Reversed Phase
Advanced Features

Dr. Shulamit Levin
Analytical Department
Medtechnica
Email: levins@medtechnica.co.il
shulal@zahav.net.il
Tel: 03-9254040
Cell: 052-448632
Fax: 03-9249977
Home page:

http://www.forumsci.co.il/HPLC
Reversed Phase HPLC

Dr. Shulamit Levin
Analytical Department
Medtechnica
Email: levins@medtechnica.co.il
shulal@zahav.net.il
Tel: 03-9254040
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Chromatographic Process
Distribution:
\[ K = \frac{C_s}{C_m} \]

Elution through the Column

Chromatogram

Reversed Phase Chromatography

Non-polar stationary phase
Polar mobile phase

Analyte Y
Non-Polar
Poorly retained
(like attracts like)

Analyte X
Polar
Well retained

Flow

RP Method Development Tools

Columns
Phenyl
MeCN
MeOH

Solvents
C18
C8
C4

Additives
pH low
low

high

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
Reversed Phase HPLC

ELUTION ORDER IN REVERSED PHASE

<table>
<thead>
<tr>
<th>TIME (MIN.)</th>
<th>RESPONSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>VOID</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

PAH Analysis with Alliance System and PDA Using a Binary Gradient

Column: HibarRT 125-4 LiChrosphere PAH
Eluent A: Water
Eluent B: Acetonitrile
Gradient: Linear A to B
11 minutes, Hold 10 minutes
Back to initial conditions
Flow Rate: 1.0 ml/min
Injection: 20ul

UV@254nm

1- Naphthalene - 20 ppm
2- Acenaphthylene - 40 ppm
3- Acenaphthene - 20 ppm
4- Fluorene - 4 ppm
5- Phenanthrene - 2 ppm
6- Anthracene - 2 ppm
7- Fluoranthene - 4 ppm
8- Pyrene - 2 ppm
9- Benzo(a)anthracene - 2 ppm
10- Chrysene - 2 ppm
11- Benzo(b)fluoranthene - 4 ppm
12- Benzo(k)fluoranthene - 2 ppm
13- Benzo(a)pyrene - 2 ppm
14- Dibenzo(a,h)anthracene - 4 ppm
15- Benzo(g,h,i)pyrene - 4 ppm
16- Indeno(1,2,3-cd)pyrene - 2 ppm

Hydrophobicity

log k' vs log P

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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IONIZABLE

MOBILE PHASE

* TYPE OF MODIFIER (MeOH, ACN)
* SOLVENT STRENGTH (% modifier)
* pH
* TYPE OF BUFFER (phosphate, acetate)
* IONIC STRENGTH (Salts, buffer concentration)
* ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)

MOBILE PHASE

* TYPE OF MODIFIER (MeOH, ACN)
* SOLVENT STRENGTH (% modifier)
* pH
* TYPE OF BUFFER (phosphate, acetate)
* IONIC STRENGTH (Salts, buffer concentration)
* ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)

OPTIMIZATION: CHOICE OF SOLVENTS

20% MeOH
20% ACN
20% EtOH
20% THF

main solvent: H₂O
**MOBILE PHASE**

- TYPE OF MODIFIER (MeOH, ACN)
- SOLVENT STRENGTH (% modifier)
- pH
- TYPE OF BUFFER (phosphate, acetate)
- IONIC STRENGTH (Salts, buffer concentration)
- ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)

**SOLVENT STRENGTH**

**Analyte Retention as a Function of % Modifier**

$k$ (retention) for each analyte changes independently as % Modifier changes. Thus, the resolution between peaks changes.

**OPTIMIZATION: % SOLVENTS**

*20% MODIFIER*

*40% MODIFIER*

*60% MODIFIER*

*80% MODIFIER*

**MOBILE PHASE**

- TYPE OF MODIFIER (MeOH, ACN)
- SOLVENT STRENGTH (% modifier)
- pH
- TYPE OF BUFFER (phosphate, acetate)
- IONIC STRENGTH (Salts, buffer concentration)
- ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)
Ionization of Acids and Bases

Dissociation of Molecule

Acid

\[ HA \rightarrow H^+ + A^- \]

(Non-ionized) (Ionized)

50% @ pKa
100% Low pH
0% High pH

50% 100% 0%

Base

\[ B \rightarrow BH^+ + OH^- \]

(Non-ionized) (Ionized)

