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A Systematic Approach for Developing High Quality LC/MS/MS Methods for Regulated Bioanalysis

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Seminar Topics

- The realities and challenges of fast method development
- Means to develop high quality method
- Case studies
- Conclusion and Summary

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Traditional Approach for Method Development

- Relies on personal experience and preference of the method development (MD) scientist
- Produce personalized method
- Conduct MD experiment linear “trail and error”
- Require longer MD time
- Result in less robust method
- Difficult to transfer
- Generate large inventory supplies and reagents

Need to change and standardize MD process!

Why Standardize Method Development Process

- Fewer variables – supplies, reagents, chemicals
- Better learning, common language, faster training
- Improve documentation
- Enhance MD team's technical expertise
- Simplify MD experiments
- Require less experienced scientific staff

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Concepts to Develop High Quality GLP Method

- Apply tighter acceptance criteria for MD data
- Understand project scope and molecule
- Standardize method development experiment
- Pre-emptive testing
- Manage phospholipids
- Evaluate linearity and carryover
- Monitor IS area response (Tandem Labs Presentation)
- Documentation and compliance

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Tighter Acceptance Criteria for MD Data

- Expect better data from MD scientist who knows the assay the best
- Raises the confidence level in the assay
- More accurate stability data
- Proposed criteria:
  - Std /QC Precision/accuracy: ≤ ± 10% (≤ ± 15% LLOQ)
  - Selectivity: 10 lots : ≤ ± 10% (≤ 15% LLOQ)

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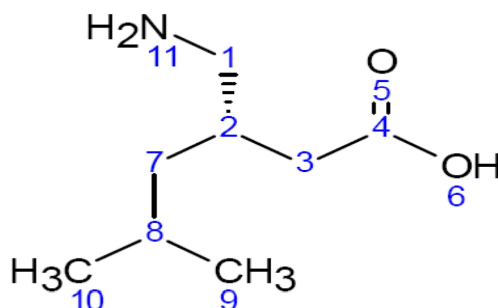
Case Study 1: Know Your Molecule: Pregabalin (zwitterionic)

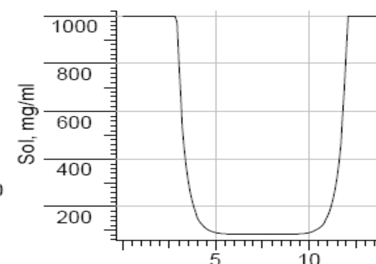


pKa Results

Acidic/Basic	Acidic/Basic	Apparent pKa Value	Error
11	MB	11.31	0.1
6	MA	4.23	0.1

Solubility Results

pH	Sol, mg/ml	Flags	%
1	1000	B	100
2	1000	B	99
5.5	84.87	Z	97
6.5	82.78	Z	100
7.4	82.6	Z	100
10	90.25	AZ	9 / 91
12	847.59	AZ	90 / 10



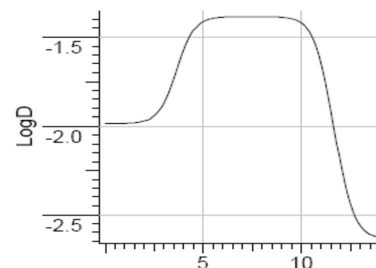
Intrinsic Solubulity, mg/ml	20.6921
Intrinsic Solubulity, log(S, mol/l)	-0.8862
Solubility in Pure Water at pH = 7.49, mg/ml	82.5956

Single-valued Properties

Name	Value	Error
LogP	1.12	0.23
MW	159.23	-
PSA	63.32	-
FRB	6	-
HDonors	3	-
HAcceptors	3	-
Rule Of 5	0	-
Molar Refractivity, cm^	44.13	0.3
Molar Volume, cm^3	159.65	3
Parachor, cm^3	396.03	4
Index of Refraction	1.46	0.02
Surface Tension, dyne/c	37.87	3
Density, g/cm^3	1	0.06
Polarizability, 10e-24 cm	17.49	0.5
Boiling Point, °C	274.03	23

LogD Results

pH	LogD
1	-1.98
2	-1.97
5.5	-1.39
6.5	-1.38
7.4	-1.38
10	-1.42
12	-2.22



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Standardization of Sample Preparation Screening

- Use protocols for each experiment
- Build a common repository of reagents, SPE/SLE plates and LC columns.
- Conduct multiple experiments in parallel
- Treat all compounds as unknown

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Case study 2: Fast MD for Guanfacine and Raltegravir

- Goal: Develop separate LC/MS/MS assays for Guanfacine and Raltegravir
- Approach: Conduct sample screening experiments together
- Time to complete: two days

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Case study 2: Fast MD for Guanfacine and Raltegravir

- Day 1: sample preparation screening protocol
- Experiment design:
  - PPE: three pHs
  - SPE: Oasis MD plate combined with A, B, N washing and elution
  - SLE: three pHs, three organic solvents
- Total samples: 78

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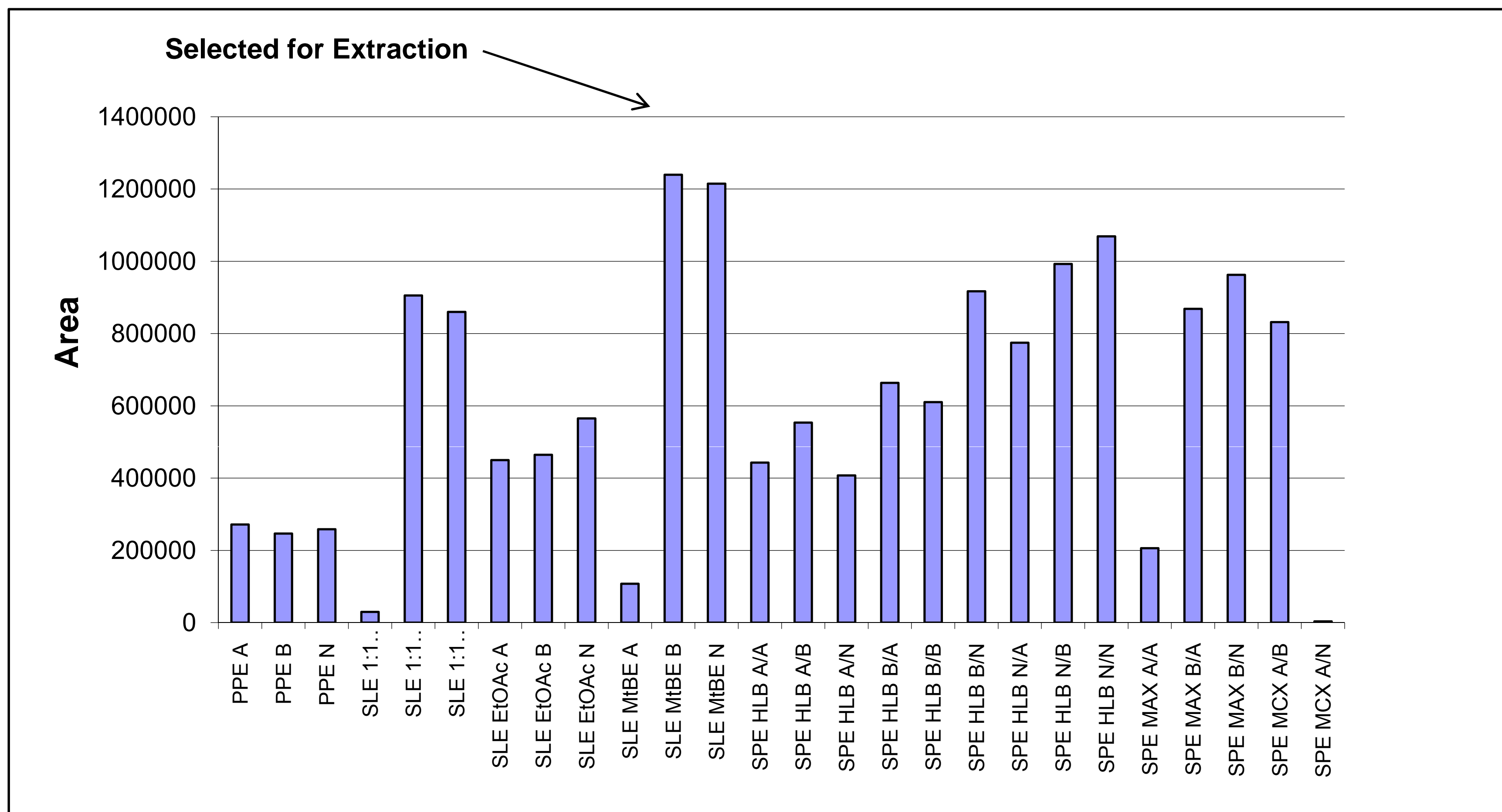
Case study 2: Fast MD for Guanfacine and Raltegravir

Guanfacine



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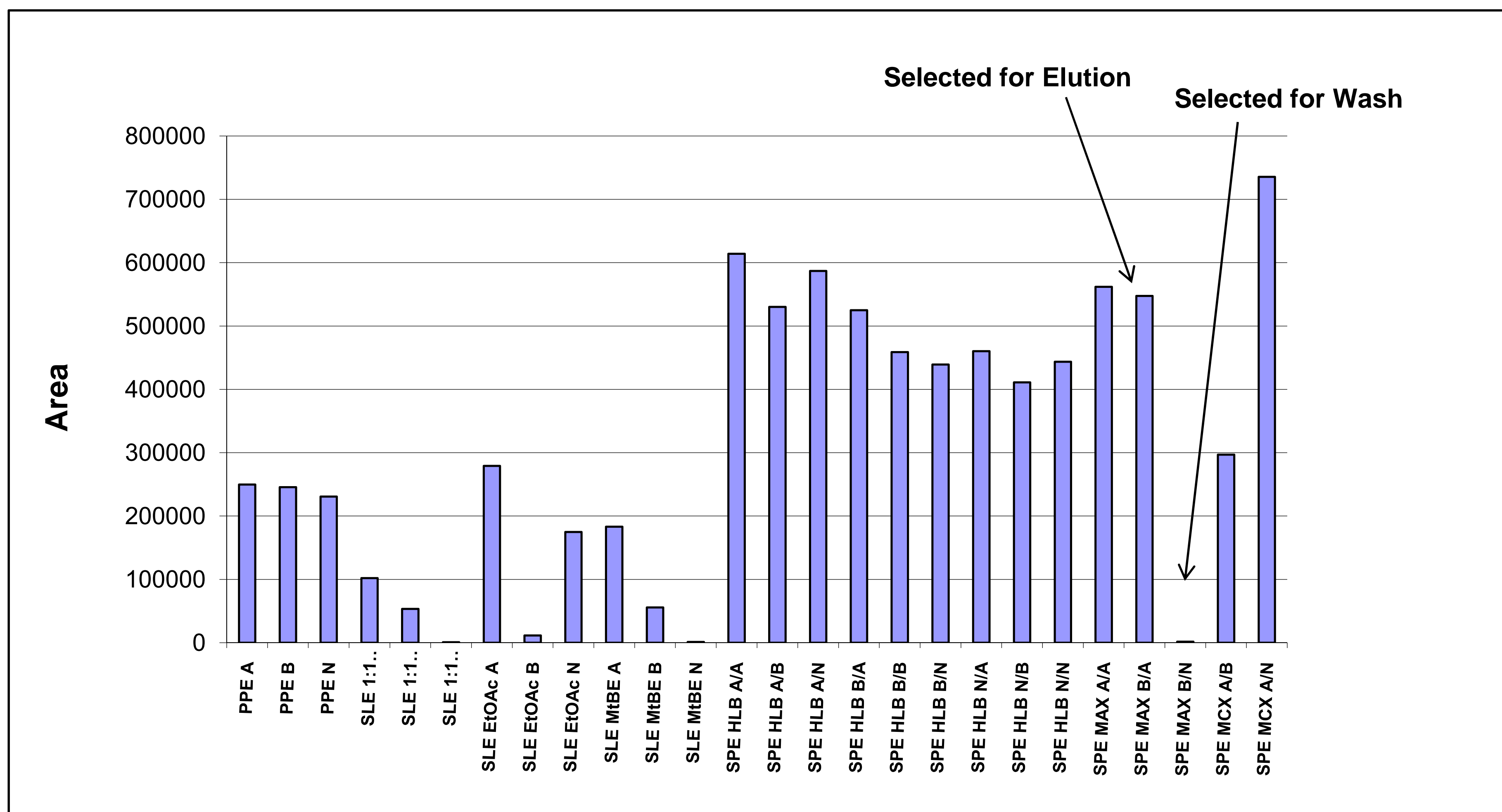
Case study: Fast MD for Guanfacine and Raltegravir



