

Simple Method (IS-MRM) to Monitor Lysophospholipids and Phospholipids During LC-MS Method Development via In-Source CID

James Little, Eastman Chemical Company, Kingsport, TN

Overview

- Phospholipids and lysophospholipids yield matrix effects in LC-MS
- These lipids present at very high levels in plasma samples after protein precipitation
- We developed a simple method, "In-source Multiple Reaction Monitoring" (IS-MRM), to monitor their elution using m/z 184 (Q1) to 184 (Q3) at low collision cell energy
- 4-5 minute methods developed to separate basic drugs analytes from these lipids
- Unexpected results indicate the concentrations of these lipids become steady-state with time if not eluted from column

Introduction

Endogenous lysophospholipids and phospholipids are present at high levels in plasma samples. These lipids are known to cause matrix effects in electrospray LC-MS analyses. Thus, it is best to avoid the coelution of drug analytes with these components when developing LC-MS methods to support DMPK/ADME studies.

All phospholipids and lysophospholipids of interest yield an intense ion at m/z 184 for the trimethylammoniummethyl phosphate ion upon in-source collision induced dissociation (CID). We refer to this approach as IS-MRM. IS-MRM was used to develop chromatographic approaches that elute the phospholipids in reasonable retention times without coeluting them with the drugs of interest.

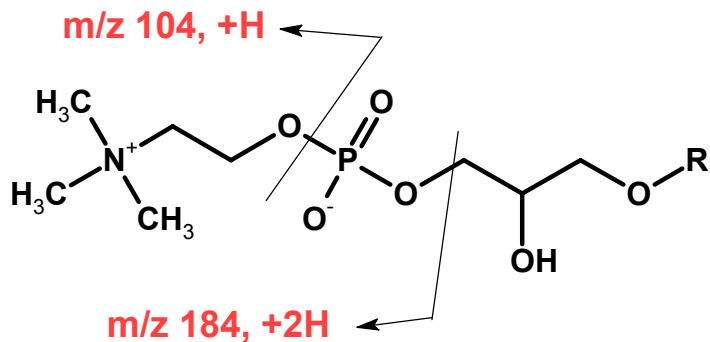
IS-MRM Experimental

- High energy *in-source* CID forms m/z 184 and 104 for lipids of interest, Q1 and Q3 set at *same* mass to pass lipid ions formed in source, collision cell at *low* energy to avoid further fragmentation
- Protein in 100 ul rat plasma precipitated with 250 ul acetonitrile
- Samples centrifuged at 1800 x g in 96 well plates, inject from *above* protein pellet
- MRM mode on Quattro Micro with Acquity HPLC, 90 V in-source voltage for lipids, 3×10^{-3} mbar Ar, 5 V collision cell energy
- Solvents methanol and/or acetonitrile, water with 10 mmolar ammonium formate and varying amounts formic acid, post column addition of aqueous buffer to ionize phospholipids at 100% organic

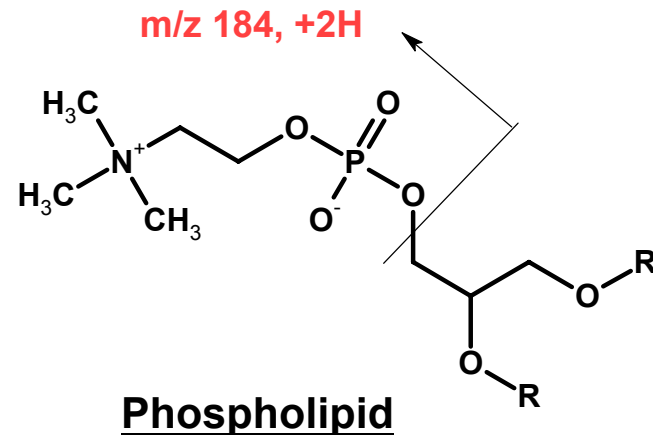
Variables Evaluated to Increase Analysis Speed and Resolution of Basic Analytes and Lipids of Interest

- **Temperature:** increasing temperature decreases analysis time
- **Solvent composition:** larger concentrations of methanol decreased analysis time versus acetonitrile
- **pH:** varying the concentration of formic acid in ammonium acetate buffer useful for resolving analytes, no effect on lipids of interest
- **Solvent strength:** organic increased to 100% immediately or slightly before elution of last analyte to speed elution of lipids
- **Solvent flowrate:** increased by factor of 3-4 slightly before last analyte eluted as increasing to 100% organic, flow diverted to waste

