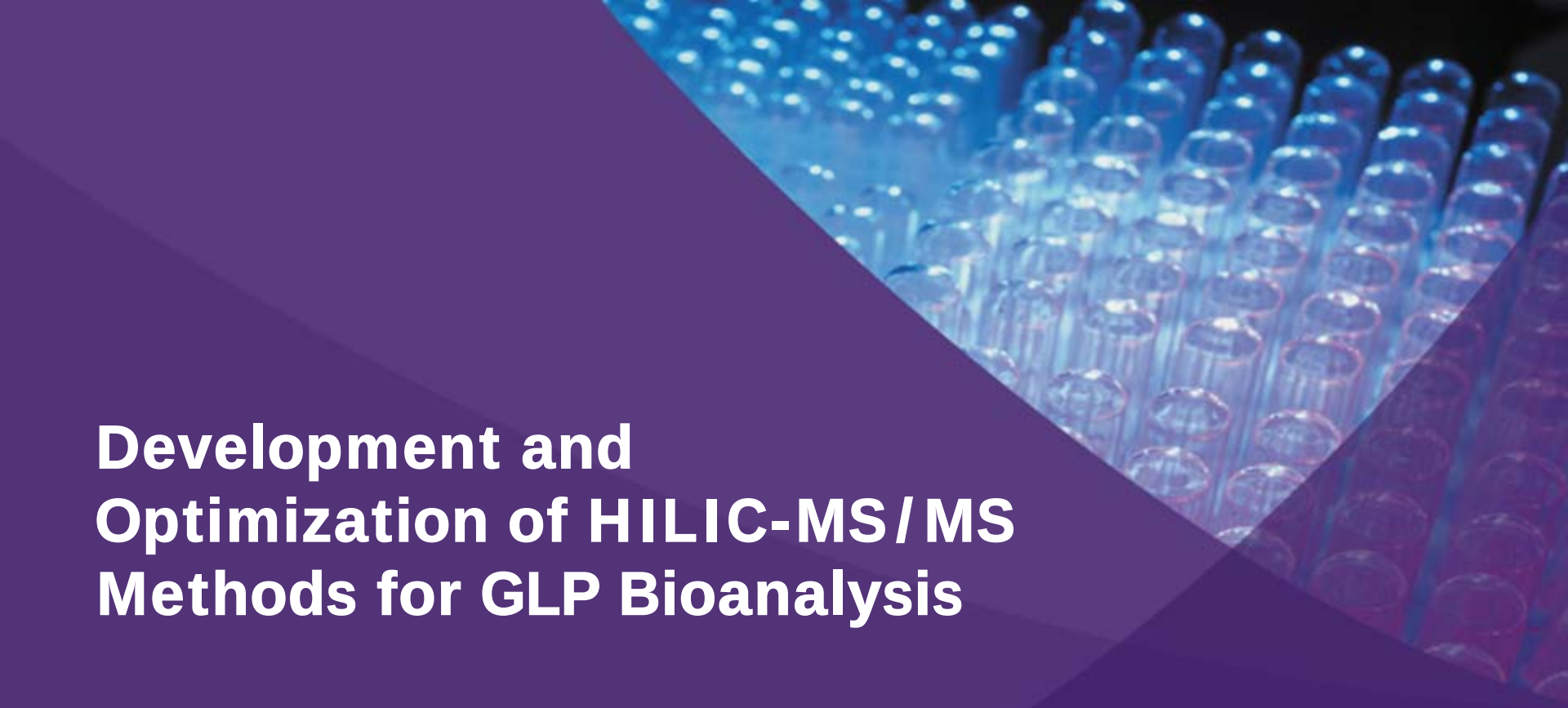

# Development and Optimization of HILIC-MS/MS Methods for GLP Bioanalysis

**William R. Mylott Jr. and Rand Jenkins**

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# Introduction

*Hydrophilic interaction chromatography (HILIC) has proven to be a powerful technique for LC-MS/MS bioanalysis of polar and ionic drugs and metabolites.*

# Introduction

*Hydrophilic interaction chromatography (HILIC) has proven to be a powerful technique for LC-MS/MS bioanalysis of polar and ionic drugs and metabolites.*

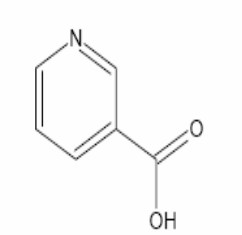
- **Enhanced selectivity for niacin assay**
- **Demonstration of high sensitivity and online cleanup of matrix interferences (phospholipids) for sufentanil**
- **Offline technique for phospholipid cleanup**
- **Online technique applied to UPLC-MS/MS**

# Why HILIC mode?

- **Good retention for polar / ionic species**
  - Hydrophilic interaction (adsorbed water layer)
  - Ion-exchange (silanols)
  - Weak reverse phase (siloxane)
- **Mobile phase composition typically >70% organic**
  - Low back pressure
  - Enhanced MS sensitivity

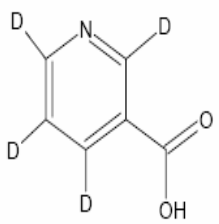
# Development challenges for niacin and NUA in human urine assay

- **Polar molecules**
- **Very low MW**
- **Endogenous**
- **Potential selectivity issues**



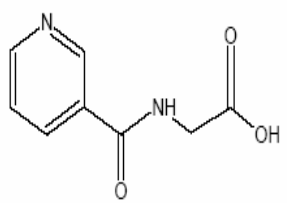
Niacin

$C_6H_5NO_2$ FW = 123.11



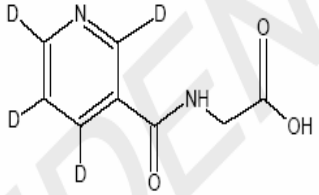
Niacin-$d_4$

$C_6HD_4NO_2$ FW = 127.11



Nicotinuric Acid

$C_8H_8N_2O_3$ FW = 180.2

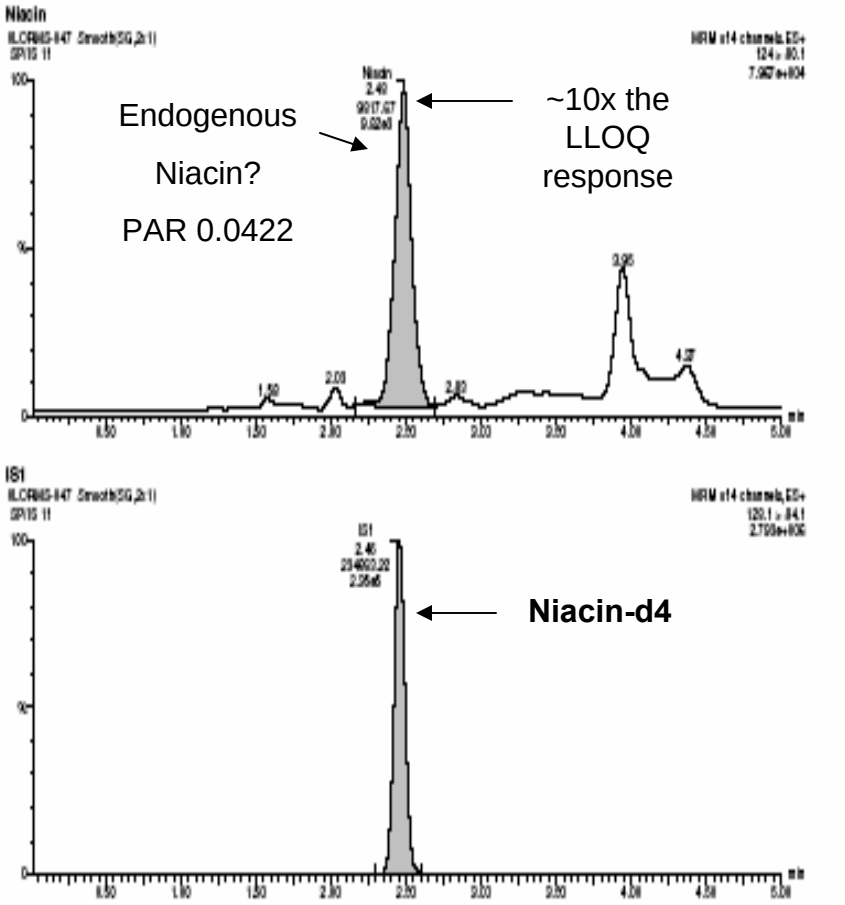


Nicotinuric Acid-$d_4$

$C_8H_4D_4N_2O_3$ FW = 184.2

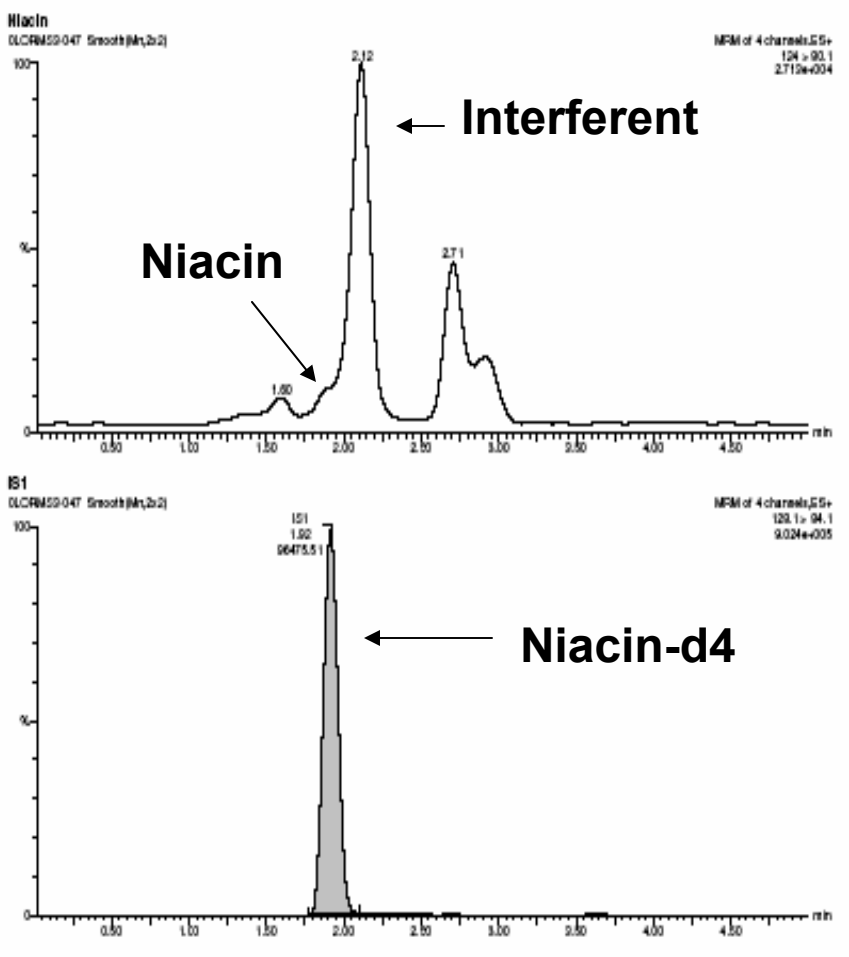
# Human urine blank with internal standard

- **Betasil Silica-100 3mm x 100 mm column**
- **95/5/0.025/0.5 ACN/water/TFA/ Acetic acid (v/v/v/v)**
- **Flow rate 0.5 mL/min**
- **Column Temp. RT**



