

# **CoSMoS ANNUAL MEETING**

**Chapel Hill, NC, July, 2007**

## **A New Approach to Retaining Hydrophilic Compounds in HPLC**

**Joseph J. Pesek**

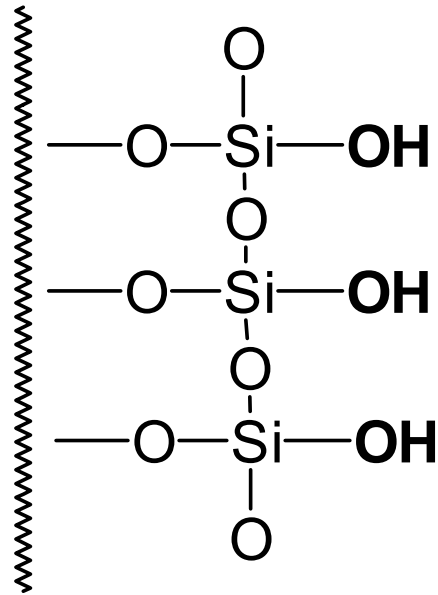
**Maria T. Matyska**

**Department of Chemistry**

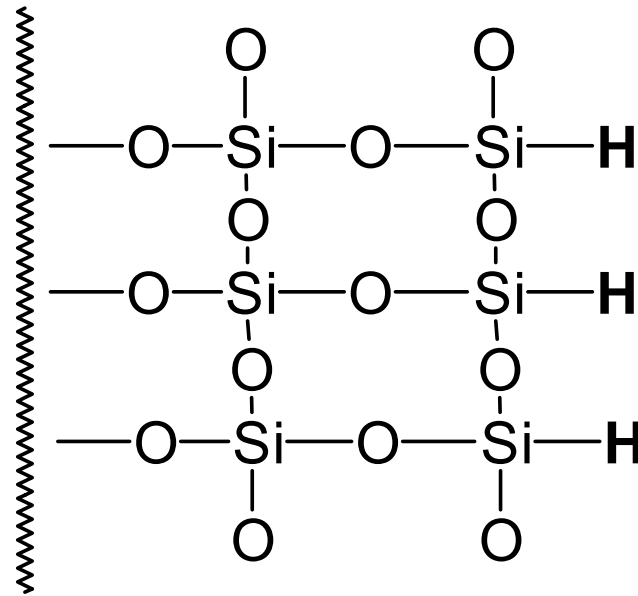
**San Jose State University**

**San Jose, CA, 95192 USA**

# ORDINARY SILICA



# HYDRIDE SILICA



**The presence of the hydride surface plus the bonded organic moiety make three modes of separation possible**

- 1. Aqueous Normal Phase**
- 2. Reversed-Phase**
- 3. Organic Normal Phase**

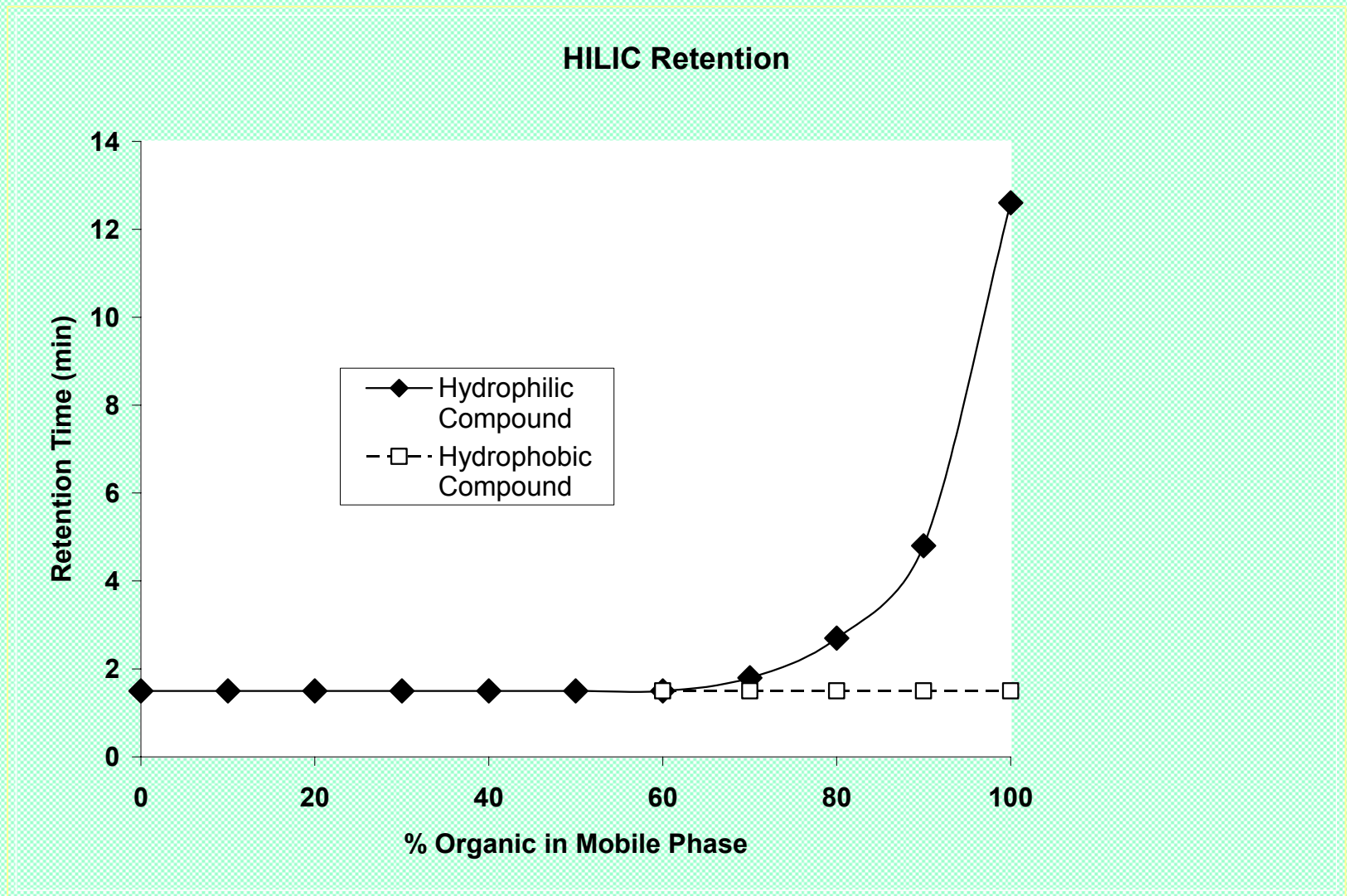
# WHAT IS AQUEOUS NORMAL PHASE RETENTION?

A continuum of retention that provides a transition from the reversed-phase to the normal phase modes with water as a constituent in the mobile phase

**Three distinct retention patterns are possible:**

- 1. No overlap of reversed phase and normal phase retention for two or more compounds.**
- 2. Overlap of reversed phase and normal phase retention for two or more compounds.**
- 3. Individual compounds that can be retained by both reversed phase and normal phase modes.**

# WHAT IS HILIC RETENTION?



# DIFFERENCES BETWEEN AQUEOUS NORMAL PHASE AND HILIC

**Aqueous Normal Phase  
Silica Hydride-Based Column**

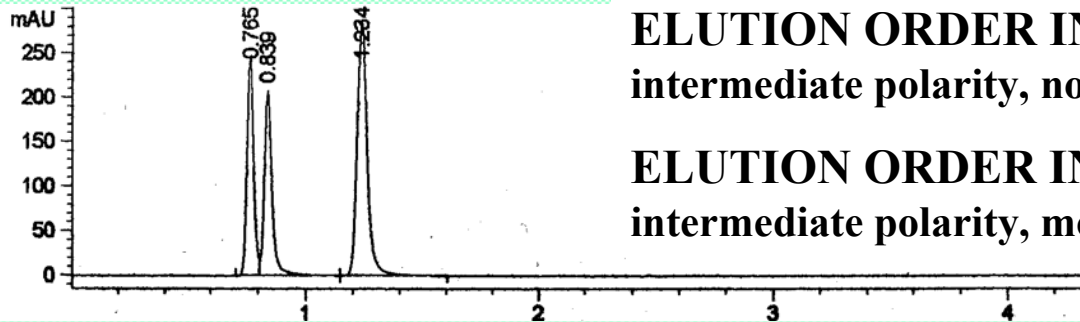
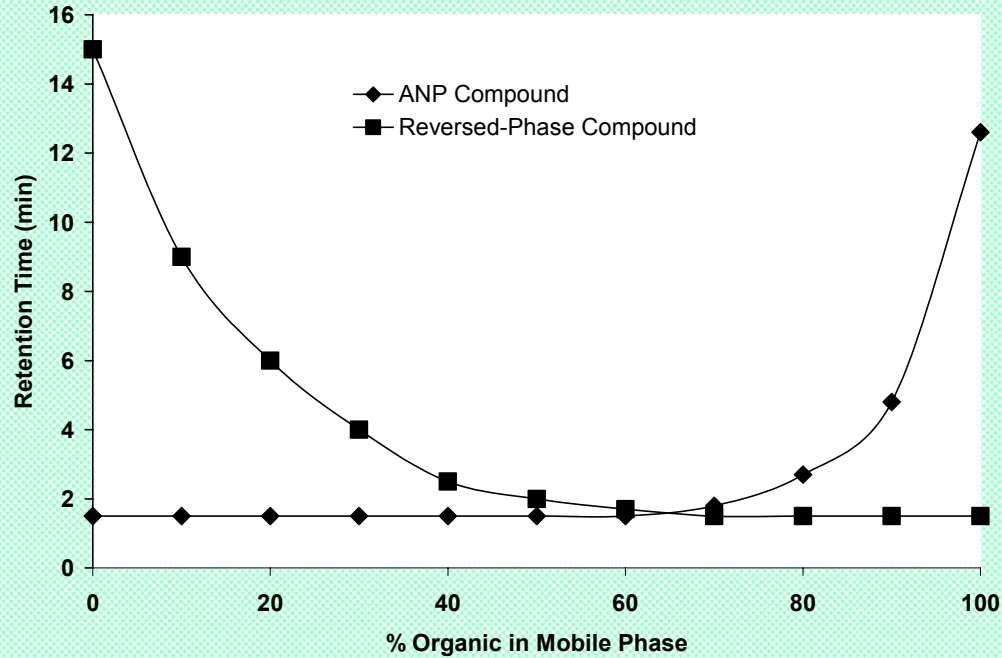
- **Retains nonpolar compounds by reversed phase mechanism**
- **Retains polar compounds by normal phase mechanism**
- **Both reversed phase and normal phase mechanisms can operate simultaneously**
- **Can separate samples with both polar and nonpolar compounds**

**Hydrophilic Interaction Chromatography (HILIC) uses ordinary Silica-Based Column**

- **Retains polar compounds by a normal phase mechanism**
- **Does not retain nonpolar compounds**
- **Cannot usually separate samples having both polar and nonpolar compounds**

# No overlap of reversed phase and normal phase retention for two or more compounds.

ANP 1

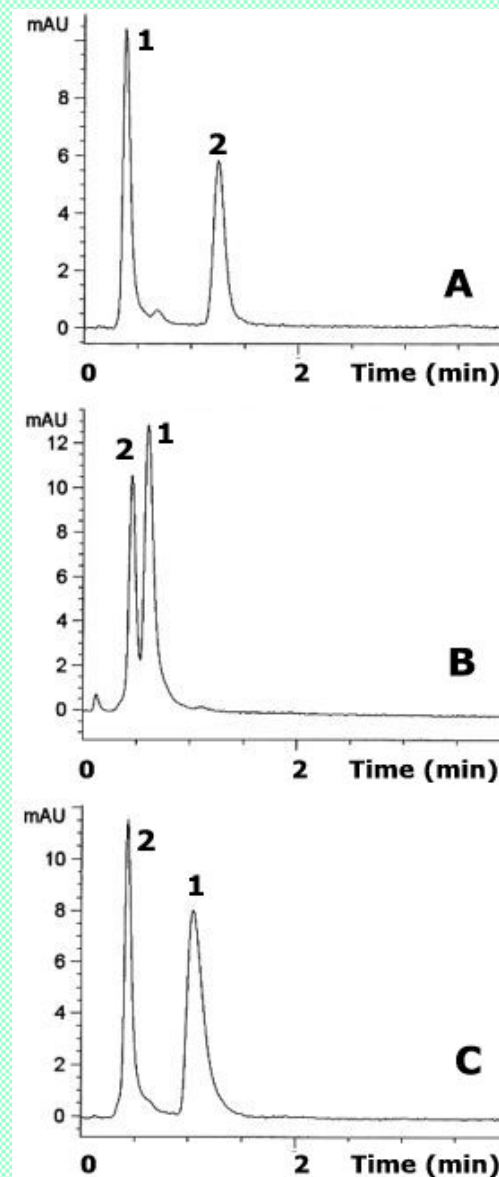
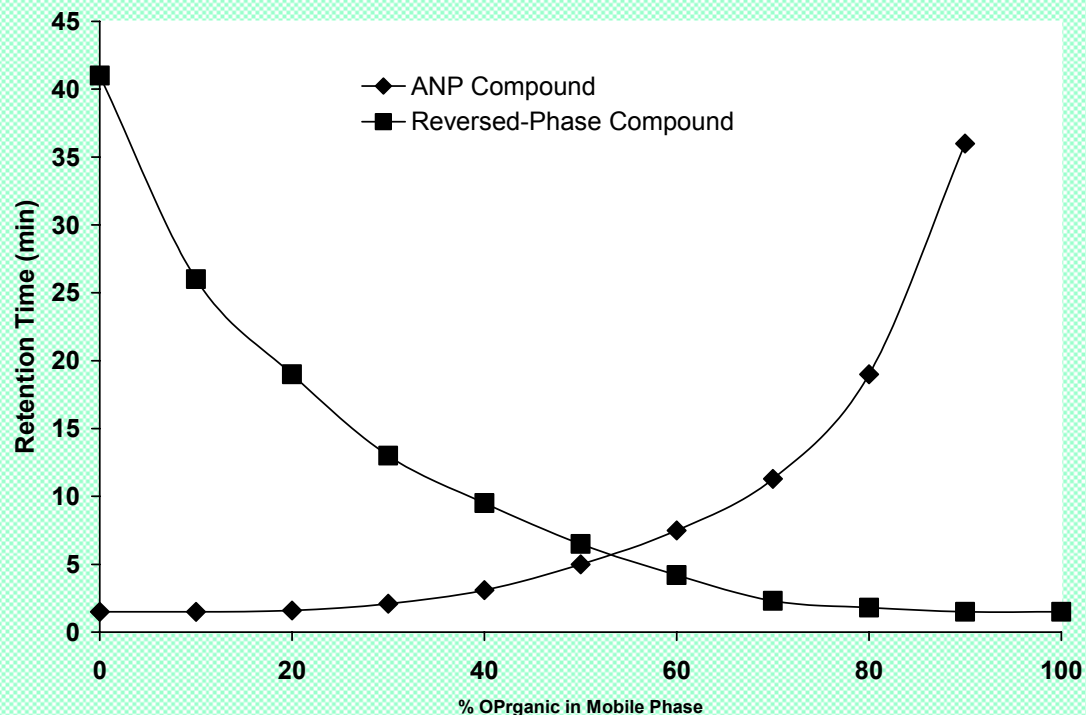