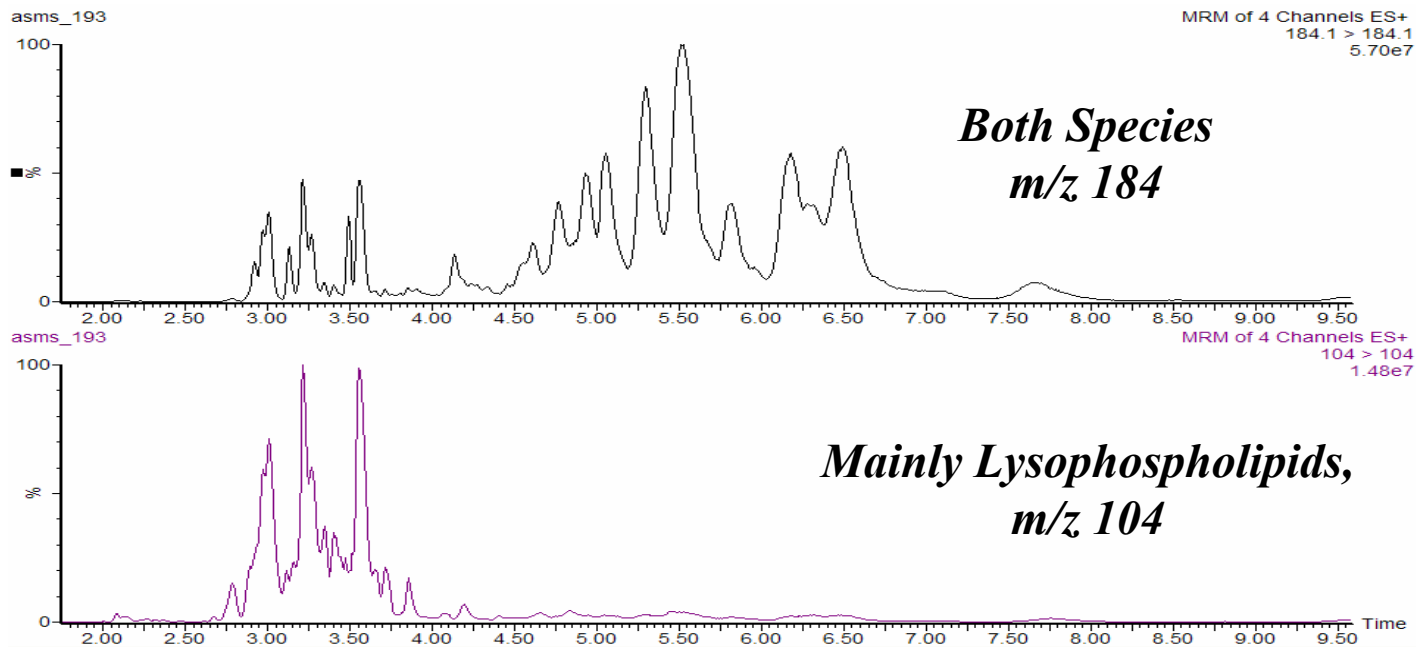
In-Source CID Ions for Lipids of Interest in Positive Ion



R=Fatty acid ester, e.g. C18:0, C18:1, C16:0, etc.

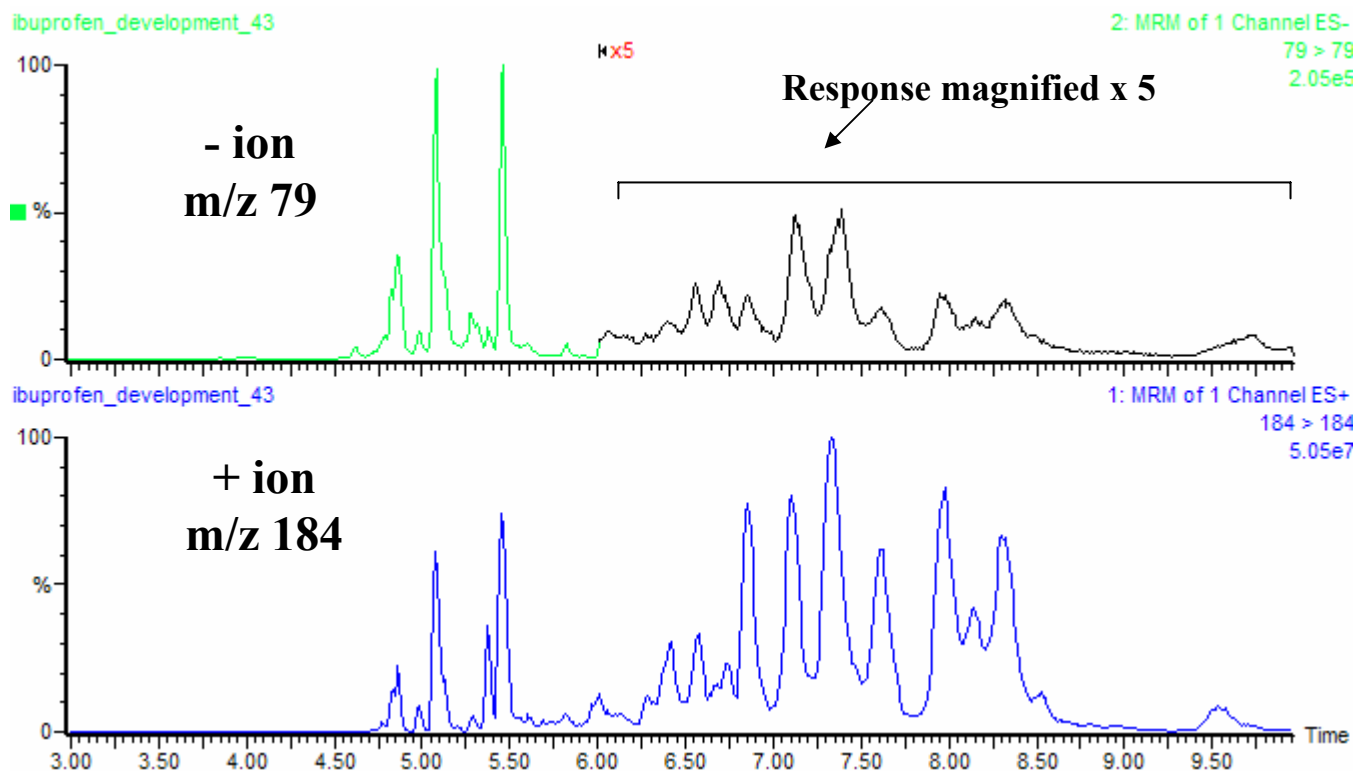


R=Fatty acid ester, e.g. C18:0, C18:1, C16:0, etc.