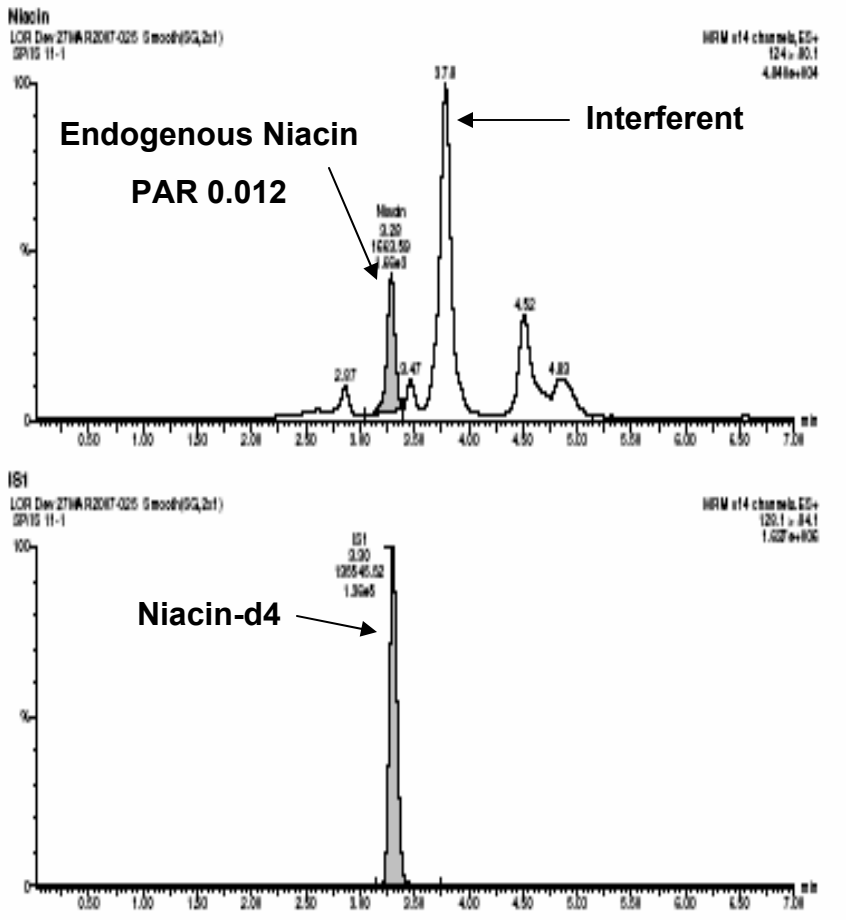
# Human urine blank with internal standard

- **Betasil Silica-100 3mm x 100 mm column**
- **95/5/12/0.025/0.5 ACN/water/*IPA*/TFA/ Acetic acid (v/v/v/v/v)**
- **Flow rate 0.5 mL/min**
- **Column Temp. 40°C**

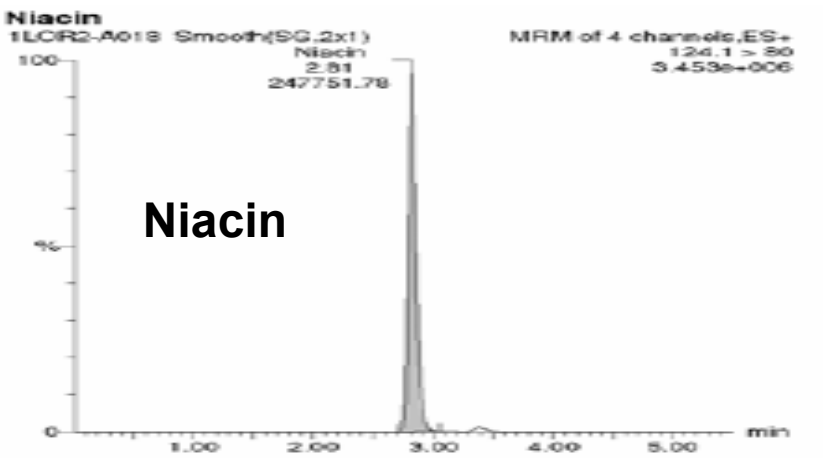


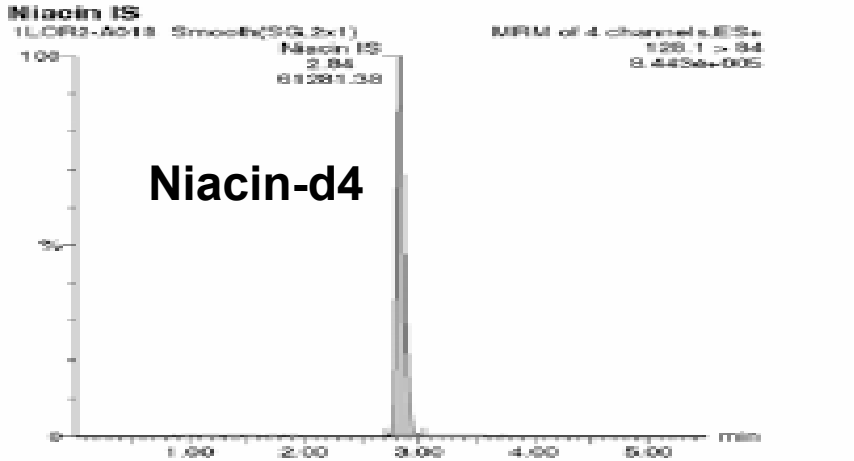
# Human urine blank with internal standard

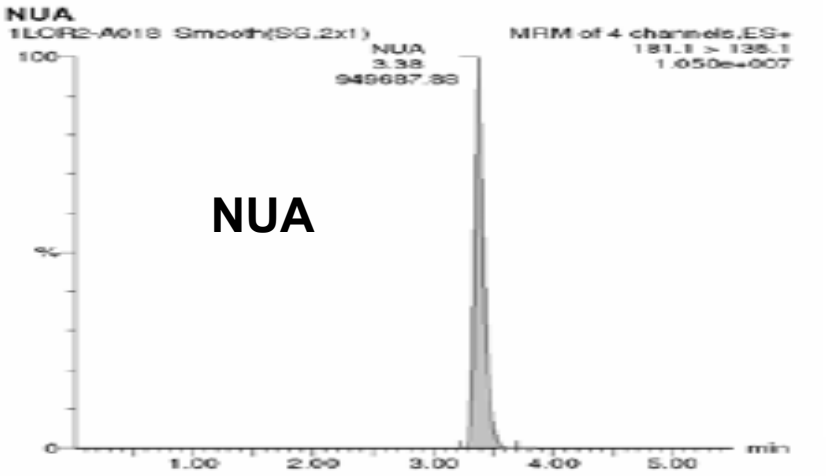
- Betasil Silica-100 3mm x 250 mm column
- 95/5/12/0.025/0.5 ACN/water/ *IPA*/TFA/ Acetic acid (v/v/v/v/v)
- Flow rate 0.75 mL/min
- Column Temp. 40°C
- Original conditions would have resulted in a 3.5x over-estimation of the actual endogenous niacin concentration

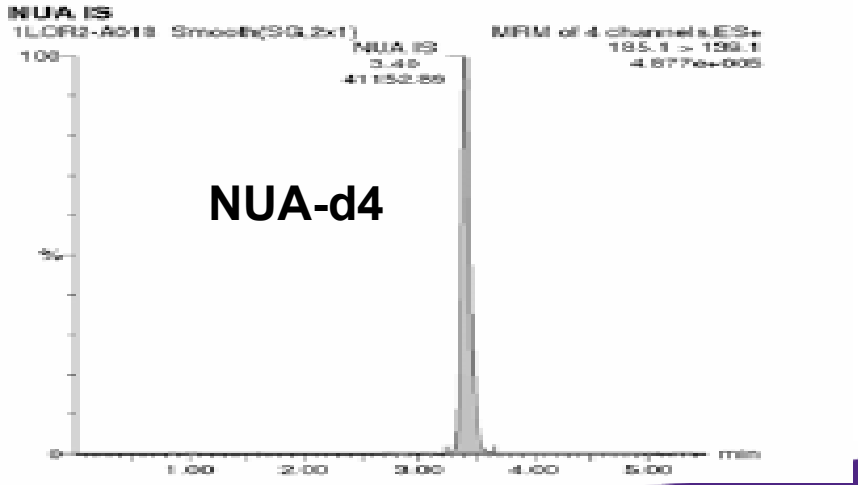


# Extracted ULOQ sample



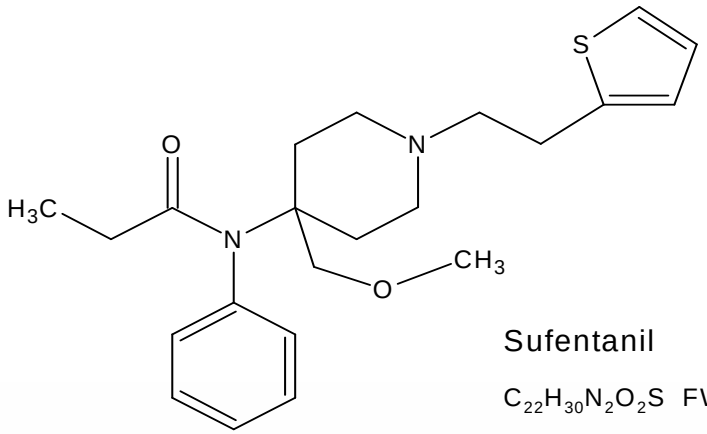



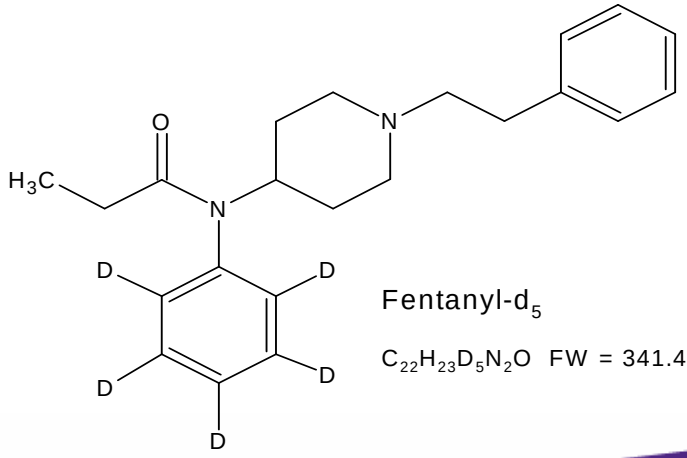
# Development challenges for sufentanil human plasma assay

- **Extremely sensitive assay required, 1.00 pg/mL**
- **Use of analogue Internal standard (fentanyl-d5)**
- **Minimize Carryover**
- **Eliminate plasma matrix effects (phospholipids)**




Sufentanil

$C_{22}H_{30}N_2O_2S$ FW = 386.56




Fentanyl-$d_5$

$C_{22}H_{23}D_5N_2O$ FW = 341.48

# Sufentanil assay summary

- Calibration range 1.00-250 pg/mL
- Sample volume 0.5 mL $K_2$EDTA Human Plasma
- Liquid/Liquid extraction with hexane/MTBE/dichloromethane
- Reconstituted with 0.25 mL Acetonitrile
- API-4000, positive ion TurboIonSpray™