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Case study 2: Fast MD for Guanfacine and Raltegravir

- Day 2: SPE wash/elution optimization for Raltegravir
- Experiment design:
  - Test MAX plate for B/A condition
  - Screen 10% organic component increments
- Bracket optimum wash/elution conditions
  - Use strongest wash that doesn't elute analyte
  - Use weakest elution that completely elutes analyte
- Total samples: 44

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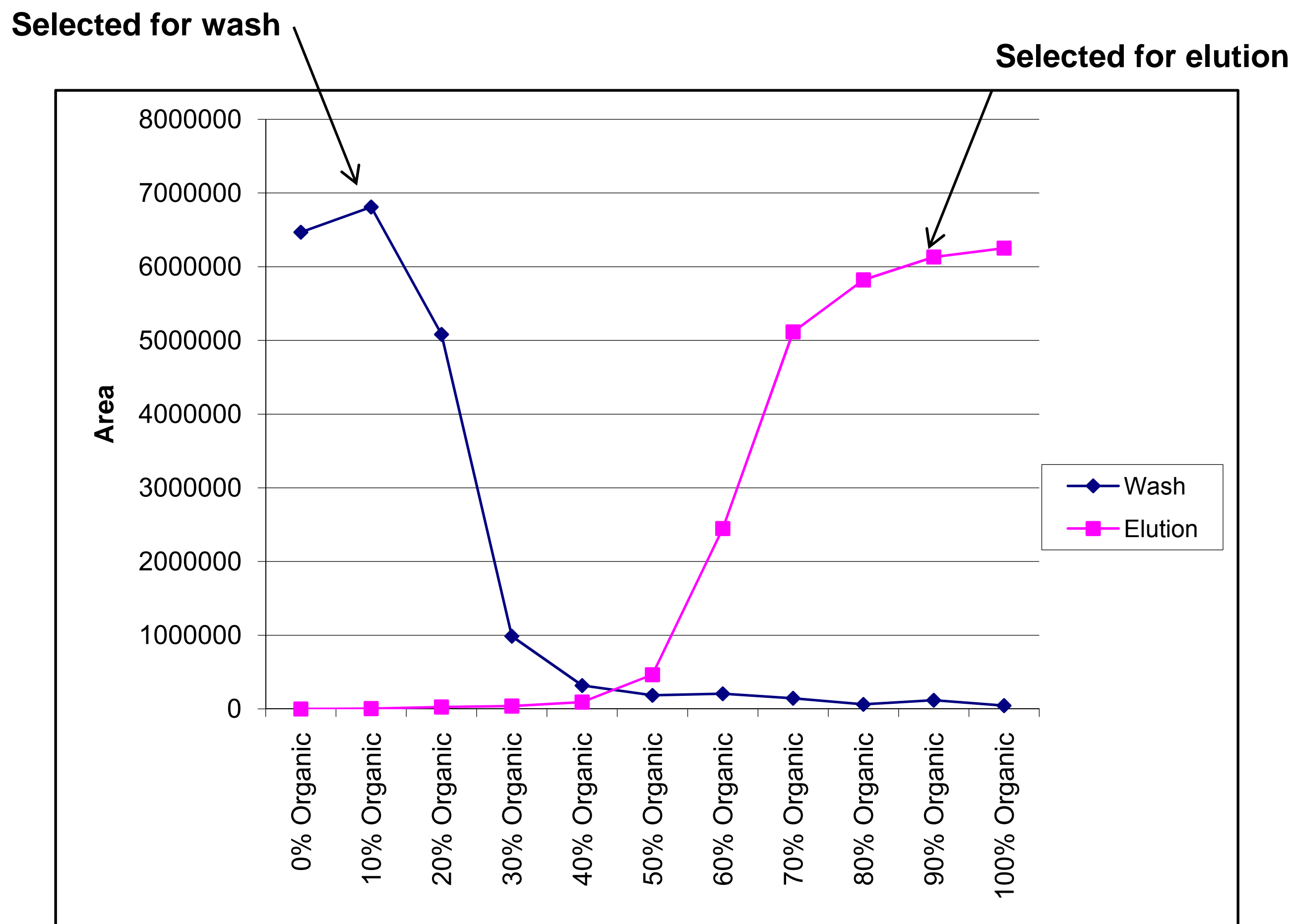
Case study 2–Fast MD for Guanfacine and Raltegravir



Standardization of LC/MS/MS development

- Standardize LC/AS cart
  - Add switching valve-diversion, backflush, trapping
- MS Tuning:
  - Tuning analyte under APCI/ESI, Neg/pos
  - Identify at least 2-3 SRM transitions
- LC column selection:
  - C18, phenyl-hexyl, Phenyl, HILIC, PFP (Pentaflourophenyl) column
  - HPLC vs. UHPLC

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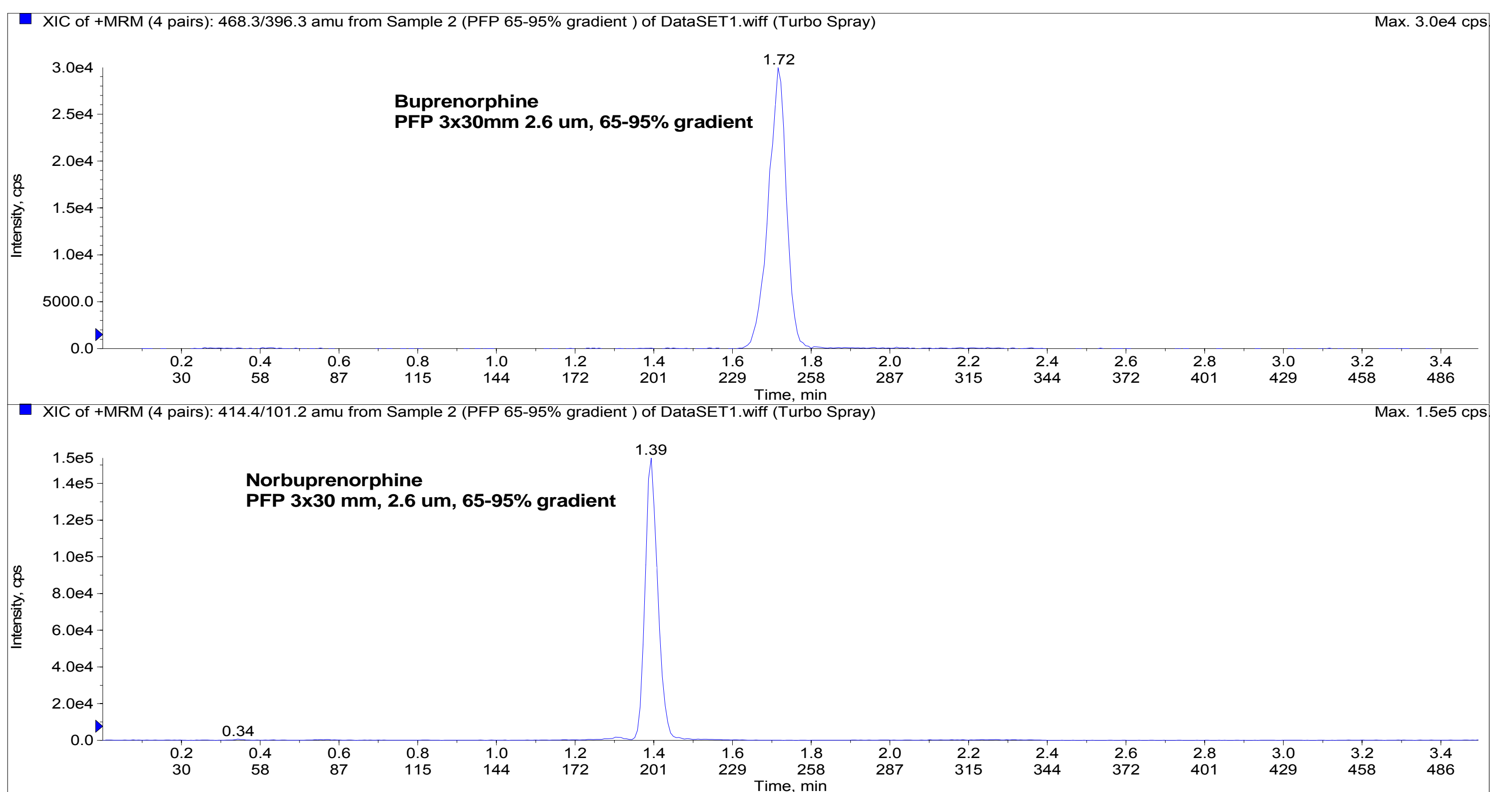
Case study 3 – PFP: Reversed vs. Normal Phase Mechanism

65-95% gradient – reversed phase chromatography



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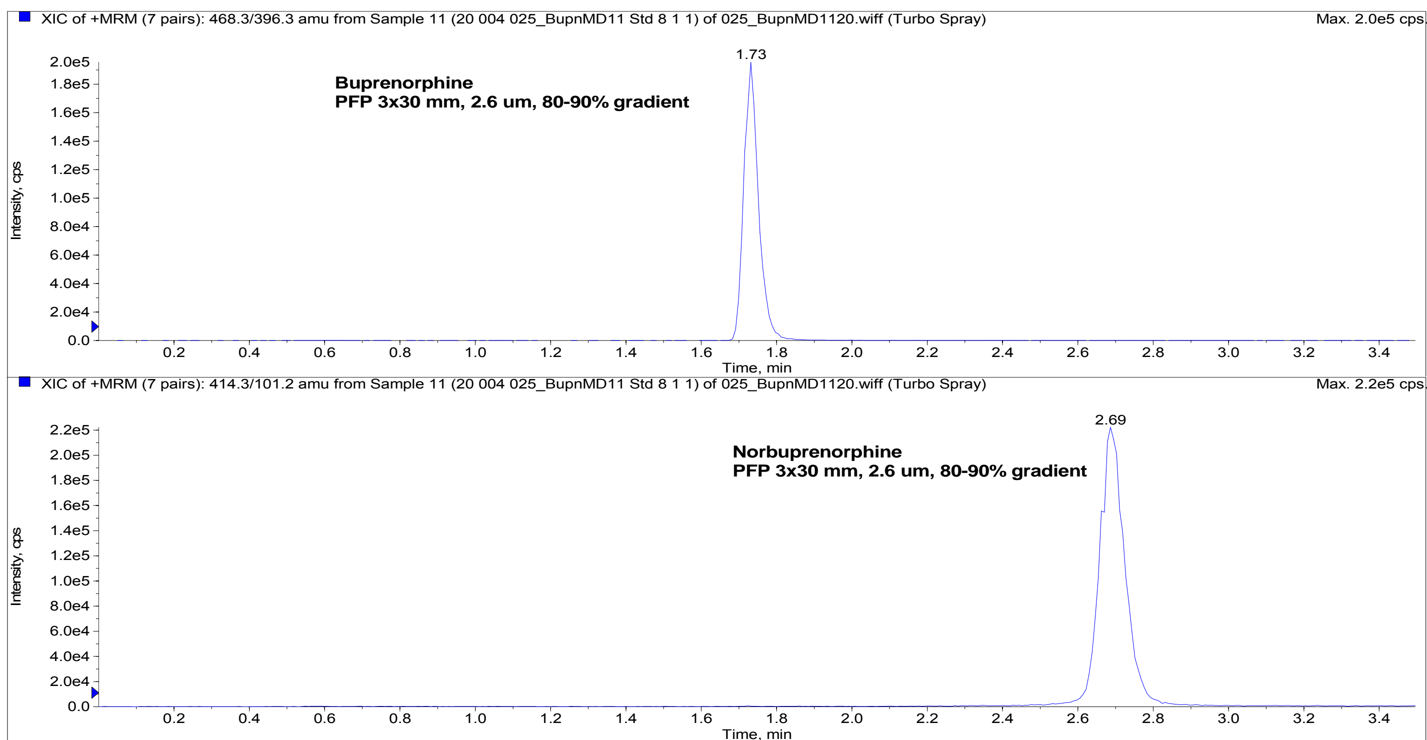
Case study 3 – PFP column: Reversed vs. normal phase