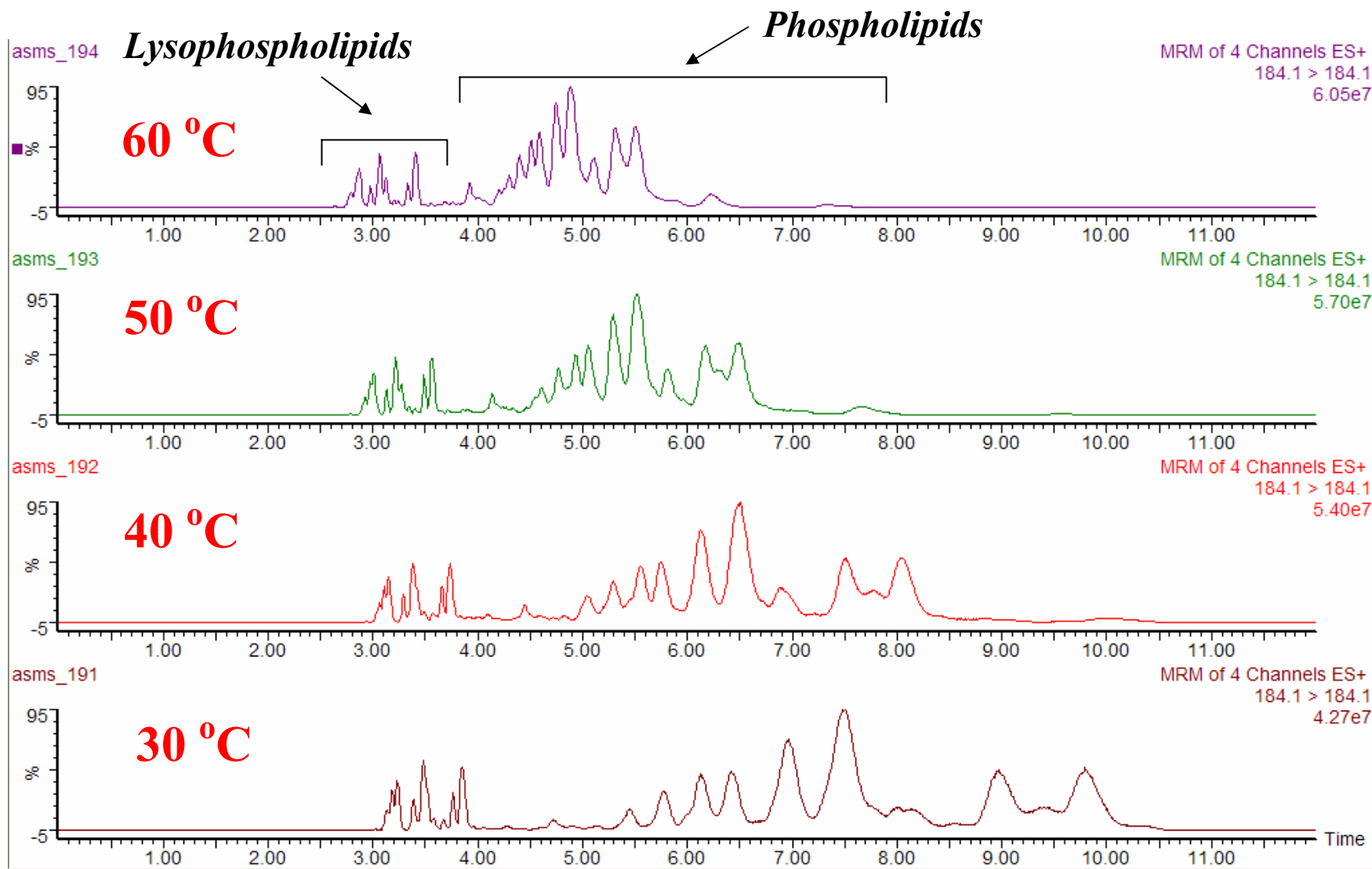


Lipid IS-MRM for m/z 184 in + Ion vs. m/z 79 in - Ion

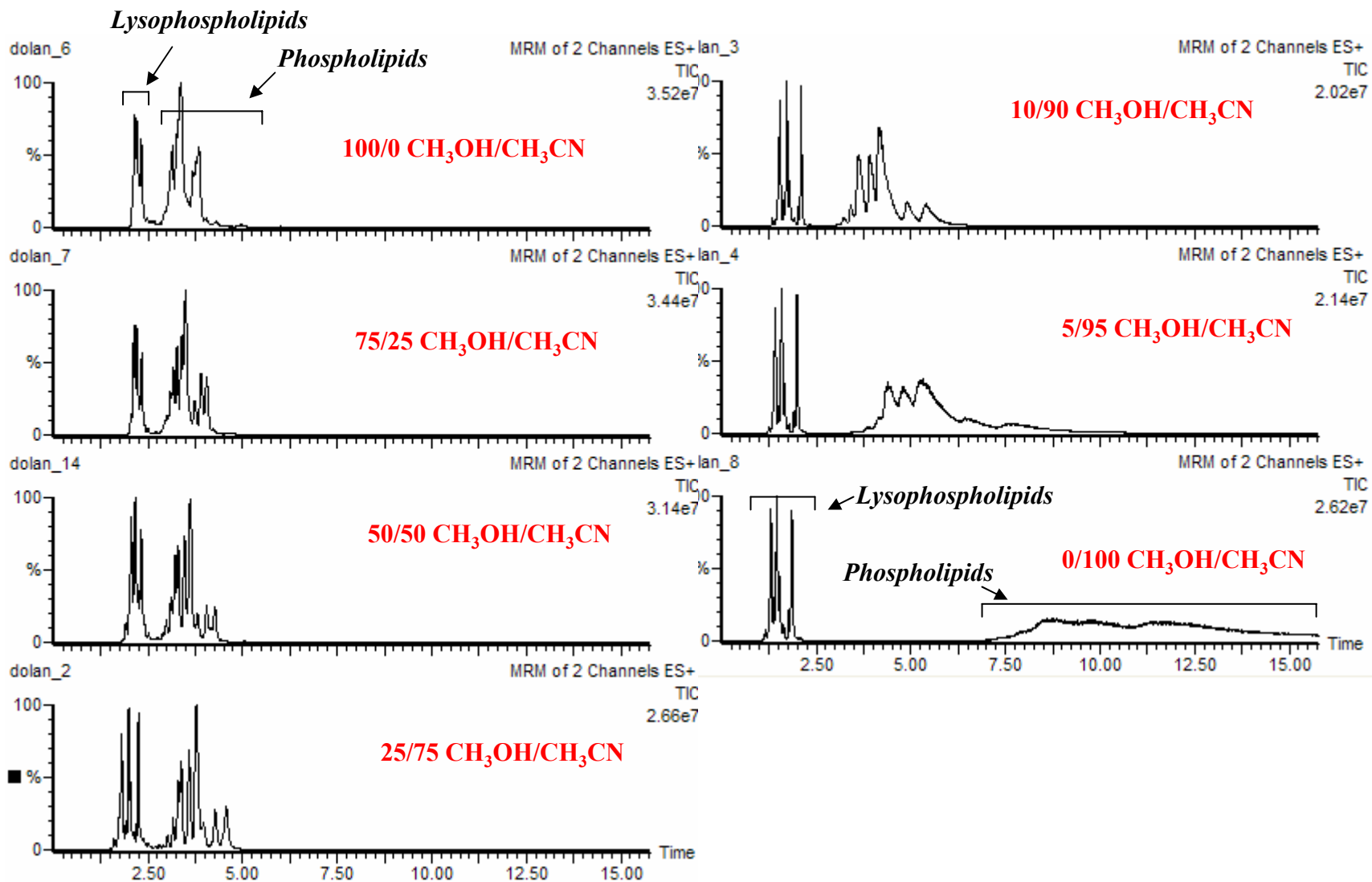
- Lipids monitored with m/z 79 (PO_3^-) by IS-MRM in negative ion mode
- Or can alternate between positive and negative ion mode for monitoring lipids and negatively ionized analytes
- Positive ion more generic for lysophospholipids and phospholipids