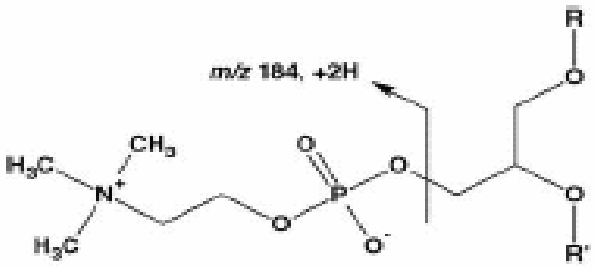
# Matrix effects

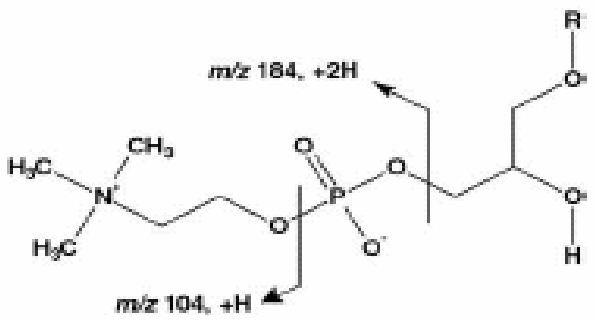
- **LLE relatively non-selective extraction**
  - ‘Dirty’ sample extracts
  - Significant matrix effect problems resulting from phospholipids

# What are phospholipids?

- **Glycerol esterified to a phosphate-containing moiety (e.g. choline) and up to two fatty acids of variable chain length**

- **The major class of phospholipids found in human plasma are the glycerophosphocholines (GPCho's)**



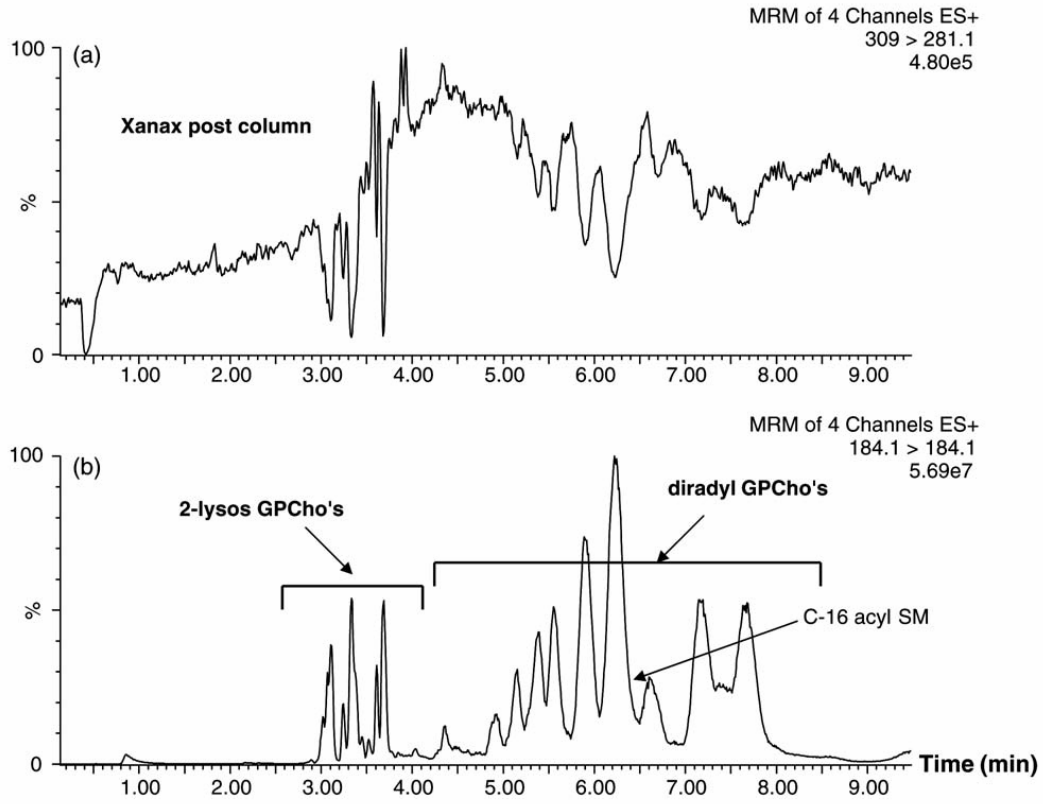



# Why are phospholipids important to consider during LC/MS assay development?

- **Co-eluting or late-eluting phospholipids are THE major source of ion suppression interference issues**
- **Chromatographically elute over a wide retention time range**
- **May build up and slowly bleed off the column during subsequent injections**
  - Variable ion suppression magnitude
  - Loss of sensitivity as run progresses
- **Accumulation on the column may alter the column chemistry**
  - Change in the retentive behavior of the stationary phase
  - Analyte retention time shifts
  - Increase the potential for column-related analyte carryover due to adsorption

***Phospholipids pose a serious ruggedness issue for assays and steps should be taken to reduce or remove them***

# Phospholipids - direct and indirect detection

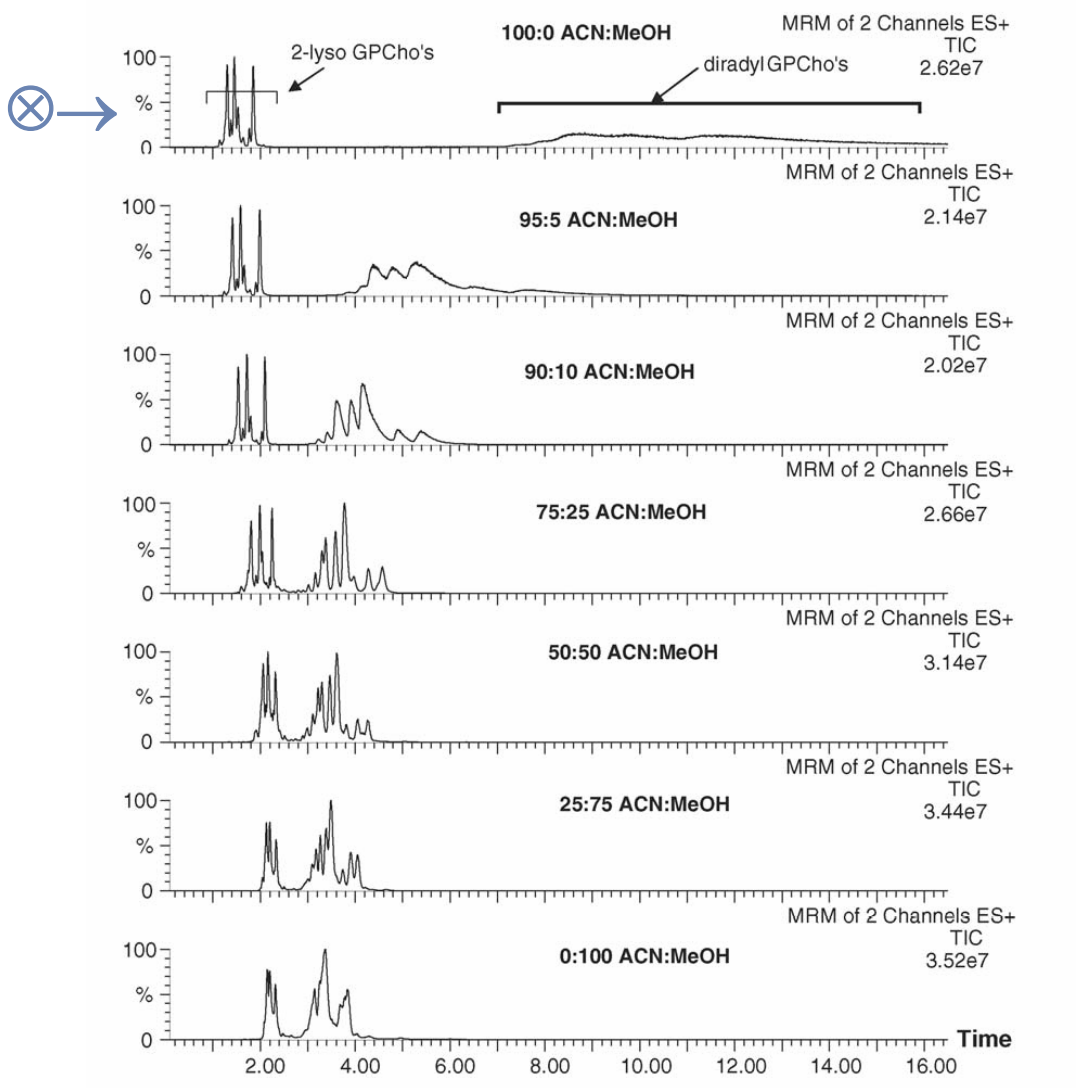



**"Matrix ionization effects shown for GPCho's with post column infusion of Xanax … and LC–MS/MS analysis of protein-precipitated rat plasma: (a) MRM transition for Xanax, *m/z* 309→281; (b) IS-MRM transition for GPCho's, *m/z* 184→184."**

**J.L. Little *et al., J. of Chrom. B* 833, (2006) 219-230**

# Phospholipids - elution patterns

**"Comparison of solvent composition for the elution of GPCho's from reverse phase HPLC column employing the same gradient elution for all separations utilizing MeOH and ACN as the organic eluents."**




J.L. Little *et al., J. of Chrom. B* 833, (2006) 219-230

# Why are phospholipids difficult to remove?

- **Amphipathic and zwitterionic character makes phospholipids extremely difficult to remove during extraction**
- **Phospholipids are a complex mixture with a wide molecular weight range, increases complexity for mass spec monitoring**
- **Common methods (PPT, Liquid/Liquid, SPE C18, C8, HLB, ion exchange) do not effectively remove phospholipids from biomatrix sample extracts**
- **Phospholipids are present at mg/mL concentrations in plasma**
  - Even if 90% removed during extraction, high µg/mL quantities still remain!
- **Reduce or eliminate phospholipids using specialized off-line cleanup**
- **Reduce or eliminate phospholipids using HPLC on-line cleanup**

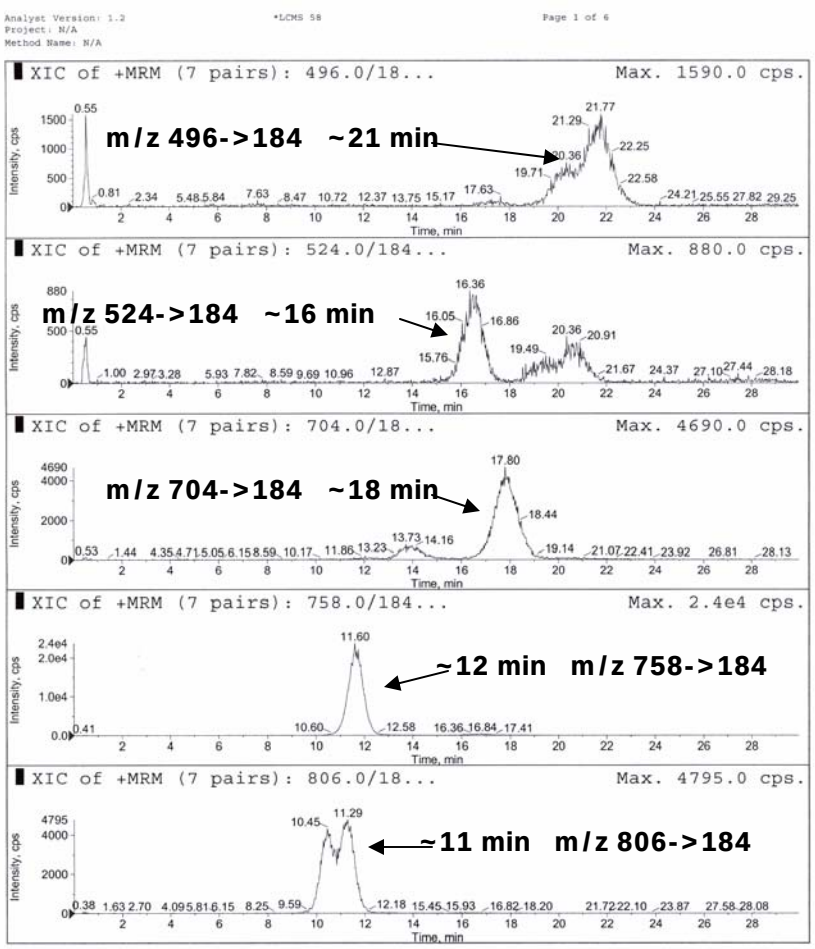
# How can phospholipids be simply monitored using LC-MS/MS?