**ELUTION ORDER IN RP:** most polar, intermediate polarity, nonpolar

**ELUTION ORDER IN ANP:** nonpolar, intermediate polarity, most polar

# Overlap of reversed phase and normal phase retention for two or more compounds.

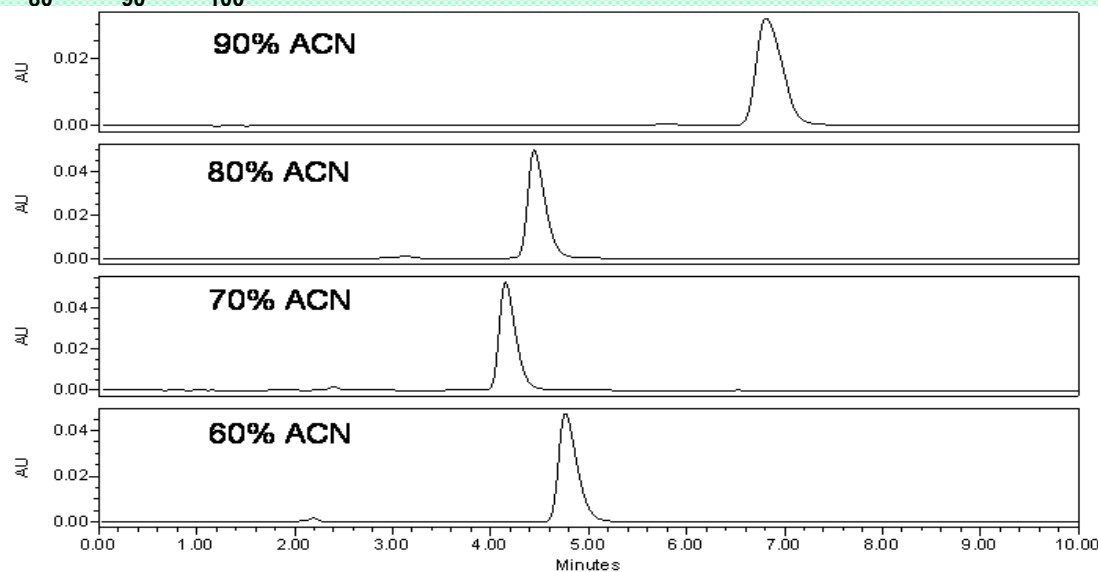
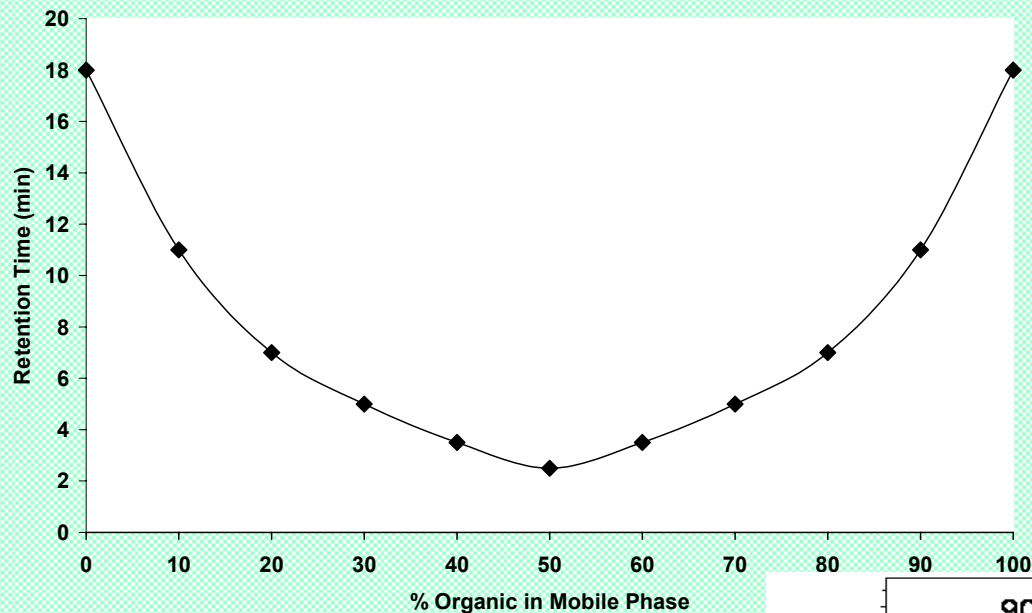
ANP 2



Aqueous normal phase (1) and reversed-phase (2) compounds at three mobile phase compositions: **A**, 50:50 acetonitrile, DI water; **B**, 80:20 acetonitrile, DI water; and **C**, 85:15 acetonitrile, DI water.

# Individual compounds that can be retained by both reversed phase and normal phase modes

ANP 3



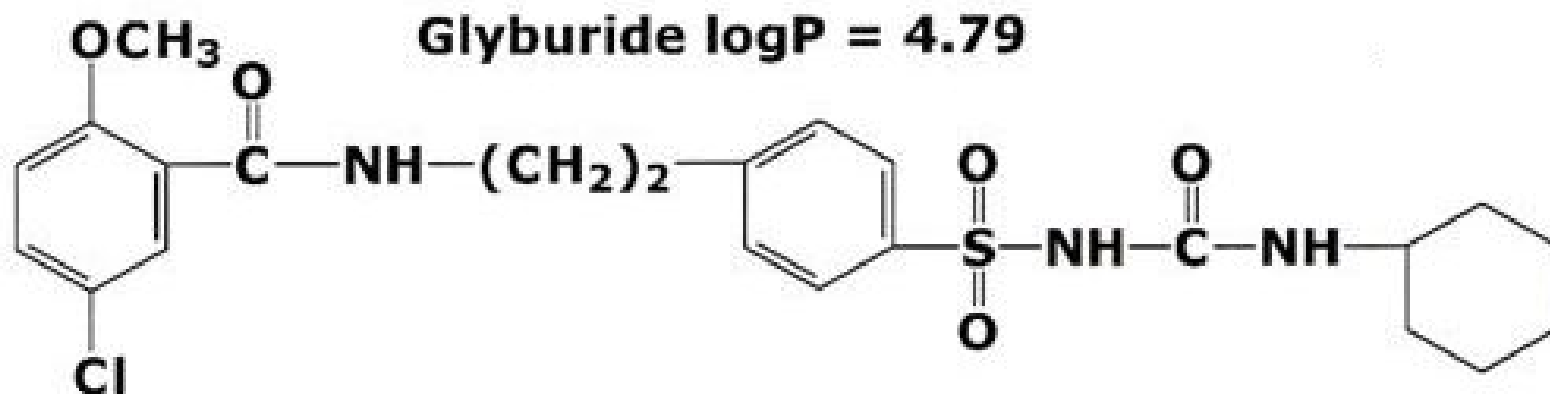
# Example of Separation Capabilities of Hydride Based Stationary Phase

**METFORMIN**

**Aqueous Normal Phase**

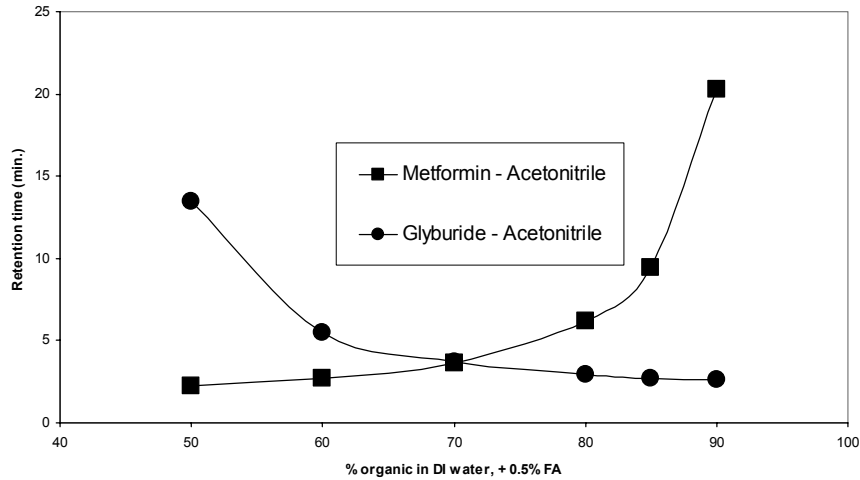
**GLYBURIDE**

**Reversed Phase**



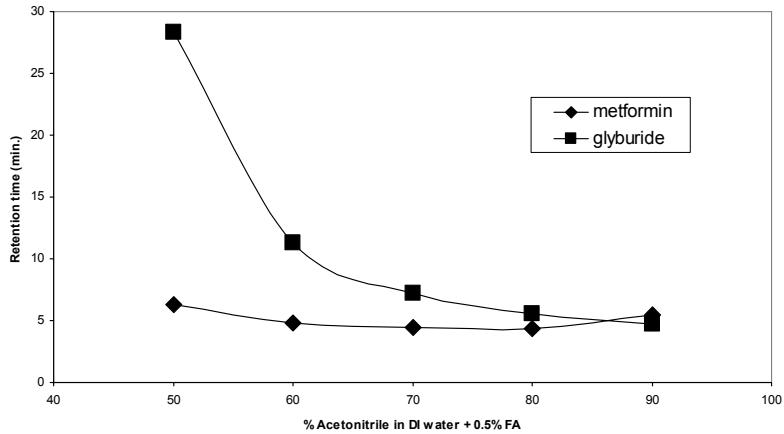
**Metformin logP = -2.64**

Hydride based BD C18, 4.6x75 mm, METFORMIN & GLYBURIDE

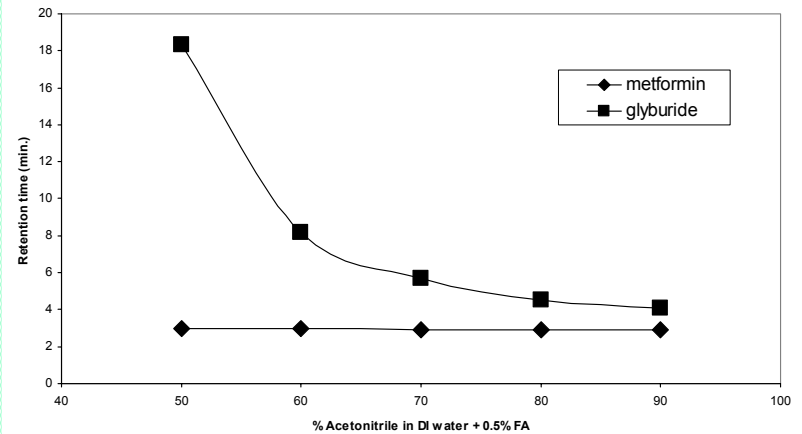


# Comparison of Hydride-Based C18 and Ordinary Silica-Based C18 Columns in the Aqueous Normal Phase Mode

LUNA C18, 4.6x150 mm



Agilent Zorbax C18, 4.6x150 mm



# METFORMIN/GLYBURIDE ON BIDENTATE C18 FAST SEPARATION WITH UV DETECTION

Column dimensions: 2.1 x 20 mm

## Mobile Phase:

A: 50:50 acetonitrile, DI water + 0.5% formic acid

B: 80:20 acetonitrile, DI water + 0.5% formic acid

C: 85:15 acetonitrile, DI water + 0.5% formic acid

**Flow rate:** 0.3 mL/minute

**Injection Volume:** 1  $\mu$ L

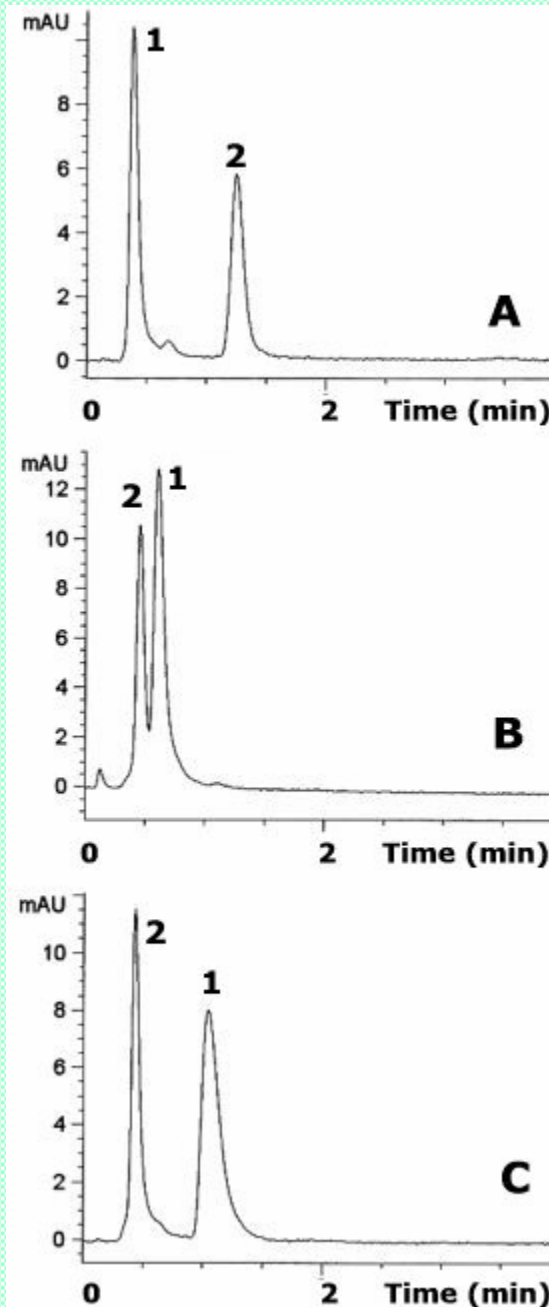
## Samples:

**1. Metformin**

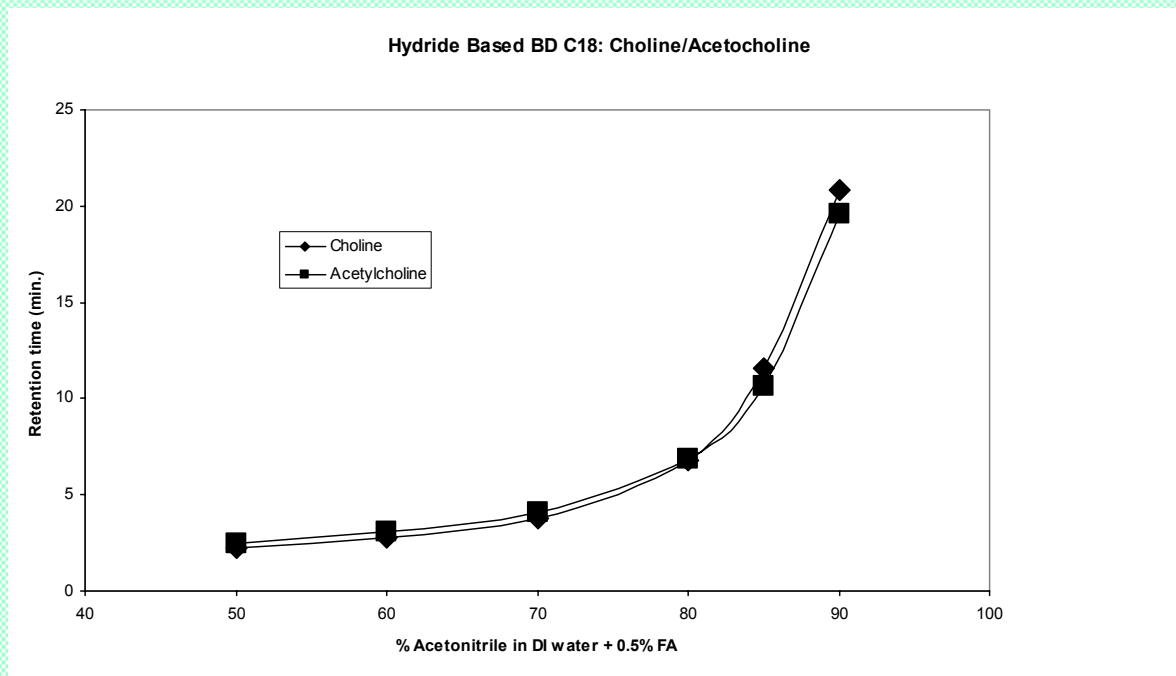
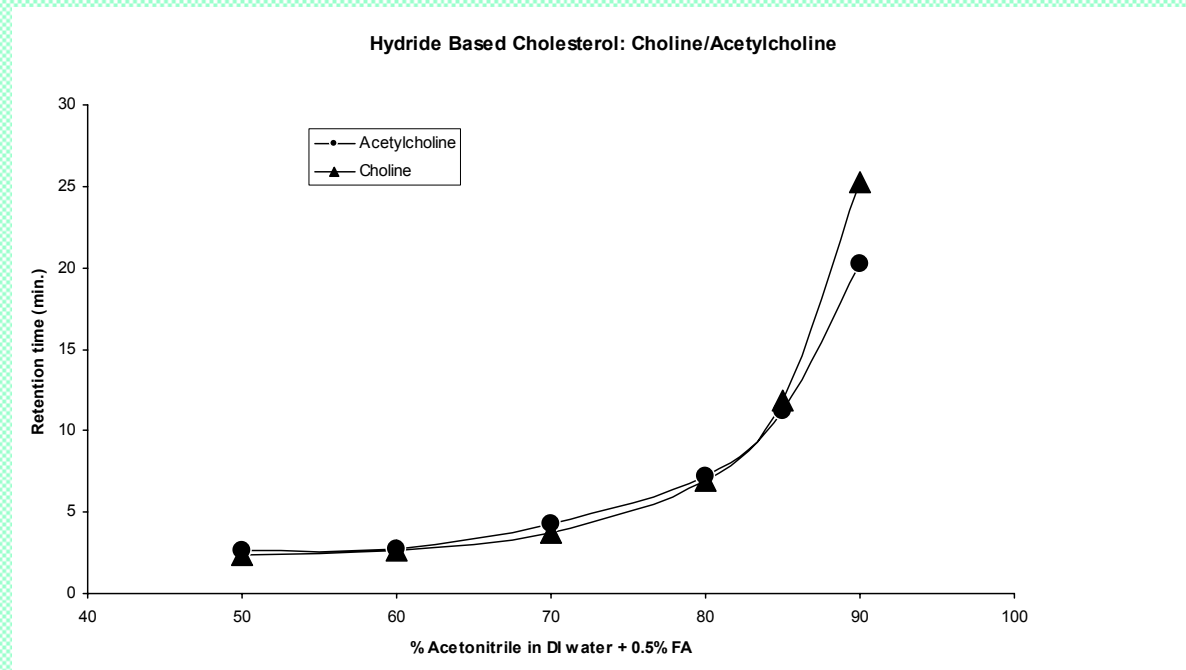
**2. Glyburide**

100  $\mu$ g/mL of each in the mobile phase

**Detection:** UV 254nm

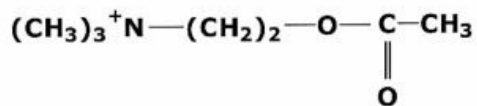


# Comparison of Choline and Acetylcholine Elution on Hydride-Based Cholesterol and BD C18 Columns under Aqueous Normal Phase Conditions

