e pg/mL) is required alternative or dual methods appears to be more appropriate
- All extraction procedures require optimisation along with chromatography assessment
- Use a Stable Isotope Labelled Internal Standard (SIL IS)
- Our increased knowledge of lipid methodology holds promise for future more efficient methodology and quicker development

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