- Take advantage of common CID fragment (m/z 184)
- Induce CID in the ion source and monitor m/z 184 →184 transition
- Monitor group of selected SRM transitions to encompass known m/z range for the mono and diradyl GPCho's

  (e.g. m/z 496 →184; 524→184; 704→184; 758→184; 806→184
- Conduct precusor ion scan vs. m/z 184 product ion

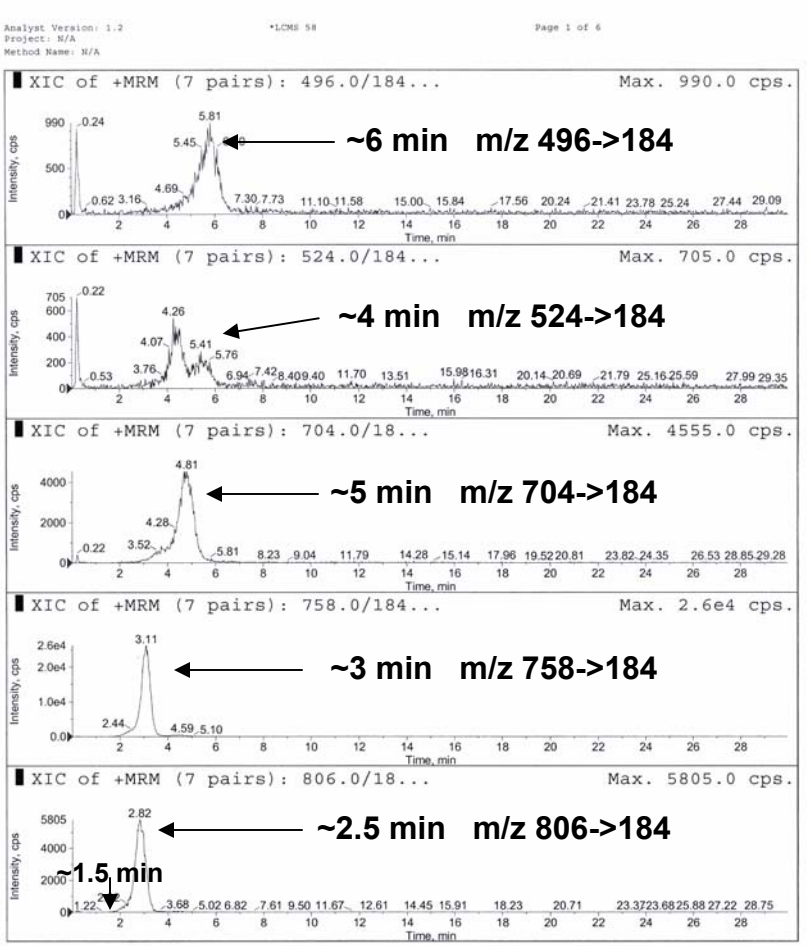
# Phospholipid profile on analytical column

- Liquid/Liquid plasma extract
- Thermo Betasil Silica 100, 2.1 x 50 mm, 5µm
- Mobile phase for first 2.5 min 80:15:5 Acn:MeOH:water with 0.8 mM ammonium formate and 0.005% formic acid
- Mobile phase after 2.5 min 50:15:35 Acn:MeOH:water with 0.8 mM ammonium formate and 0.005% formic acid
- Phospholipids elute over a long period of time in order opposite that of RP
- Increased possibility of late eluting ion suppression effects



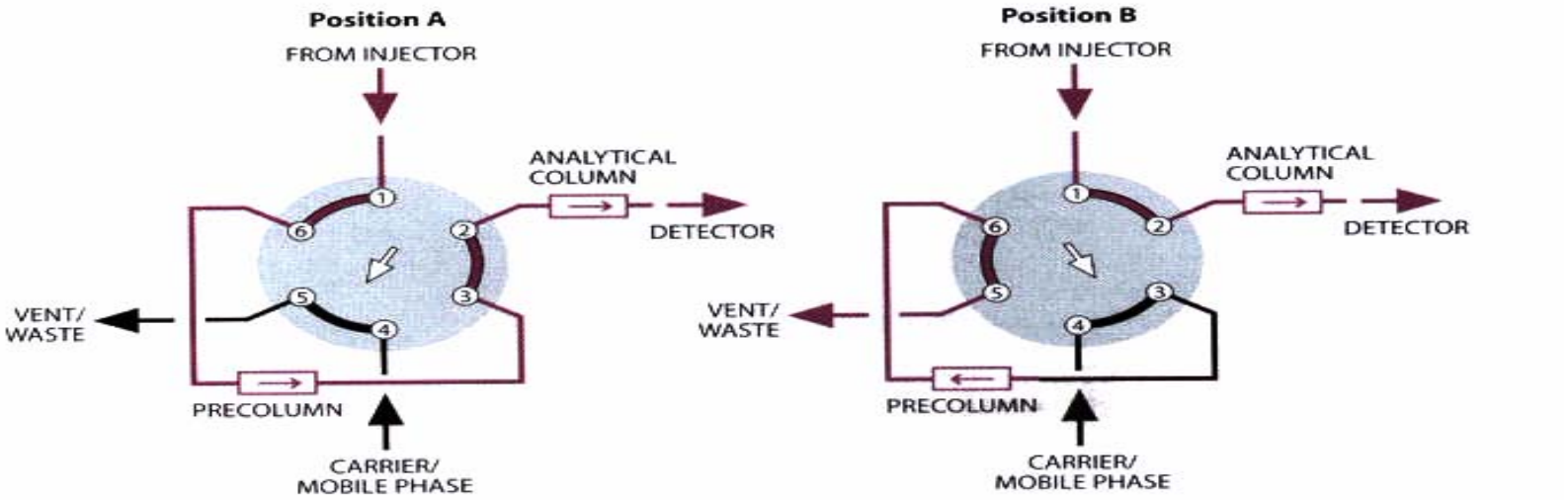
# Phospholipid profile on guard column

- Liquid/Liquid plasma extract
- Thermo Betasil Silica 100, 2.1 mm x 10 mm, 5 µm
- Same conditions as used for the analytical column
- Analyte and IS elute at ~0.6min from the guard while the phospholipids are “trapped”
- Use divert valve to flush phospholipids to waste
- Eliminate phospholipids without extending analysis time

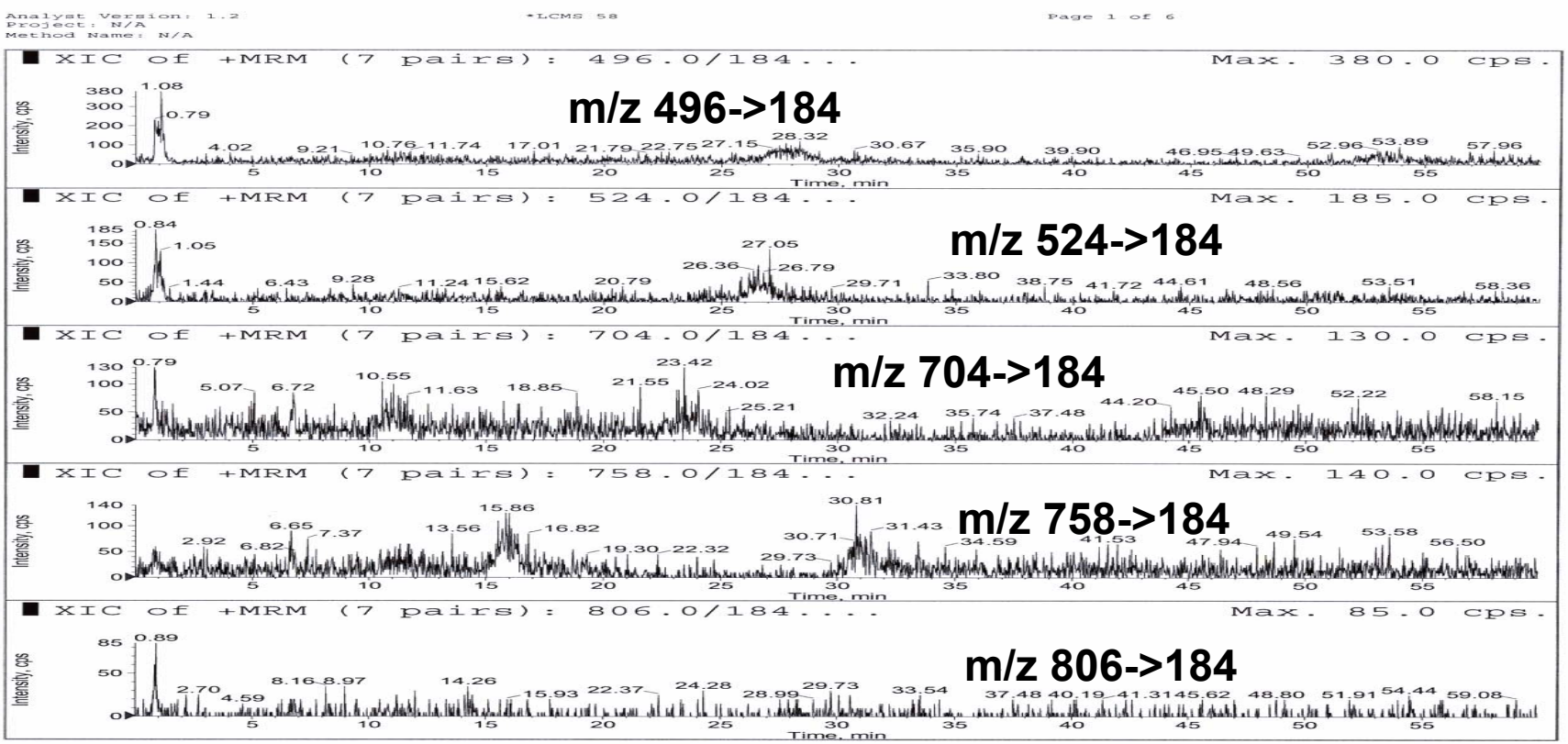


# Setup for on-line phospholipid cleanup

- 0-1.0 min: guard and analytical columns in line
- At 1.1 min: valve switches to flush guard column in reverse direction
- At 3.9 min: valve switched for re-equilibration