50% @ pKa
100% Low pH
0% High pH

50% 100% 0%

Retention Factor versus pH for Acids, Bases and Neutrals

Resolution of Two Acidic Compounds at Different Mobile Phase pH's

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
Enhanced Resolution of Basic Compounds at High pH

Dependence of Selectivity on pH

Impact of pH on the Retention of a Zwitterionic Compound

UV/Vis Spectral Change Between Ionized and Non-ionized Forms
Reversed Phase HPLC

Typical Chromatograms for pH Failure of an Ordinary C_{18}-Silica Column

1. Uracil
2. Propranolol
3. Naphthalene
4. Acenaphthene
5. Amitriptyline

MOBILE PHASE

- TYPE OF MODIFIER (MeOH, ACN)
- SOLVENT STRENGTH (% modifier)
- pH
- TYPE OF BUFFER (phosphate, acetate)
- IONIC STRENGTH (Salts, buffer concentration)
- ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)

Relevant Buffers for pH’s 2-7

<table>
<thead>
<tr>
<th>Additive or Buffer</th>
<th>pK_a</th>
<th>pH range (± 1 pH unit)</th>
<th>Volatile or Non-Volatile</th>
<th>Recommended for use with Extended pH Packings</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA</td>
<td>0.2</td>
<td>1.15 – 3.15</td>
<td>Volatile</td>
<td>Yes</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>3.76</td>
<td>3.76 - 5.76</td>
<td>Volatile</td>
<td>Yes</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>3.75</td>
<td>2.75 - 4.75</td>
<td>Volatile</td>
<td>Yes</td>
</tr>
<tr>
<td>Acetate</td>
<td>4.76</td>
<td>4.76</td>
<td>Non-Volatile</td>
<td>Yes (1-10mM) NH_4^+, Na, K</td>
</tr>
<tr>
<td>Formate</td>
<td>3.75</td>
<td>2.75 - 4.75</td>
<td>Non-Volatile</td>
<td>Yes (1-10mM) NH_4^+, Na, K</td>
</tr>
<tr>
<td>Phosphate</td>
<td>2.15</td>
<td>1.15 - 3.15</td>
<td>Non-Volatile</td>
<td>Yes</td>
</tr>
<tr>
<td>7.20 6.20 - 8.20</td>
<td></td>
<td>Non-Volatile</td>
<td>No for pH’s &gt;7.0 (lower the temperature the longer the column lifetime)</td>
<td></td>
</tr>
</tbody>
</table>
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Types of Buffers and Ionic Strength

- pH 10: Borate
  - 20 mM H$_3$BO$_3$
- pH 7: Phosphate
  - 20 mM K$_2$HPO$_4$
- pH 4-5: Acetate
  - 10 mM CH$_3$COONH$_4$
  - 100 mM CH$_3$COOH
- pH 2-3.5: Phosphate
  - 20 mM H$_3$PO$_4$ - KH$_2$PO$_4$

MOBILE PHASE

- TYPE OF MODIFIER (MeOH, ACN)
- SOLVENT STRENGTH (% modifier)
- pH
- TYPE OF BUFFER (phosphate, acetate)
- IONIC STRENGTH (Salts, buffer concentration)
- ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)

Ion Pair Reagent

Alkylamines

Alkylsulfonates

The larger alkyl, the longer are retention times

Concentration of Ion-Pair Reagent in the Mobile Phase

The larger alkyls saturate the stationary phase at lower concentrations

k'

Conc. of Ion Pair Reagent in the Mobile Phase

5 mM

C8

C7

C6

C5

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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Stationary Phase Characterization

The Evolution of the Silica Gel Particle Platform

- 1960’s Pellicular native silica
- 1970’s Irregular 10 µm native silica
- 1980’s Spherical 5 µm native silica
- 1990’s Spherical 3-5 µm high purity silica
- 2000’s Hybride Silica-Gel (co-polymer organic/inorganic) high purity silica

Improvement in Peak Shape for Bases

Not all C18’s are the same!

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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Different Columns – Different Chromatograms

Symmetry<sup>®</sup>C<sub>18</sub>

SymmetryShield<sup>®</sup>RP<sub>18</sub>

YMC ODS AQ

“Relative” Ranking of C18 Columns Using a Standardized Test

- There are no bad C18 columns.
- There are only different C18 columns.