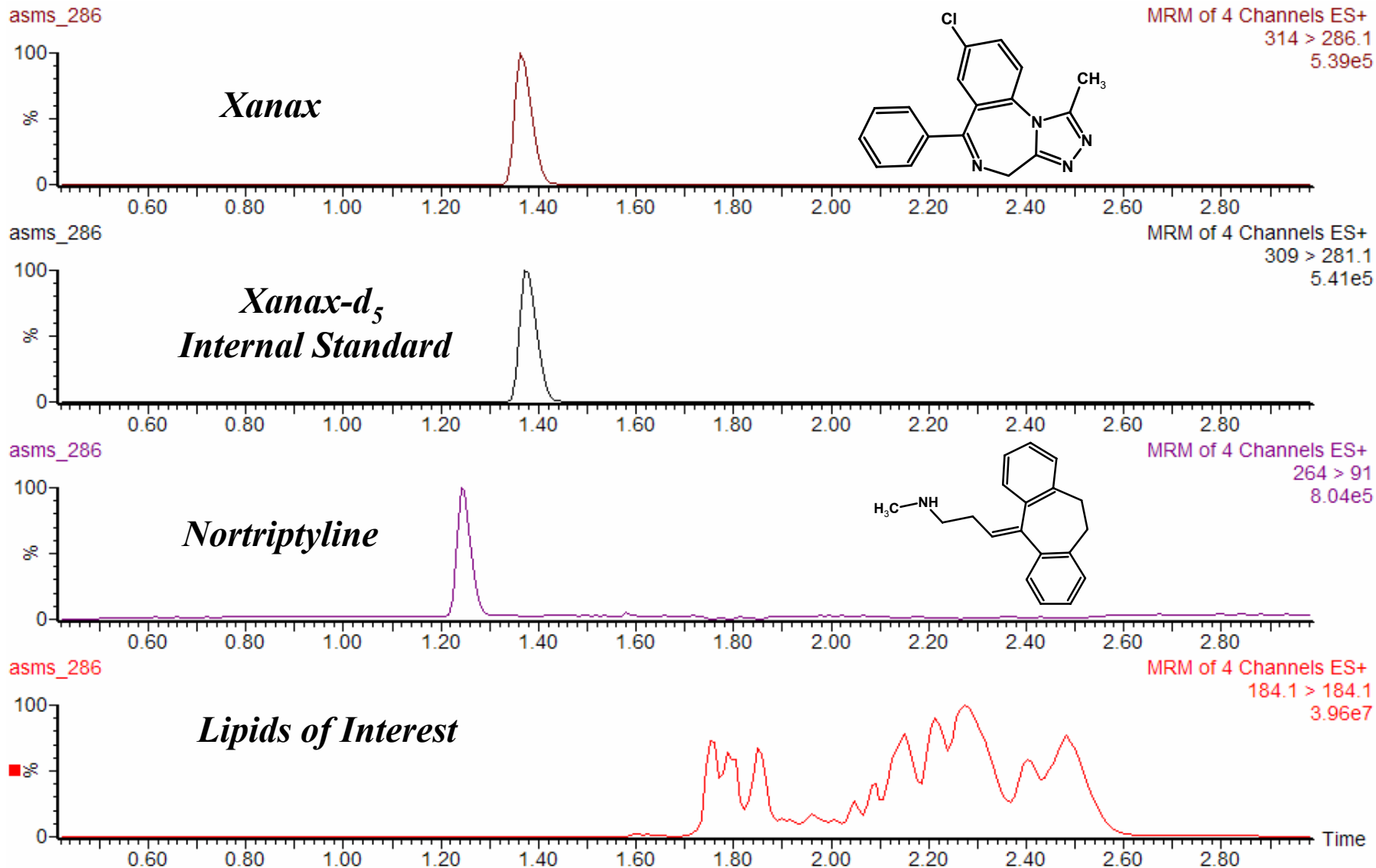
Elution of Lipids of Interest Versus Temperature