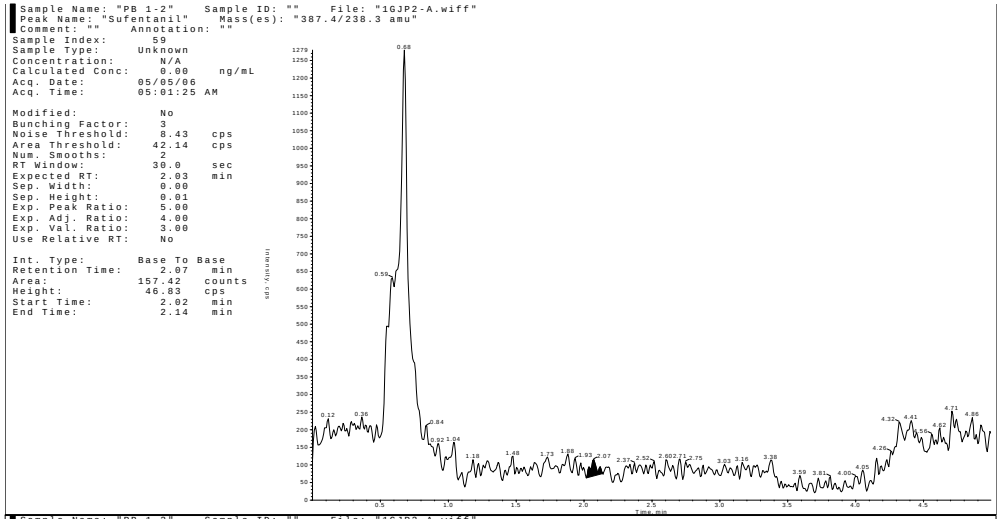


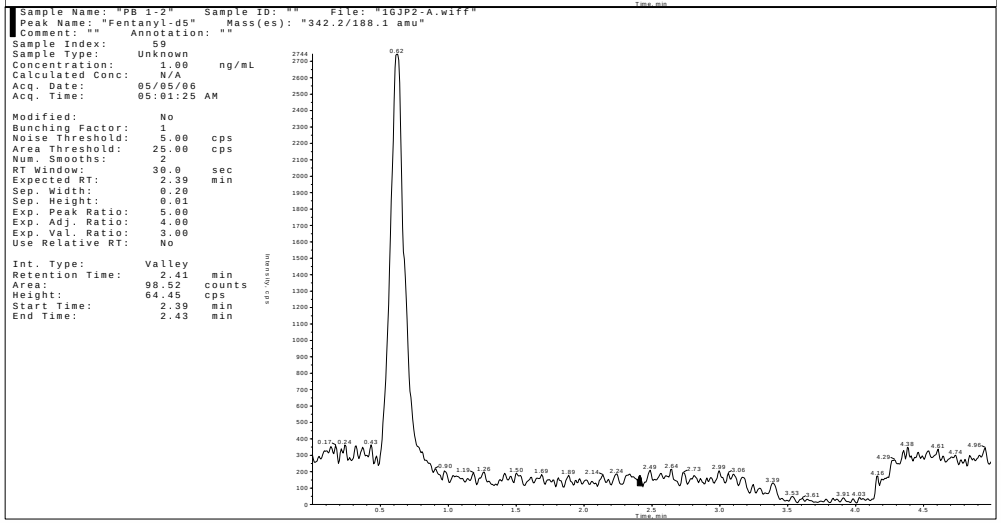
# Phospholipid profile with optimized conditions

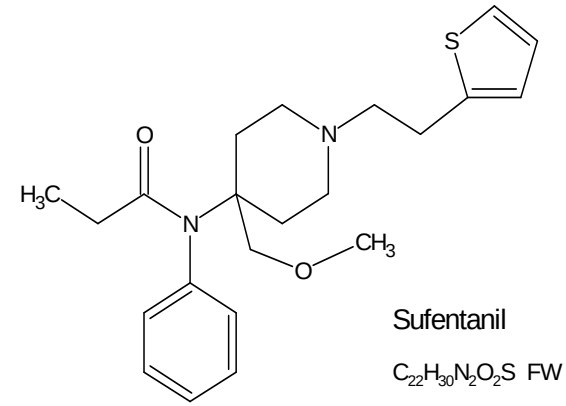



- **Phospholipids are effectively removed!**

# Blank human plasma extract

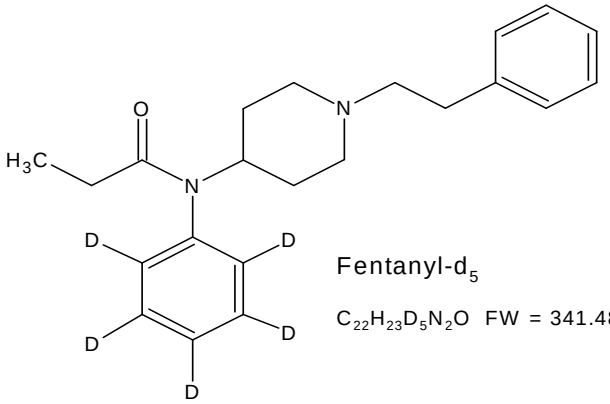



Sufentanil

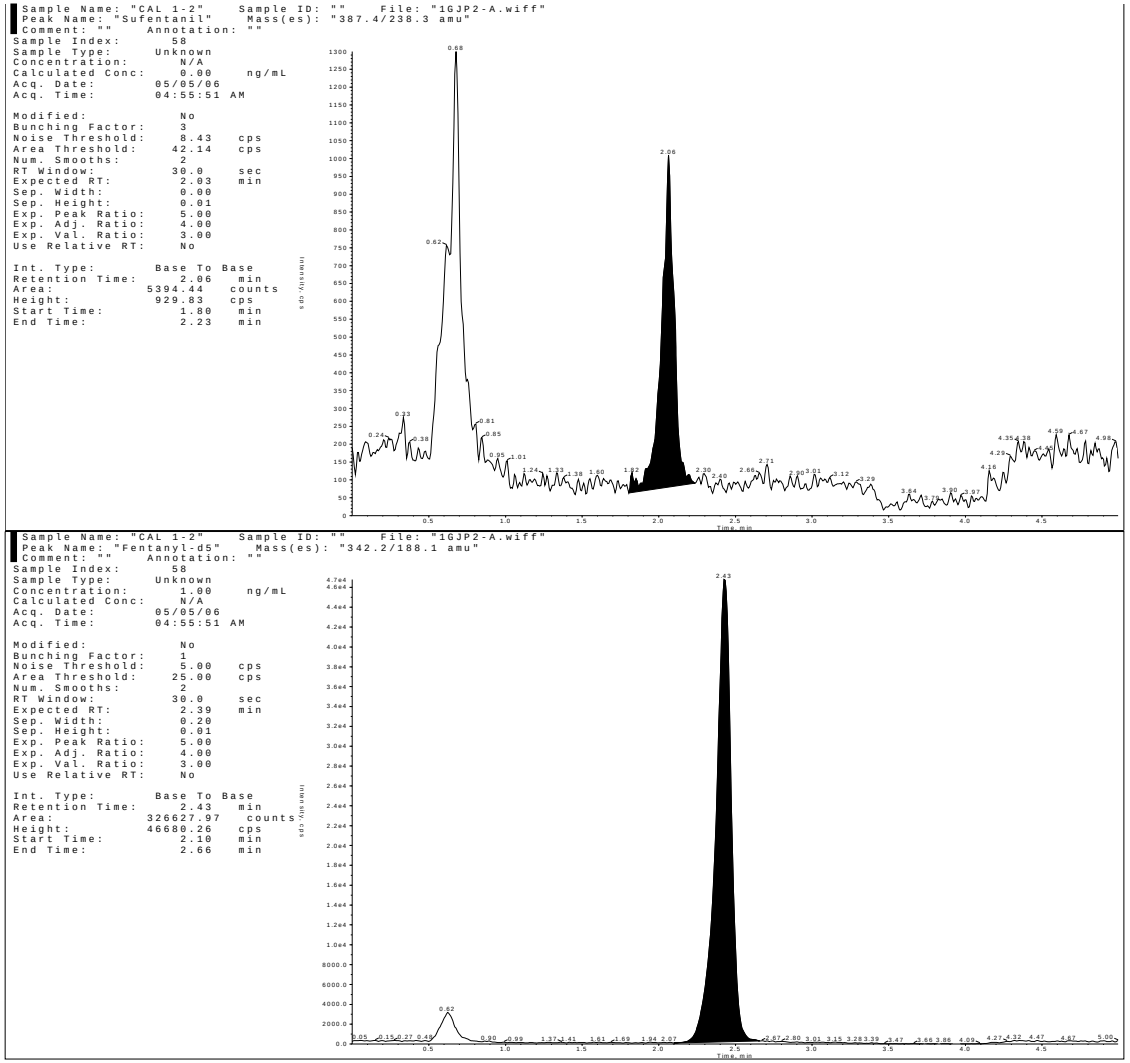
$C_{22}H_{30}N_2O_2S$ FW = 386.56




Fentanyl-$d_5$

$C_{22}H_{23}D_5N_2O$ FW = 341.48

# Extracted LLOQ (1.00 pg/mL) sample




- **Sufentanil**
  **m/z 387->238**
- **RT ~2.0 min**

- **Fentanyl-d5**
  **m/z 342->188**
- **RT ~2.4 min**

# Offline “HILIC”??? phospholipid cleanup evaluation

- **Human plasma extracted using protein precipitation with ACN/MeOH (4:1, v/v)**
  - Known to contain very high concentrations of phospholipids
- **Human plasma extracted using supported Liquid Extraction (Biotage Isolute SLE$^{+}$)**
  - Diatomaceous earth (largely consists of silica)

# Extraction summaries

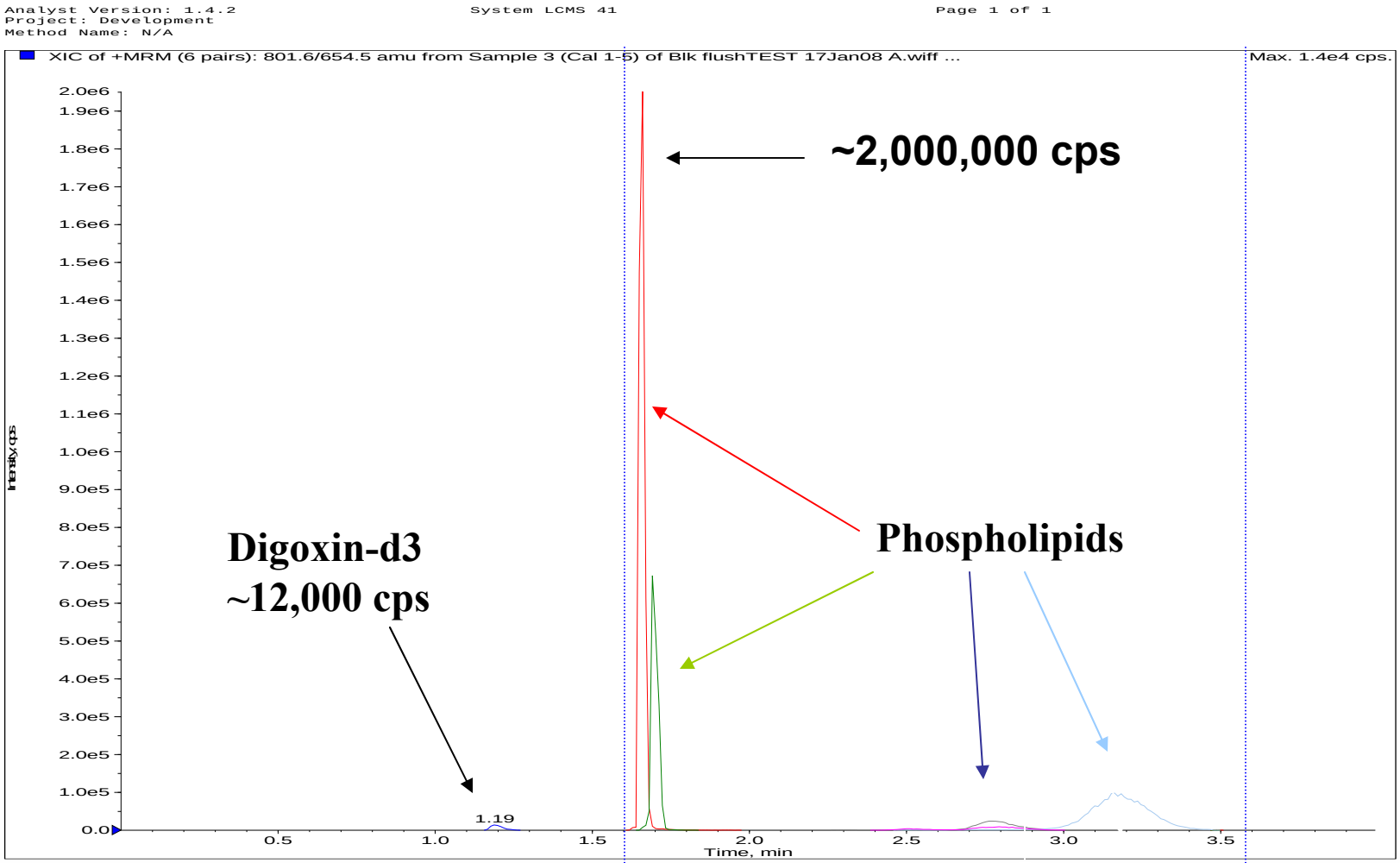
- **PPT**
  - **100 µL plasma sample + 50 µL buffer + 50 µL I.S., vortex**
  - **300 µL of 4:1 ACN:MeoH, vortex, centrifuge**
  - **Evaporate to dryness and reconstitute with 250µL**
- **SLE+**
  - **100 µL plasma sample + 50µL buffer + 50µL I.S., vortex**
  - **Transfer 200 µL to SLE+ plate, allow to absorb into bed and equilibrate for 5 min**
  - **Add 400 µL of DCM and allow to equilibrate for 5 min. Apply slight vacuum.**
  - **Repeat previous step and combine extracts**
  - **Evaporate and reconstitute with 250 µL**