Making a Bonded Phase Material: Monofunctional Synthesis

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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Surface of a Silica Gel
Bonded-Phase Packing Material

Mixed-Mode Retention:
- Hydrophobic Interaction with Bonded Phase
- Ion exchange Interaction with Charged Sites

Stationary Phase Properties

CHEMISTRY:
- BONDED HYDROCARBON:
  - C-18, C-8, C-4, C-1, CN, phenyl
- % COVERAGE
- TYPE OF SILICA GEL

GEOMETRY
- SPHERE-IRREGULAR
- PARTICLE DIAMETER
- POROSITY

Stationary Phase Ligands

<table>
<thead>
<tr>
<th>Stationary phase</th>
<th>Functionality</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(_{18})</td>
<td>(-\text{Si(CH}_3\text{)}_2\text{C}_18\text{H}37)</td>
</tr>
<tr>
<td>C(_{8})</td>
<td>(-\text{Si(CH}_3\text{)}_2\text{C}_8\text{H}17)</td>
</tr>
<tr>
<td>C(_{4})</td>
<td>(-\text{SiCH}_3\text{)}_2\text{H}8)</td>
</tr>
<tr>
<td>Aminopropyl</td>
<td>(-\text{Si(CH}_3\text{)}_2\text{NH})</td>
</tr>
<tr>
<td>Cyanopropyl</td>
<td>(-\text{Si(CH}_3\text{)}_2\text{(CH}_2\text{)}_2\text{CN})</td>
</tr>
<tr>
<td>Diol</td>
<td>(-\text{Si(CH}_3\text{)}_2\text{OCH}_2\text{CH(OH)CH}_2\text{OH})</td>
</tr>
</tbody>
</table>

Retention time

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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Neutral Compounds: C18 versus C4 (Same Brand - Different Ligands)

Minutes
0 4 8 12 16 20 24

YMC-Pack™ Pro C18™
YMC-Pack™ Pro C4™

Acenaphthene
Naphthalene
Butylparaben

Note: Similar selectivity due to same silica particle.

Type of Ligands

Conventional Alkyl
Aqueous C18
Ultra IB

Reversed-Phase Packing with an Embedded Polar Ligand

Embedded Polar Group Ligand

Traditional, Straight Chain Alkyl Ligand

Commercial Phases with Embedded Polar Group

SymmetryShield RP (Waters)
Discovery RP Amide16 (Supelco)
Prism, Spectrum (Keystone)
Bonus RP (Hewlett Packard)

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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**Embedded Polar Ligand: Possible Mechanism**

- Polar group increases water concentration in surface layer
- Shields Negative Silanols
- No additional hydrophobic retention
- Reduced retention of bases
- Reduced peak tailing

**Embedded Polar Goups**

- Mixed-Mode Retention: Hydrophobic Interaction with Bonded Phase
- Ion exchange Interaction with Charged Sites

**Embedded Polar Goups vs Linear Alkyl Ligand on Silica Gel**

- Mobile Phase pH < 3
- Mobile Phase pH > 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>SymmetryShield™ RP18 TF USP</th>
<th>Symmetry® C18 TF USP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amitriptyline</td>
<td>1.13</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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Impact on Selectivity - Retention

B. A. Alden

Selectivity Difference: Furazolidone Impurities

SymmetryShield™ RP8
Symmetry® C8

Stationary Phase Properties

CHEMISTRY:
* BONDED HYDROCARBON:
  - C-18, C-8, C-4, C-1, CN, phenyl
* % COVERAGE
* TYPE OF SILICA GEL

GEOMETRY
* SPHERE- IRREGULAR
* PARTICLE DIAMETER
* POROSITY

CARBON LOAD

Increasing carbon load on a similar geometrical shaped particles increases retention.

Retention time
Surface of a Silica Gel Bonded-Phase Packing Material

- Residual silanols
- Endcap
- Alkyl chains

Silica based "bonded phases"

- Bulky alkylsilane ligands cannot react with all available silanols due to steric hindrance.