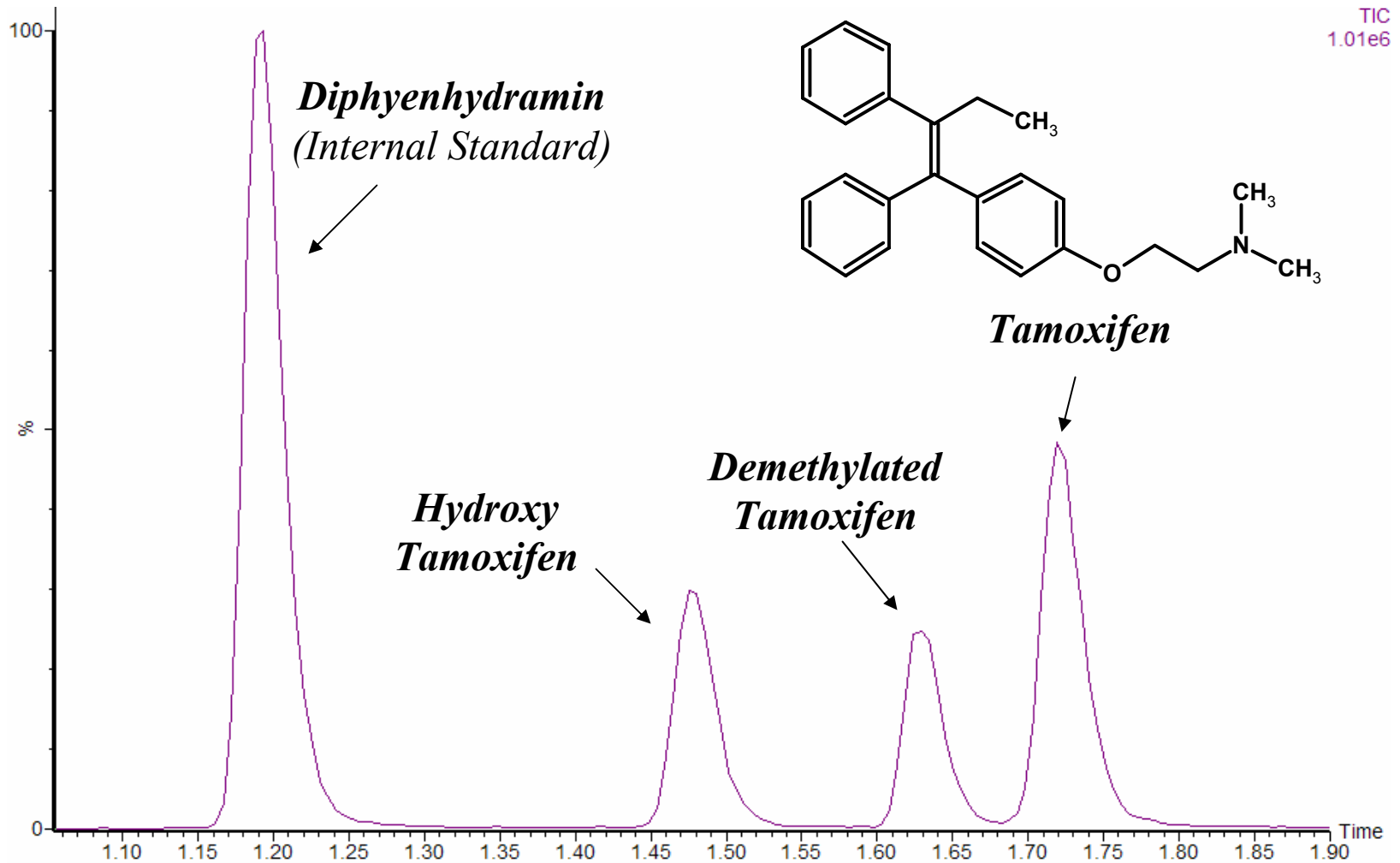
Elution of Lipids of Interest versus MeOH/CH₃CN Content