# Instrument conditions

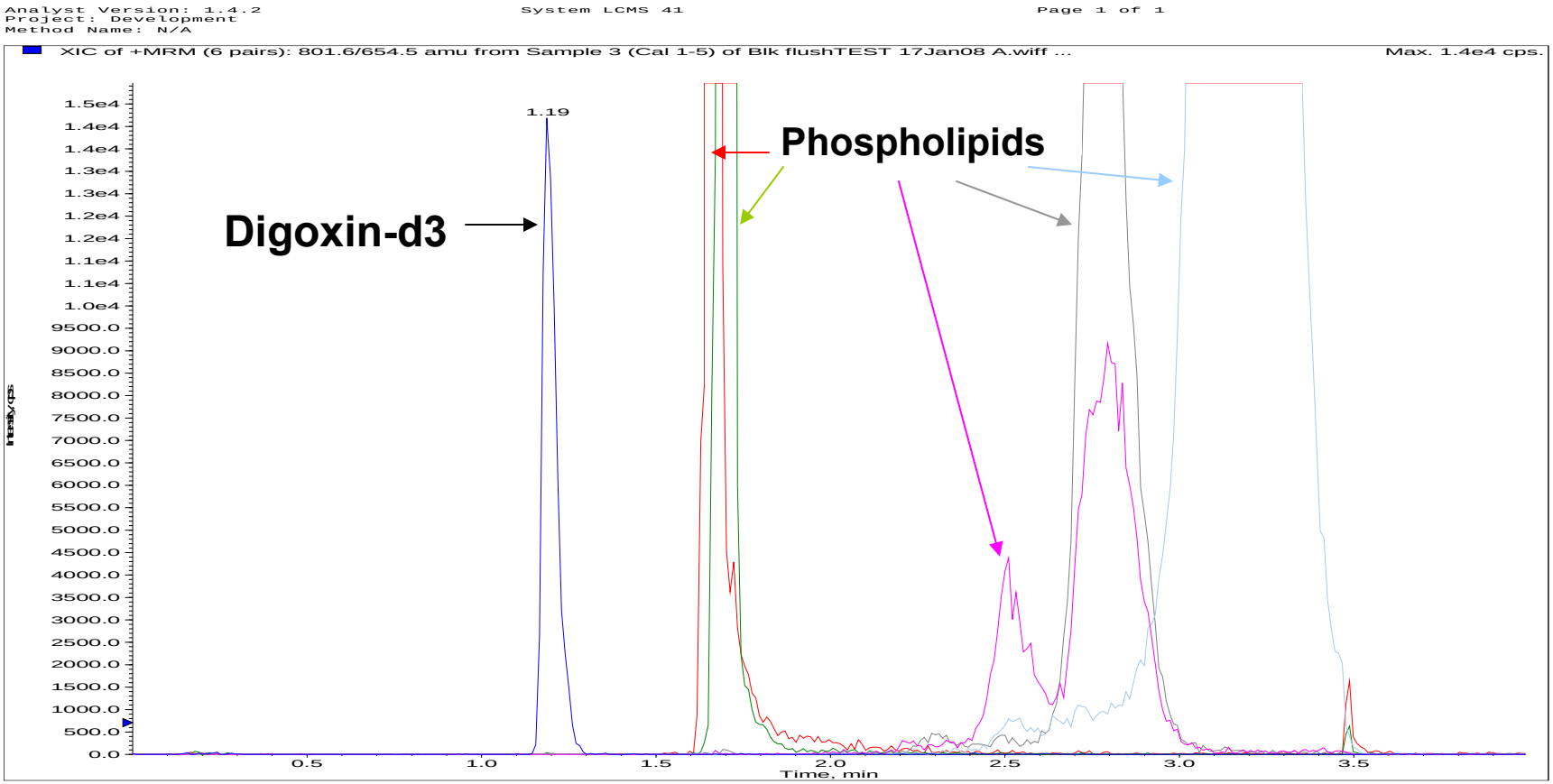
- **Column: MetaGuard C18,2x10 mm**
- **Gradient Elution**
- **Mobile Phase A: 5 mM ammonium acetate**
- **Mobile Phase B: 70/25/5/0.5 ACN/MeOH/Water/1 M Ammonium acetate (v/v/v/v)**
- **Sciex API-4000, positive ion TurboIonSpray™, MRM**

| Analyte | ~ $t_R$ (min) | Dwell Time (ms) | Q1 m/z | Q3 m/z | DP | CE | CXP | EP |
|---|---|---|---|---|---|---|---|---|
| Digoxin-d3 | 1.2 | 100 | 801.6 | 654.5 | 63.25 | 20.69 | 24.47 | 10 |
| Phospholipid | 1.7 | 100 | 496.0 | 184.0 | 63.25 | 20.69 | 24.47 | 10 |
| Phospholipid | 1.7 | 100 | 524.0 | 184.0 | 63.25 | 20.69 | 24.47 | 10 |
| Phospholipid | 2.8 | 100 | 704.0 | 184.0 | 63.25 | 20.69 | 24.47 | 10 |
| Phospholipid | 3.2 | 100 | 758.0 | 184.0 | 63.25 | 20.69 | 24.47 | 10 |
| Phospholipid | 2.8 | 100 | 806.0 | 184.0 | 63.25 | 20.69 | 24.47 | 10 |

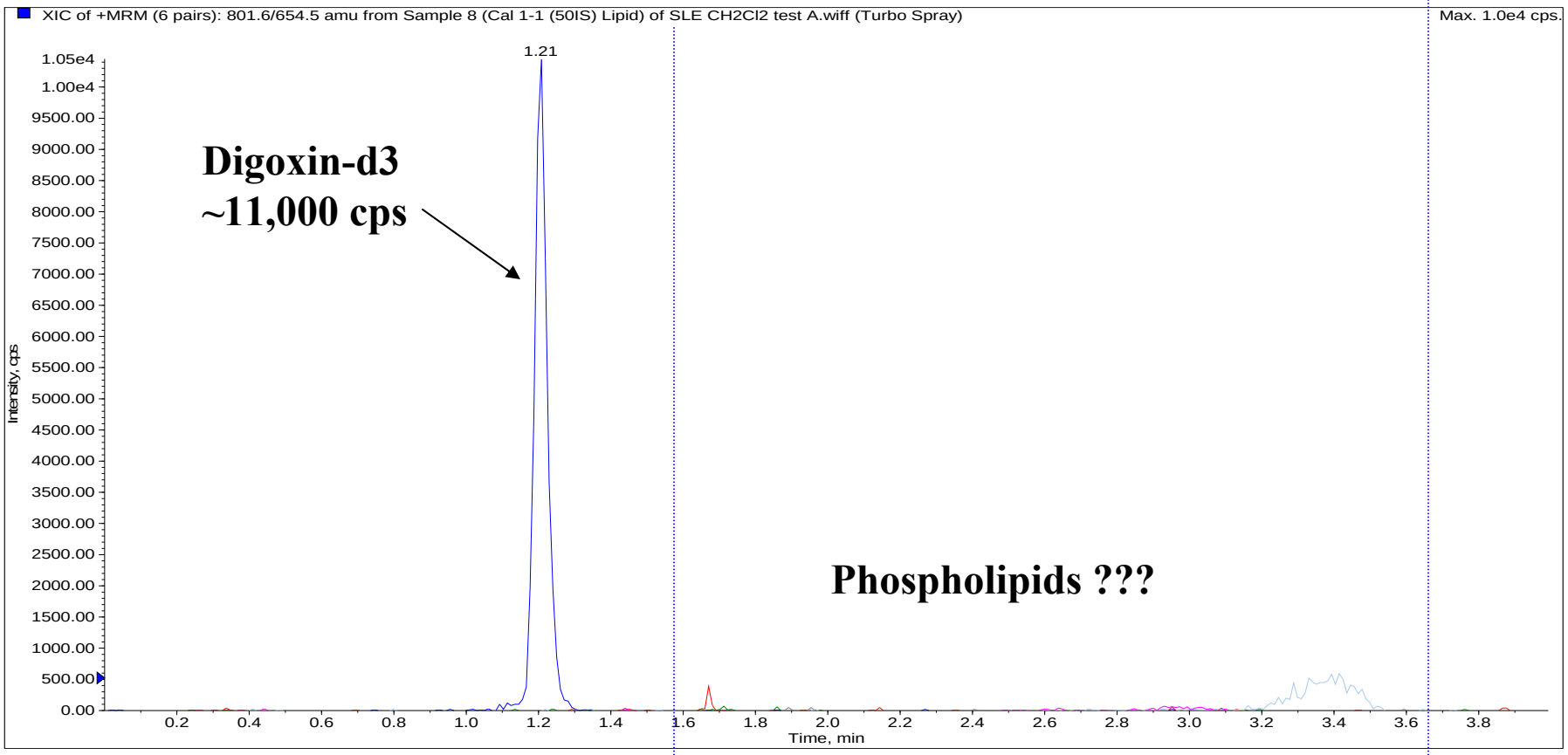
# Phospholipid profile (PPT procedure, TIC)