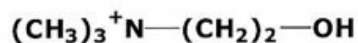


# SEPARATION OF ACETYLCHOLINE/CHOLINE ON STANDARD AND SHORT CHOLESTEROL COLUMNS

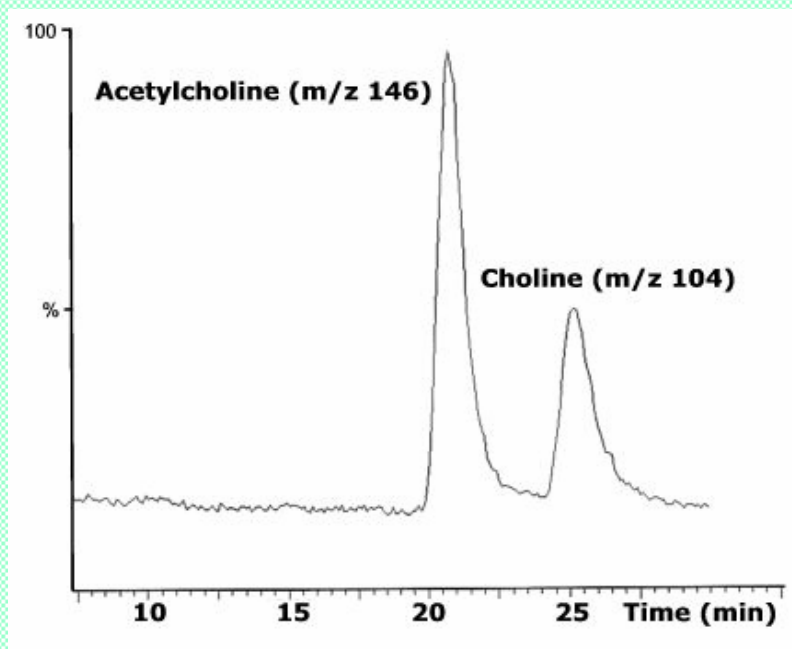
Acetylcholine



Choline

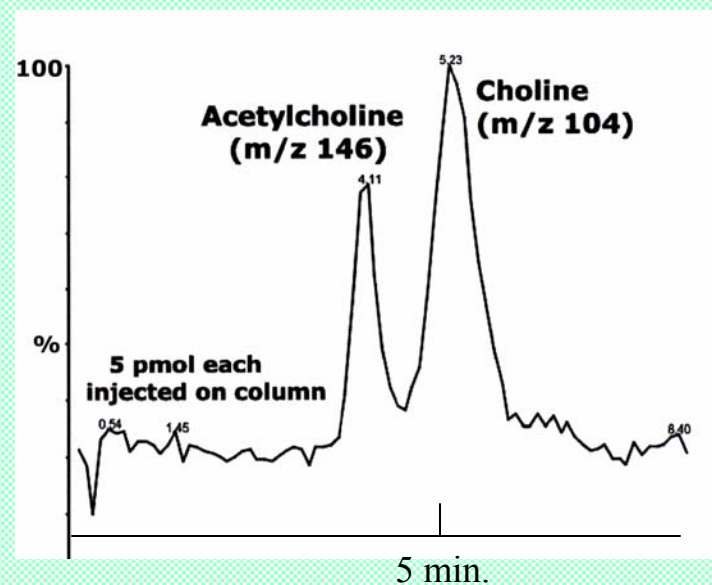


On standard 4.6x75 mm column using 90% ACN + 10% DI water with 0.5% formic acid



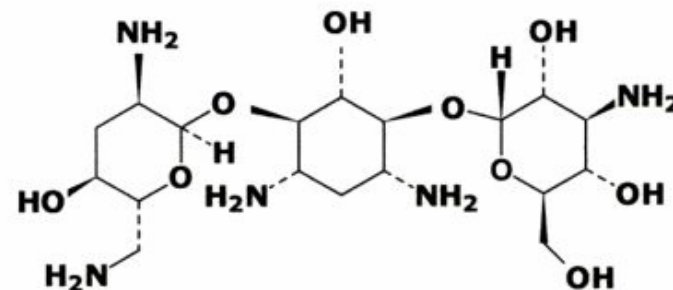
Challenging quaternary amine compounds

On 2.1 x 20 mm Column in 92% ACN + 8% DI water with 0.5% formic acid

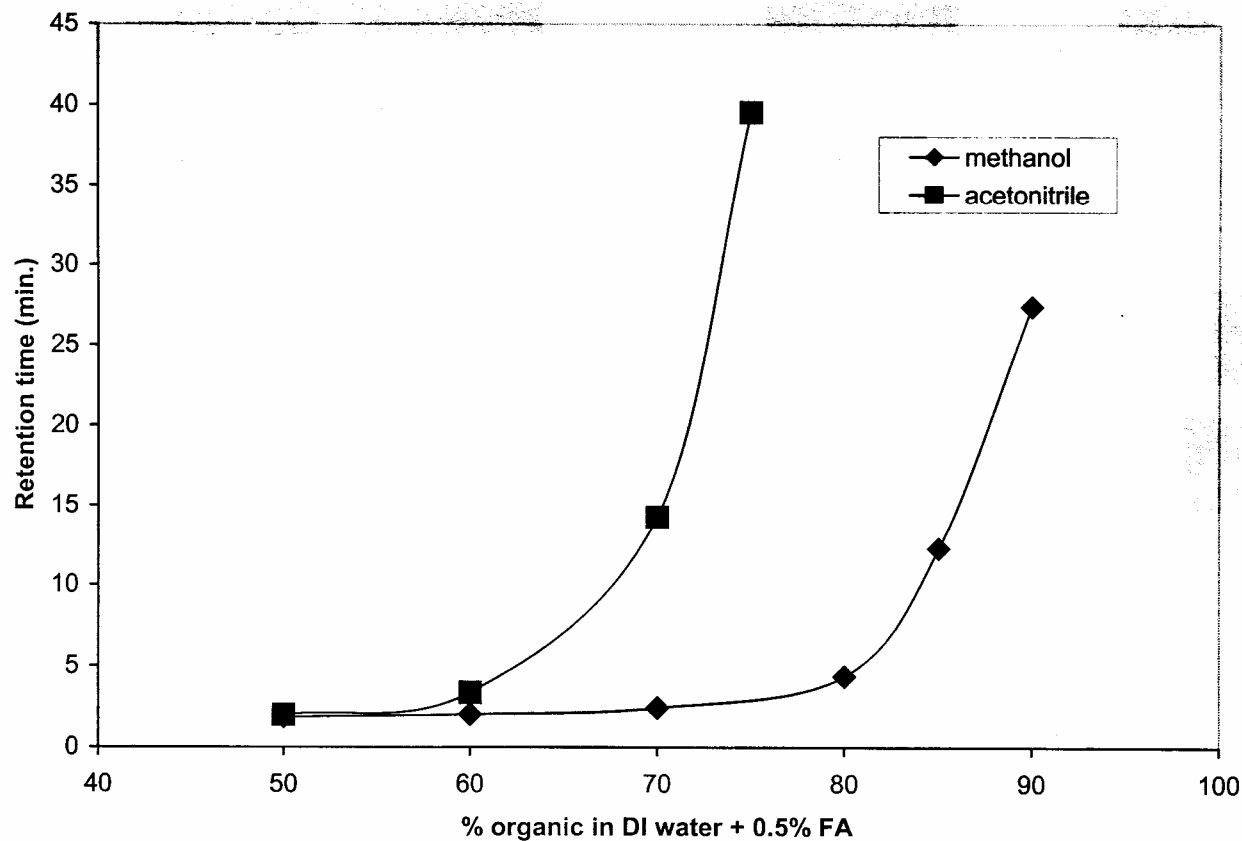


# Aqueous Normal Phase Retention of the Basic Drug Tobramycin

**TOBRAMYCIN F.W. 467.51**

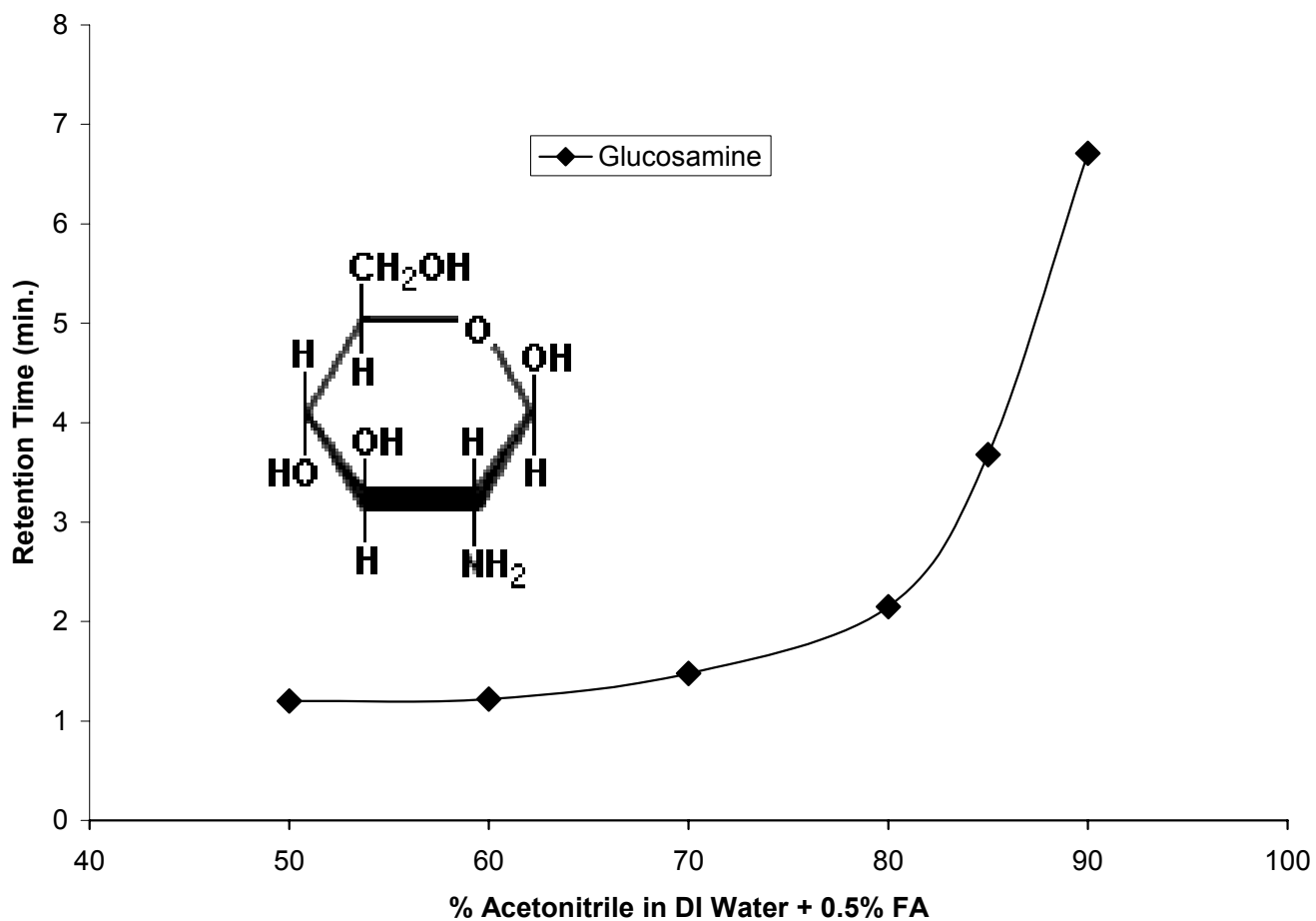


Hydride Based Cholesterol, 4.6x75 mm Sample: TOBRAMYCIN

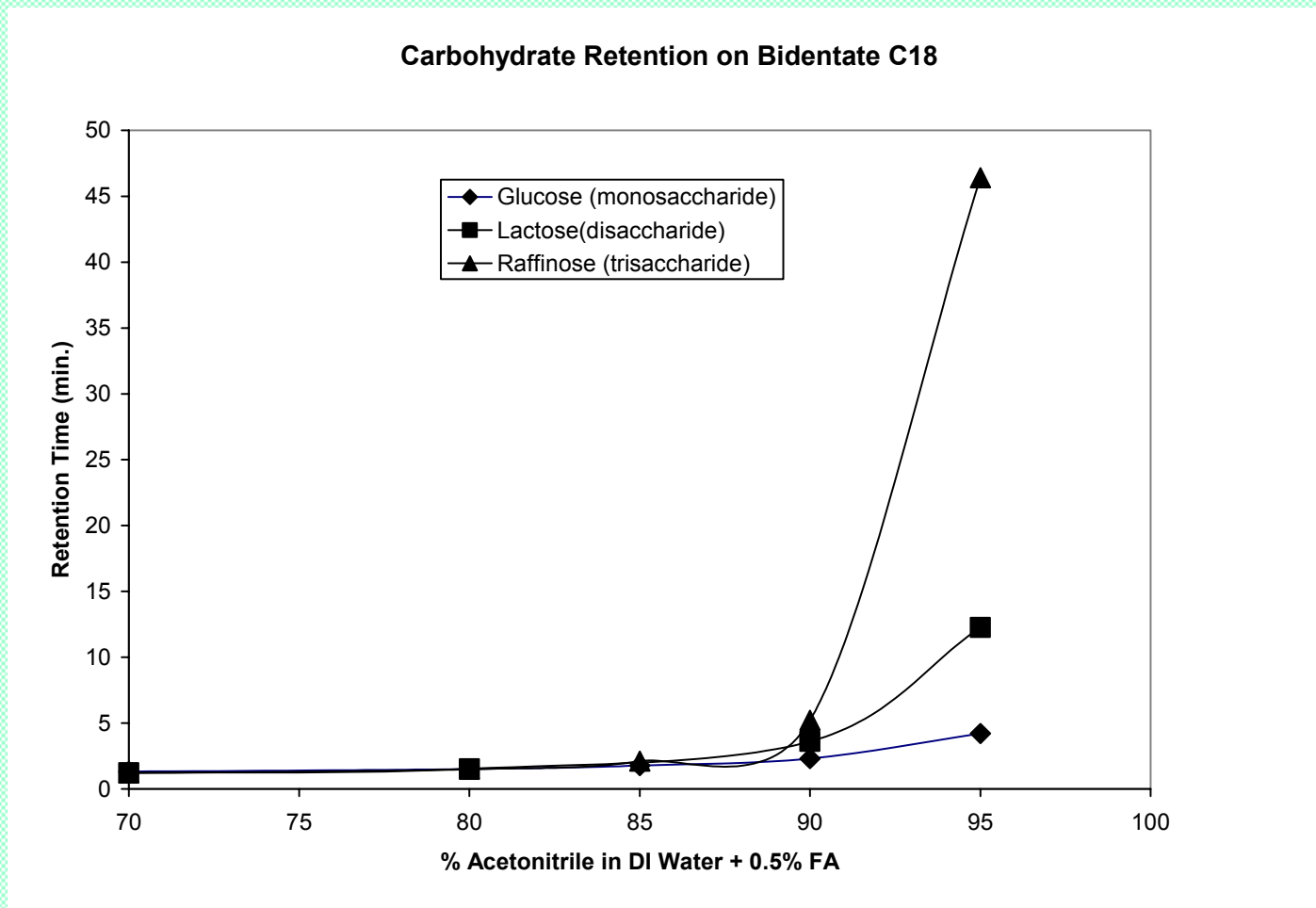


# GLUCOSAMINE BIDENTATE C18

Glucosamine on Bidentate C18



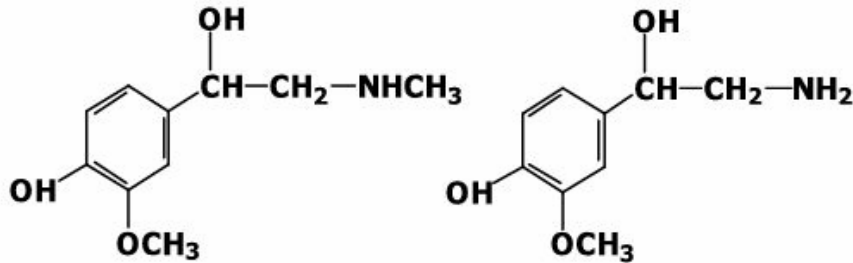
# AQUEOUS NORMAL PHASE RETENTION OF CARBOHYDRATES ON A HYDRIDE-BASED COLUMN



Separation based on size

Detection by MS in the APCI+ mode

# ANP Clinical Application 1



Metanephrine M.W. 211

Normetanephrine M.W. 197

## Method Conditions

Column: Cogent Bidentate C18, 4 $\mu$ m, 100A.

Catalog No.: 40018-75P

Dimensions: 4.6 x 75 mm

Mobile phase: A. 90:10 Acetonitrile/DI Water + 0.5% formic acid

B. 85:15 Acetonitrile/DI Water + 0.5 % formic acid

Flow rate: 0.5 mL/min.

Injection Volume: 10  $\mu$ L

Samples:

1. Triacetylnormetanephrine (m/z 166.2)

2. Triacetylmetanephrine (m/z 180.2)

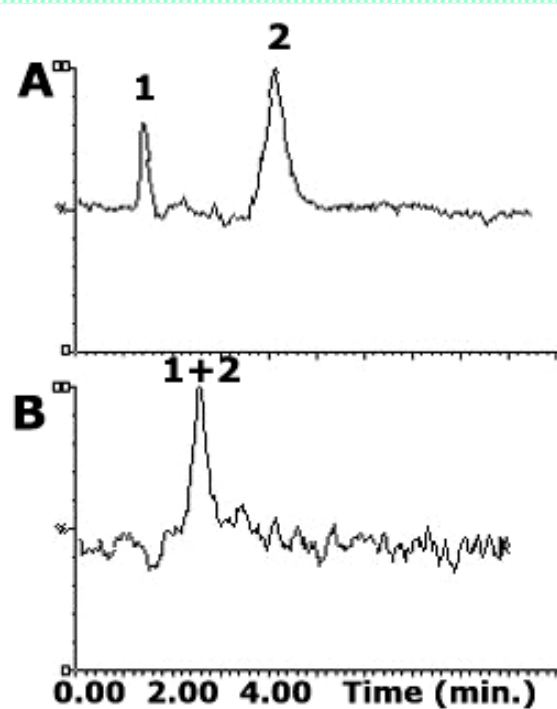
200 ng of each sample was dissolved in 1 mL of reverse osmosis water

Detection: Atmospheric Pressure Chemical

Ionization in positive mode: APCI+

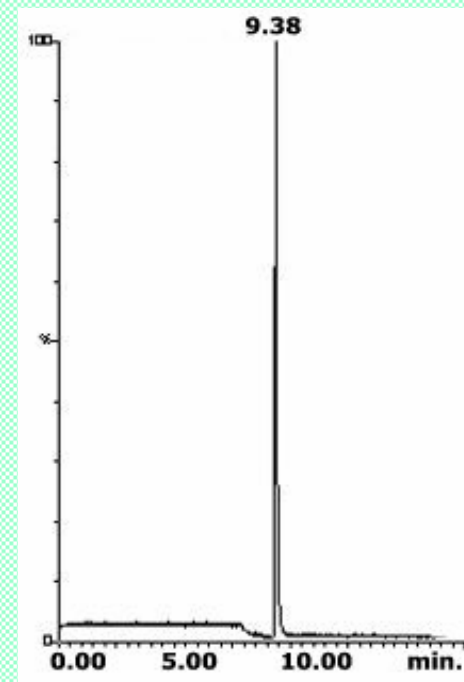
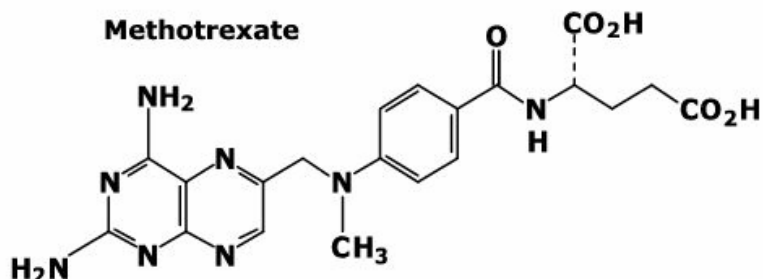
Single Ion Monitoring

In detection single ion monitoring (SIM) was used. Mass transition of m/z 310.2 to m/z 166.2 (triacetylnormetanephrine) and m/z 324.2 to m/z 180.2 (triacetylmetanephrine) that correspond to the fragmentation of the (M+H<sup>+</sup>) ions were monitored.



# ANP Clinical Application 2

The powerful anticancer drug, methotrexate (4-amino-N10-methylpteroyl glutamic acid) acts as an antimetabolite and is used for the treatment of many neoplastic diseases including acute leukemia, osteosarcoma, non-Hodgkins lymphoma, and breast cancer. There is a great interest in pharmacological studies and clinical monitoring of methotrexate.