80-90% gradient – normal phase chromatography



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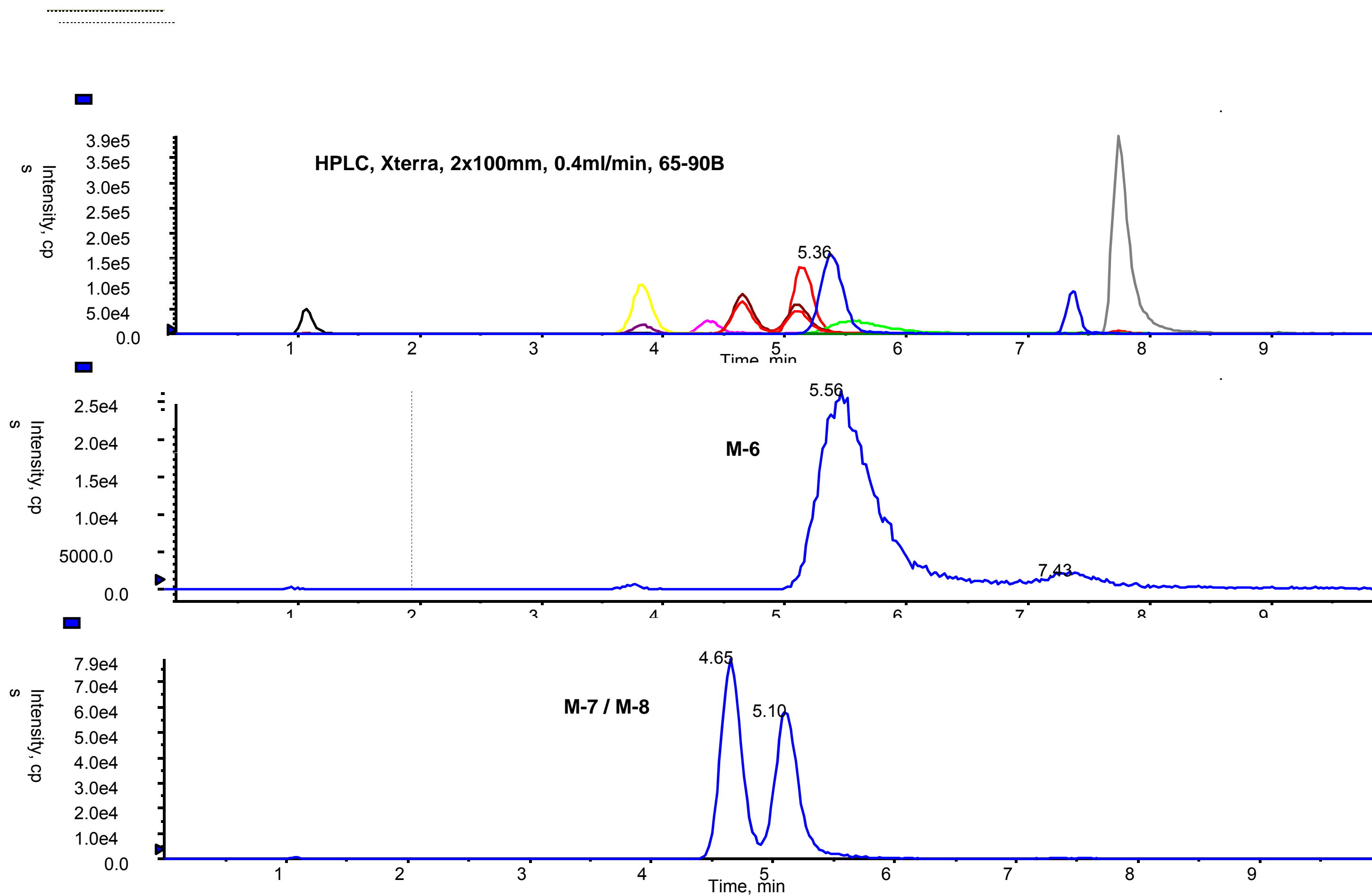
Case study 4: HPLC vs. UHPLC-Improving Resolution and Efficiency

HPLC, 10 minutes



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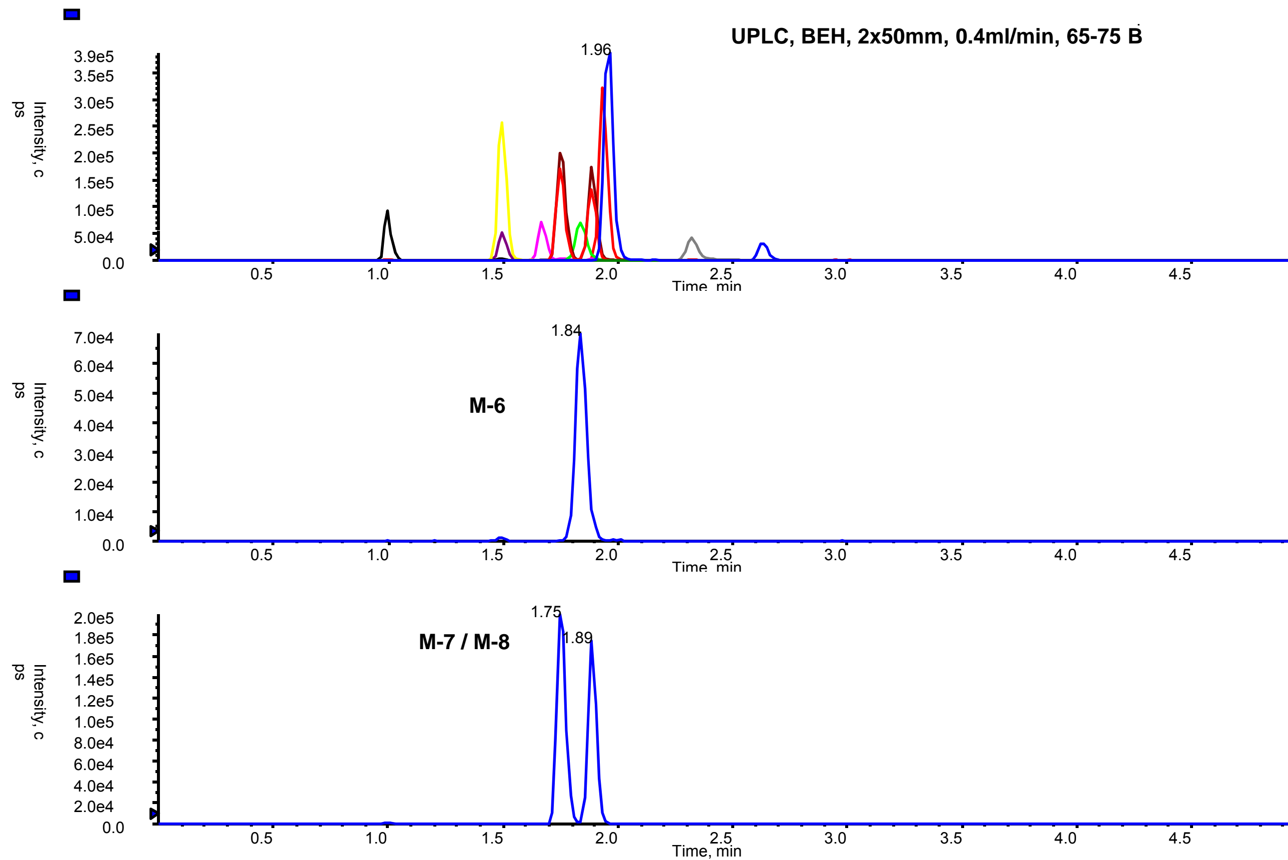
Case study 4: HPLC vs. UHPLC-Improving Resolution and Efficiency

UHPLC, 4.5 minutes



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Known Matrix Effectors

- Salt (problem in urine; L/L, SPE and turbulent flow remove)
- Proteins, peptides (most removed by all techniques)
- Phospholipids - “The Biggest Problem”
- Others
  - Other lipids (e.g., fatty acids – negative mode effects)
  - Dosing formulations
  - Unknowns...

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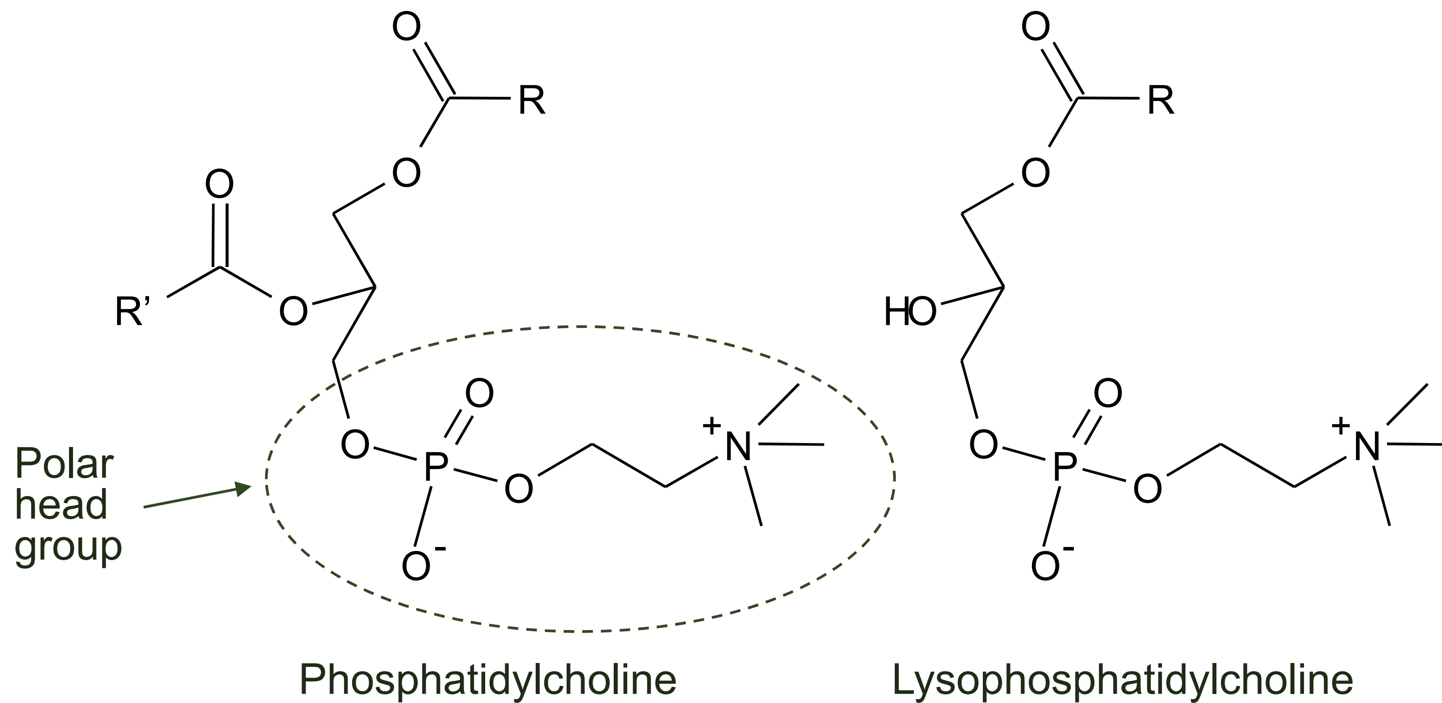
Phospholipids



R, R' = $C_{12} – C_{18}$

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Phospholipids Characteristics

- Ubiquitous (present in all species)
- High-levels (5-20 mg/ml in mammalian plasma)
- Chromatographically disperse; overall, relatively late-eluters in RP
- Accumulate on/bleed off LC column
- Surfactant behaviors– significant “matrix effectors”, especially in ESI/MS
- Matrix-variable (e.g., human diets)
- Unstable – degrade to fatty acids and head groups (effect on incurred sample repeat analyses)

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Managing phospholipids

- Monitor major PL transitions all the time:
  - 496, 524 → 184 (LysoPC)
  - 704,758,806 → 184 (PC)
- Standardize LC/AS cart with two switching valves
- Decide if need to set up:
  - Guard column trapping
  - Column backflush
  - Column forward flush
  - Diversion

