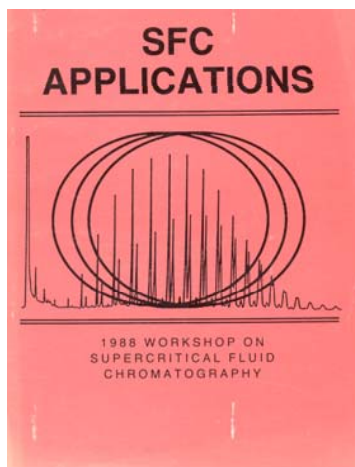


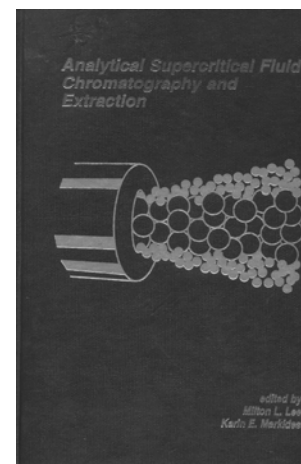
# **Applicability of SFC for Separation of Amine Salts, Phospholipids, and Polypeptides**

**Larry T. Taylor and Mehdi Ashraf-Khorassani  
Department of Chemistry  
Virginia Tech  
Blacksburg, VA 24061-0212 USA**

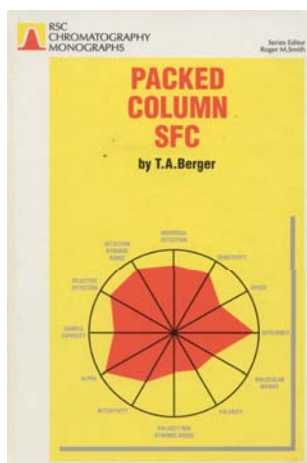
# A Look Back at SFC



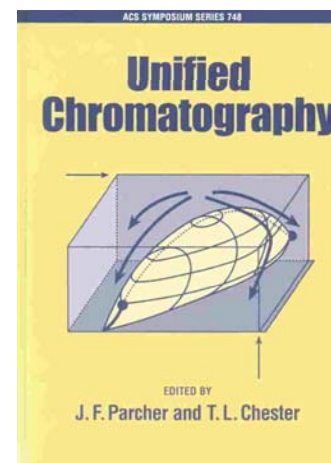
**Atlas of Chromatograms  
1988 – Park City, UT**



**Text by Committee  
Open Tubular Columns  
1990**



**Packed Column SFC  
Terry Berger  
1995**



**SFC Provides the  
Continuum between GC and HPLC  
2000**

# **Can Supercritical Fluid Chromatography Be More Than a “Nitch” Technique?**

**“Above a certain temperature and pressure, matter can be transformed into a bizarre form of matter called a supercritical fluid ...not ordinarily seen on earth”**

**The New York Times  
May 19, 1987**

**SFC is like a singer who dreams of performing in giant arenas in front of sold-out crowds but can only land small gigs. In 2002 the technique was said to be on the verge of a comeback. Six years later, it has yet to share the spotlight with HPLC, but it has been able to gather a small, devoted following in industry.**

**R. Mukhopadhyay  
Anal. Chem., 2008, p. 3091**

# **Routes for Expanding the SFC Sample Base to More Polar and Ionic Compounds**

- **More polar pure fluid - no**
- **Polar solvent modified CO<sub>2</sub> - no**
- **Polar mobile phase additive - yes**
- **Deactivated stationary phase - possible**

# Role of Additives

(Added to the modifier at levels up to ~0.5% )

- **Suppress solute ionization.**
- **Enhance solvating power of the mobile phase.**
- **Modify stationary phase.**
- **Deactivate solid support.**
- **Form ion pairs with ionic analytes**
- **Alter enantio-selectivity**

---

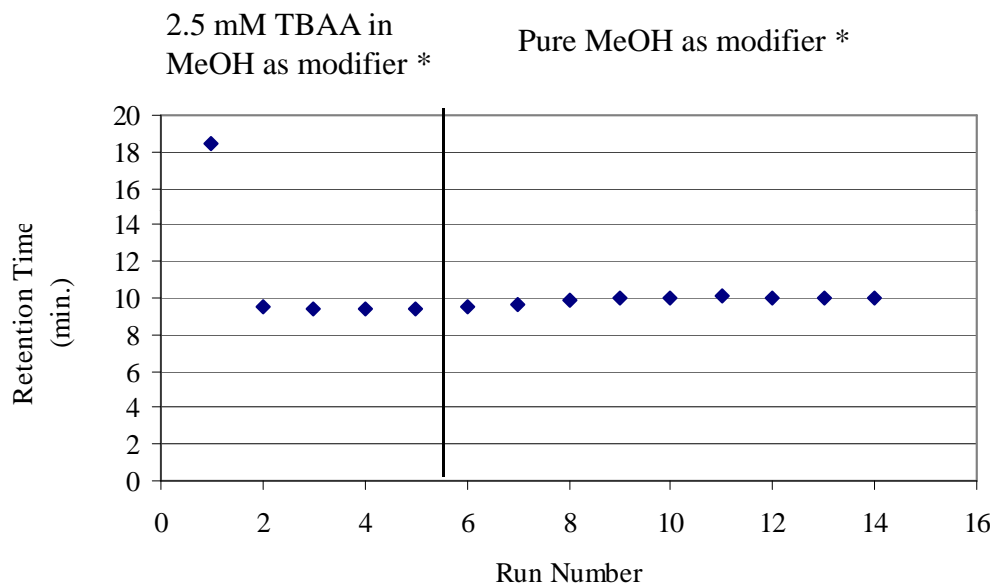
## Most common additives:

- **Bases (diethylamine, isopropylamine)**
- **Acids (trifluoroacetic, alkyl sulfonic, citric)**
- **Salts (ammonium acetate, sodium alkylsulfonate)**

# Our Compound Focus

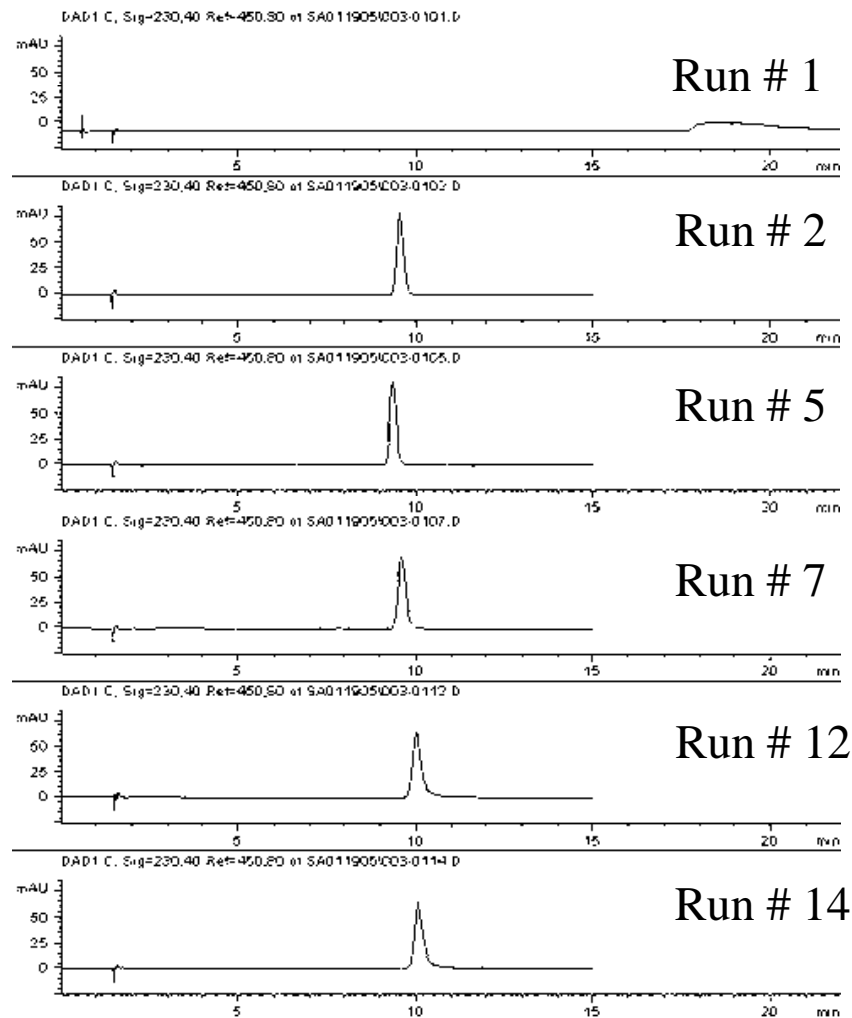
- Ionizable: carboxylic acids, sulfonic acids, primary and secondary amine
- Ionic: cationic quaternary amines, anionic phosphate and sulfonate salts
- Zwitterionic: phospholipids, peptides

# Separation of Sodium p-Toluene Sulfonate on Silica Column with TBAA – Stationary Phase Modified

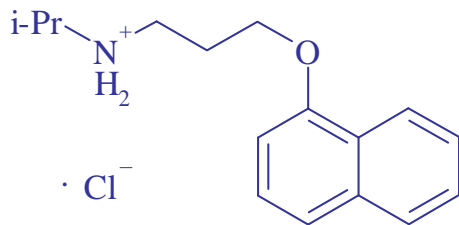


**Strong memory effect/  
similar with AA and TMAA**

\*15% modifier in M.P.; 2 mL/min; 120 bar; 40 °C



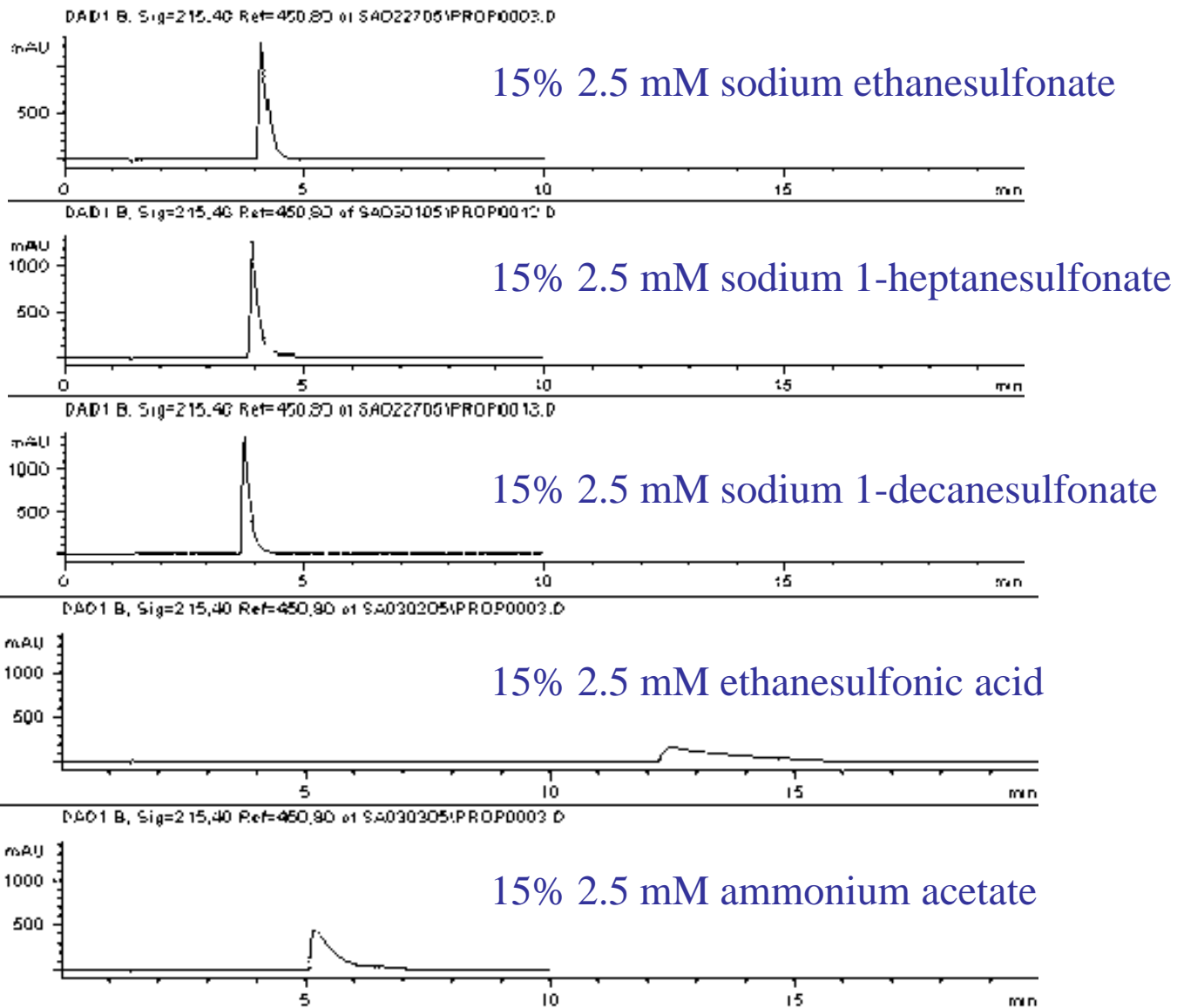
# Ion Pair or Ion Suppression SFC?