## Inverse Gradient Program

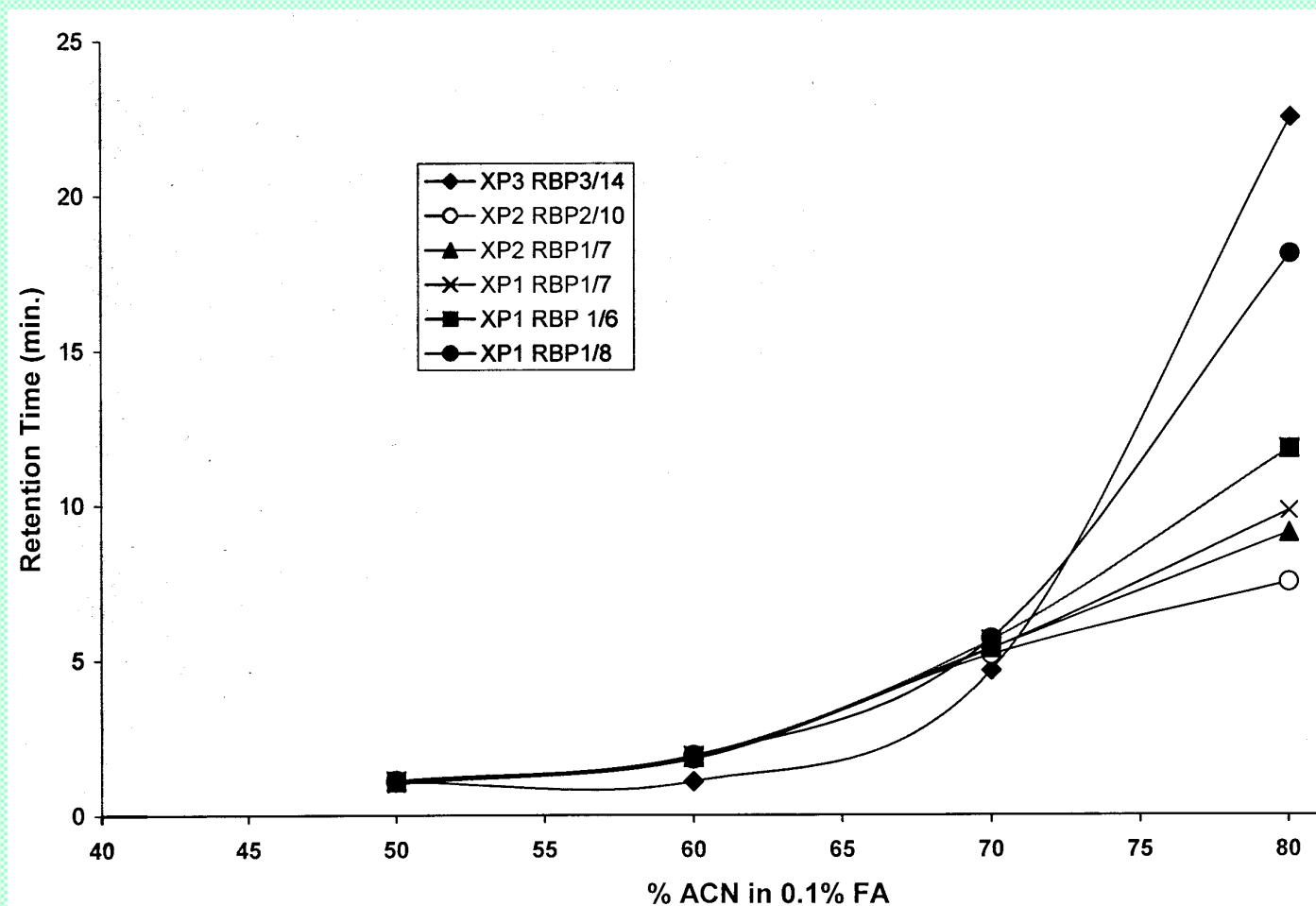
**A: DI Water + 0.5% Formic Acid**

**B: Acetonitrile**

Time (min.)	%A	%B
0.00	10.0	90.0
1.00	10.0	90.0
5.00	80.0	20.0
10.00	80.0	20.0
10.01	10.0	90.0
12.00	10.0	90.0

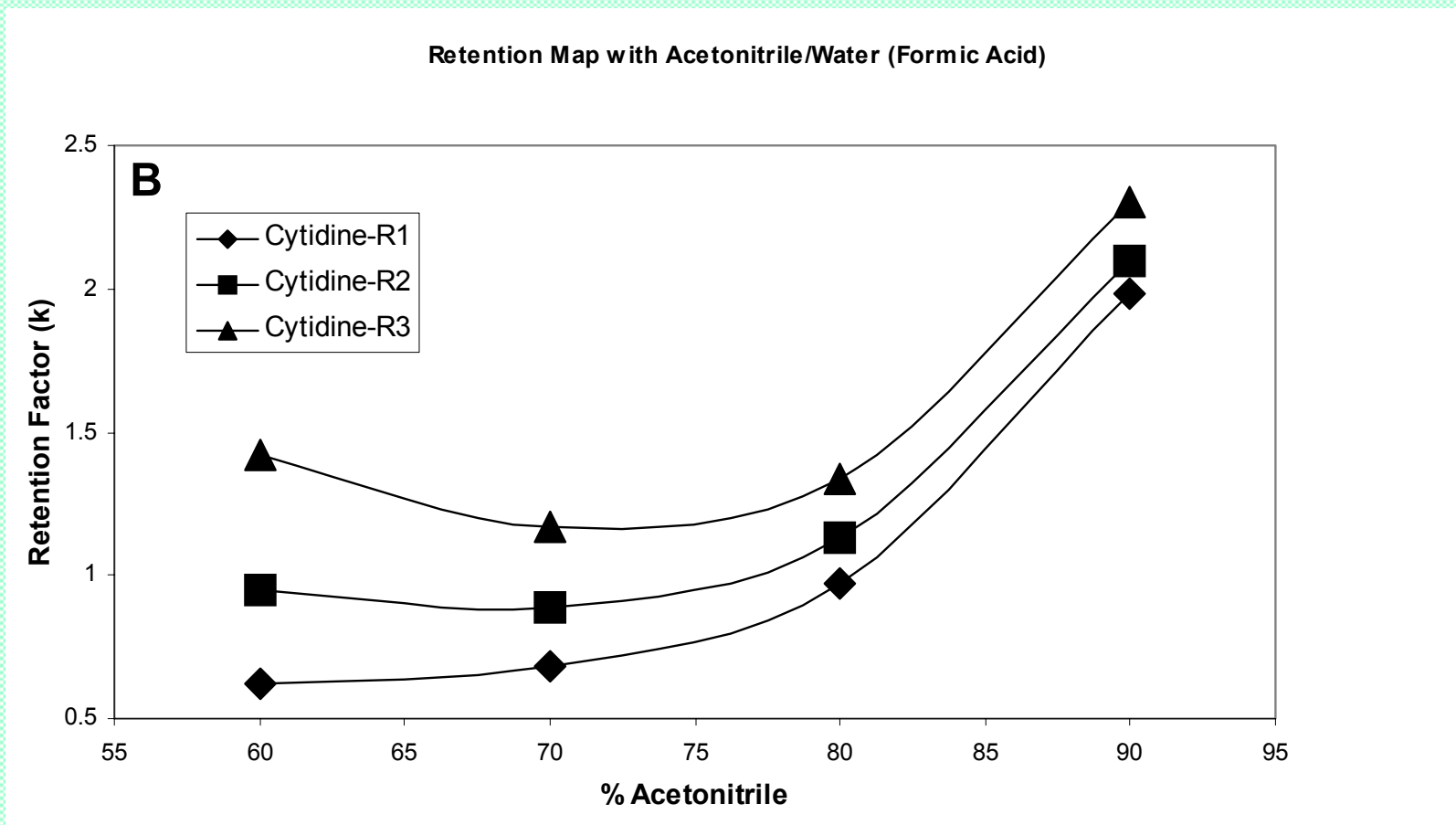
A quadrupole mass spectrometer operating in the positive - ion mode and an atmospheric pressure ionization (API) source was used for selective detection and assured that no interfering peaks affect the quantitative results. A bidentate C18 column was the column of choice for the ANP gradient analysis of the drug. The retention of the methotrexate is more than sufficient. The LC-MS method developed assures both high specificity and sensitivity.

# ANP RETENTION CAN BE OBSERVED FOR HYDROPHILIC PEPTIDES



GENERAL PEPTIDE STRUCTURE: Ac-AXEXAHKAY-NH<sub>2</sub>

# SOME COMPOUNDS DISPLAY BOTH REVERSED PHASE AND ANP BEHAVIOR ON THE SAME COLUMN



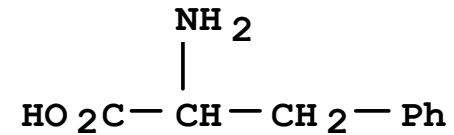
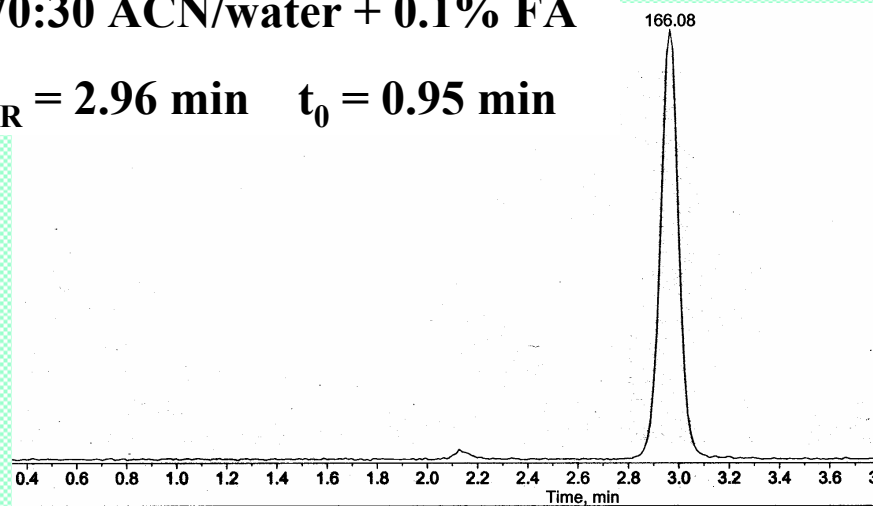
**USE OF TOF MS IN AQUEOUS  
NORMAL PHASE WITH A SILICA  
HYDRIDE COLUMN FOR DETECTION  
OF METABOLITES**

# ANP RETENTION ON DIAMOND HYDRIDE COLUMN

## AMINO ACIDS - PHENYLALANINE

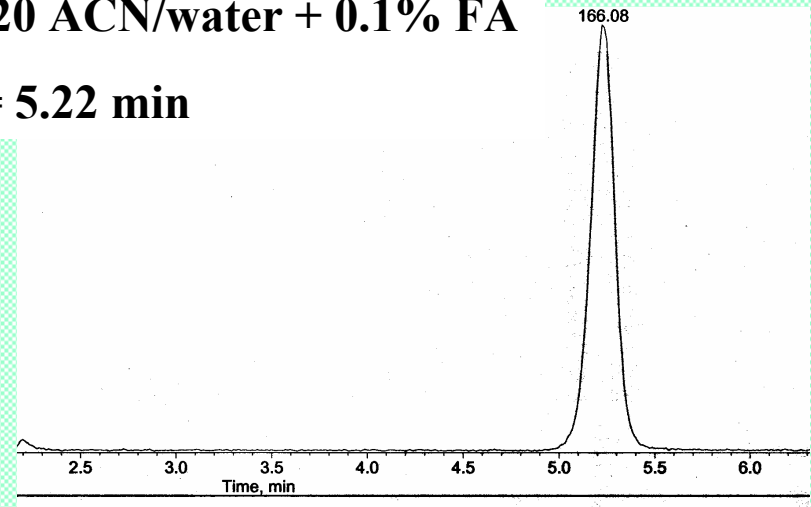
70:30 ACN/water + 0.1% FA

$t_R = 2.96$  min    $t_0 = 0.95$  min



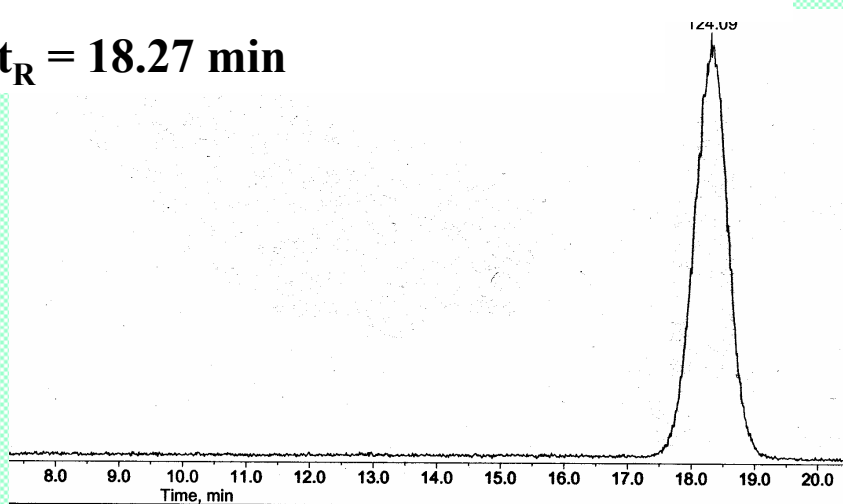
80:20 ACN/water + 0.1% FA

$t_R = 5.22$  min

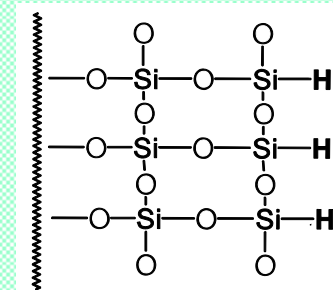


90:10 ACN/water + 0.1% FA

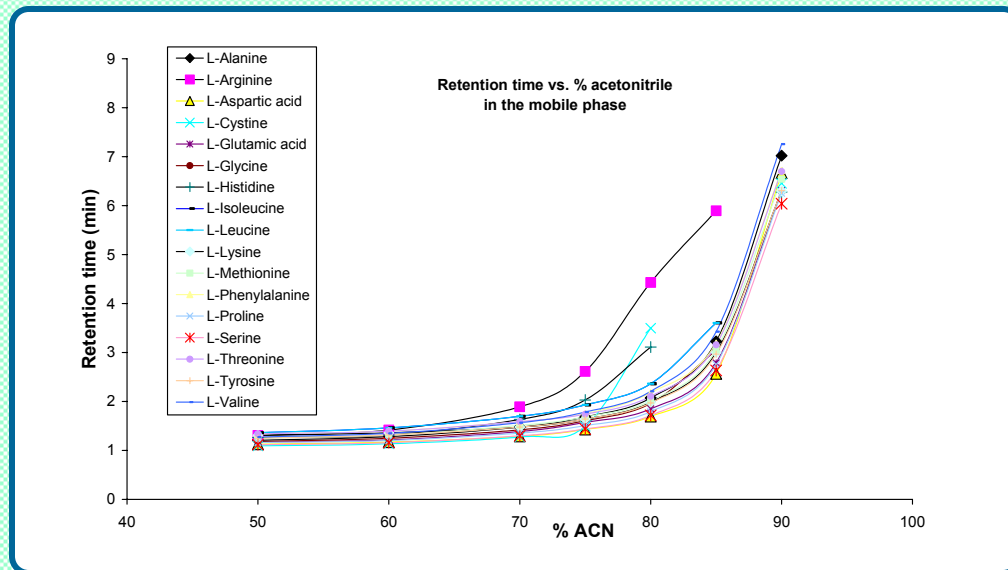
$t_R = 18.27$  min



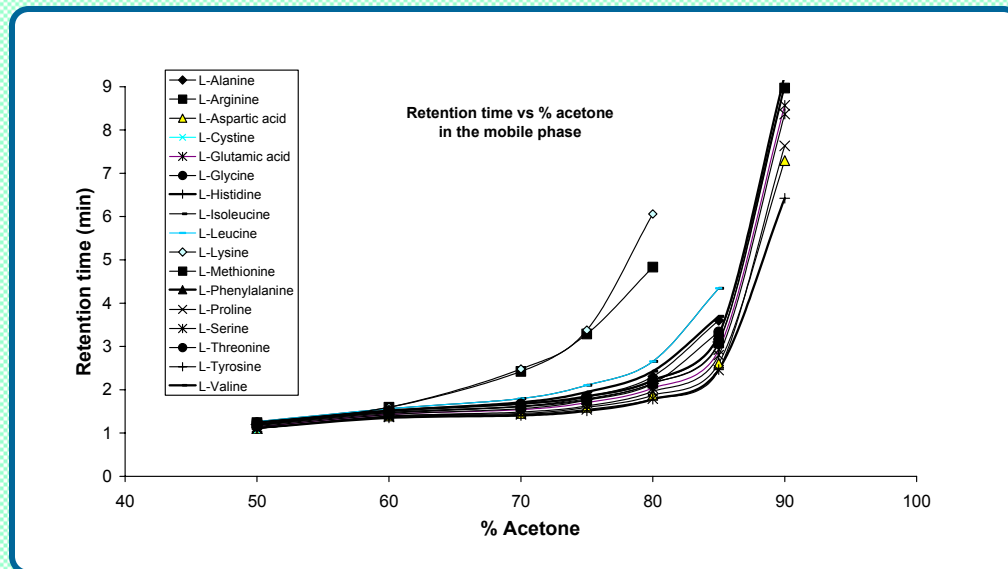
**Diamond Hydride: essentially an unmodified hydride surface**