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Case Study 5: Elution of phospholipids-forward flush

- Proprietary assay in human plasma (eight analytes and one IS)
- Protein precipitation sample preparation
- Issues: PL bleed
- Solution: LC column forward flush to remove phospholipids

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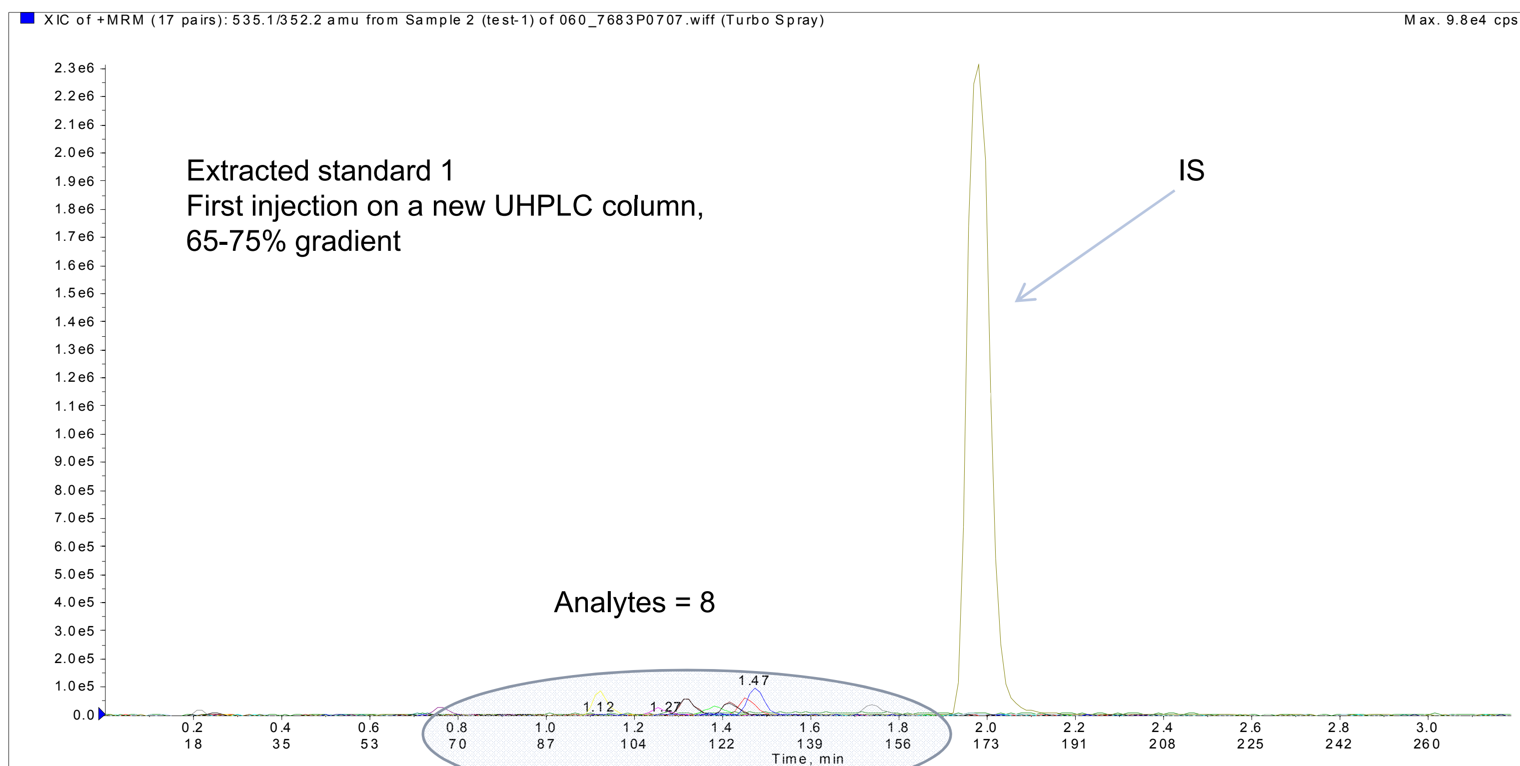
Case Study 5: Elution of phospholipids-forward flush

First injection on a brand new UHPLC column (65-75%)



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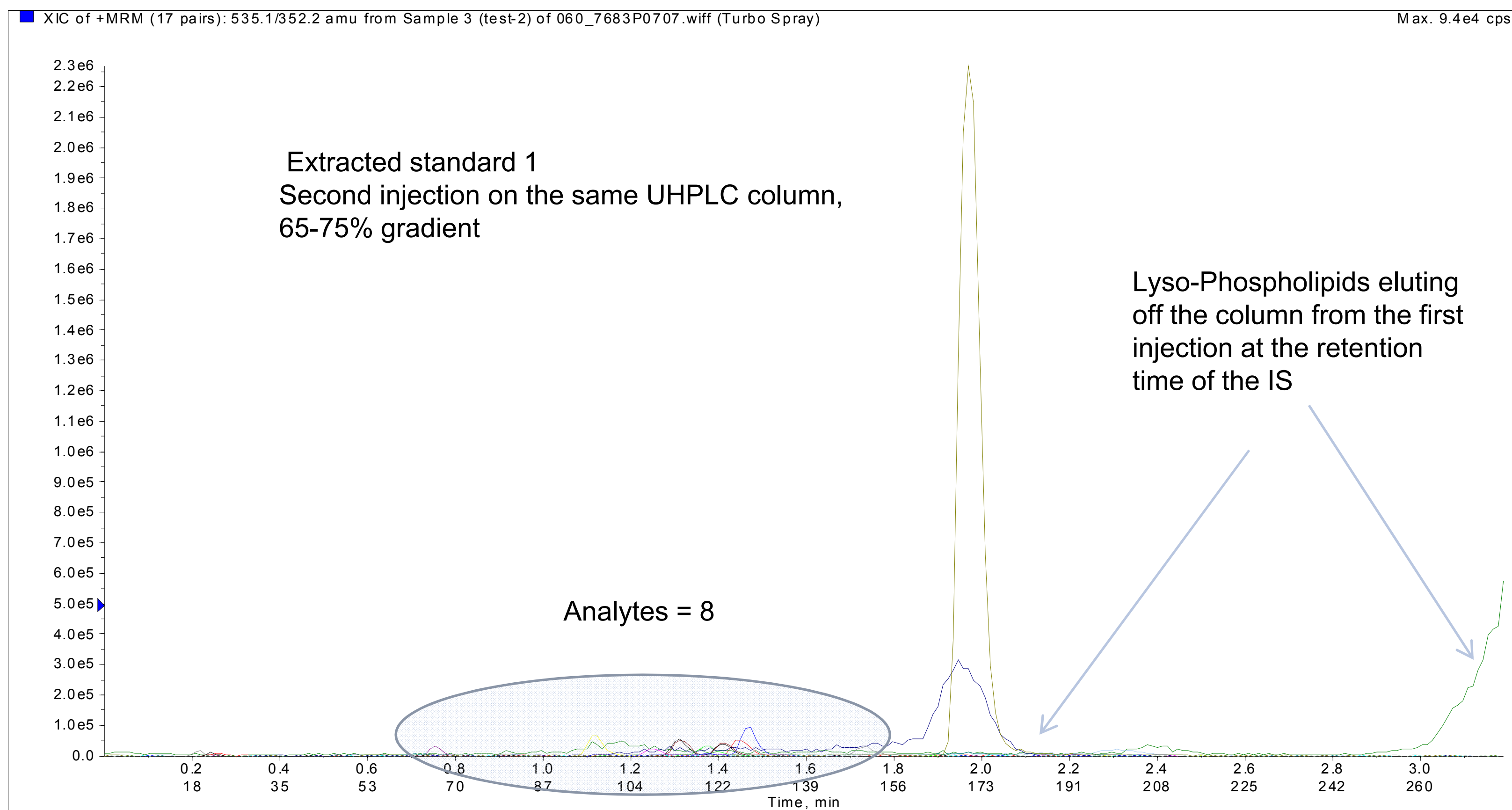
Case Study 5: Elution of phospholipids-forward flush

Second injection on the same column (65-75%)



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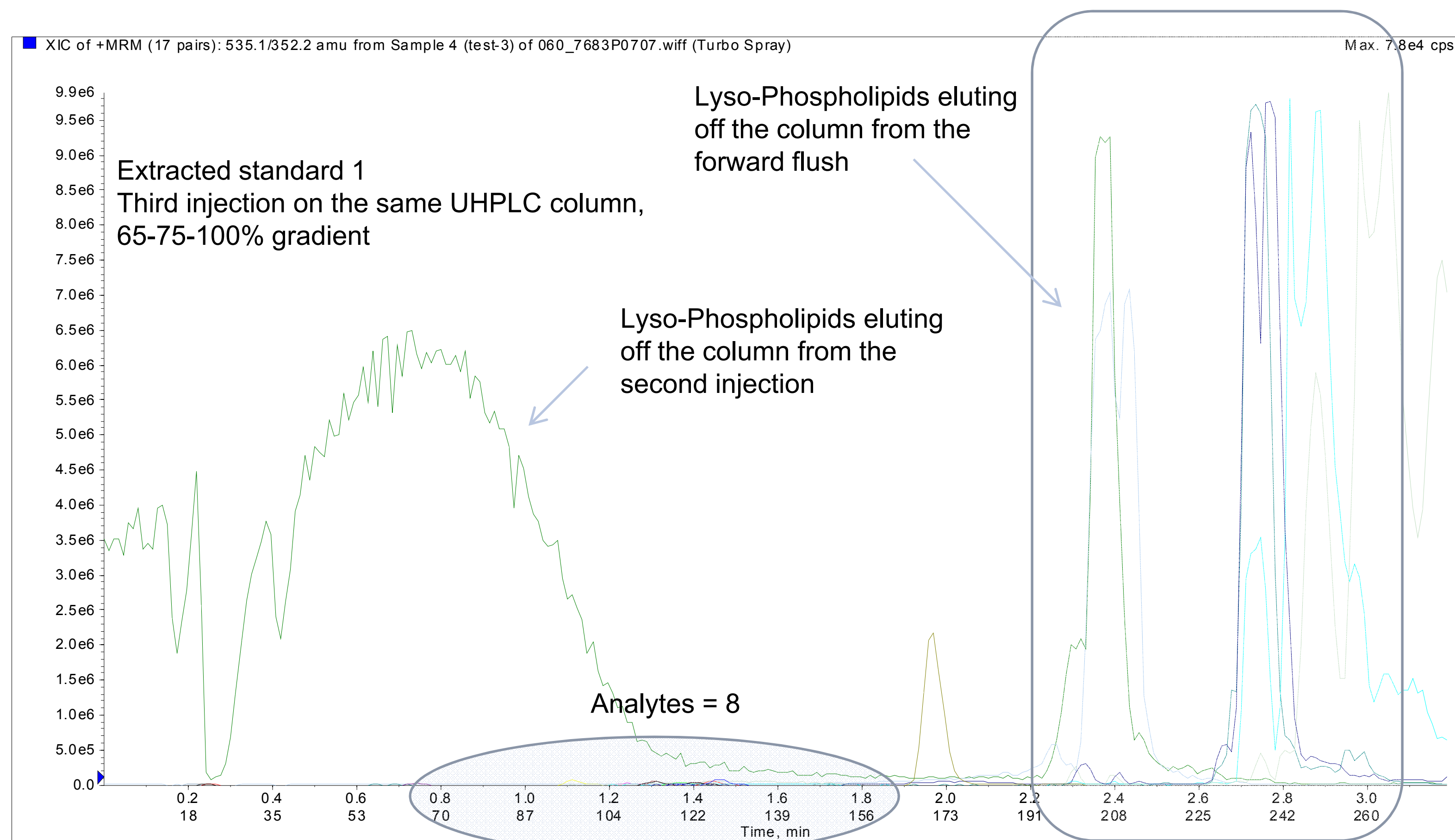
Case Study 5: Elution of phospholipids-forward flush

Third injection on the same column (65-75-100%)