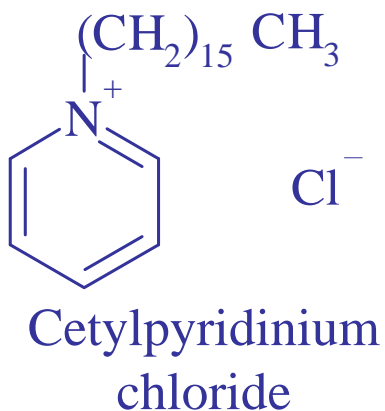
Propranolol  
Hydrochloride

No elution with  
pure methanol

Temperature: 40 °C;  
Pressure: 120 bar; UV  
detection: 215 nm.  
**Deltabond CN**

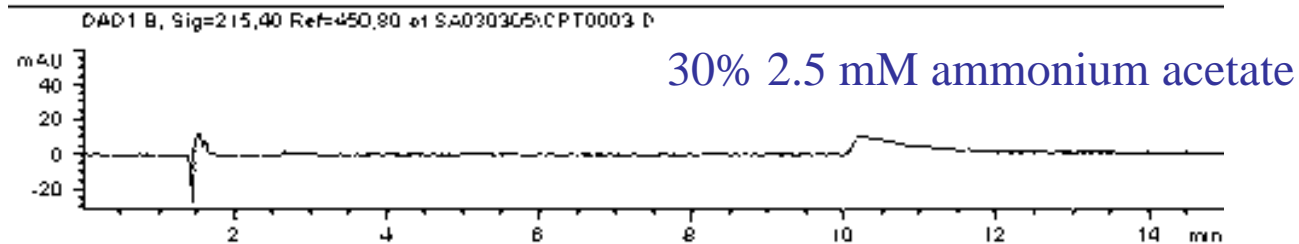
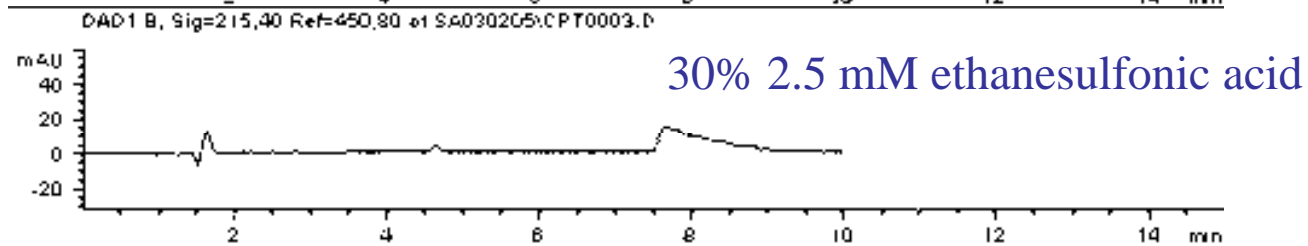
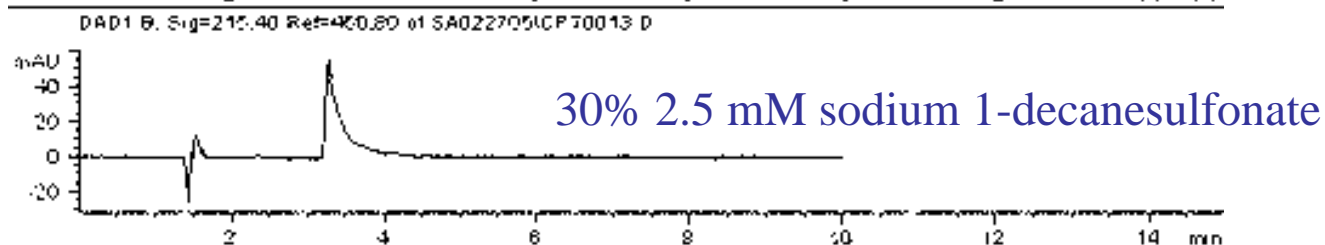
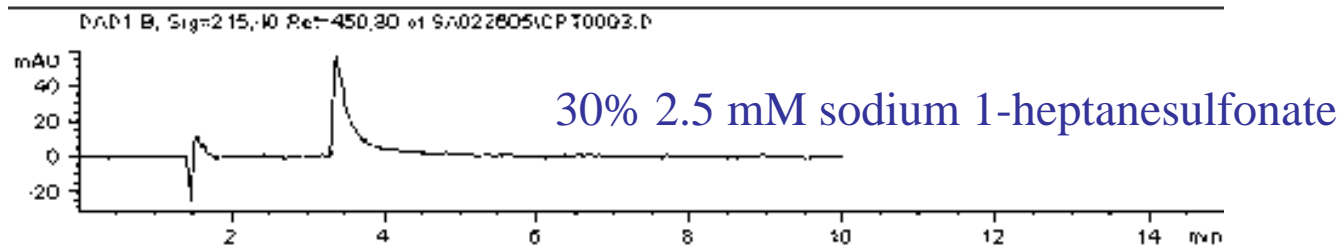
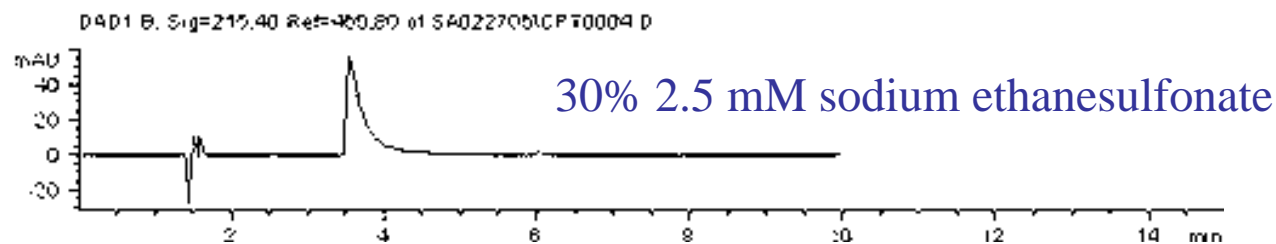


# Ion Pair SFC - Deltabond CN column

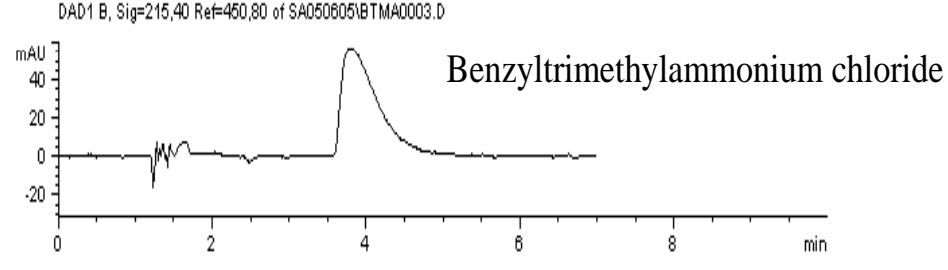
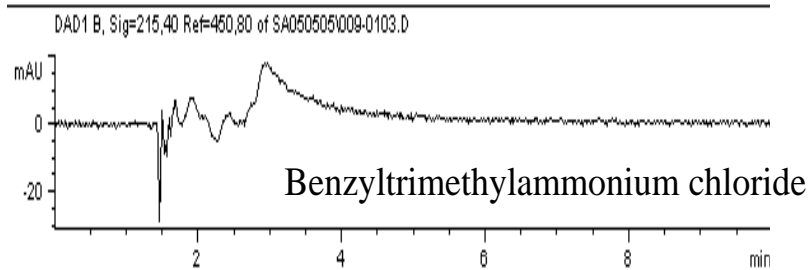
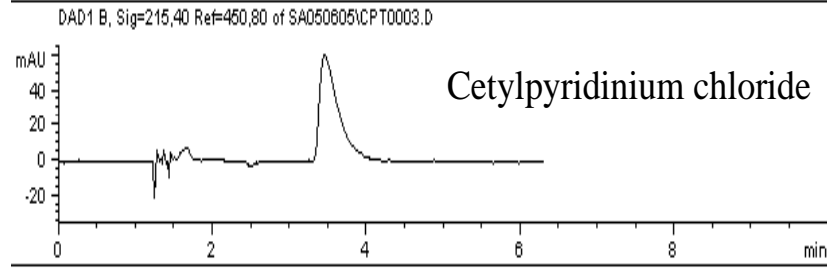
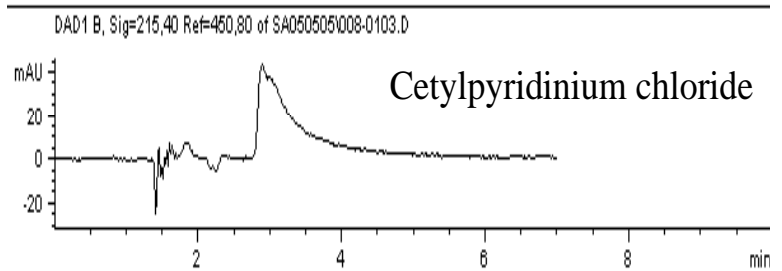
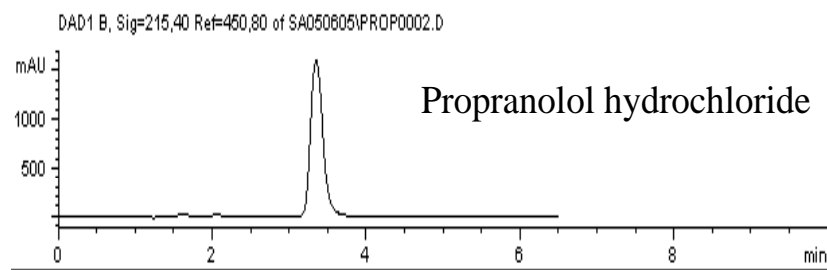
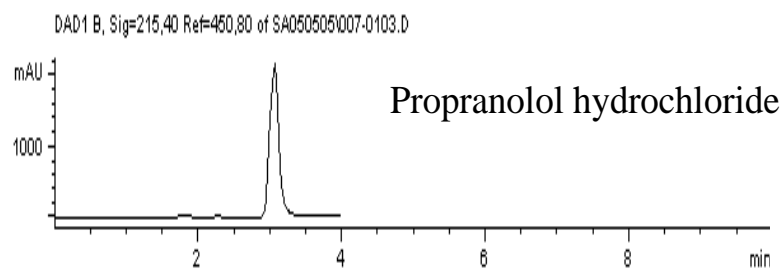


No elution with pure methanol

Temperature: 40 °C;  
Pressure: 120 bar;  
UV detection: 215 nm.