Analysis, Lipids *Not Diverted* for Illustration



Analysis of Tamoxifen (25 ng/ml) and Major Metabolites, Lipids *Diverted*

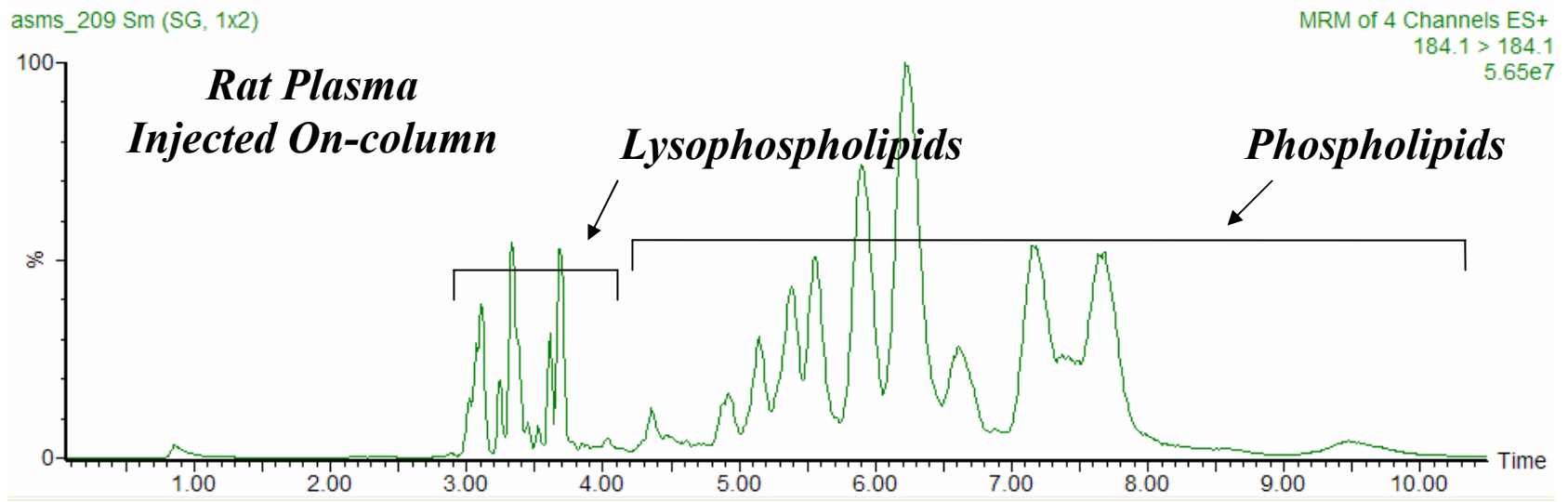
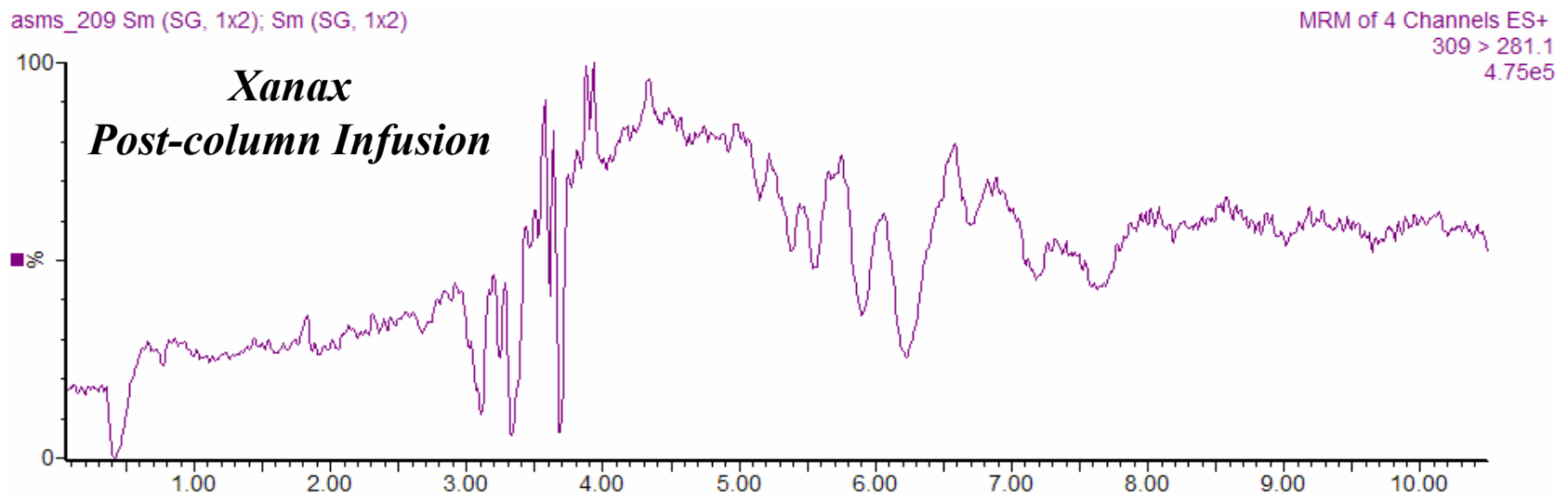


Typical Conditions, e.g. Tamoxifen and Metabolites

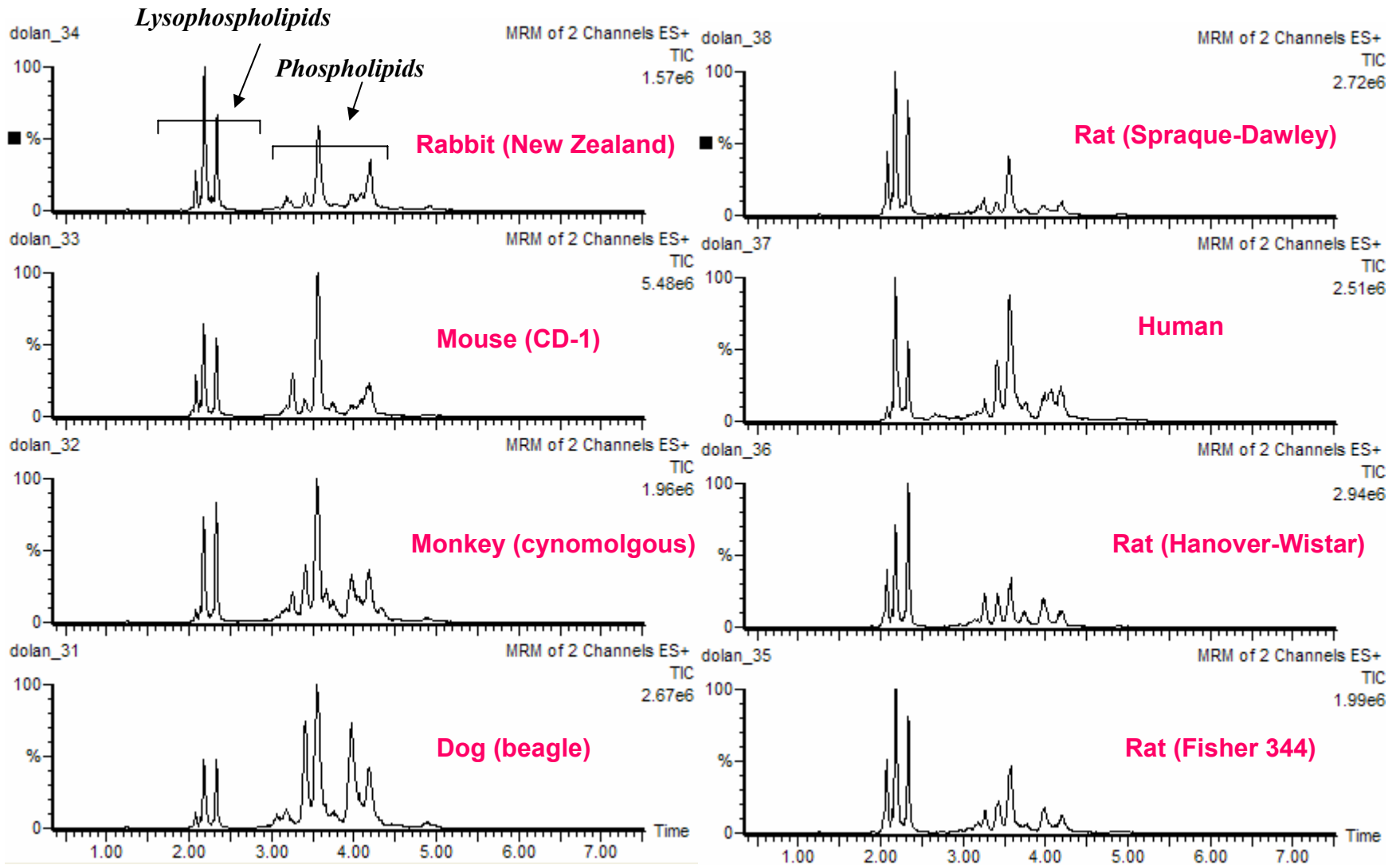
- 50 x 2 mm Varian Monochrom C18, 0.5 μ precolumn filter in holder
- Solvent A, water with 10 mmolar ammonium formate adjusted with formic acid to pH ~4.5, 30 ml of methanol to slow microbial growth
- Solvent B methanol
- pH *critical* to separation, pH 3.5 *no* separation of tamoxifen and demethylated metabolite!
- Flow diverted before and after analytes of interest
- Column temperature 40 °C, sample 4°C, 5 μ l injection
- 0.5 minutes for autosampler sequence, ***total 4.5 min cycle time***