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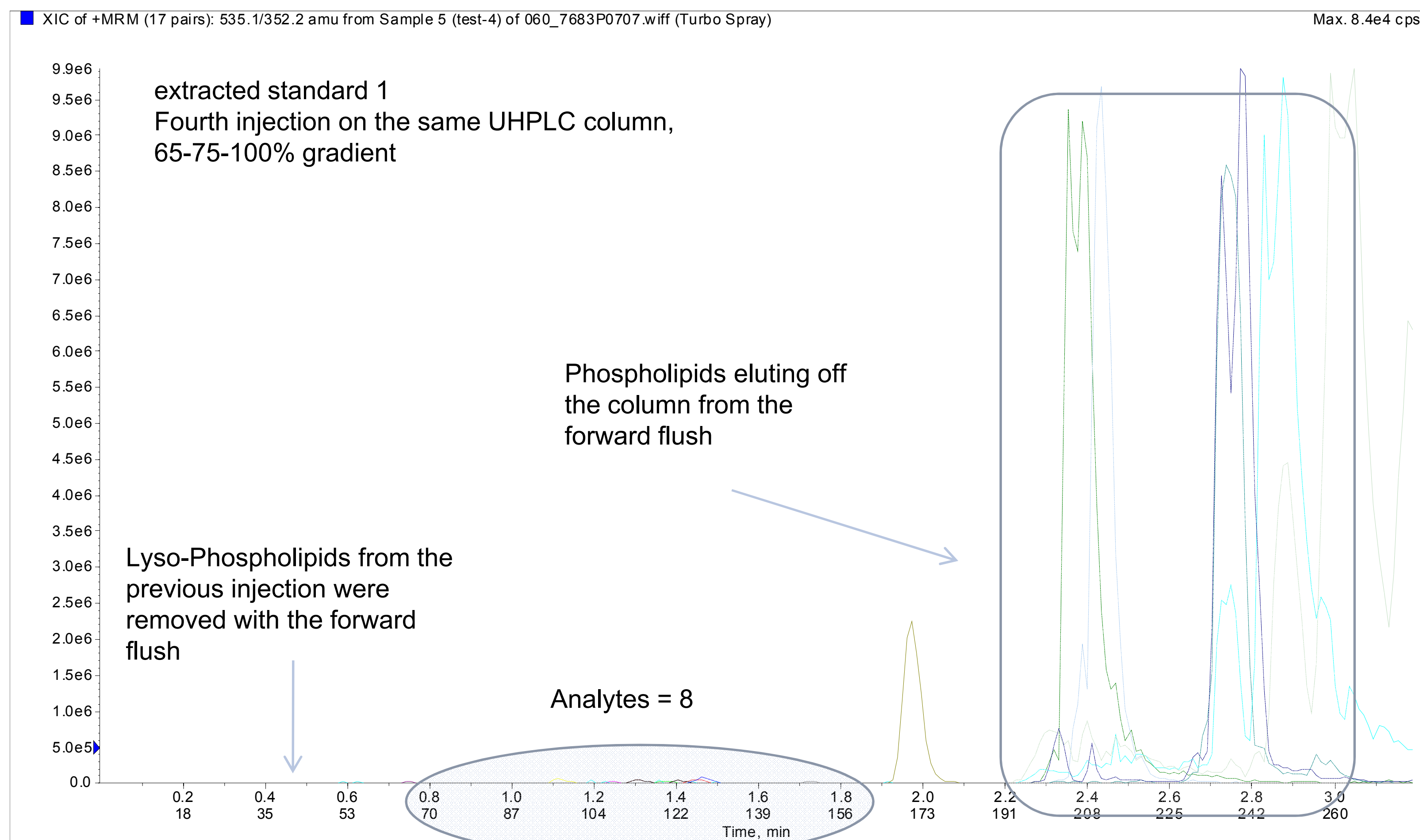
Case Study 5: Elution of phospholipids-forward flush

Fourth injection on the same column (65-75-100%)



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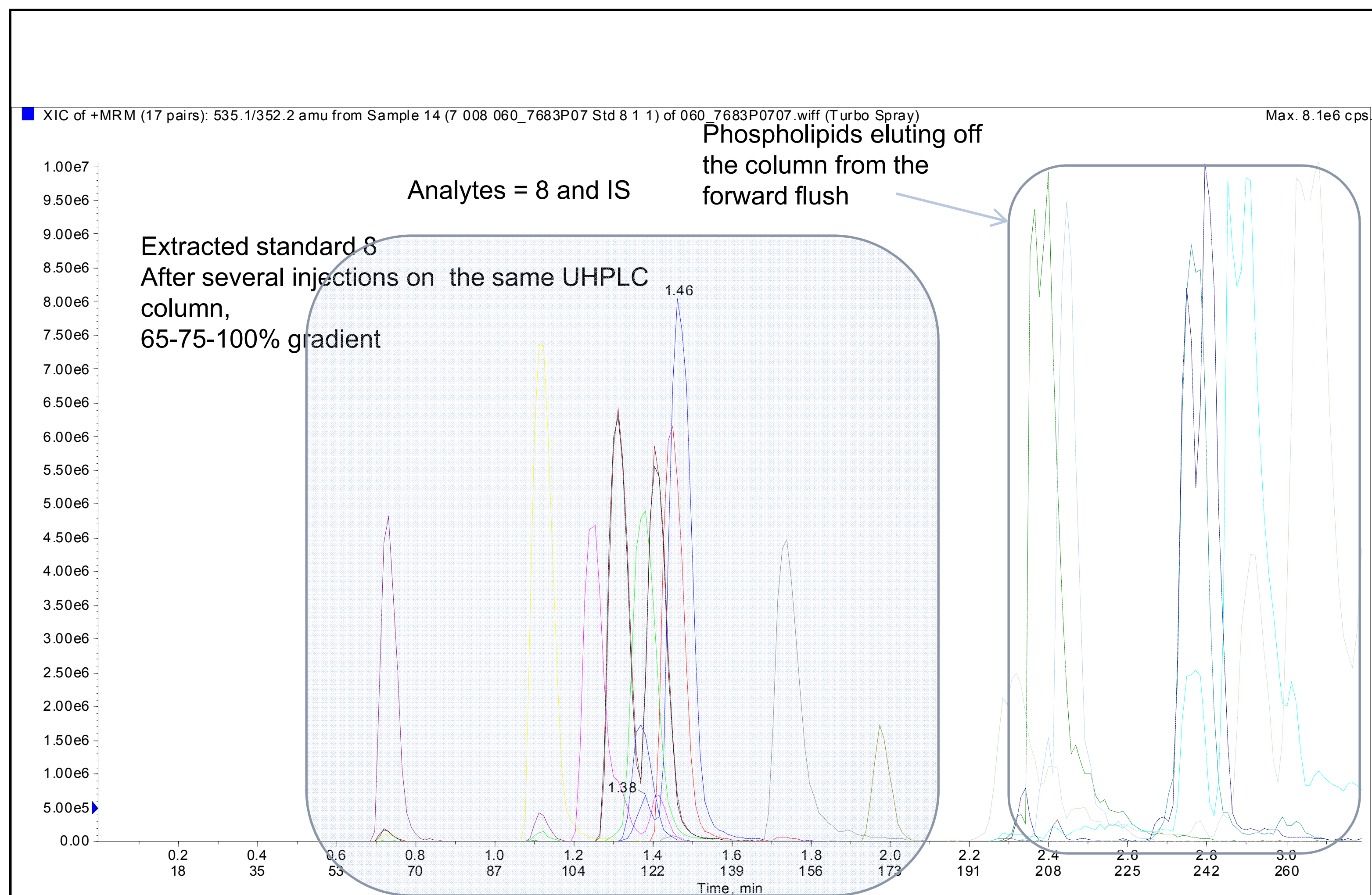
Case Study 5: Elution of phospholipids-forward flush

Final condition, High Std, 65-75-100%



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Case study 6: Splitting curve and increased PL background

- Midazolam and hydroxymidazolam in human plasma
- Protein precipitation sample preparation
- Issues: Splitting curve and raised PL background
- Solution: LC column backflush to remove phospholipids (10:90 water/acetone)

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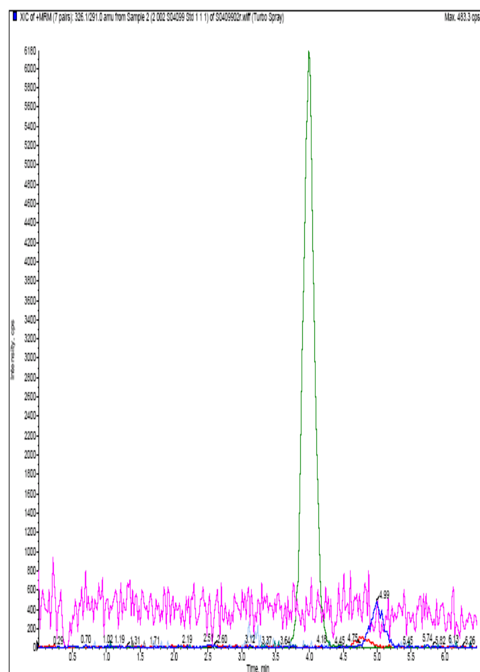
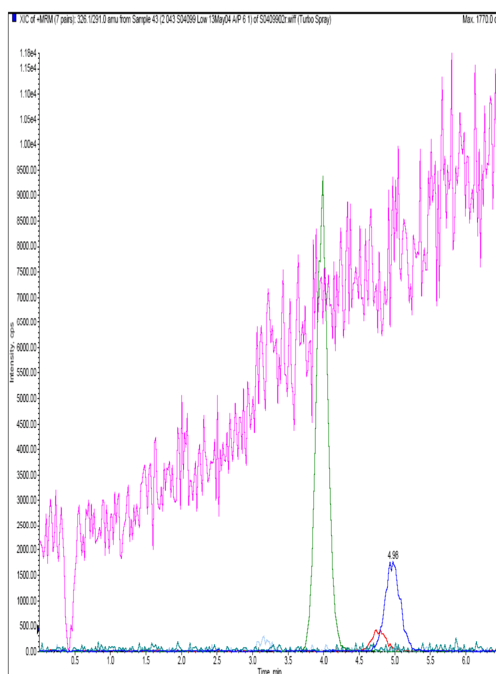
Case study 6-Splitting Curve and Raised PL Background