# Elution of Amine Salts on Ethylpyridine Column - Additives Help



**20% MeOH containing 0.5% IPA**

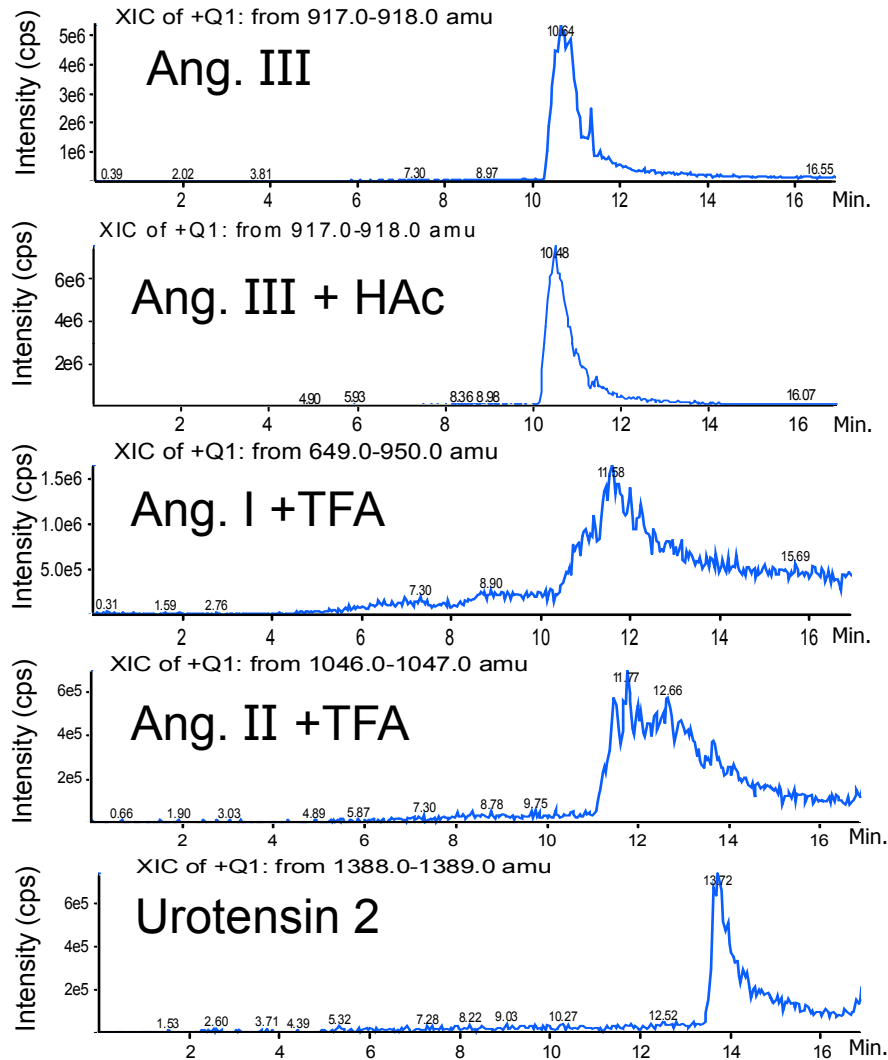
**20% MeOH containing 2.5 mM AA**

# Polypeptides

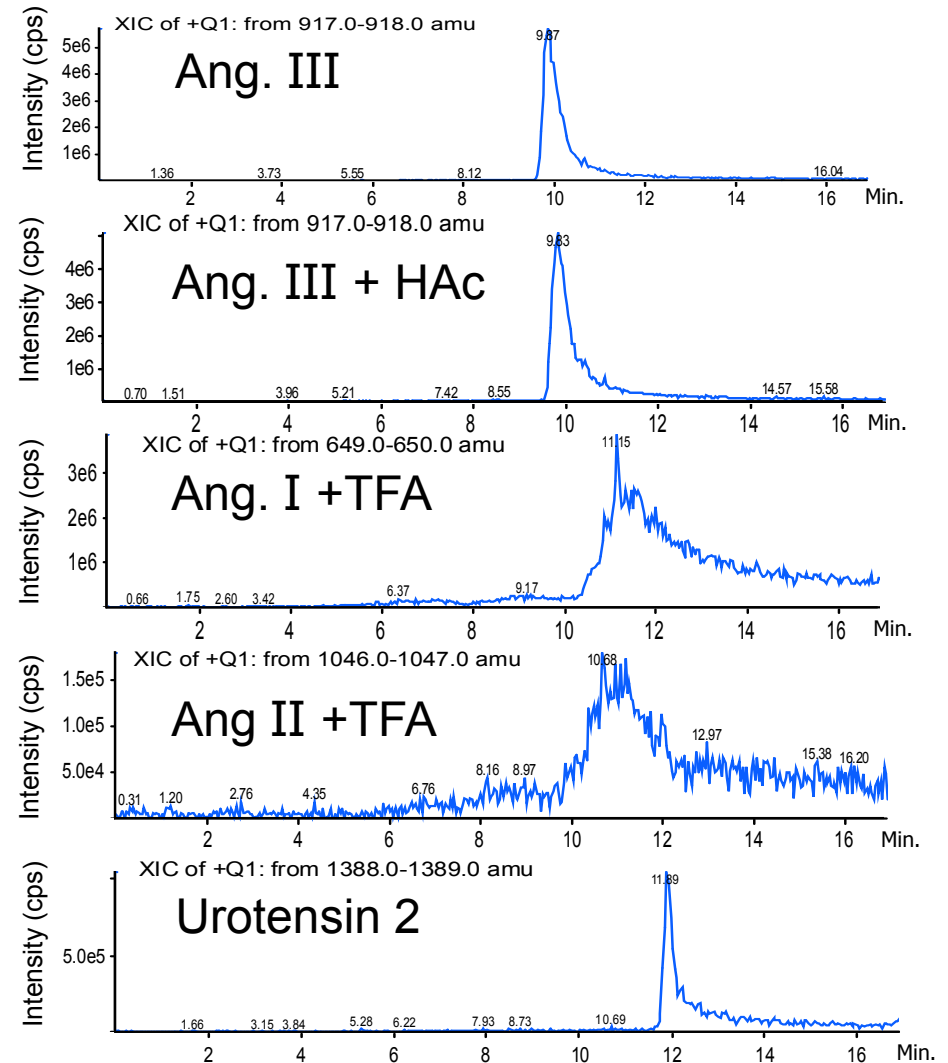
- *Angiotensin I, human (acetate salt)* HAsp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-LeuOH M.M.=1295.7 Da
- *Angiotensin II, human (acetate salt)* HAsp-Arg-Val-Tyr-Ile-His-Pro-PheOH M.M.= 1045.5 Da
- *[Val<sup>8</sup>] Angiotensin III, human (acetate salt)* HArg-Val-Tyr-Val-His-Pro-PheOH M.M.= 916.5 Da
- *Sauvagine*  
PyrGPPISIDLSLELLRKMIEIEKQEKEKQQAANNRLLLDTI-NH<sub>2</sub> M.M.= 4599.4 Da
- *Urotensin II, human (hydrochloride salt)* H-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH•HCl  
M.M.= 1388.6 Da