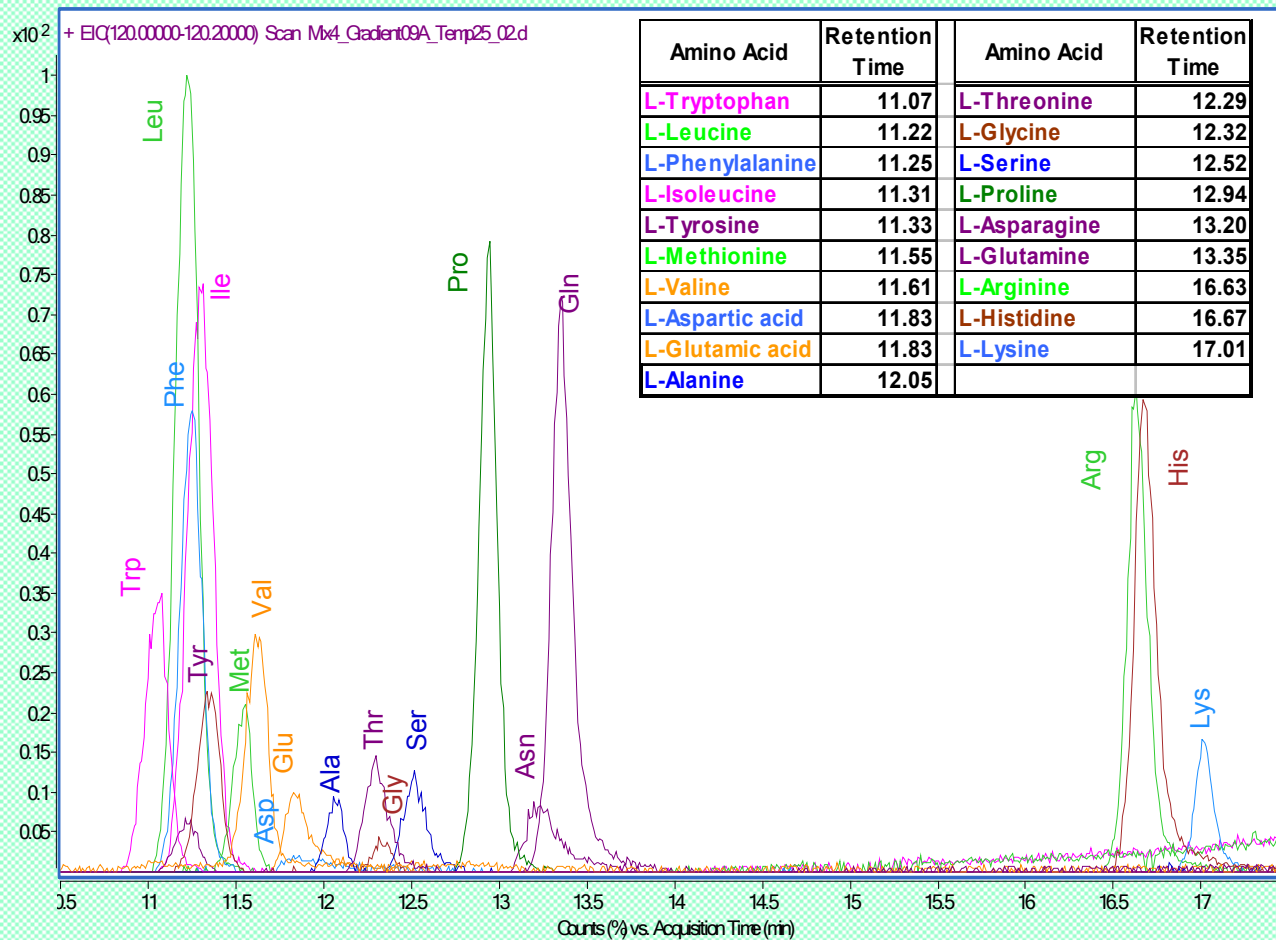
**Detection: Agilent TOF-MS  $m/z = 166$**



Retention Time With Acetonitrile



Retention Time With Acetone



## Extracted Ion Chromatogram Of Nineteen Amino Acid Separation

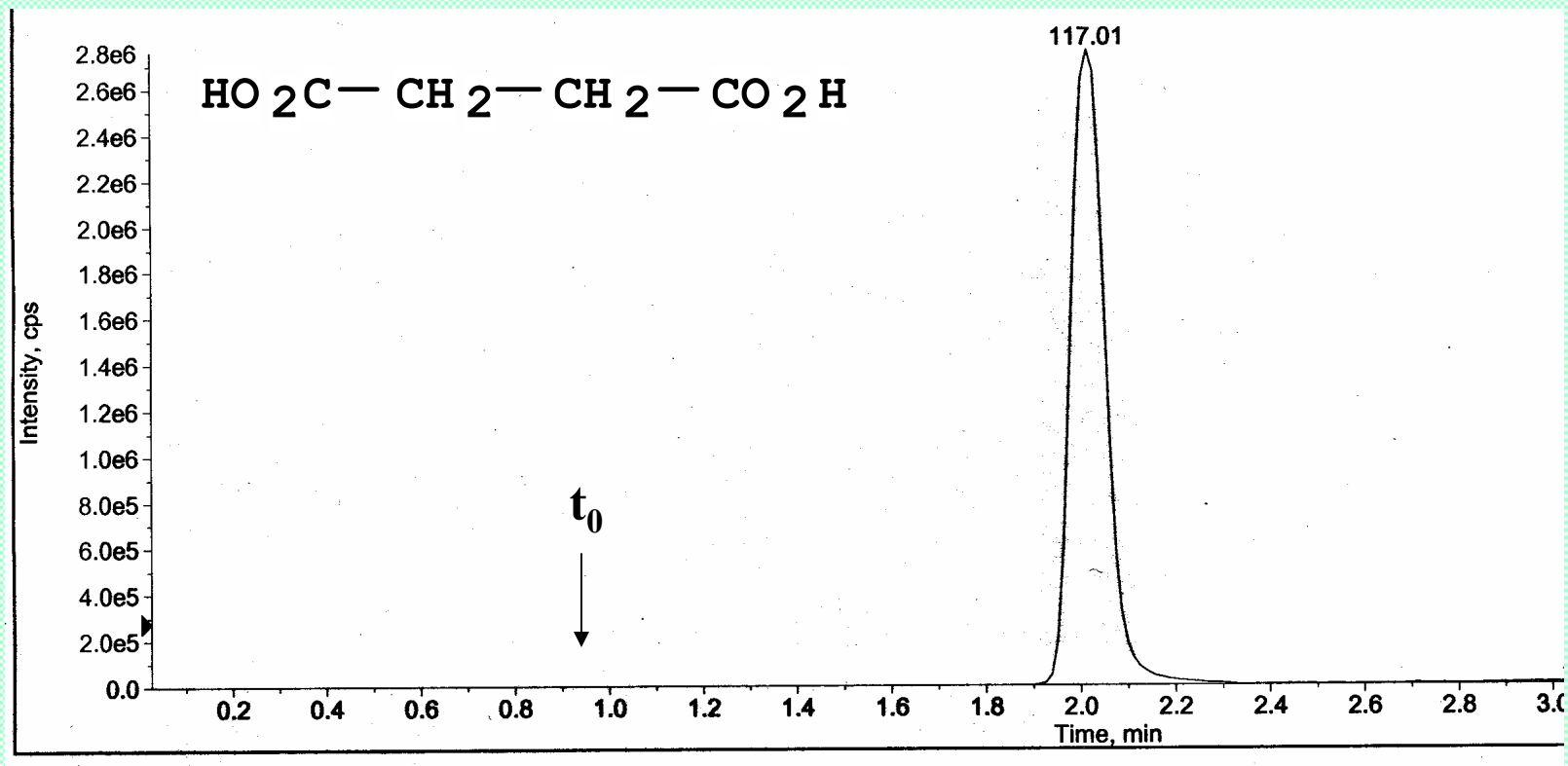
All of the critical amino acid pairs (those that are isobaric or have masses within one mass unit ) are separated under these conditions except for the Leucine / Isoleucine pair. At present, the maximum separation is approximately 0.15 min with 0.30 minutes needed for resolution with the peak widths obtained for these two amino acids.

# GRADIENT REPRODUCIBILITY

Amino Acid	G 1B	G 1B inj 2	Gr 1B inj 3	Gr 1B inj 4	%RSD	Gr 1B	Gr 1B inj 2	Gr 1B inj 3	Gr 1B inj 4	%RSD
Retention time	15 °C	15 °C	15 °C	15 °C		30 °C	30 °C	30 °C	30 °C	
L-Alanine	9.654	9.622	9.637	9.633	0.14	9.671	9.705	9.678	9.687	0.15
L-Glutamine	10.961	10.929	10.955	10.940	0.13	10.911	10.933	10.917	10.938	0.12
L-Histidine	12.178	12.180	12.183	12.180	0.02	12.162	12.173	12.168	12.178	0.06
L-Methionine	8.771	8.751	8.754	8.762	0.10	8.856	8.900	8.873	8.905	0.26
L-Phenylalanine	8.369	8.360	8.363	8.360	0.05	8.532	8.576	8.527	8.559	0.27
L-Proline	10.647	10.628	10.650	10.610	0.17	10.495	10.507	10.496	10.511	0.08
L-Serine	9.932	9.935	9.935	9.928	0.03	9.948	9.971	9.971	9.986	0.16
L-Threonine	9.731	9.745	9.746	9.738	0.07	9.725	9.770	9.736	9.774	0.25
L-Tyrosine	8.491	8.483	8.495	8.498	0.08	8.641	8.686	8.641	8.679	0.28

The table shows retention time reproducibility for nine amino acids at two temperatures. Four replicates were performed at each temperature. The reproducibility was 0.28% or better for the amino acids. This is a significant improvement over what is usually observed for most HILIC analyses, especially considering this is gradient data with only a 5 minute re-equilibration time between runs.