No column backflush

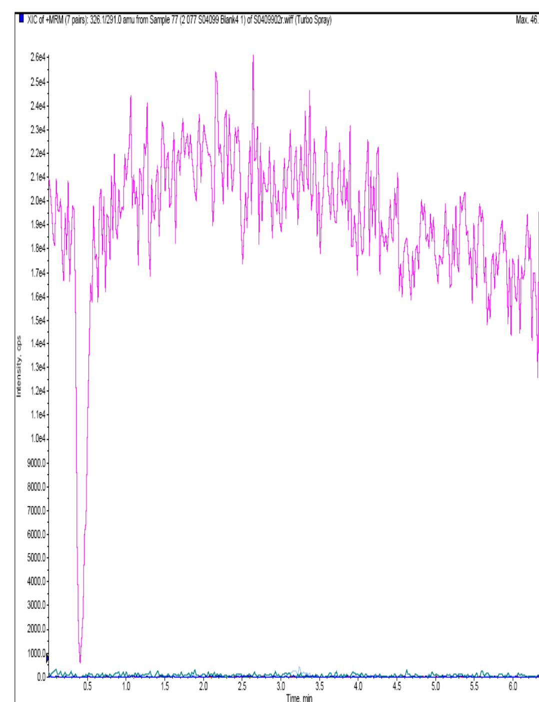
Sample #1



Sample #47



Sample #77



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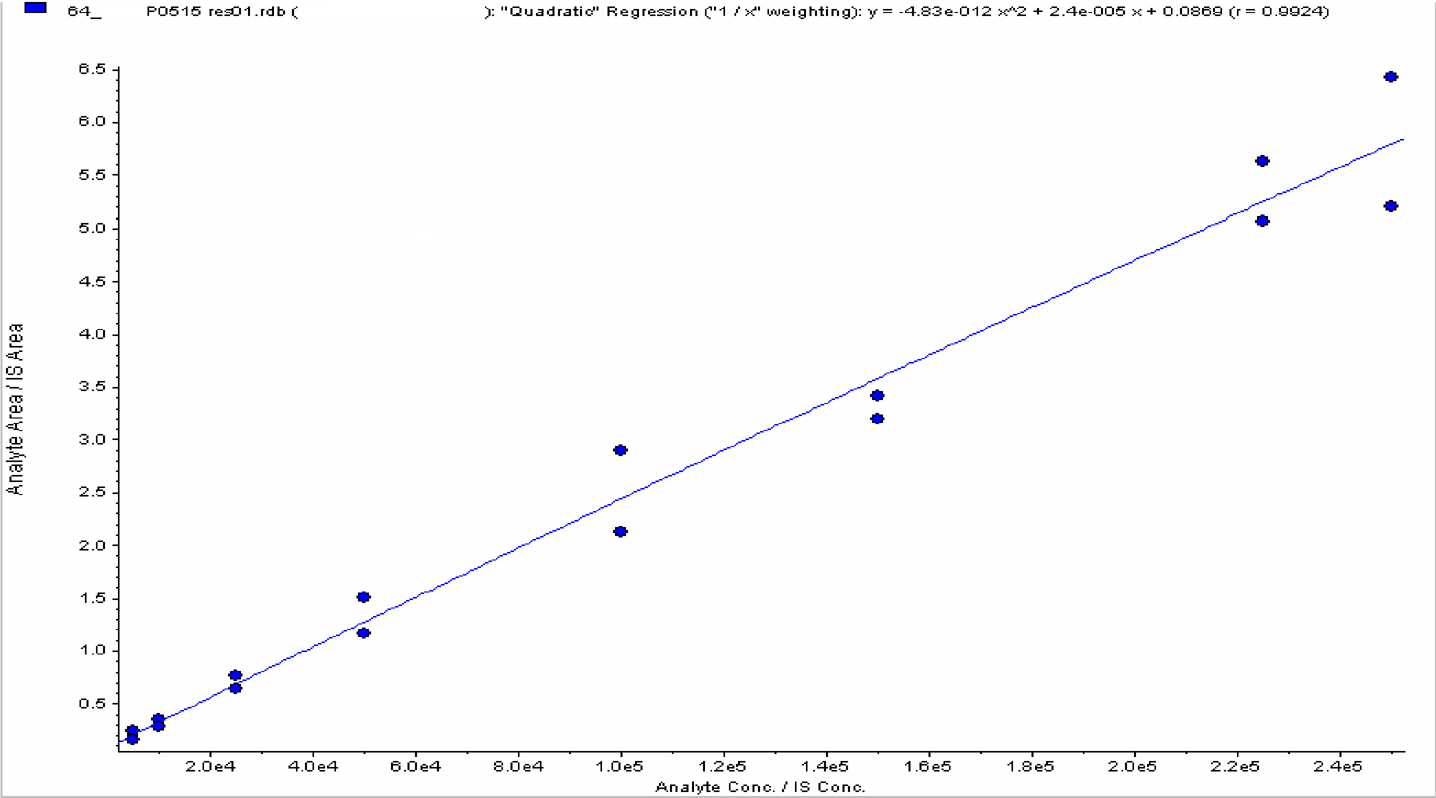
Case study 6-Splitting Curve and Raised PL Background

No column backflush



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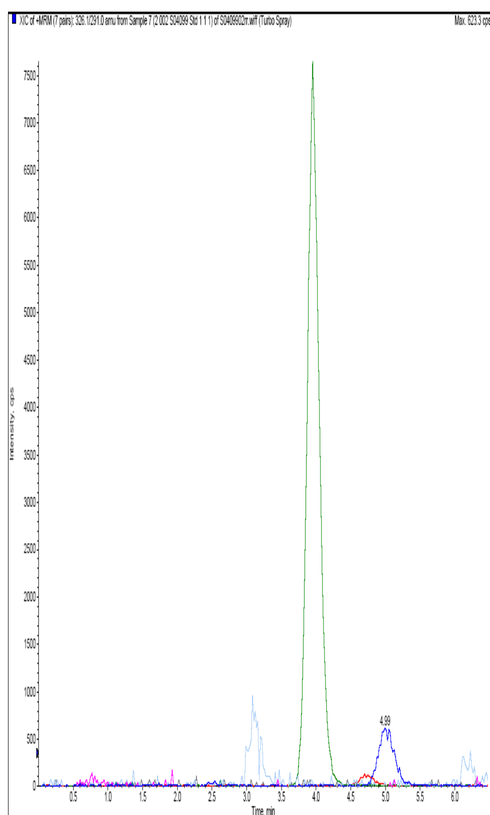
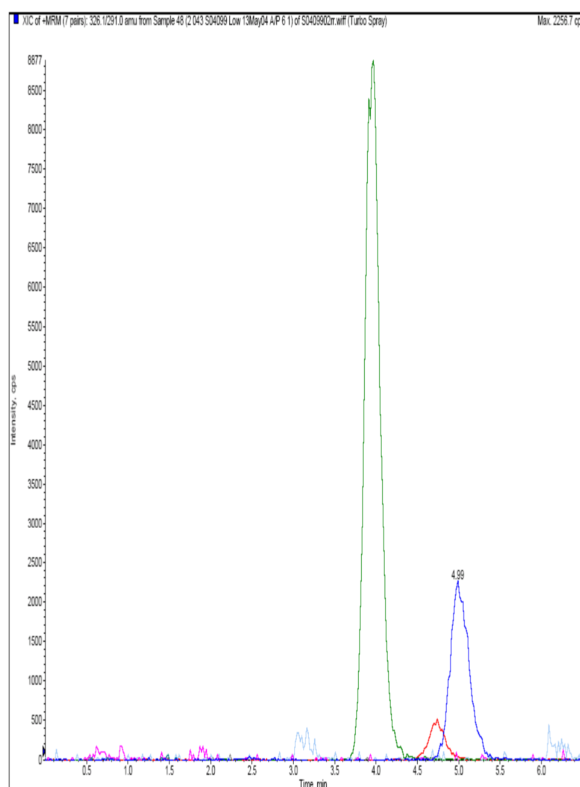
Case study 6-Splitting Curve and Raised PL Background

With column backflush

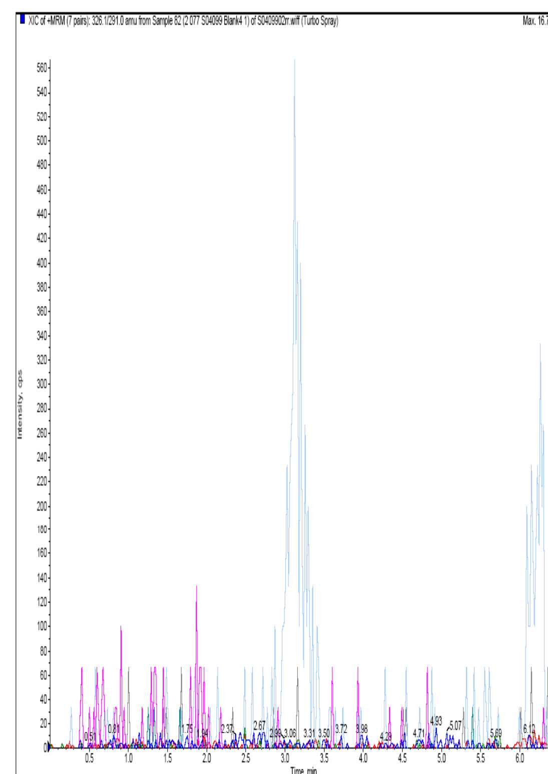
Sample #1



Sample #44



Sample #77



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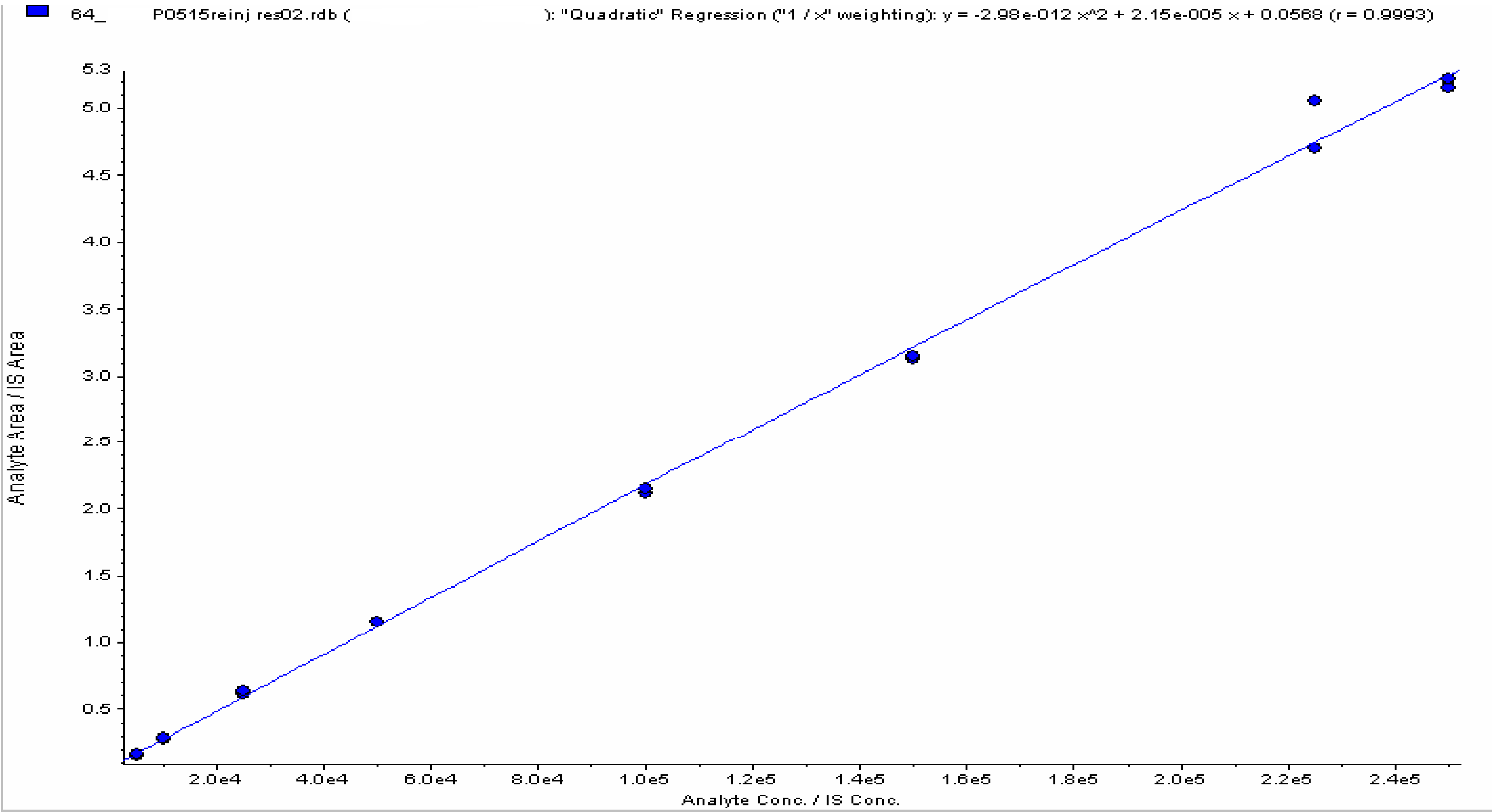
Case study 5-Splitting Curve and Raised PL Background

With column backflush



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Evaluate linearity, carryover and stress test

Linearity:

- APCI is preferred over ESI
- Narrow linear range if possible

Carryover:

- Use isocratic or shallow gradient if possible
- Identify if injector Vs. LC column carryover

Stress test:

- Evaluate linear range at 2x below target LLOQ and 2x above ULOQ
- Inject test batch consecutively at 5µL, 10µL, 20µL or 30µL

Documentation and Compliance

- Regulated bioanalysis involves in multiple personnel and department.
- It is important to capture of all information for each critical stage of process.
- Utilization of three-tiered checklists:
  - Pre-method development (Pre-MD) checklist
  - Pre-method validation (Pre-MV) checklist
  - Post-method validation (Post-MV) checklist

Pre-MD Checklist

Gather information:

- Species, anticoagulant, aliquots, target LLOQ
- co-admin drugs, reference material info, ISTD info, metabolite info
- calibration range, previous methodology, stability, stickiness info
- If structure is available, use software or others to chemical-physical property
- Pay attention to specific functional groups