# SFC-MS of Polypeptides

Pure methanol as modifier

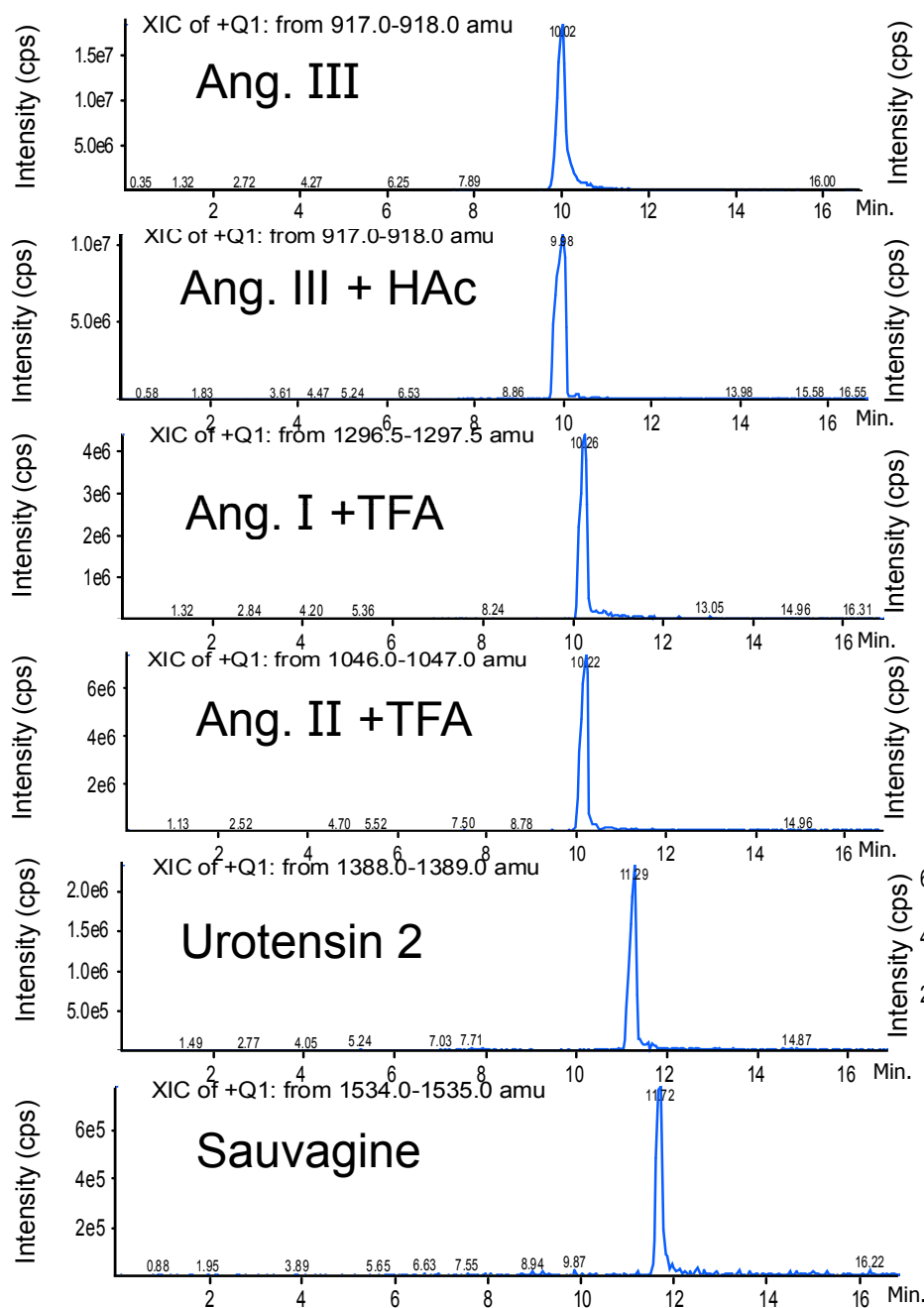


5 mM TFA/MeOH as modifier

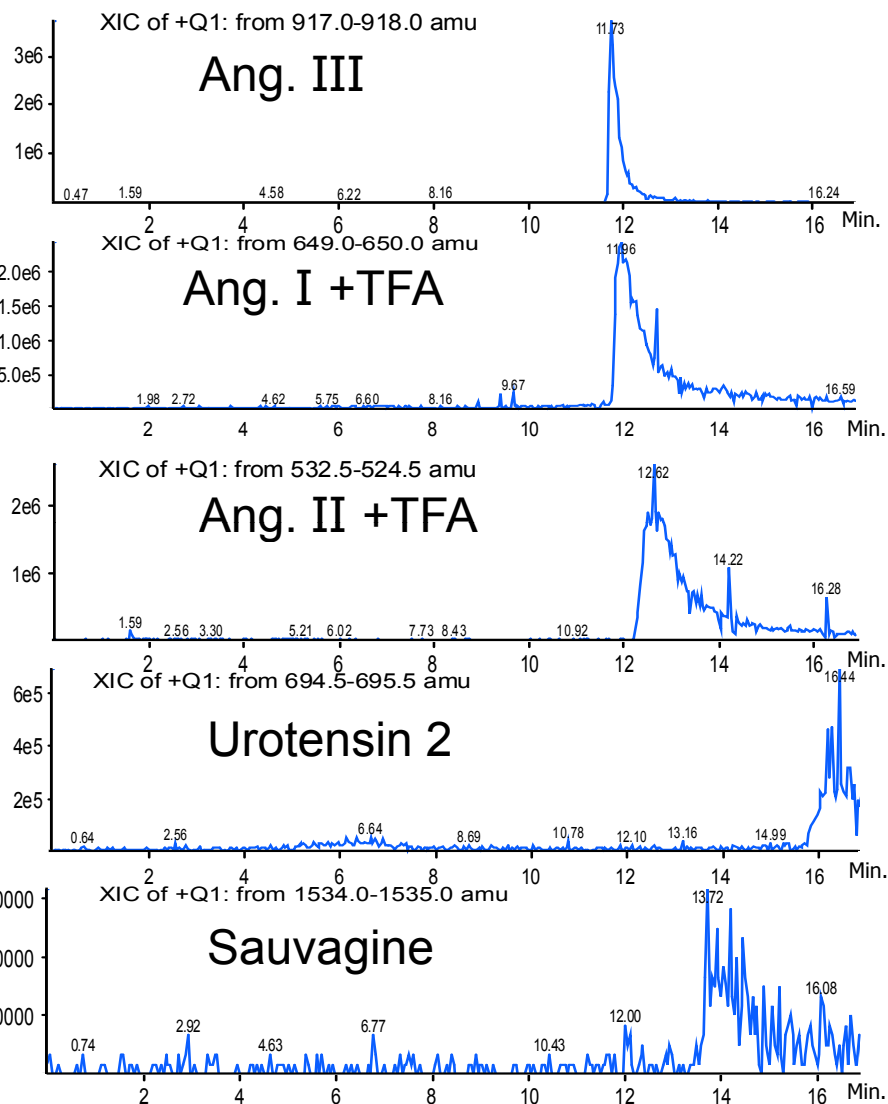


Sauvagine was strongly retained in both cases.

## 13 mM TFA/MeOH as modifier



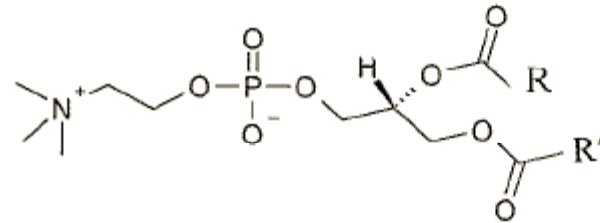
## 13 mM ATFA/MeOH as modifier



13 mM TFA yielded the best result due to its high acidity.

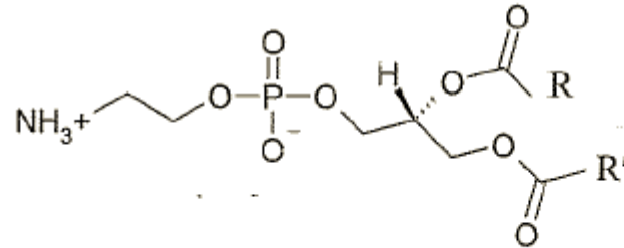
# Phospholipids

PC



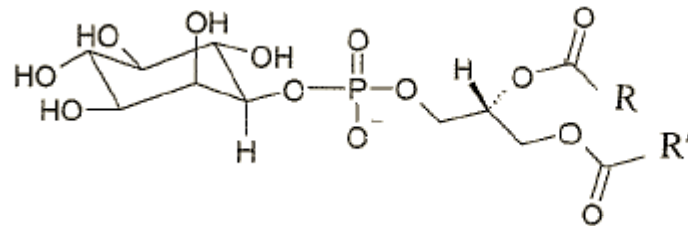
Phosphatidylcholine

PE



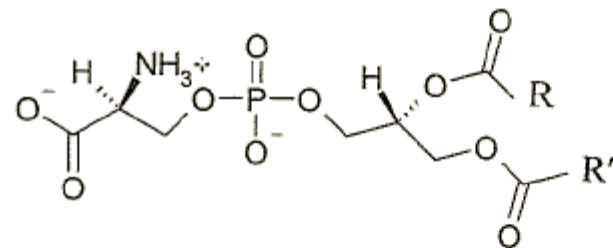
Phosphatidylethanolamine

PI



Phosphatidylinositol

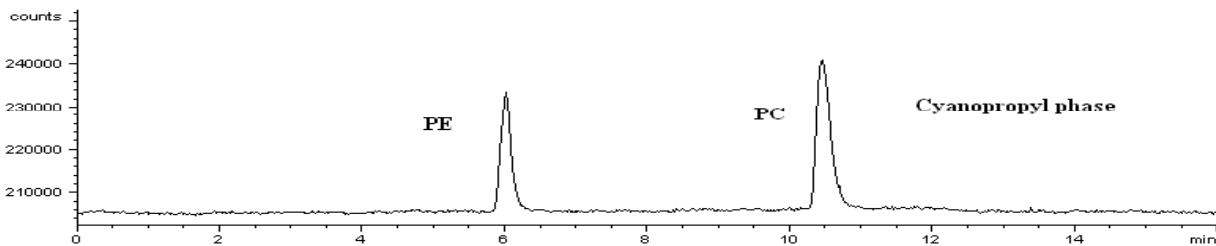
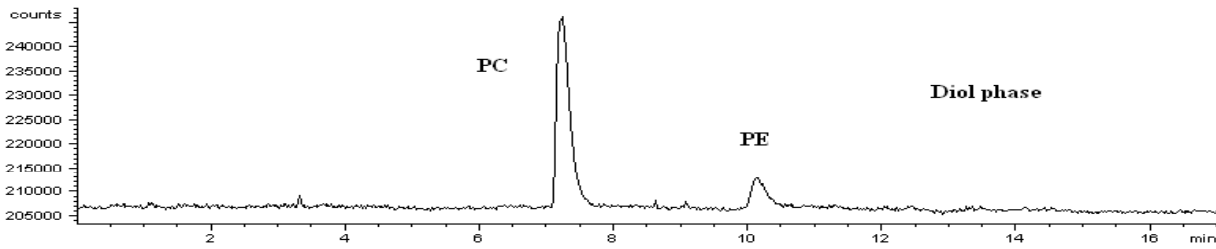
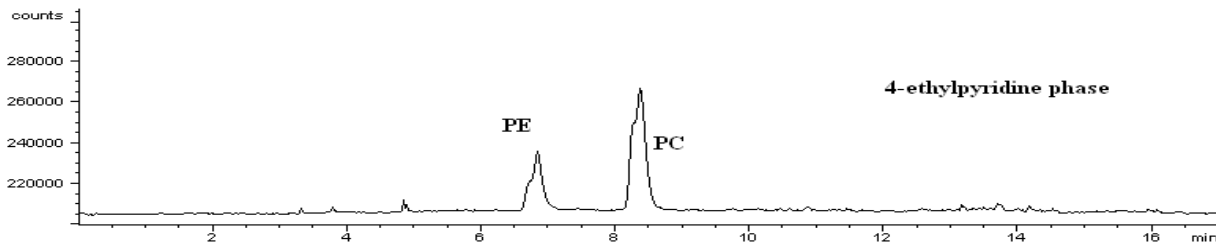
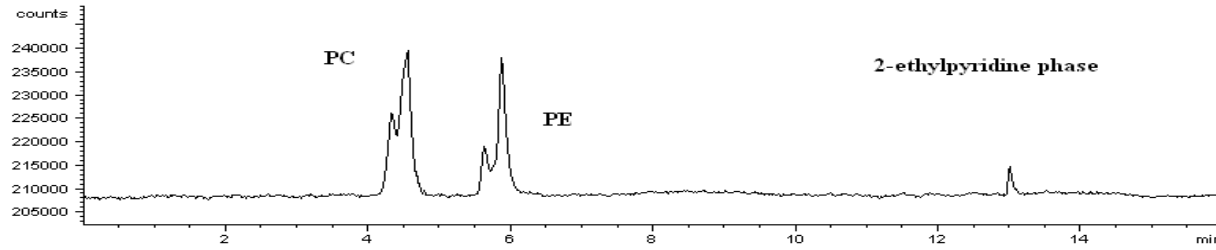
PS



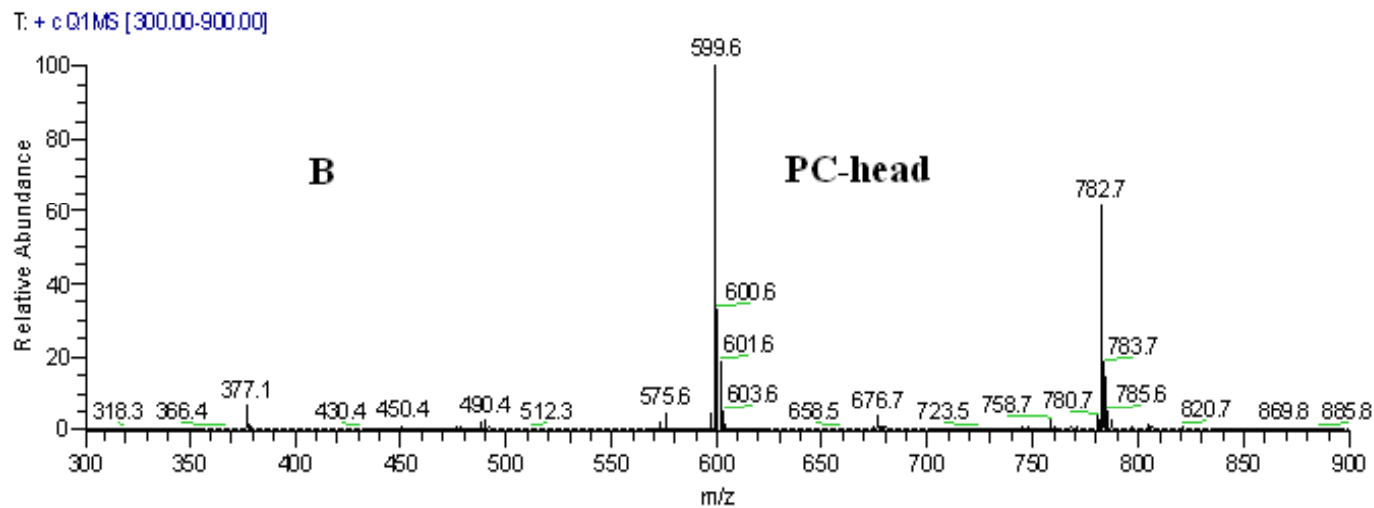
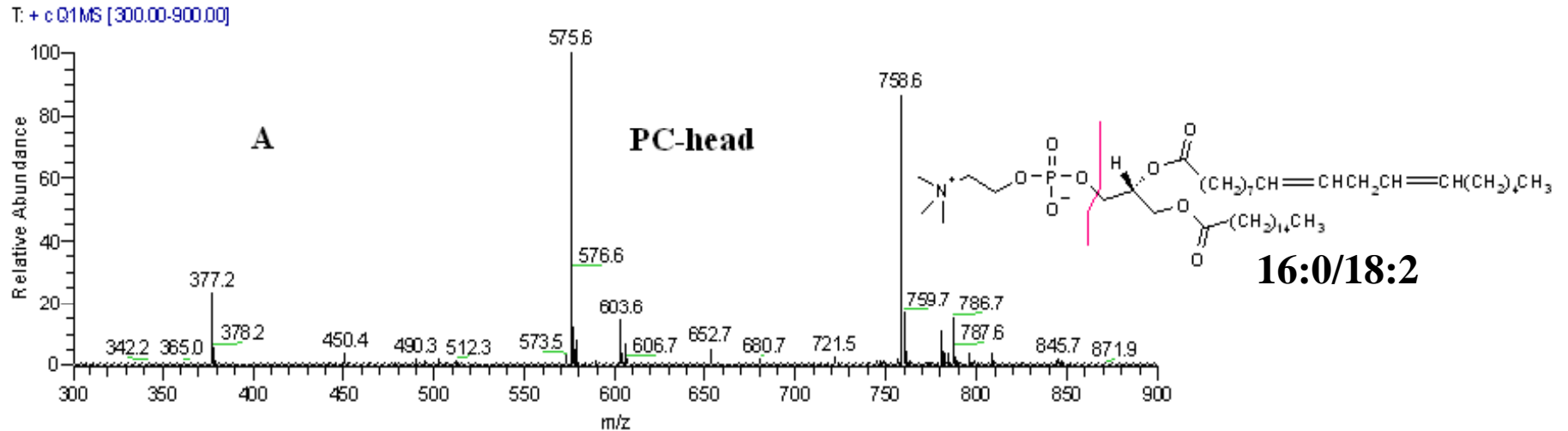
Phosphatidylserine