Time (min)	Flow (mL/min)	%A	%B
1. Initial	0.4	80	20
2. 0.20	0.4	80	20
3. 0.30	0.4	50	50
4. 1.30	0.4	0	100
5. 1.90	1.2	0	100
6. 3.51	1.2	0	100
7. 3.52	0.8	80	20
8. 4.00	0.4	80	20

Matrix Suppression Shown by Post-Column Infusion of 180 ng/ml Solution of Xanax and Injection of Rat Plasma Sample

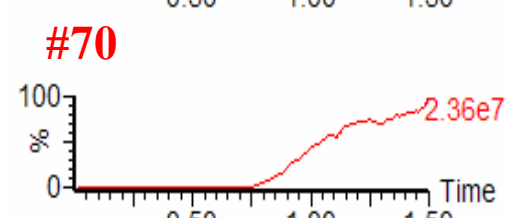
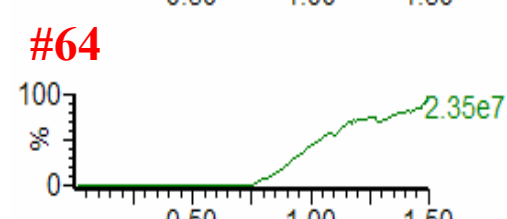
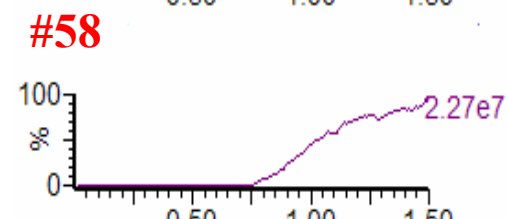
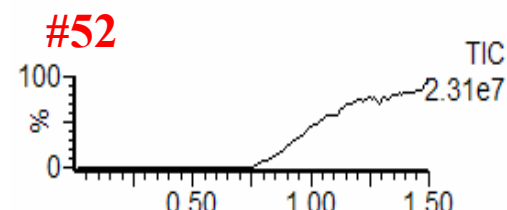
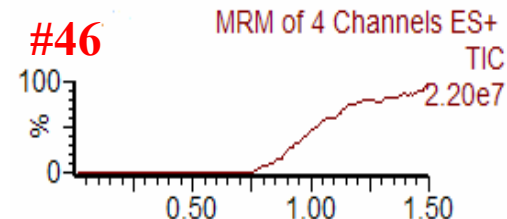
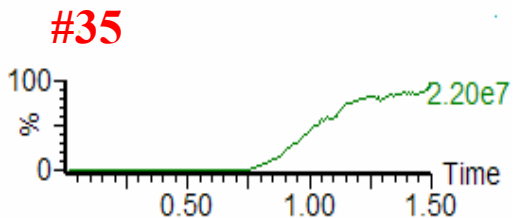
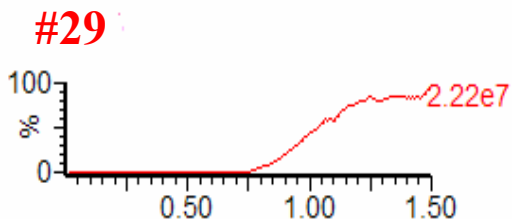
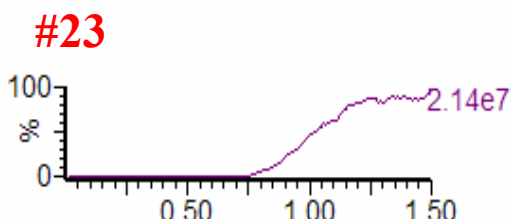
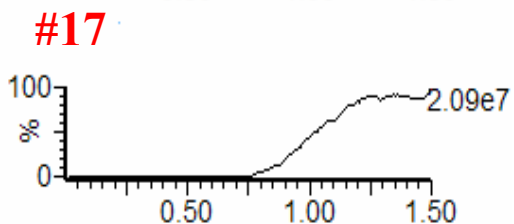
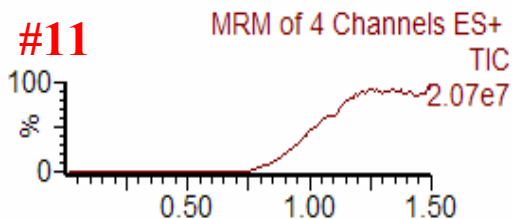
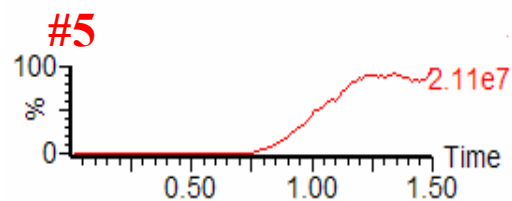
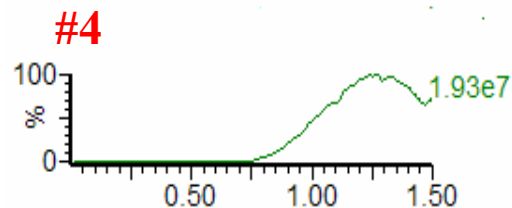
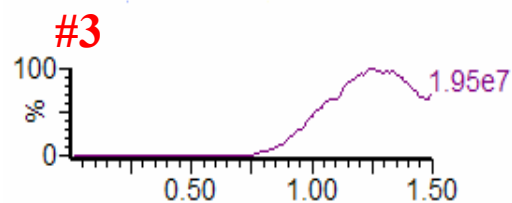
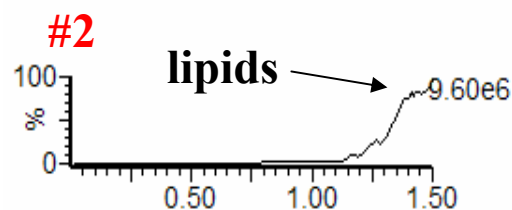
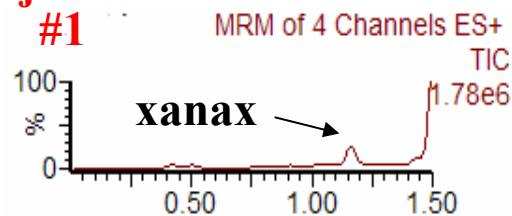