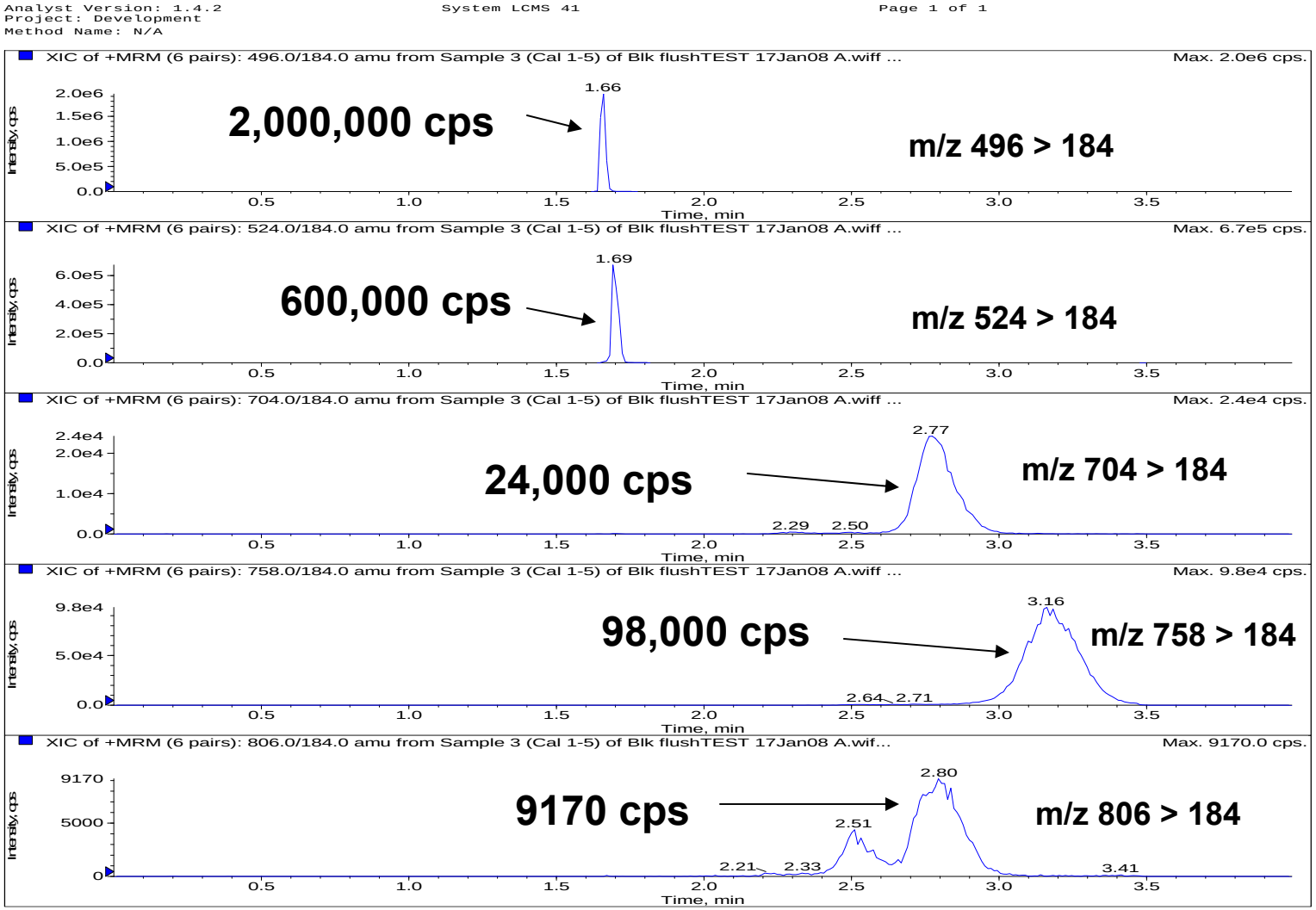
# Normalized to digoxin-$d_3$ response



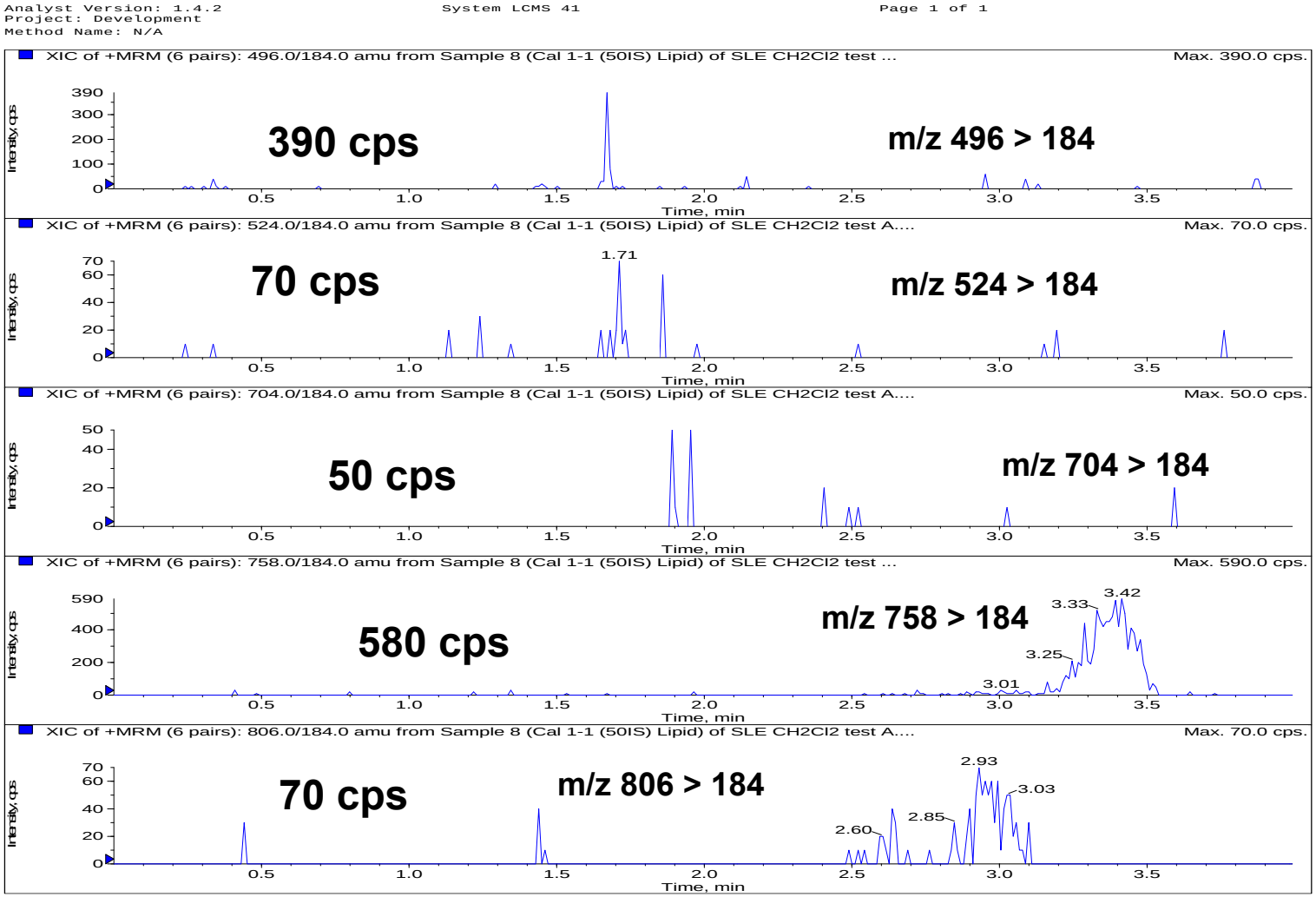
# Phospholipid profile (SLE procedure, TIC)



# Phospholipid profile (PPT procedure, MRM)



# Phospholipid profile (SLE procedure, MRM)



# Phospholipid evaluation using UPLC/MS/MS

- Aliquot volume: 0.25 mL human plasma
- Extraction solvent: 85:15 Ethyl acetate/IPA
- Reconstitution solvent: 0.2 mL of 40:60:0.1 ACN:water:acetic acid

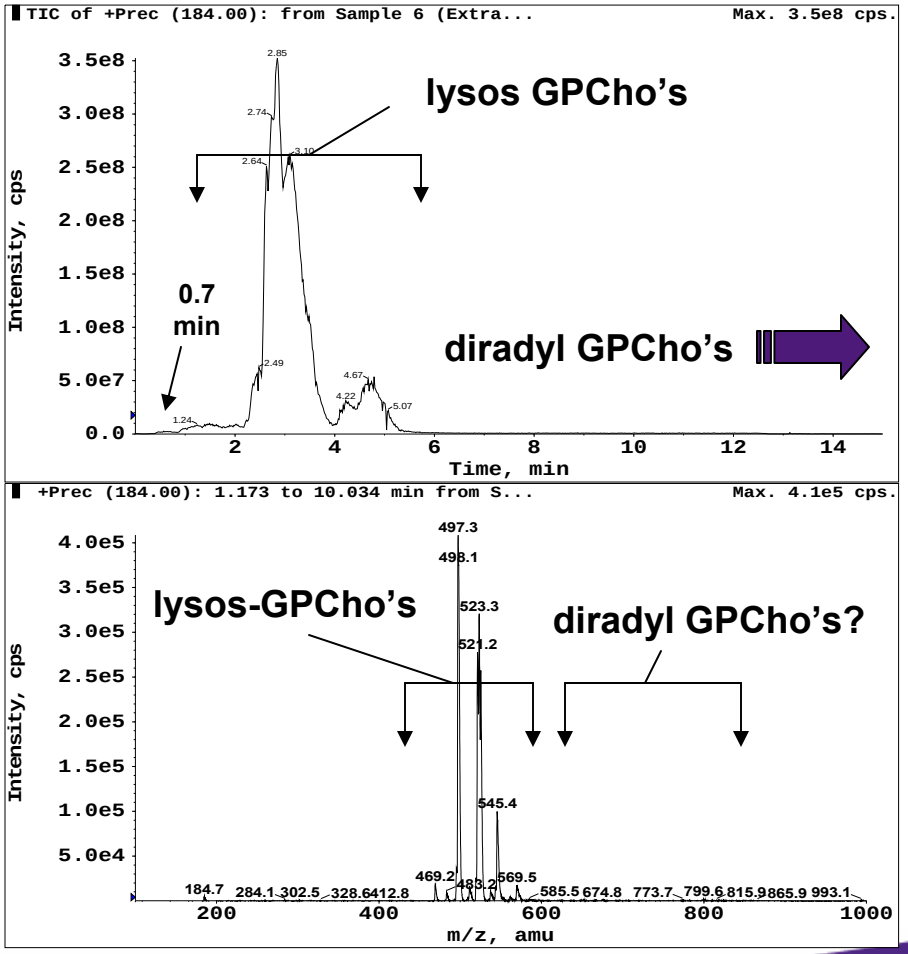
# Instrument conditions

- **Column: Waters VanGuard Acquity UPLC BEH C18, 1.7 µ, 2.1 x 5 mm**
- **Gradient Elution**
- **Mobile Phase A: 45:55 2mM ammonium actetate pH 4 / methanol**
- **Mobile Phase B: 30:70 2mM ammonium actetate pH 4 / methanol**
- **Sciex API-4000, positive ion TurboIonSpray™, Precursor scan of m/z 184**

| Step | Total Time (min) | Flow Rate (µL/min) | Composition | |
|---|---|---|---|---|
| | | | A (%) | B (%) |
| 0 | 0 | 400 | 100 | 0 |
| 1 | 1.1 | 400 | 100 | 0 |
| 2 | 1.5 | 400 | 0 | 100 |
| 3 | 11.9 | 400 | 0 | 100 |
| 4 | 12 | 400 | 100 | 0 |
| 5 | 15 | 400 | 100 | 0 |

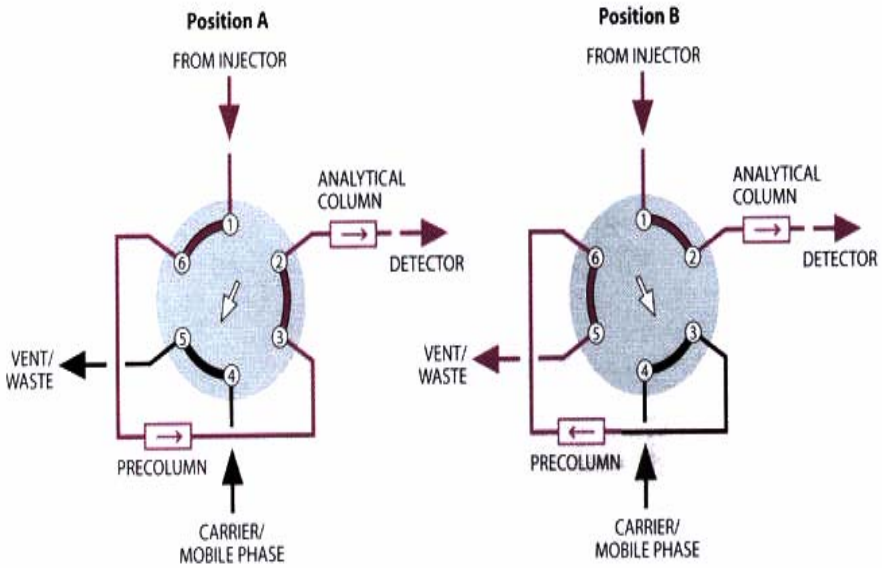
# Phospholipid profile in “forward” flush direction

- Extracted Plasma sample
- Compound X elutes at 0.7 min
- Lysos GPCho’s strongly retained but can be eluted
- Diradyl GPCho’s did not elute!
- Add divert valve and backflush column after compound X elutes.