# SFC-ELSD of phospholipid mixture with methanol modified CO<sub>2</sub> on various stationary phases

- PS and PI did not elute
- Partial separation of PE & PC observed on pyridine columns

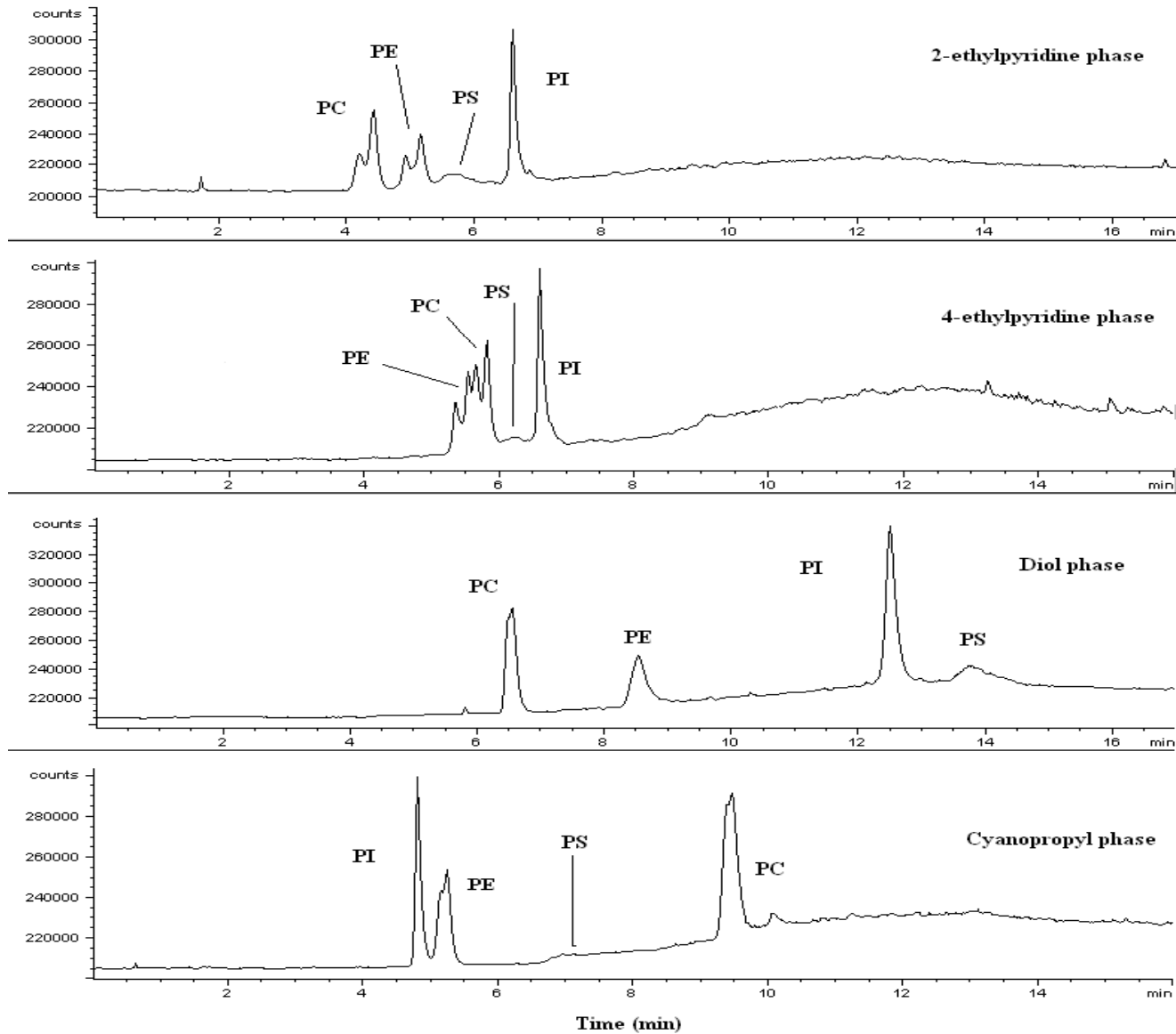


# MS of different PC peak components with only methanol as the modifier



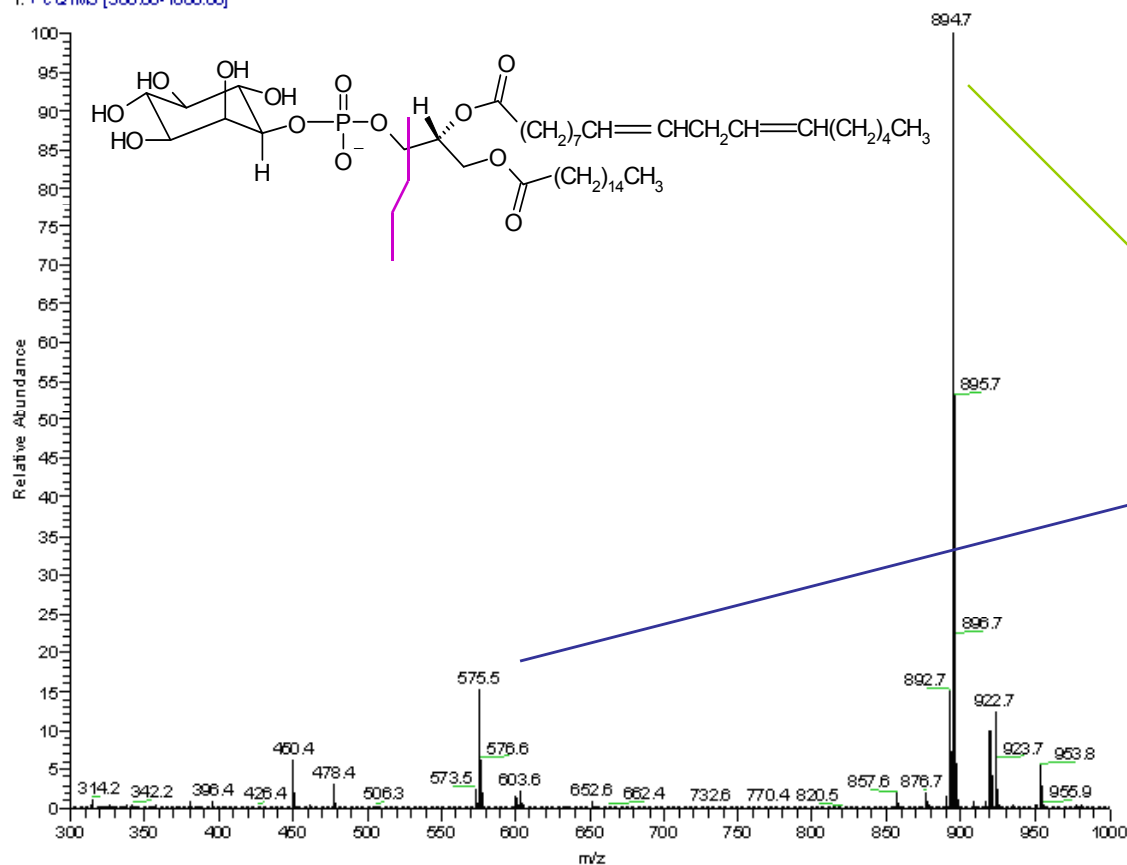
Column : 4-ethylpyridine phase

# SFC-ELSD of PLs with IPA on various stationary phases



# Mass spectrum of PI peak component with Isopropylamine (5mM) as additive

T: + e Q1MS [300.00-1000.00]

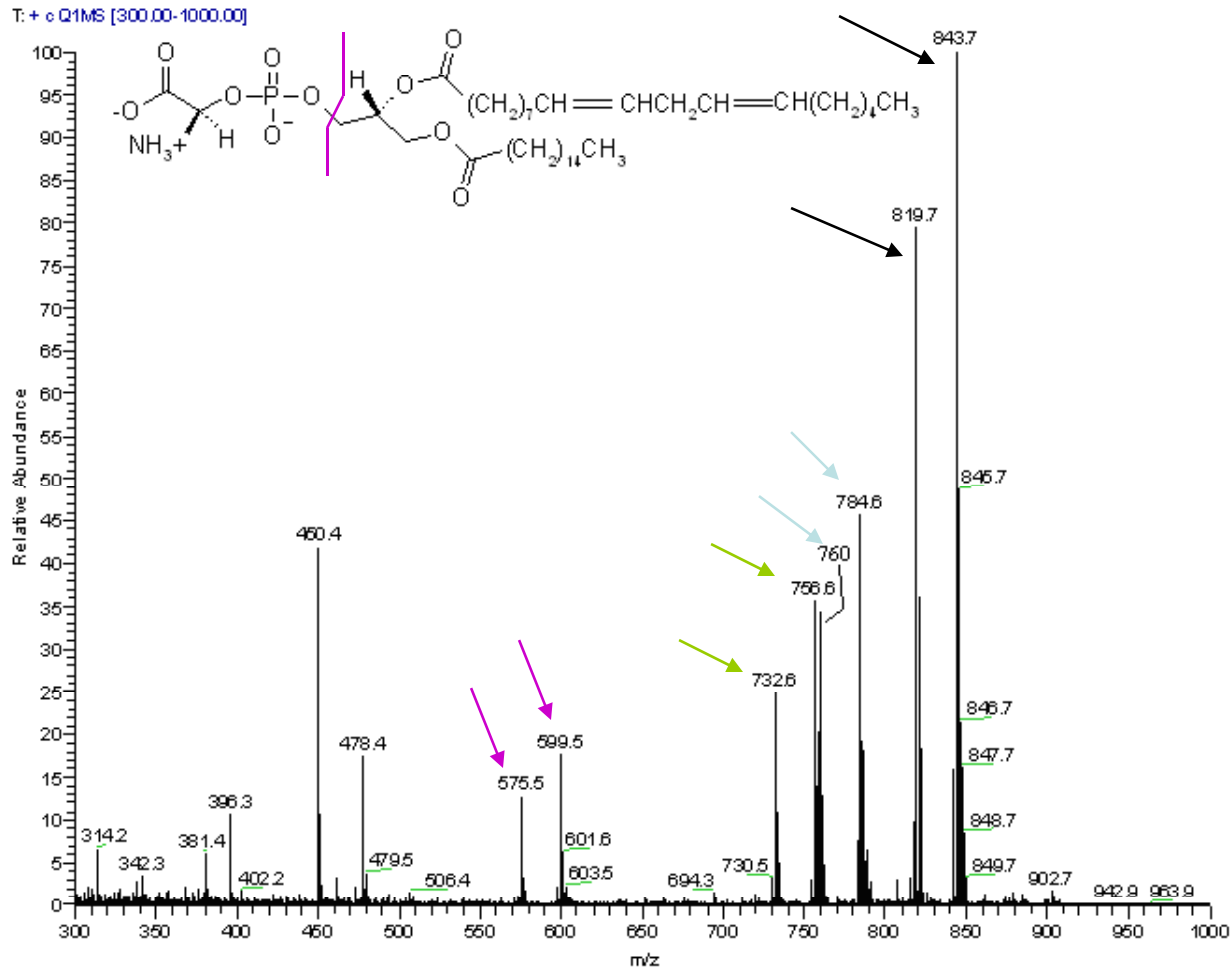


The parent peak is  $m/z$  894 (PI (16:0/18:2) anion + proton + IPA neutral + proton)