Ligand Density (Surface Coverage)

- Silica Silanols: 6 - 8 μ moles/m²
- Highest Bonding Reported: 4.2
- Residual Silanols (Best Case): 2.0
- Residual Silanols (Typical): > 3.5

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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Better Way to Compare: Ligand Density (Surface Coverage)

\[ \chi = \frac{\% C}{100 \cdot SA \cdot nC \cdot 12 \cdot \left( \frac{\% C}{100} \cdot \frac{MW - 1}{nC \cdot 12} \right) \} = \mu\text{moles/m}^2 \]

SA = Specific Surface Area
MW = Molecular Weight of Ligand
\%C = % Carbon Load
nC = # of Carbon Atoms in Ligand

Ligand Density Retention Silanols
Surface Area % C Ligand Density

Acidic Compounds: C18 versus C8 (Same Brand - Different Ligand)

Note: Similar selectivity due to same silica particle.

Relative Hydrophobicities of General Purpose Analytical Packings

Stationary Phase Properties

CHEMISTRY:
BONDED HYDROCARBON:
C-18, C-8, C-4, C-1, CN, phenyl

% COVERAGE

TYPE OF SILICA GEL
Native/Synthetic-Pure

GEOMETRY
* SPHERE- IRREGULAR
* PARTICLE DIAMETER
* POROSITY

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levins@medtechnica.co.il
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Types of Silica

- Silanols
- pH stability
- Metal content
- Temperature stability

Structure of Silica Gel

- Amorphous, porous matrix of silicon atoms joined together with oxygen atoms to form "siloxane bonds" = (Si - O - Si)

What are Silanols?

- Residual unreacted surface hydroxyl groups left over from polymerization
- Reactive sites for use in bonding ligands (C18) to the silica gel surface

Silanol (Si-O-H)

Surface Silanols Found on Silica Gel

- Vicinal (Bridged)
- Geminal (Silanediol)
- Lone (most active)
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Mixed-Mode Retention:
- Hydrophobic Interaction with Bonded Phase
- Ion exchange Interaction with Charged Sites

Mobile Phase pH < 3
- Si – OH

Mobile Phase pH > 3
- Si – O^–

Hybrid versus Silica Gel Particle

Silica Gel C18 Materials
1/2 free silanols

XTerra™ C18 Materials
1/3 free silanols

Bonded Phase on Particles

Bonded Hybrid versus Bonded Silica Gel Surfaces
- C18-Bonded Silica Gel
  - 1/2 -OSi(CH₃)₂R
  - 1/2 -OH
- C18-Bonded Hybrid
  - 1/3 -OSi(CH₃)₂R
  - 1/3 -CH₃
  - 1/3 -OH

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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**PERFORMANCE BY ONE PEAK**

Retention Factor or Capacity Ratio:

\[ k' = \frac{t_R - t_0}{t_0} \]

\[ k' = \phi \frac{C_m}{C_w} \]

Number of Theoretical Plates:

\[ N = 16 \left( \frac{t_R}{W} \right)^2 \]

**Asymmetry Factor**

\[ A_f = \frac{t_{90\%}}{t_0} \]

Tail Factor:

\[ T_f = \frac{A + B}{2A} \]

**Tailing Factor USP**

Integration Errors Caused by Tailing

- T = 1.00
  - Recovered Peak Areas
    - 99.9%
    - 99.8%
    - 99.6%

- T = 1.58
  - Recovered Peak Areas
    - 97.6%
    - 95.3%
    - 92.3%

Types of Silica

- Silanols
- pH stability
- Metal content
- Temperature stability

Amitriptyline Peak Tailing Over Extended pH Range 1-12

Buffer pH

- Hybrid C18
- Pure Silica C18
- Conventional C18

Integration Errors Caused by Tailing

- T = 1.00
  - Recovered Peak Areas
    - 99.9%
    - 99.8%
    - 99.6%

- T = 1.58
  - Recovered Peak Areas
    - 97.6%
    - 95.3%
    - 92.3%

Types of Silica

- Silanols
- pH stability
- Metal content
- Temperature stability

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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pH Limitations of Silica Based Packing Materials

Hydrolysis of Bonded Ligand

Typical Chromatograms for Reference C<sub>18</sub>-Silica Column

Initial

1. Uracil
2. Propranolol
3. Naphthalene
4. Acenaphthene
5. Amitriptyline

After failure

1. Uracil
2. Propranolol
3. Naphthalene
4. Acenaphthene
5. Amitriptyline

Types of Silica

- Silanols
- pH stability
- Metal content
- Temperature stability

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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**Metal Content in Silica**