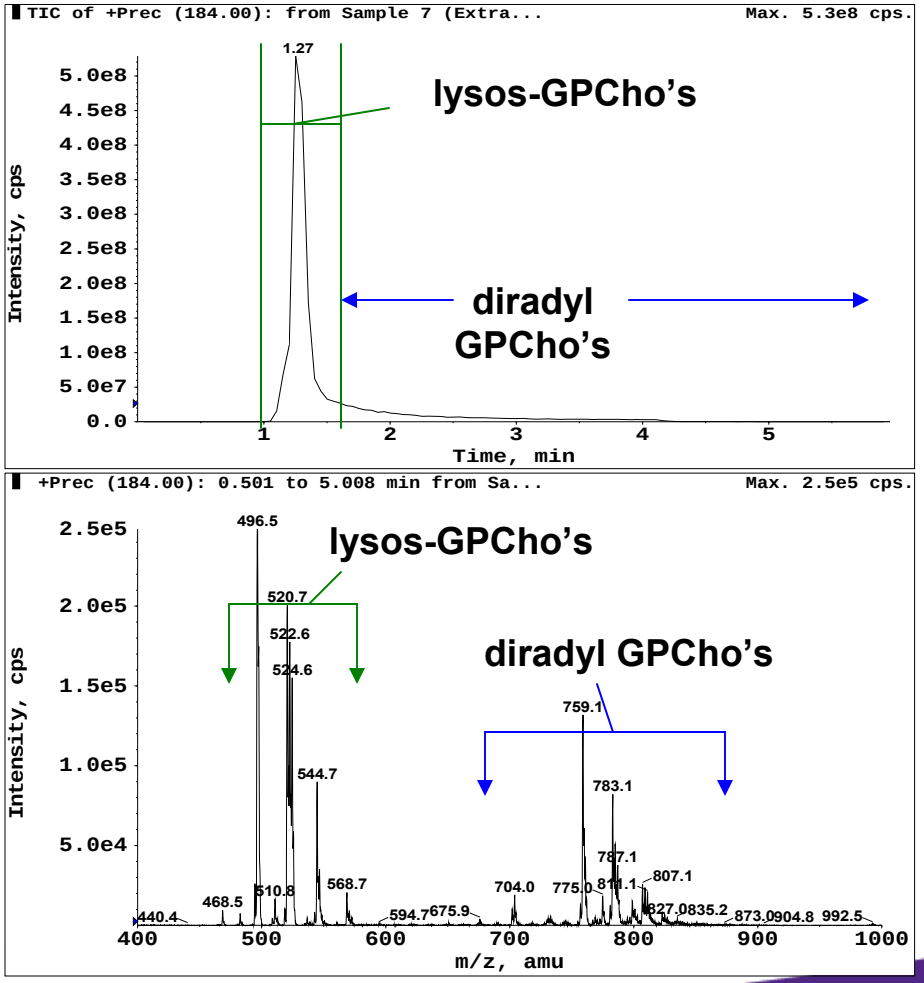
# Phospholipid profile in “back” flush direction

- **Compound X’s elute at 0.7 min and phospholipids are retained**
- **At 1 min valve switches, reversing flow to guard flushing phospholipids to waste**
- **At 5 min valve switches back to original position for requilibration**



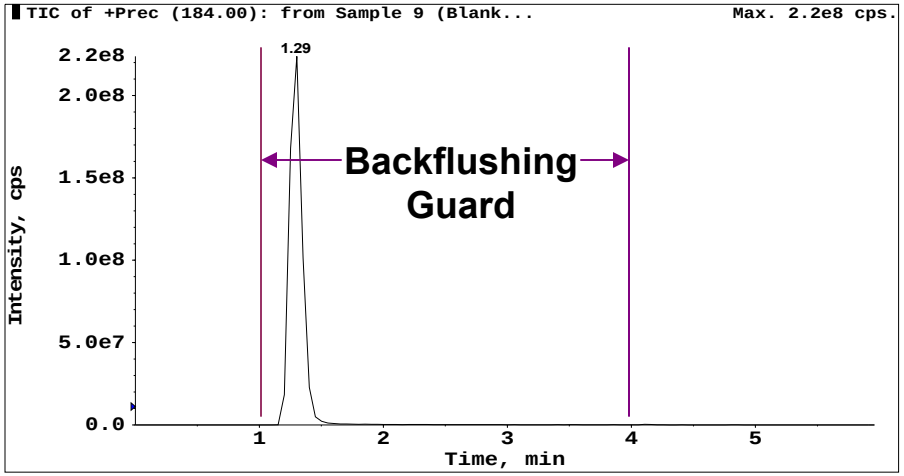
# Guard column backflush monitored for phospholipids

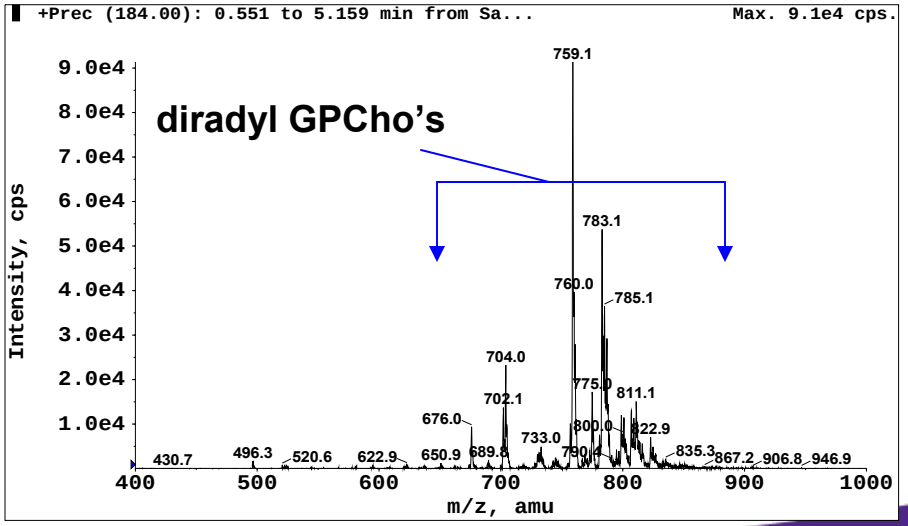
- Extracted plasma sample
- From 1-4 min the guard column is backflushed with 30/70 2mM ammonium actetate pH 4 / methanol at 0.8 mL/min
- The Lysos GPCho's are eluted quickly and the diradyl GPCho's are eluting over a long period of time
- Needs further optimization



# Guard column backflush monitored for phospholipids

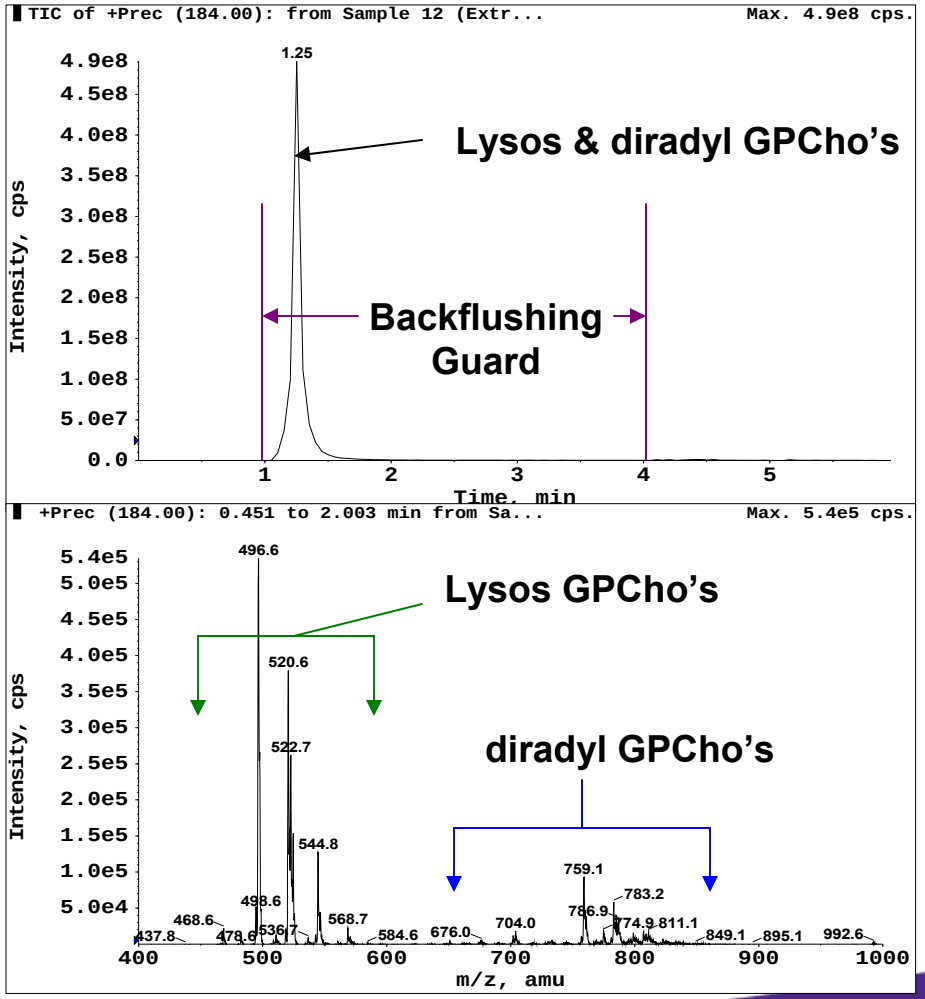
- Injected reconstitution solvent immediately after an extracted plasma sample
- From 1-4 min the guard column is backflushed with 10/90 2mM ammonium actetate pH 4 / methanol at 0.8 mL/min
- Confirms diradyl GPCho's were accumulating on the guard column and 30/70 2mM ammonium actetate pH 4 / methanol is not a strong enough wash
- Confirm phospholipids removed using plasma extract



# Guard column backflush monitored for phospholipids

- Extracted plasma sample
- From 1-4 min the guard column is backflushed with 10/90 2mM ammonium actetate pH 4 / methanol at 0.8 mL/min
- Confirms both the Lyso-GPChos and GPChos are effectively being washed to waste with 10/90 2mM ammonium actetate pH 4 / methanol



# Conclusion

- **HILIC is a powerful technique for LC-MS analysis of polar analytes**
- **Inherent high organic mobile phase is ideal for enhanced MS sensitivity**
- **SLE is an effective technique for off-line removal of phospholipids before HILIC or RP analysis**
- **Column switching on-line removal of phospholipids is flexible and highly effective**

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