The loss of PI head group (260 Da) and IPA additive (59 Da)

- Column : diol phase

# Mass spectrum of PS peak components with Isopropylamine (5mM) as additive



Four pairs of protonated molecules, each pair separated by 24 mass units:  
**m/z 819/843 – IPA adduct;**  
**m/z 760/784 – No adduct;**  
**m/z 732/756 – No adduct;**  
**And m/z 575/599 - Fragment**

**m/z 732/760 and m/z 756/784**  
**The mass difference is 28**  
**suggested by the loss of two methylene groups on the PS chain**

- **Column : diol**

# Conclusions

- **Sulfonate additives enhance the elution of amine salts and vice versa from conventional bonded phases.**
- **Ethylpyridine stationary phase is probably protonated in methanol-modified CO<sub>2</sub> thus promoting the elution of cationic amine salts without the need for additives. Anionic analytes are irreversibly retained.**
- **Separation of polypeptides is enhanced when charged.**
- **Pyridine phases separate phospholipids by polarity completely and in part by hydrophobicity; while cyano and diol phases separate by only polarity**

# Acknowledgments

**Princeton Chromatography Inc.**

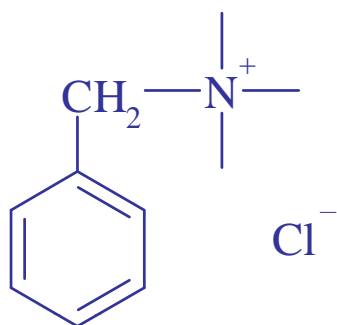
**J. David Pinkston – Procter & Gamble Co.**

**Jun Sally Zheng – Abbott Laboratories**

**Henry Yip – Schering Plough**



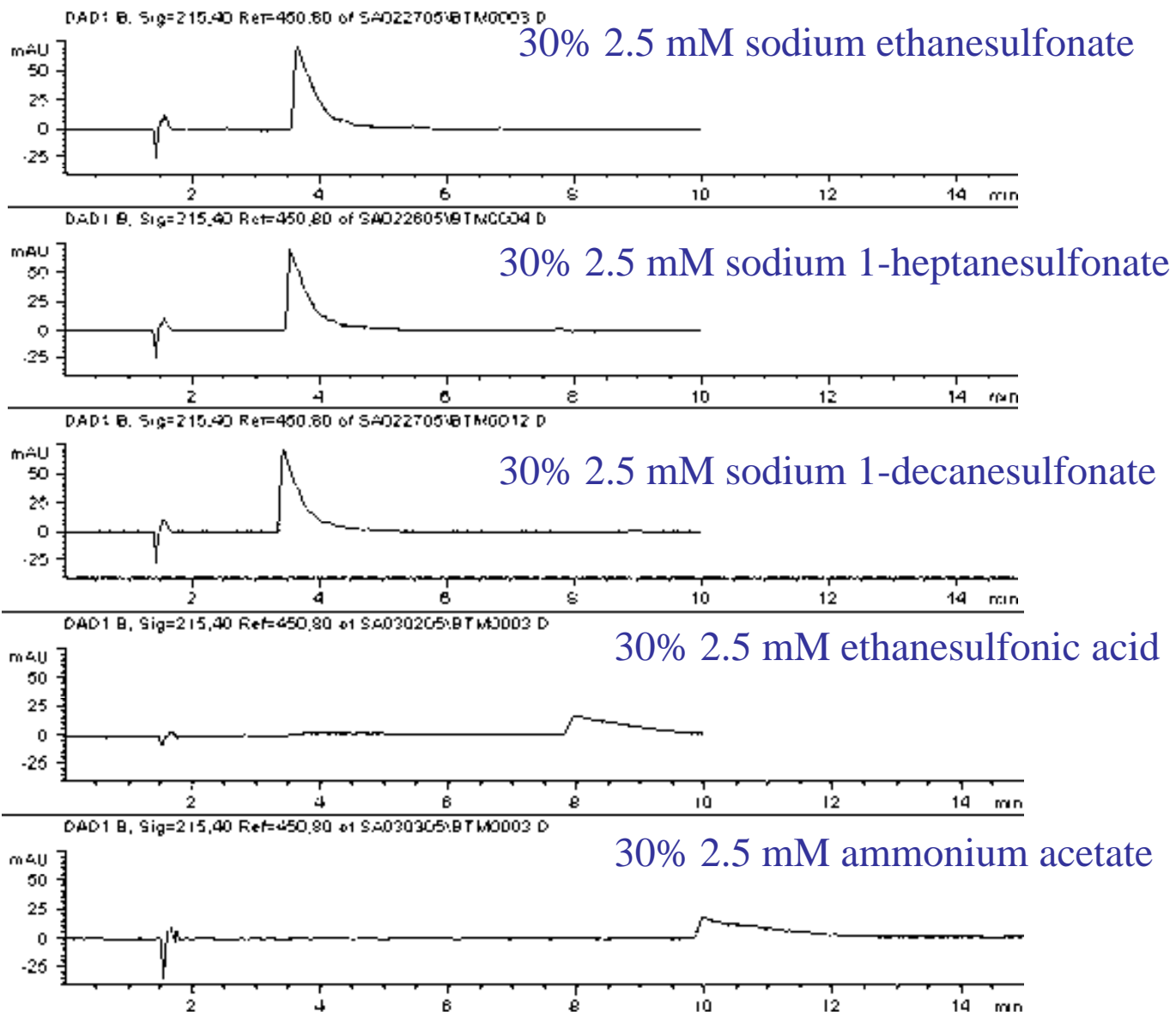
# Ion Pair SFC - Deltabond CN column



Benzyltrimethylammonium chloride

No elution with pure methanol – irreversible adsorption

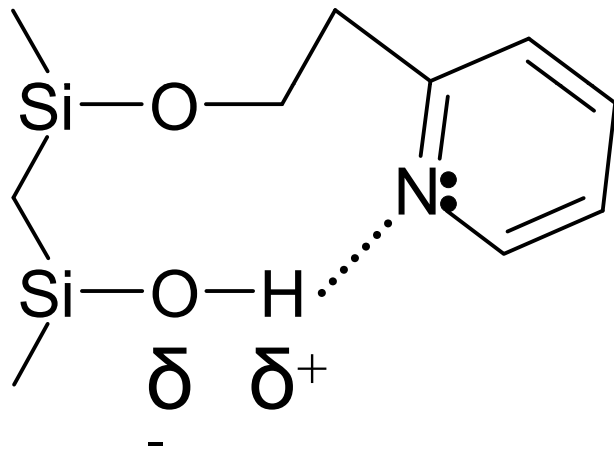
Temperature: 40 °C;  
Pressure: 120 bar;  
UV detection: 215 nm.



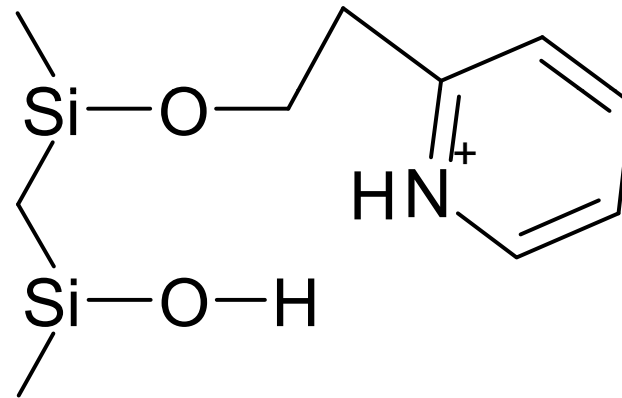
# Background

- 20<sup>th</sup> Anniversary of SFC conferences
- SFC extends GC sample base to higher molecular weight analytes
- Mobile phase modifier extends SFC sample base to more polar analytes
- SFC provides the continuum between GC and HPLC (“extreme case” of liquid chromatography)

## Multiple sites for 2-ethylpyridine phase in methanol-modified CO<sub>2</sub>



Deactivated silanol active site by hydrogen bonding and steric hindrance



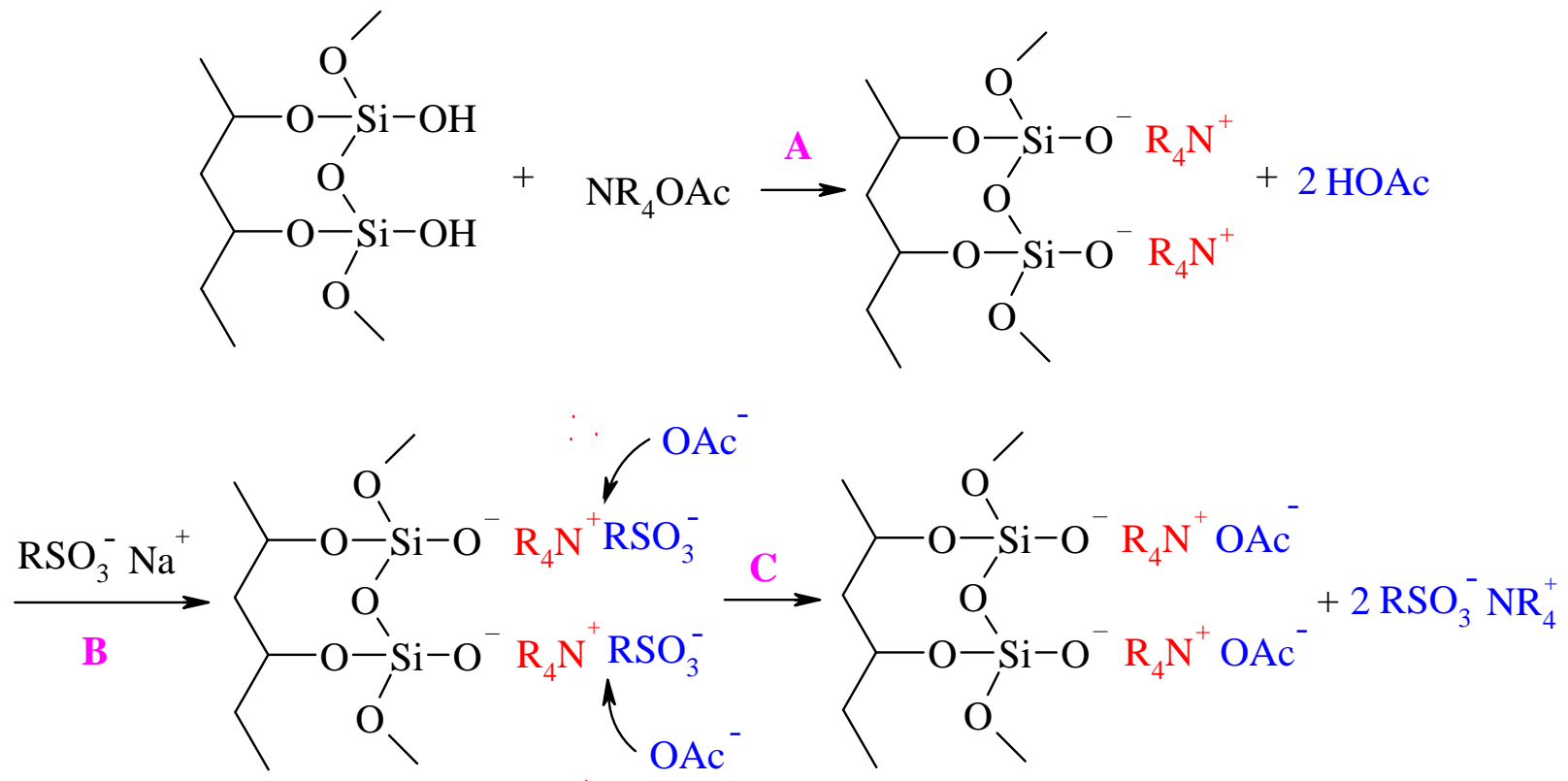
Positively charged stationary phase - CO<sub>2</sub>/MeOH is acidic

Normal Phase Chromatography  
Supercritical Fluid Chromatography  
Hydrophilic-Interaction Chromatography

- More Polar Stationary Phase
- Less Polar Mobile Phase

Is it really chromatography if the separation is governed by polar forces?