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Pre-MV Checklist-Part 1

MD scientist self checklist

- Preemptive experiments
- Qualification batch with validation QCs
- Selectivity evaluation with 10 lots
- Carryover and linearity assessment
- Recovery evaluation
- Interference evaluation (analyte only and/or co-administered drug)
- Consecutive reinjection
- Phospholipid profile assessment

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Pre-MV Checklist-Part 2

MV Qualification data

- Chromatogram prints:
  - LLOQ (S/N should be > 10:1)
  - Carryover Blank (contribution< 20% of LLOQ)
  - Analyte Only (contribution < 5% of mean I.S.)
  - QC0 (contribution < 20% of LLOQ)
- Statistic prints:
  - Calibration curves
  - IS area Chart (Back to back injection)
  - Watson Std/QC from qualification run

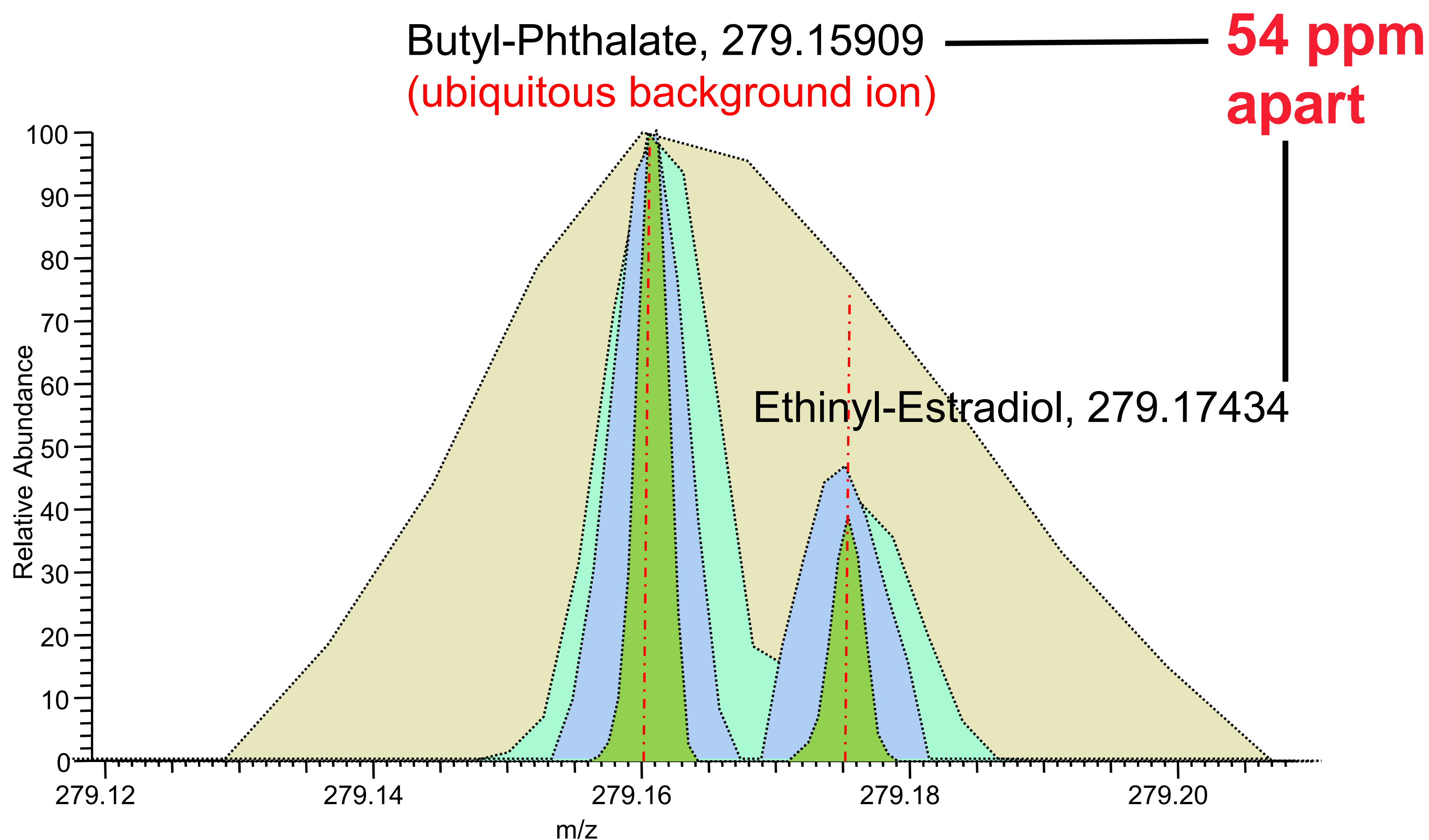
Conclusions

- Simple sample preparation and fast chromatography do not correlate to a robust and reliable bioanalytical method
- Protocol and screening driven MD approach allows rapid MD, less variability and more effective use of resources
- Phospholipids are important matrix effectors and monitoring them during MD is essential
- Proper documentation of all MD work is critical (for future MD, troubleshooting, auditing)

Next Generation Methods -HRMS

- Q Exactive – HRMS
  - Simpler method development
    - tSIM, Full Scan MS, SRM
  - Greater confidence in data - specificity
  - Troubleshooting for triple quadrupole based methods
    - Matrix effects
    - Metabolites
    - Co-eluting peaks
  - Qual Quan workflows
    - Metabolites
    - Metabolite stability
    - Biomarkers
  - Large and small molecule quantitation

Specificity = Resolution + Mass Accuracy



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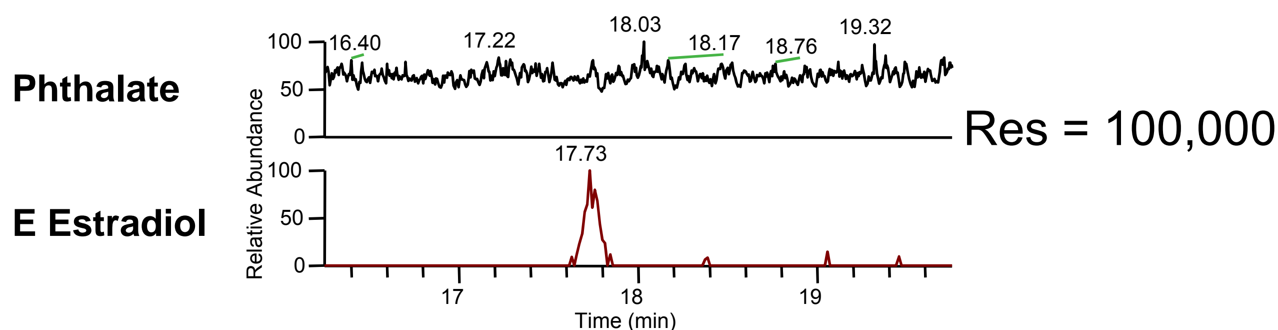
100 ppb Ethinyl-Estradiol – 100k vs 10 K Res

h Resolution and Mass Accuracy Esse

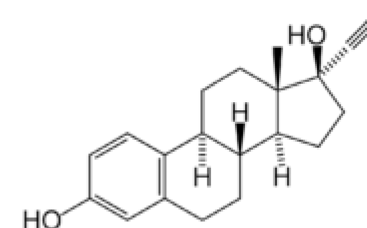
Ethinyl-Estradiol

Phthalate

E Estradiol

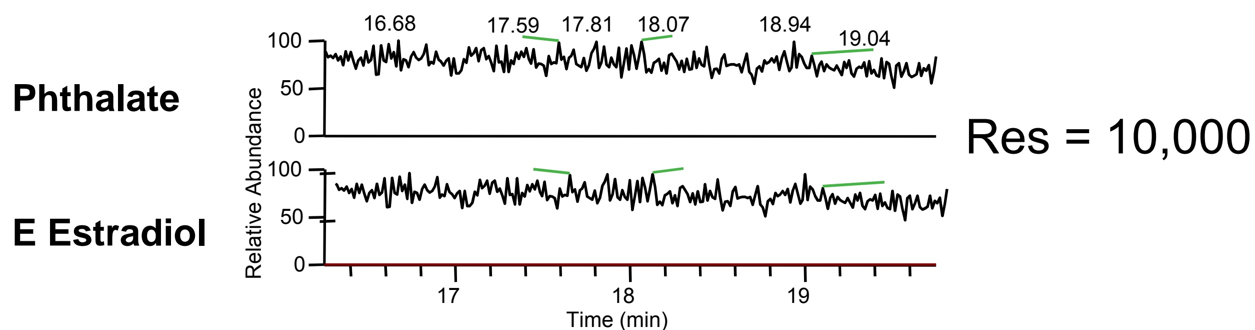


Res = 100,000



Phthalate

E Estradiol



Res = 10,000

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Summary Table

Compound	TSQ		QE Full Scan (70K)		QE tSIM (70K)	
	LOD	LLOQ	LOD	LLOQ	LOD	LLOQ
Oxycodone	1	**5**	5	10	1	**5**
Buprenorphine	50	100	50	**50**	50	**50**
Paroxetine	1	10	1	**5**	10	10
Ketoconazole	50	100	1	**50**	1	**50**
Clonazepam	1	**10**	5	50	10	10
Verapamil	1	**1**	5	5	1	5
Alprazolam	1	**5**	5	10	10	50
Reserpine	1	**10**	10	50	5	**10**
Clopidogrel	1	**5**	50	50	5	10

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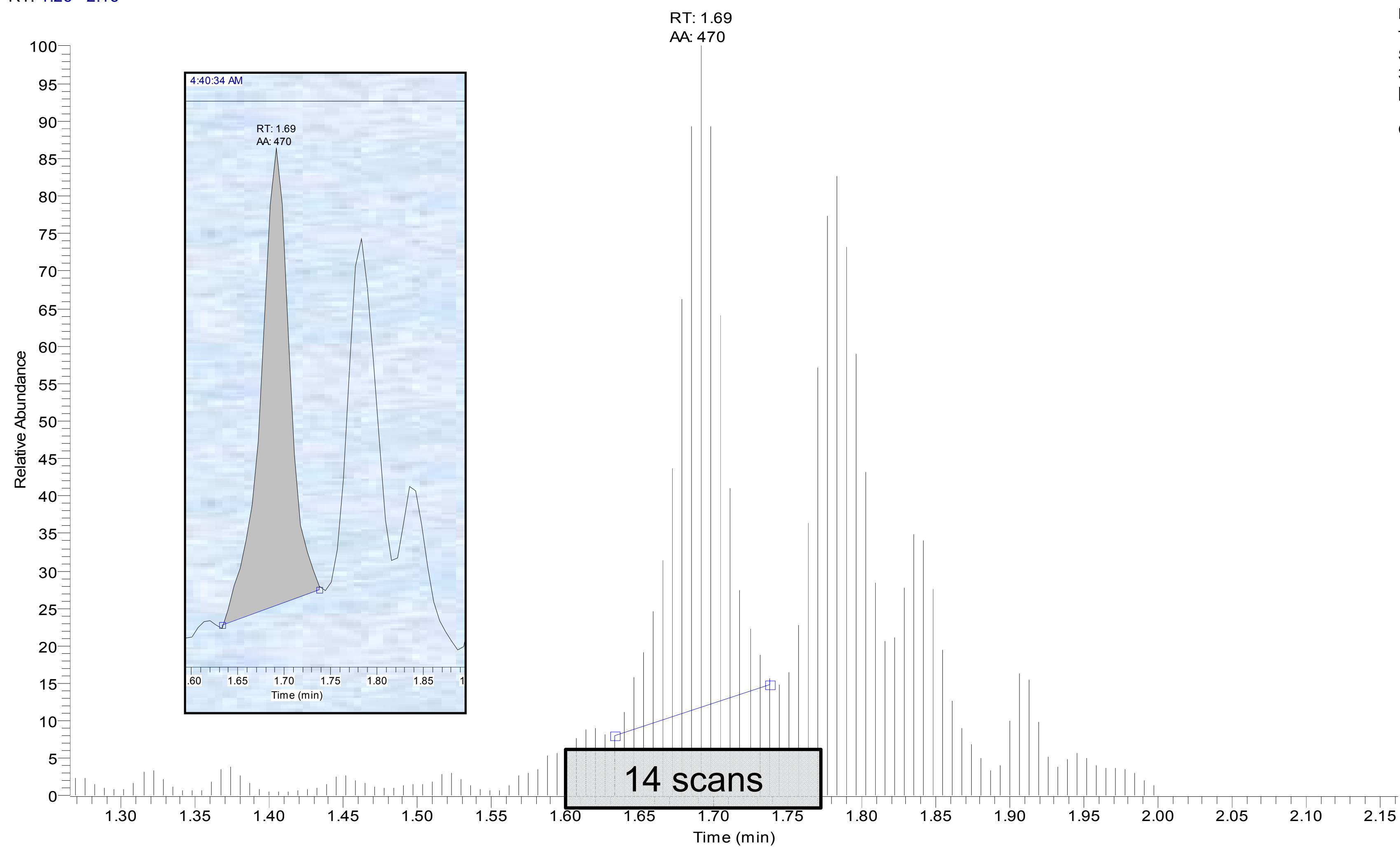
TSQ (SRM) Oxycodone LLOQ 5pg/mL