# ANP RETENTION OF SUCCINIC ACID ON DIAMOND HYDRIDE COLUMN

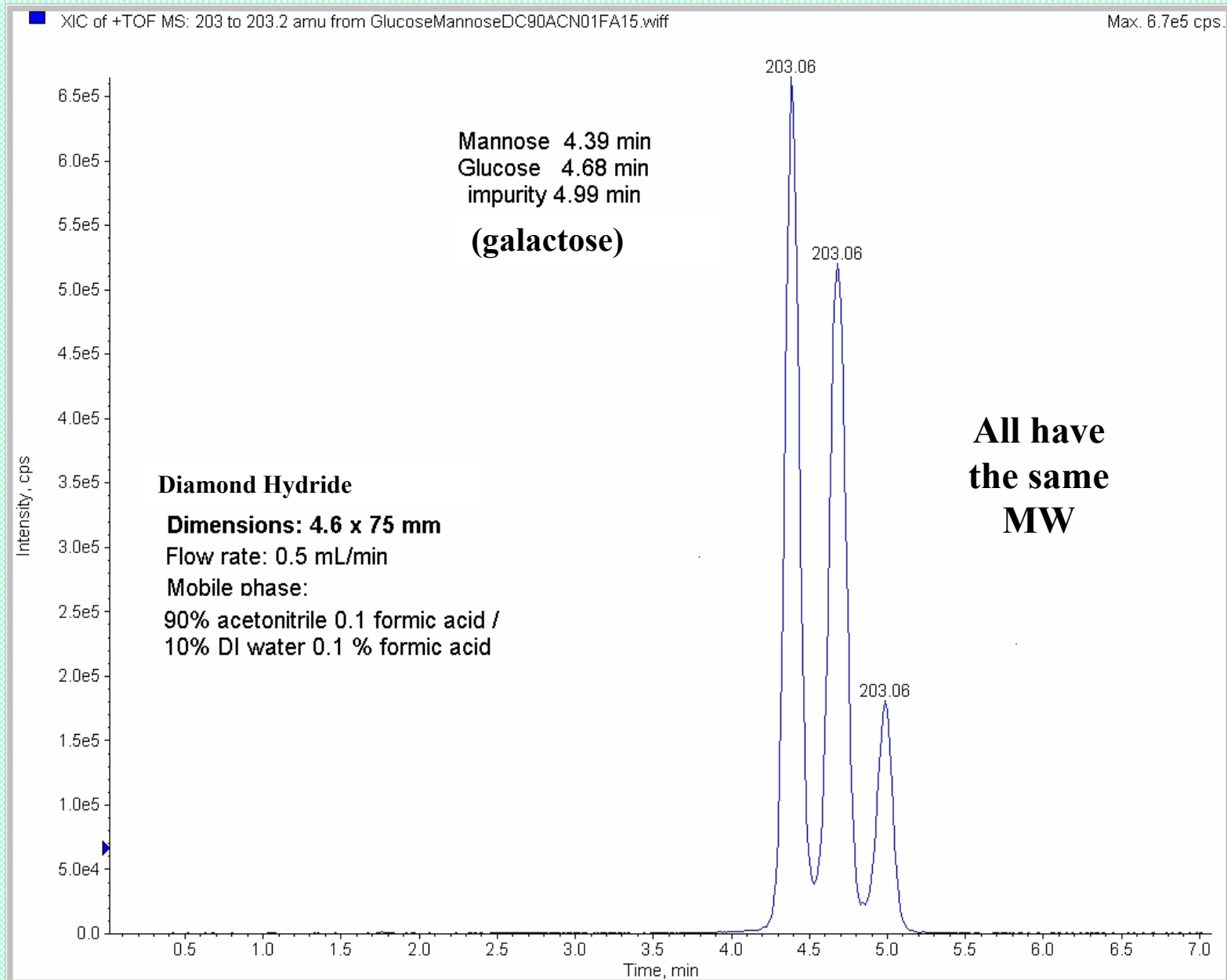


**Mobile Phase: 95:5 ACN/water + 0.1% FA**

**Detection: TOF-MS @  $m/z = 117$**

**Retention increases to 5 min in 0.1% Ammonium Acetate**

# ANP RETENTION OF CARBOHYDRATES ON DIAMOND HYDRIDE COLUMNS

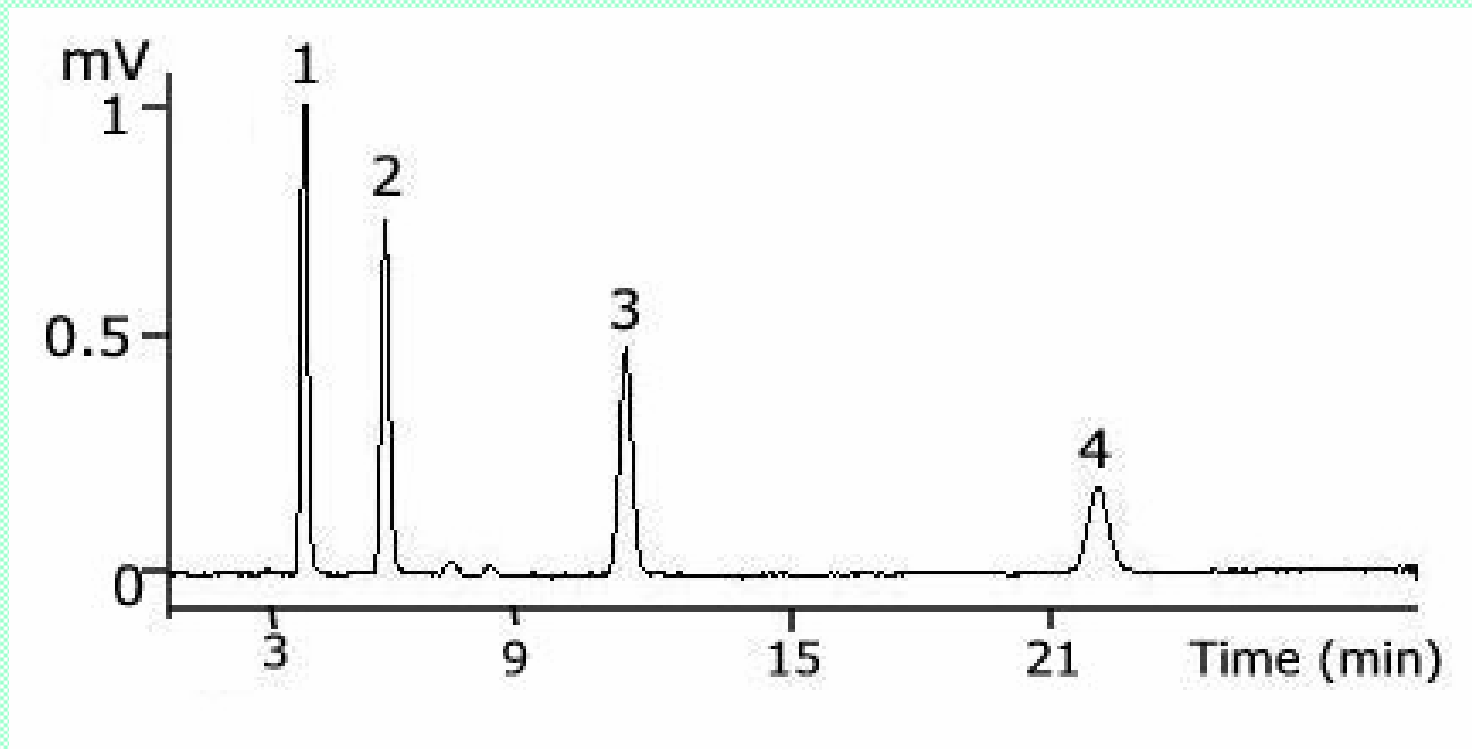


# **FEATURES OF DIAMOND HYDRIDE FOR METABOLITE ANALYSIS**

- 1. Retains amino acids, small organic acids and carbohydrates in the Aqueous Normal Phase Mode in acidic aqueous/organic mobile phases containing greater than 60% acetonitrile or acetone.**
- 2. Higher temperature results in increasing retention time – opposite to what is typically observed in reversed phase retention.**
- 3. Excellent reproducibility is obtained in either isocratic or gradient elution with retention time RSD better than 0.3%**
- 4. Re-equilibration time is rapid (< 5 min) which is similar to or better than observed in most reversed phase applications and superior to re-equilibration times obtained in the HILIC mode.**

**USE OF HYDRIDE BASED  
STATIONARY PHASES FOR  
REVERSED PHASE SEPARATIONS**

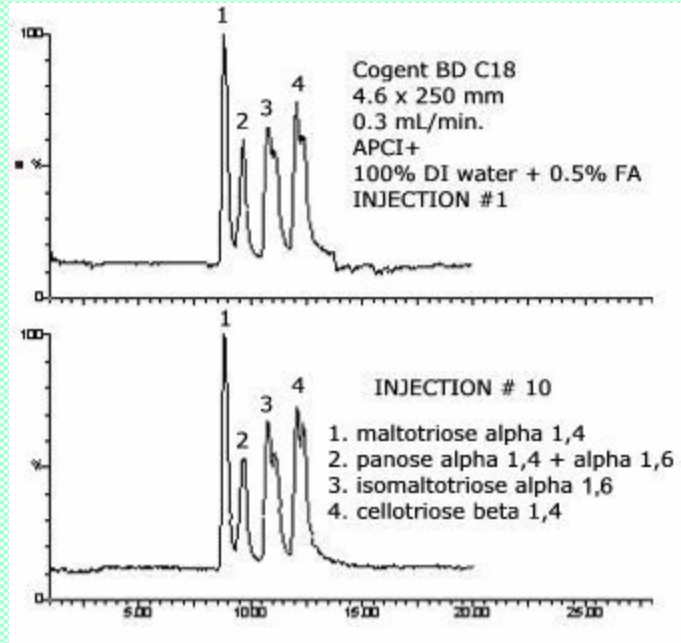
# CAPILLARY LC SEPARATION OF STEROIDS IN REVERSED PHASE MODE



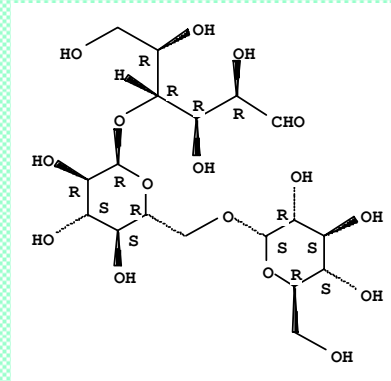
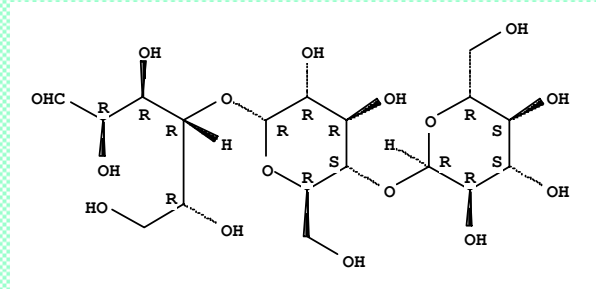
Column: Cogent Bidentate C18 capillary HPLC Column, 4  $\mu\text{m}$ , 100  $\text{\AA}$   
Dimensions: 50  $\mu\text{m}$  i.d. x 40 cm packed (50 cm total length),  
Mobile phase: 70:30 acetonitrile/DI water + 0.1% formic acid  
Flow rate: 0.010 mL/min.

1. Prednisolone; 2. Corticosterone; 3. Norgestel; 4. Progesterone

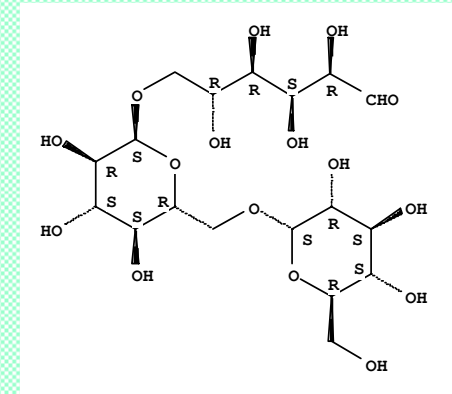
# SEPARATION OF CARBOHYDRATE STRUCTURAL ISOMERS



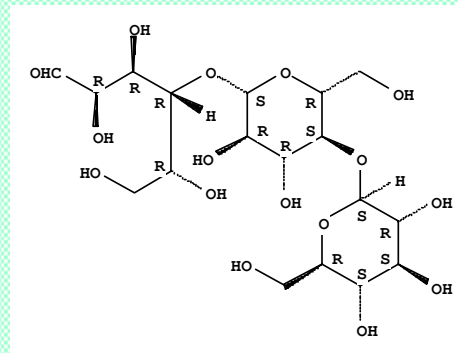
maltotriose  $\alpha$  1,4



panose  $\alpha$  1,4 +  $\alpha$  1,6



isomaltotriose  $\alpha$  1,6



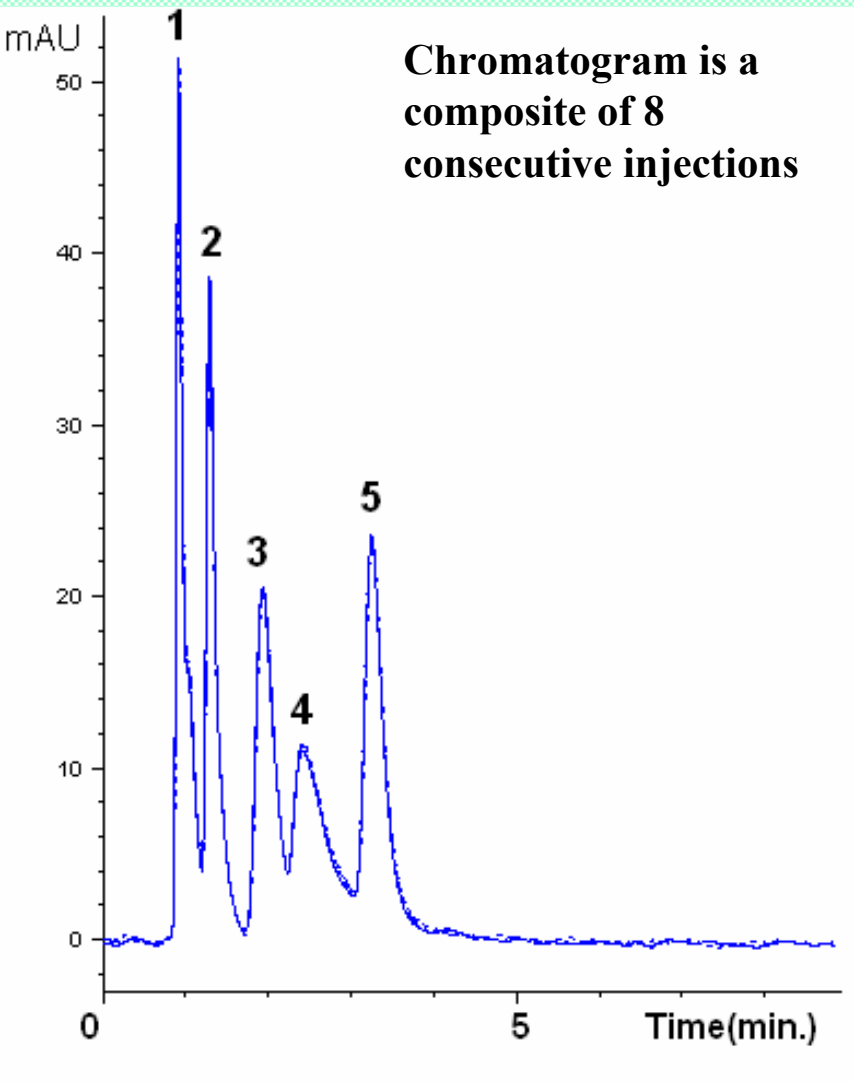
cellotriose  $\beta$  1,4

Mobile Phase: 100% water

All compounds have MW = 504

Each compound detected by MS in APCI+ mode with single ion monitoring (SIM) using a specific fragment ion

# Peptides: simple isocratic RP – HPLC analysis



An HPLC peptide standard mixture was resolved in under 5 minutes using a short (4.6 x 75 mm) Cogent BD C18 column and a simple isocratic RP-HPLC method. The separation was very reproducible. To achieve the separation presented on a conventional HPLC column, 4.6 x 250 mm, a gradient method is required.

If higher resolution is desired a longer column should be used. Columns from leading brands of manufacturers were evaluated using the same conditions and compounds 3 and 4 were never separated under RP-HPLC isocratic conditions.