# Proposed Elution Mechanism on *Silica Phase* for Sulfonates with Ammonium Additives

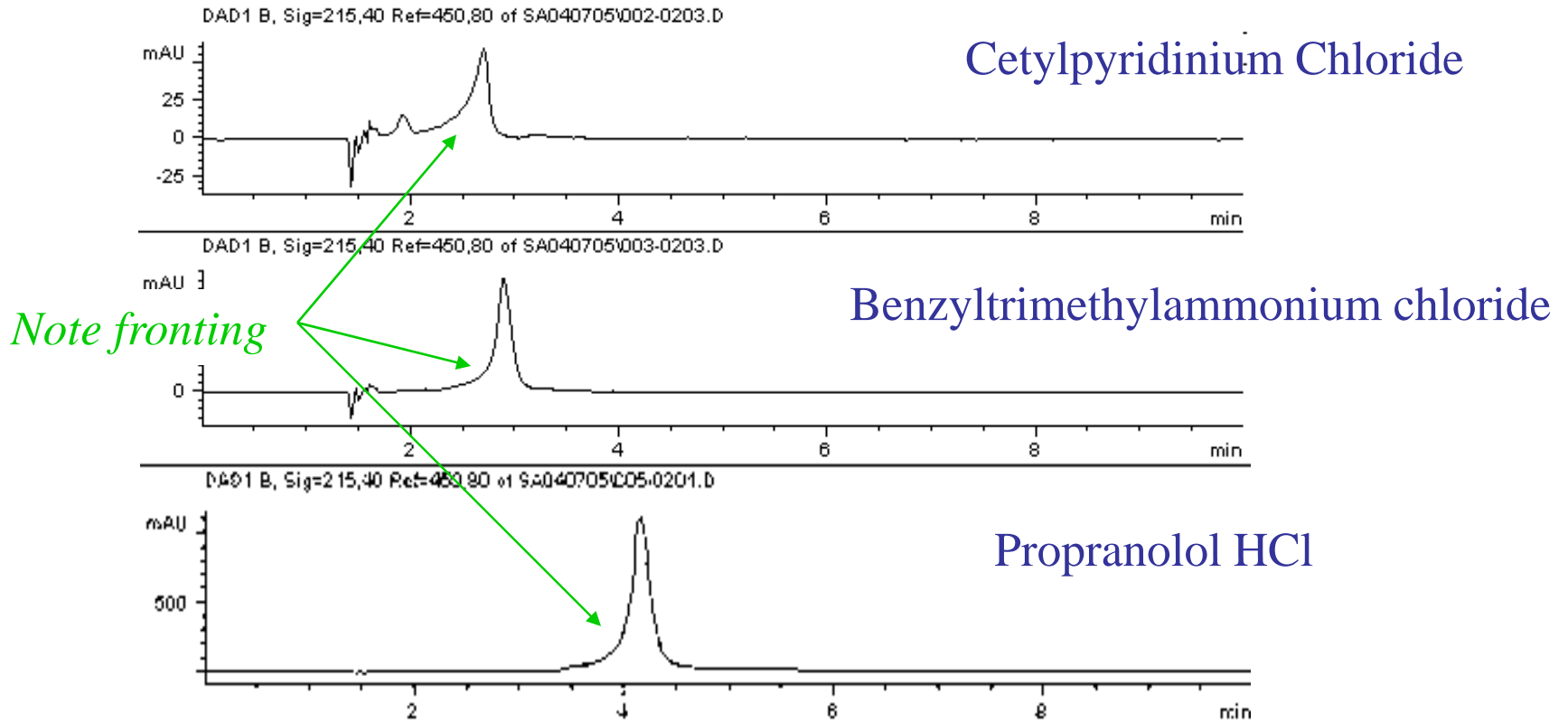


**A** = modification of stationary phase

**B** = charged phase – sulfonate interaction

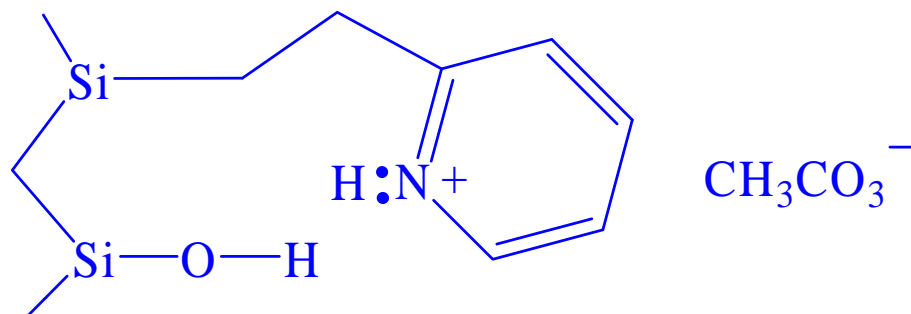
**C** = ion exchange

# Elution of Amine Salts on Ethylpyridine Column – No Additive



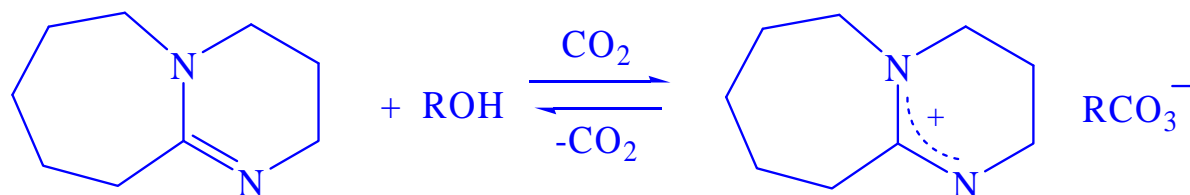
20% pure methanol as modifier; 120 bar; 40 °C

# Suggested Protonation of Ethylpyridine Bonded Phase in Methanol-Modified CO<sub>2</sub>



**Sulfonates are strongly retained on the pyridine column.**

**Quaternary amine salts are not retained on the pyridine column.**



“Reversible Nonpolar – to – Polar Solvent”, P. G. Jessop, D. J. Heldebrant, X. Li, C. A. Eckert, and C. Liotta, Nature, 436, 1102 (2005).

# Summary

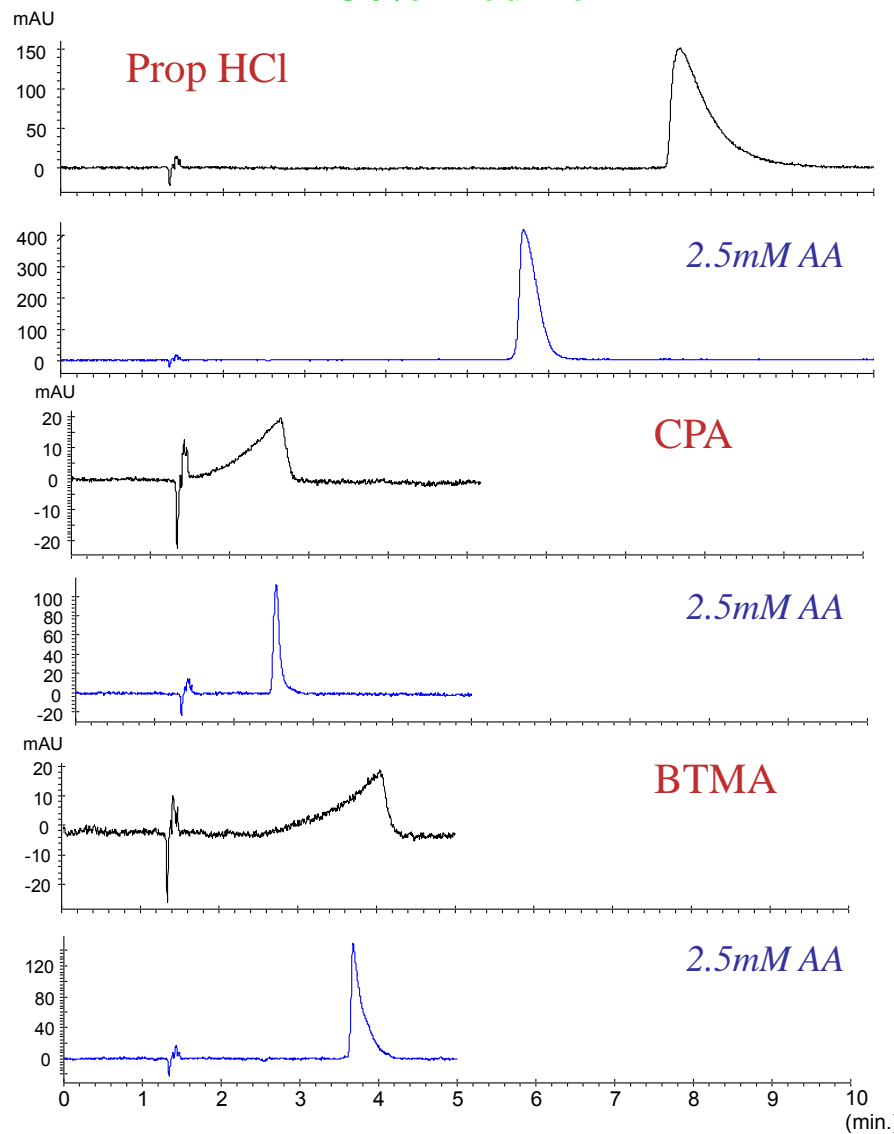
- ✚ Sulfonate additives helped the elution of amine salts and amine salts helped the elution of sulfonates from **Deltabond cyano** phase
- ✚ **Ethylpyridine** column is probably protonated in methanol-CO<sub>2</sub> mobile phase thus promoting the elution of cationic amine salts without the need of additives
- ✚ Similar electrostatic repulsion mechanism is effective between the amine salts and **SAX** column
- ✚ **Ammonium acetate** improved the peak shape of analytes on the **SAX** column by **deactivating silanol groups and interacting with propyltrimethylammonium groups**