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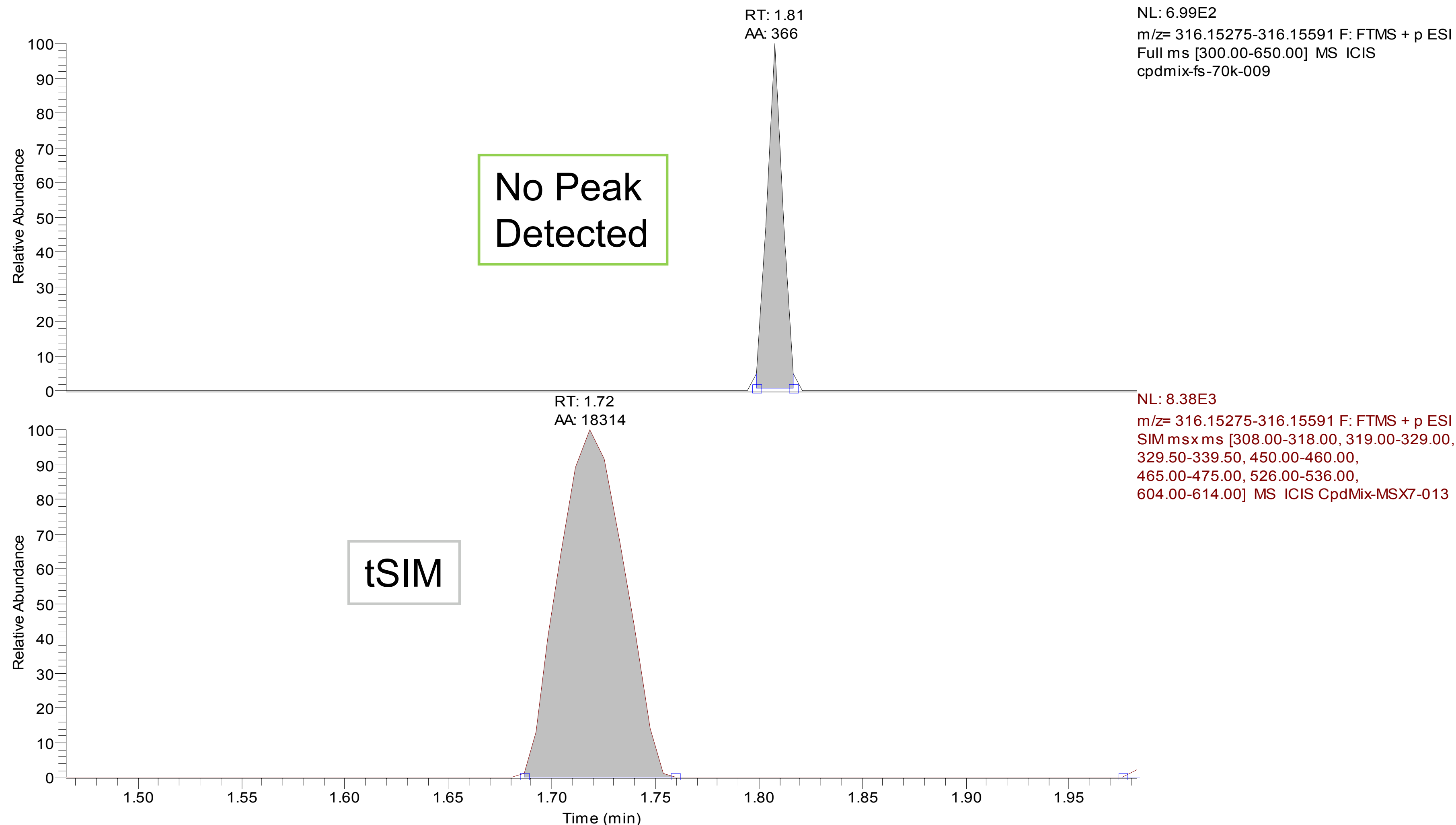
FS (5pg/mL) tSIM (5pg/mL) Comparison



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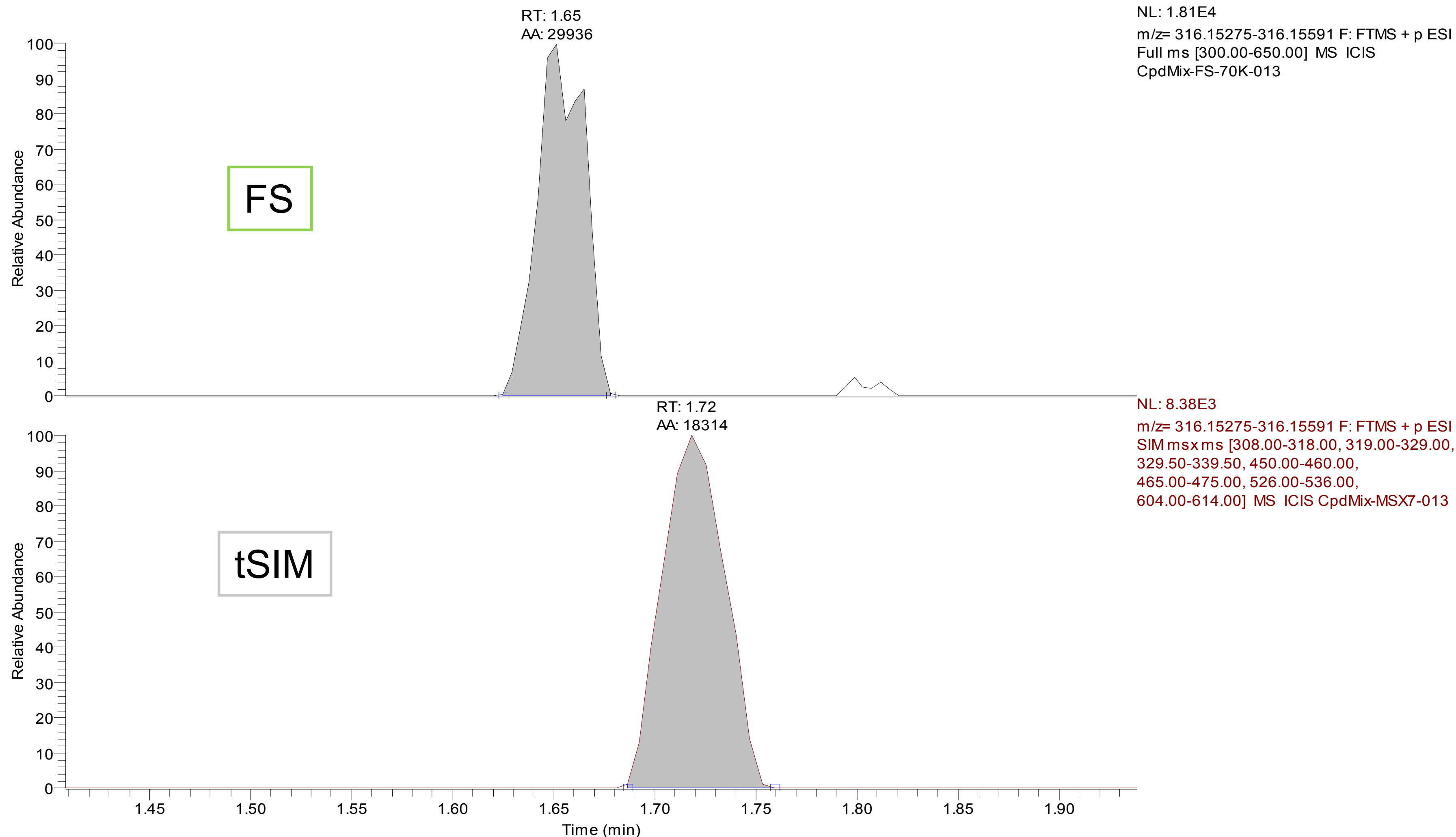
tSIM (5pg/mL) & FS (10pg/mL) Comparison



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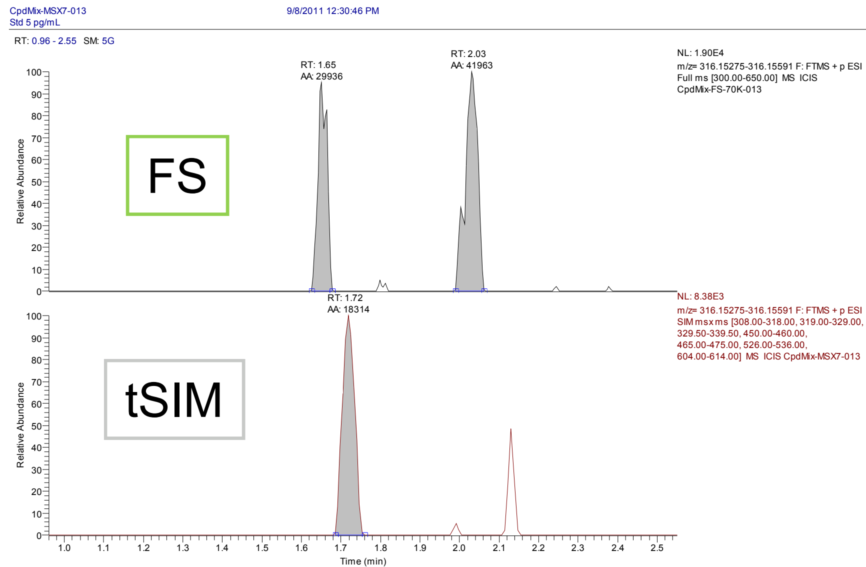
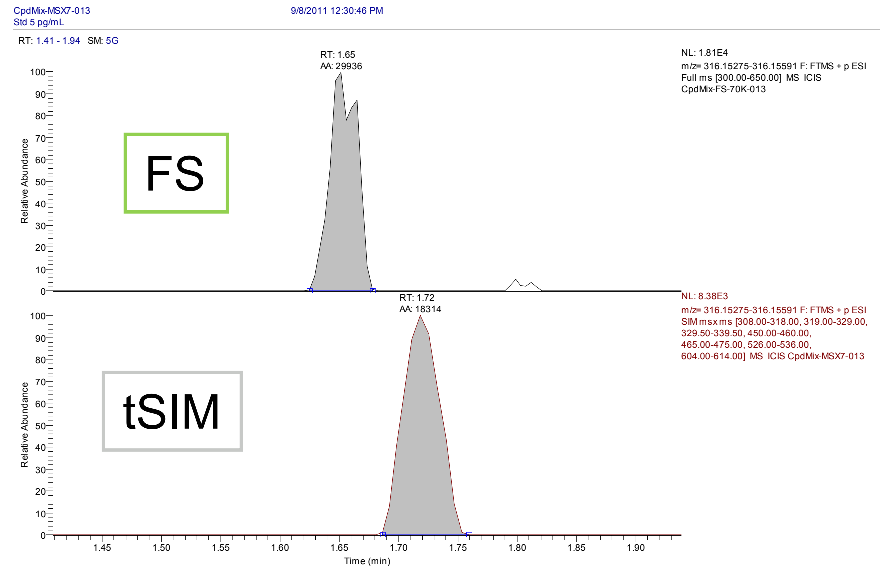
tSIM (5pg/mL) & FS (10pg/mL) Comparison





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