Comparison of Different Species IS-MRM of m/z 184 and 104 Summed



What If Lipids Not Eluted from Column with Full Gradient?

Injection



What If Lipids Not Eluted from Column with Full Gradient?

- Used small gradient to focus basic analytes xanax, xanax-d₅, and nortriptyline
- Solvent strength too weak to elute all lipids in one analyses
- Compared reproducibility and calibration curves results to those obtained with complete separation of analytes from lipids
- Similar conditions used for tamoxifen, but pH 3.5

Time (min)	Flow (mL/min)	%A	%B
1. Initial	0.4	80	20
2. 0.20	0.4	80	20
3. 0.21	0.4	40	60
4. 1.50	0.4	10	90
5. 1.51	0.4	80	20
6. 2.50	0.4	80	20

(Cycle time 3 min with autosampler sequence)

Summary of Results from *Not* Eluting Lipids of Interest

- Peak shape and retention times of basic analytes unaffected in 70 injections
- Sensitivity (area counts) for basic analytes decreased 15-35%
- After 6 injections, the response for lipids of interest became essentially "steady-state," ratio m/z 184:104 essentially constant
- Analysis time could be decreased 30-50%
- Curve fit, r^2 of ~ 0.995 , was very similar when analytes separated or not separated from lipids
- 2.3% relative standard deviation for Xanax (external std, n=70)
- 1.7% relative standard deviation for Xanax (vs. internal d₅ std, n=70)

Possible Problems with *Not* Eluting Lipids

- The concentration and profile of lipids can vary with individual rat with time, work needs to be performed to determine the effect on precision and sensitivity in an ADME study
- Current results with only one rat plasma sample!
- The variation of lipids could be more problematic for precision and accuracy using either external or internal standard methods employing non-labeled standards
- The number of samples injected at some point could affect the peak shapes of basic analytes, currently only 70 injections evaluated before column "cleaned."

Conclusions

Phospholipids and lysophospholipids found at high concentrations in plasma samples suppress the response of basic analytes in LC-MS analyses. Characteristic fragment ions are formed by the high energy in-source fragmentation (IS-MRM) these lipids at m/z 184 and/or 104. These ions can be monitored in MRM methods to develop fast separations of basic analytes from these matrix interferences.

Solvent composition, strength, temperature, and flowrate were found to significantly decrease the retention time of these lipids in reverse phase C18 separations. Typically 4-5 minute (total cycle time) analyses could be developed for a variety of basic analytes.

Preliminary work was performed to determine the effect on precision and sensitivity for basic analytes if these lipids *were not* eluted from the column. A loss in sensitivity was noted, but the precision was not significantly affected. Further comparisons need to be made in an ADME study with a variety of rat samples. Therefore, at the present all studies in our laboratory attempt to separate the majority of the phospholipids and lysophospholipids from basic analytes.

Available Information

Presentation and associated experimental detail available at:

"A Little Mass Spectrometry and Sailing,"

<http://users.chartertn.net/slittle/>

Website also contains information on:

- Identification of surfactants by LC-MS
- Using silylation and methylation reagents for GC-MS
- Accurate mass measurements by magnetic and TOF MS
- Versatile CI manifold for mixing and using gases
- GC-MS CI reagent gas selection
- NIST software for EI and MS/MS searches and data storage
- Polycarbonate and Polyester Analyses by GC-MS

Acknowledgements

- M. Wempe, S. Haynes, C. Cleven, C. Buchanan, J. Skoty, and L. Cunningham, Eastman Chemical Company
- Jennifer Jones, Cari Randles, Tanya Jenkins from Waters for useful information on operation of Quattro Micro/Acquity UPLC
- Patrick Bennett *et al*, Tandem Labs, for information on phospholipids and lysophospholipids:
www.tandemlabs.com/capabilities_publications.html
- John W. Dolan for useful chromatography advice, wide variety of "HPLC Tips" in LCGC:
www.lcgcmag.com/lcgcmag/
- D. Humphries *et al*, column selection for basic analytes, J Chrom B, 810 (2004) 229-234.
- P. Rudewicz *et al*, plasma analysis method, J. Chrom B, 799 (2004) 271-280.