**Mobile Phase:** 25% acetonitrile/75% DI water + 0.1% formic acid

**Flow rate:** 1 mL/minute

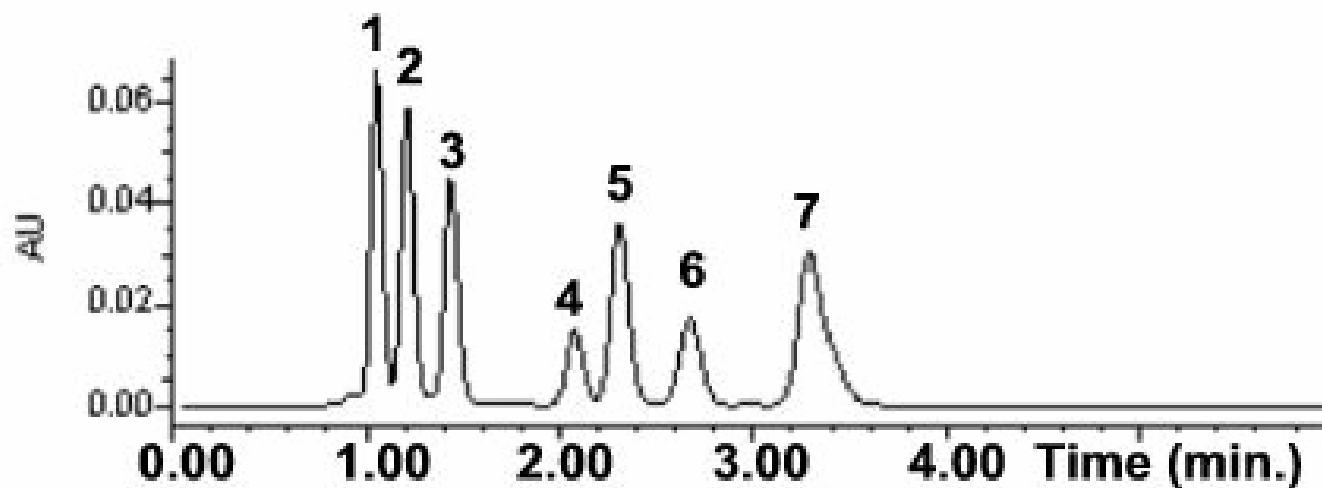
**Detection** UV 214 nm

1. Gly – Tyr; 2. Val – Tyr – Val; 3. Met-enkephalin  
4. Leu-enkephalin; 5. Angiotensin II

# SEPARATION OF A MIXTURE OF POLAR AND NONPOLAR COMPOUNDS

Compound No.	Compound Type	Mol Wt.	ApKa	BpKa	Log P
1	Cytidine-R1	397	12.12	3.73	1.54
2	Cytidine-R2	454	12.07	3.73	3.66
3	Cytidine-R3	425	12.09	3.73	2.66
4	Quinolinedione-R1	536	8.65, 8.68	-	3.46
5	Tetramic acid	536	7.91, 10.98	5.36	2.96
6	Quinolinedione-R2	520	8.64	-	2.74
7	Benzopyran	396	-	-	2.80

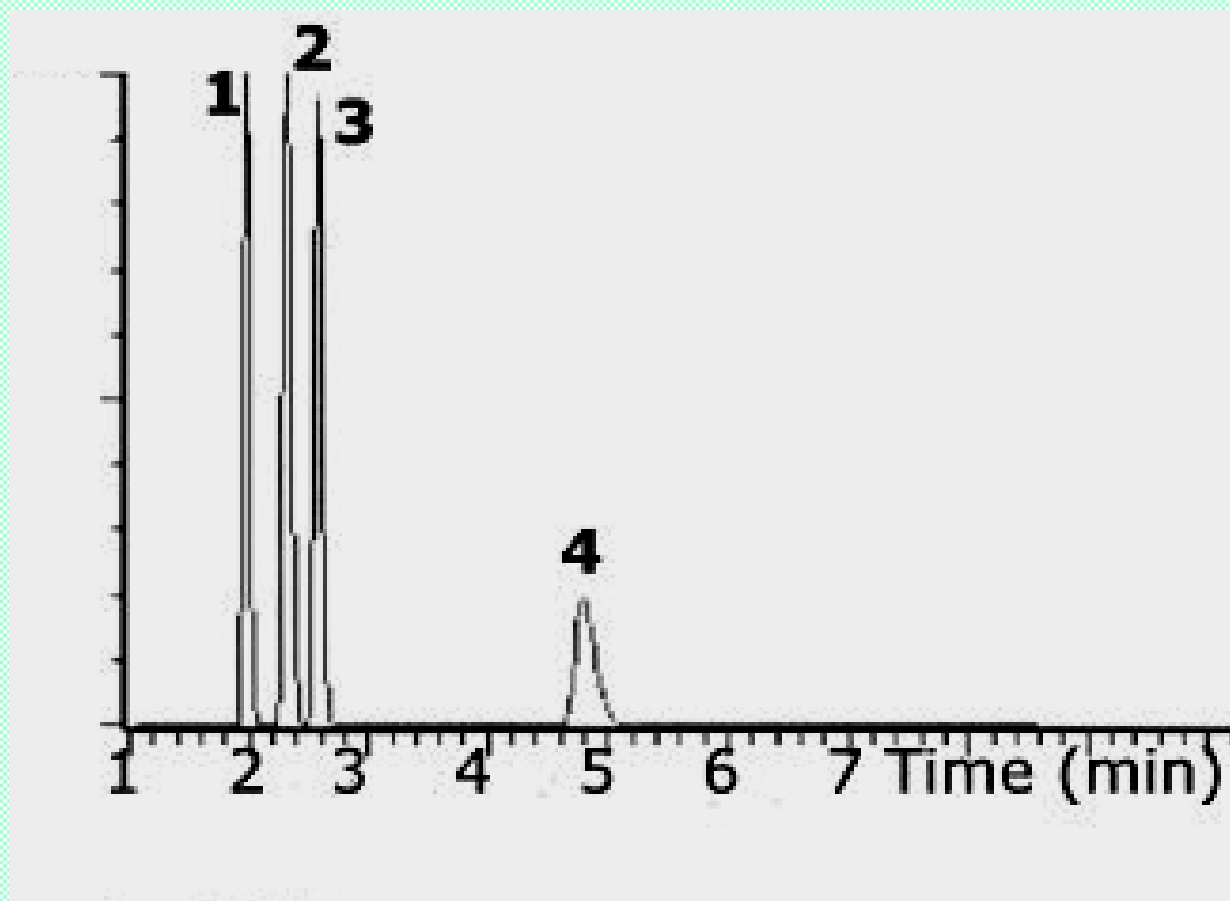
Mobile Phase: 60:40 acetonitrile/water



**ANOTHER UNIQUE AND INTERESTING  
FEATURE OF HYDRIDE BASED  
STATIONARY PHASES**

**ORGANIC NORMAL  
PHASE RETENTION**

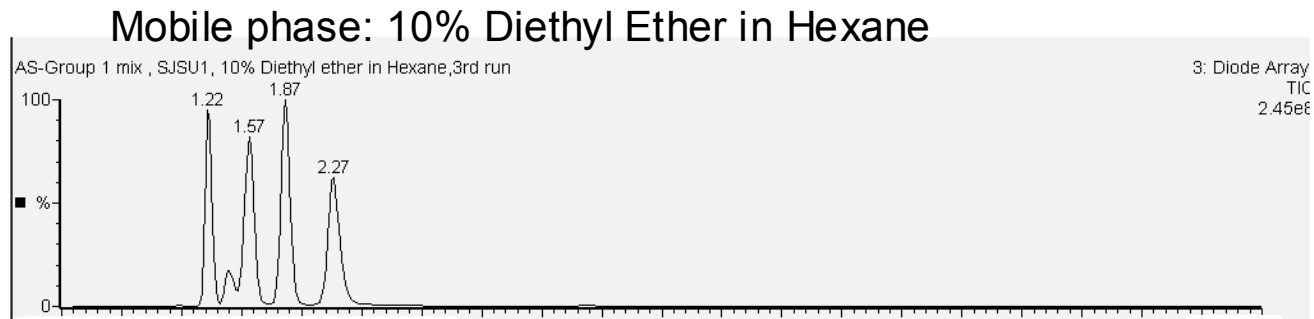
# NORMAL PHASE SEPARATION OF SUBSTITUTED PHENOLS



**Atmospheric Pressure Chemical Ionization in positive mode - APCI+**  
**Column: Bidentate C18, mobile phase: 95:5 Hexane/Ethyl Acetate**  
**Flow rate: 1.0 mL/min.**

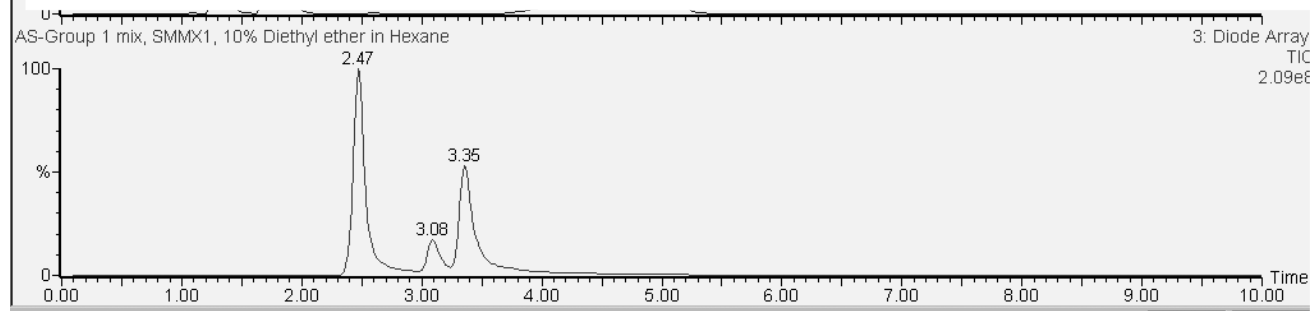
**Samples: 1 – phenol with aldehyde, 2 – parent phenol,**  
**3 – phenol with ketone, 4 – phenol with acid**

# USE OF SILICA HYDRIDE COLUMN IN NORMAL PHASE MODE



A: HYDRIDE COLUMN

B: COMMERCIAL SILICA COLUMN

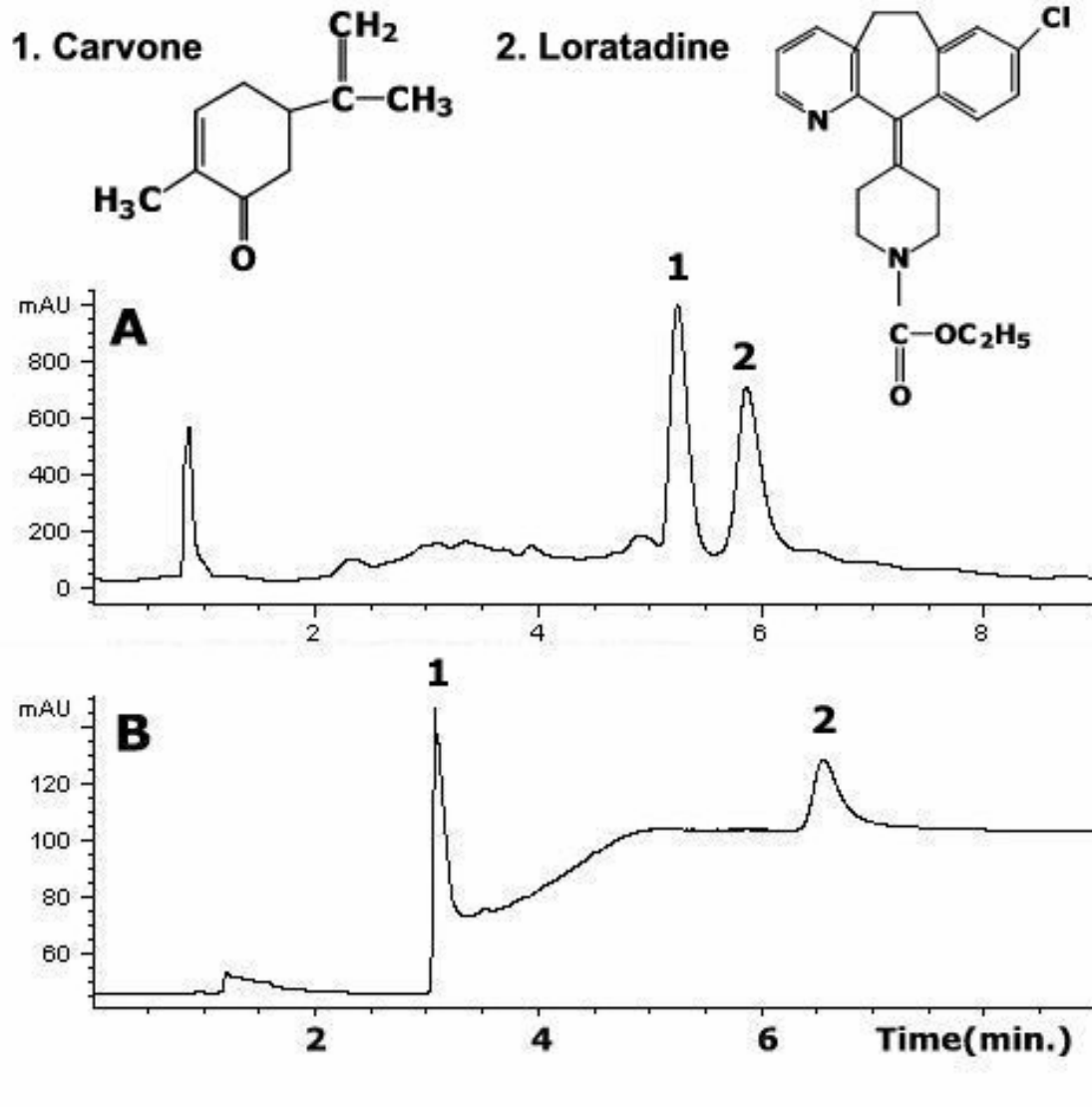


**Stationary phase is unmodified silica hydride**

# NORMAL PHASE GRADIENT SEPARATIONS

A: 0-0.5 min 100% hexane; 0.5-7 min to 50:50 hexane/dichloromethane; 50:50 hexane/dichloromethane to 10 min.

B: 0.0 to 1.0 min 100% dichloromethane; 1.0 to 3.0 min to 100% ethyl acetate



A: Bidentate C18 B: Hydride Silica

# **IMPORTANT FEATURES OF HYDRIDE BASED STATIONARY PHASES**

- 1. Hydride phases operate in the normal phase, aqueous normal phase and reversed phased modes**
- 2. Hydride phases can be used in 100% aqueous mobile phases with no stationary phase collapse**
- 3. Bases are retained at low pH so high pH mobile phases may not be necessary for many applications**
- 4. Surface absorbs very little water so there is reproducible retention in the normal phase and rapid equilibration for gradient separations**
- 5. Bidentate phases can be used over a broad range of pH and can be used at high temperatures**

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Hydride Columns**