# Summary

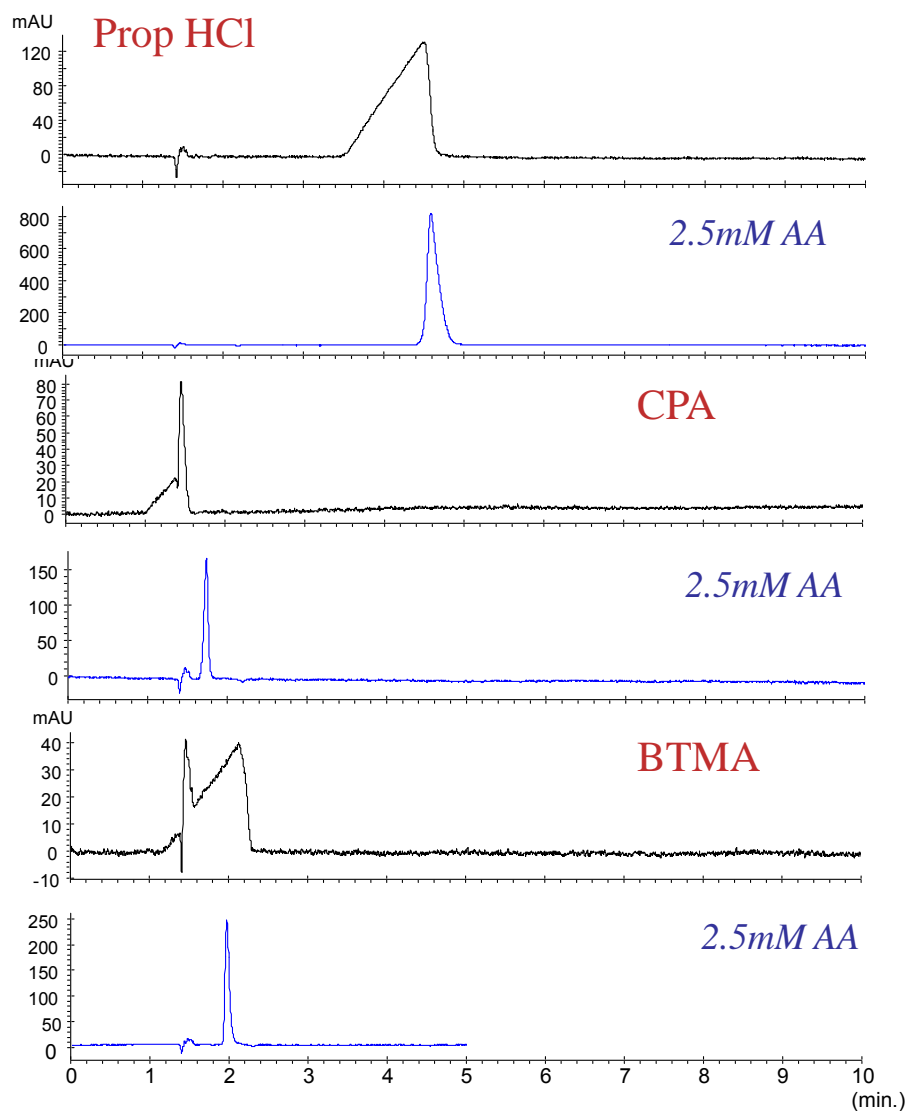
- **No elution of Anionic PLs with methanol modified CO<sub>2</sub>**
- **Pyridine phases separate PC and PE by polarity completely and in part of by hydrophobicity; while cyano and diol phases only separate by polarity**
- **IPA gave better results compared to acidic and ionic additives. The best resolution was found on the diol column.**

# Elution of Amine Salts from SAX Column

30% modifier



40% modifier



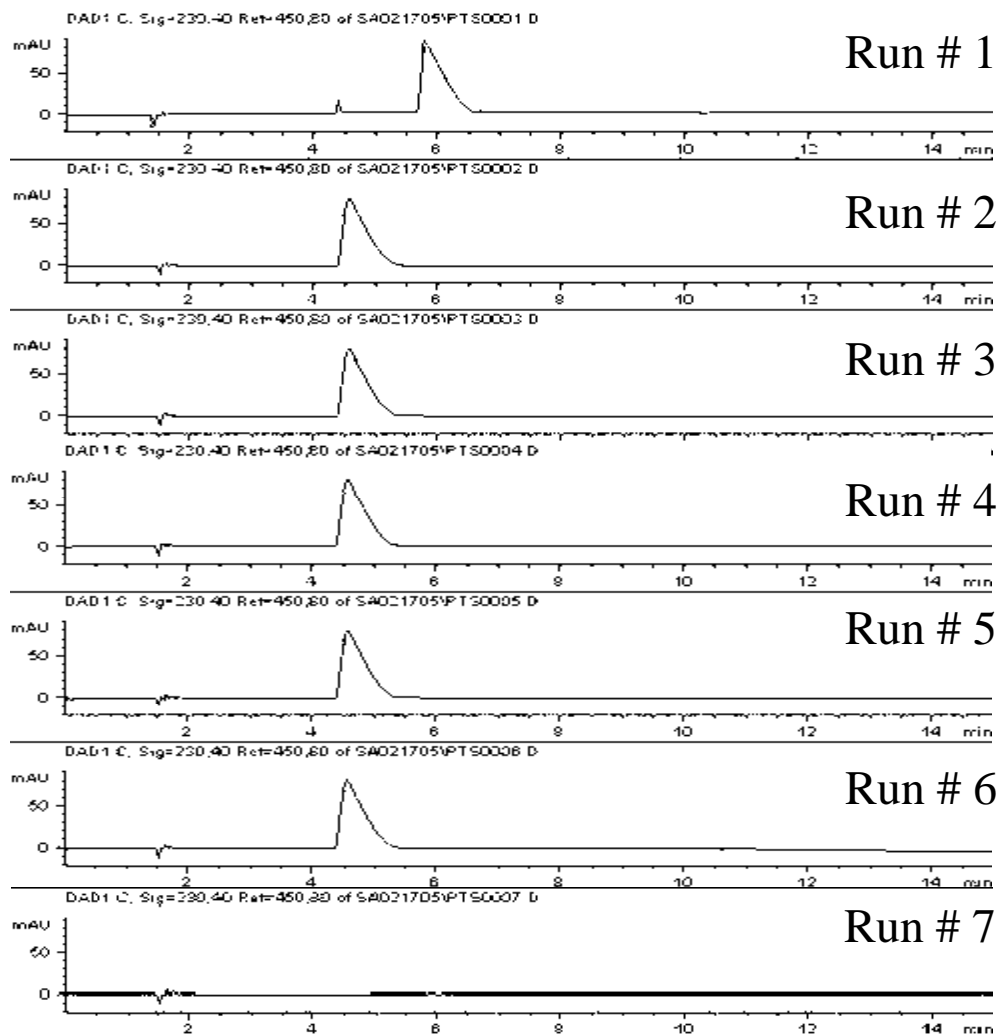
# Can the sample base for SFC be enhanced?

**“Additives will provide a key to the separation of more polar solutes by SFC”, T. A. Berger and J. F. Deye, J. Chromatography, 1999, 547, 377.**

# Separation of Sodium p-Toluene Sulfonate on Deltabond CN Column with AA – Ion Pair SFC

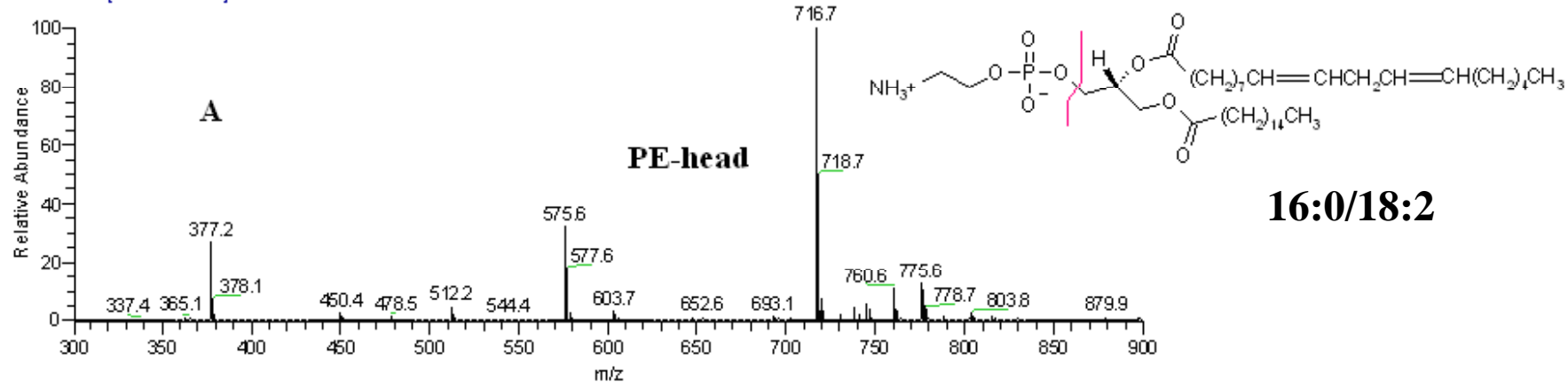
No memory effect after AA was removed from the mobile phase/  
similar with TMAA and TBAA

The modifier was changed to pure MeOH at run #6. The new modifier reaches the column after approximately 8 minutes at 2 mL/min of 15:85 Modifier/CO<sub>2</sub>.

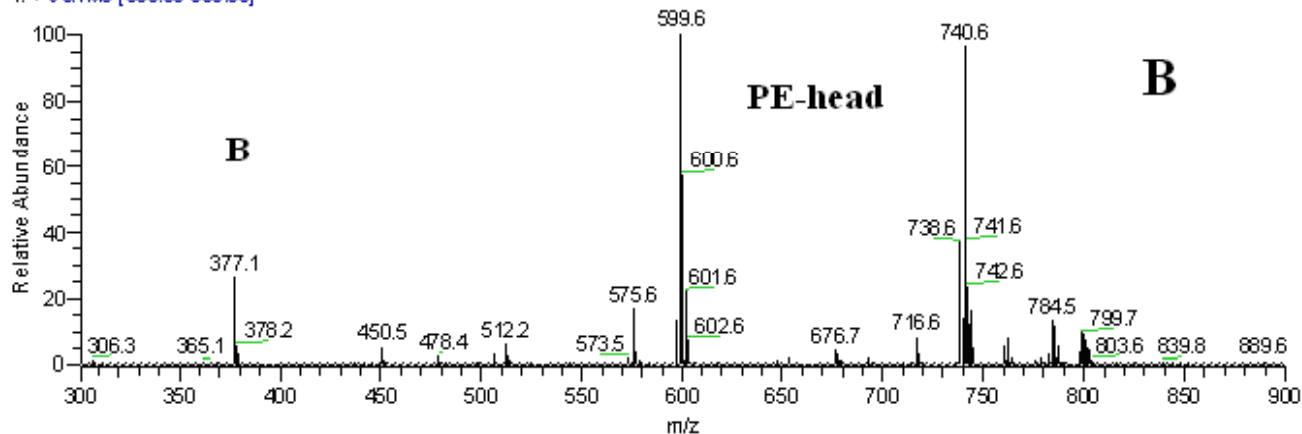


# MS of different PE peak components with only methanol as the modifier

T: + c Q1MS [300.00-900.00]



T: + c Q1MS [300.00-900.00]

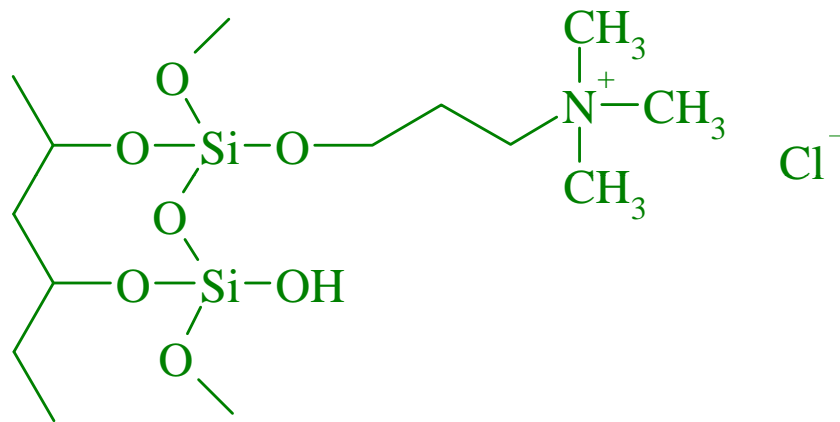


Column : 4-ethylpyridine phase

# Conclusions

- Polypeptides were successfully eluted from Ethylpyridine column **with, and sometimes without, mobile phase additives.**
- The **strength** of the acidic additives was critical to achieve good peak shapes.
- We speculate that protonation of the pyridine functional groups on the stationary phase and of the amine groups of the peptides helped the elution of peptides due to **repulsion between the stationary phase and the analytes.**
- When salts were used as additives, tailing instead of fronting peak shapes indicated **more interaction among the analytes, the additive, and the stationary phase.**

# Elution of Amine Salts from Strong Anion Exchange Column



Supelcosil LC-SAX1

- ❖ Will we be able to elute the analytes without the use of ion-pair reagents?
- ❖ Will there be fronting peak shapes for those permanently charged quats?
- ❖ What would ammonium acetate do on this phase?