**Aluminum in the Silica Gel Lattice**

Bronsted Acid

3D top view of silica particle surface with silanols pointing upward

Metal available for chelation

**Correlation Between Base Tailing and Aluminum Content of Silica Gel**

- **Analyte:** Chlorpheniramine
- **Mobile Phase:** Acetonitrile/KH$_2$PO$_4$ pH 3.0 (20:80)

**Correlation between Metal Content of Silica Gel and Peak Retention and Shape**

**Peak Shapes of Chelating Agent (Hinokitiol)**

**Waters Symmetry® C18 Methanol**

- **Al conc. = 10 ppm**
  - **TF USP = 1.95**

**Waters Nova-Pak® C18 Methanol**

- **Al conc. = ~375 ppm**
  - **TF USP = 6.5**

**Low metals**

**High metals**

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
Types of Silica

- Silanols
- pH stability
- Metal content
- Temperature stability

Temperature Effects on Resolution

Resolution can be temperature dependent

Temperature can be a critical parameter to control in order to achieve reproducible separations.

K’ vs Temperature

K’ vs Temperature

Column Temperature (C)

Effect of Temperature (Isocratic separations)

Higher Temperature:
- Shorter Run Time
- Sharper Peaks
- Better Sensitivity
- Lower Back Pressure

Higher Temperature: 60°C, 1160 psi
50°C, 1160 psi
40°C, 1160 psi
30°C, 1920 psi

N=2250
N=1680

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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**Effect of Temperature on Column Efficiency**

![Graph showing the effect of temperature on column efficiency.](image)

**Dependence of Retention on Temperature**

Conditions:
- Column: XTerra™ MSC, 18 Å, 2.1 X 50 mm, 2.6 µm
- Mobile Phase: 25% ACN/75% buffer (10 mM, pH5, NH4AC)
- Flow Rate: 0.6 mL/min
- Injection Vol. 3 µL
- Detector: 210 nm

Analyte Conc. (µg/ml):
1: doxepin 0.5
2: imipramine 1.0
3: amitriptyline 3.0

Higher Temp., Shorter Run Time, Higher Signal

**Temperature Effects on Resolution - Gradient**

![Graph showing temperature effects on resolution.](image)

**High Temperature Phosphate Buffer Test**

The chart below shows the comparison of resolution at different temperatures and days. The test was conducted using a phosphate buffer with the conditions:
- Column: XTerra™ RP-18, 150 mm, 5 µm
- Mobile Phase: 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B)
- Flow Rate: 0.75 mL/min
- Injection: 20 µL
- Gradient: 0-45 min., 0-95% B
- Detection: 214 nm

Analyte Tailing Factor:
1. Doxepin 1.2
2. Nortriptyline 1.1
3. Amitriptyline 1.1

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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Stationary Phase Properties

CHEMISTRY:
- BONDED HYDROCARBON:
  C-18, C-8, C-4, C-1, CN, phenyl
- % COVERAGE
- TYPE OF SILICA GEL

GEOMETRY
- SPHERE-IRREGULAR
- PARTICLE DIAMETER
- POROSITY

Spherical and Irregular particles


Resolution - Time Diagram

- Resolution (Arbitrary Units)
- Analysis Time [min]
- Length / dp
  - 30 cm / 10 µm
  - 15 cm / 5 µm
  - 9 cm / 3 µm

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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Comparison of the van Deemter Plots for 5 µm and 2.5 µm XTerra® MS C18 Particles

(50/50, acetonitrile / water mobile phase)

Smaller Particles Higher Flow Rate provide Shorter Run Time Higher Efficiency

5 µm XTerra® Particle

2.5 µm XTerra® Particle

Linear Velocity (mm/sec)

H (µm)

Challenge of producing smaller particles

Contains a proportion of 2 µm particles

Both are commercial 2 µm packings

Centered at 2.4 µm Narrower distribution (Waters proprietary technology)

Fast Gradient Application

Columns: XTerra™ MS C18 2.1 x 20 mm, 2.5 µm; XTerra™ MS C18 2.1 x 50 mm, 5 µm
Mobile Phase: A: 0.1% TFA in water, B: 0.08% TFA in MeCN
Gradient: 5% B to 50% B in 45 seconds and 120 seconds
Column Temperature: 60 °C
Flow Rate: 1.5 mL/min.
Detector: 254 nm
Injection Volume: 1 µL

Fast Gradient of 12 Standards

Column: XTerra™ MS C18 2.1 x 20 mm, 2.5 µm
Gradient 0% B to 100% B in 2.5 minutes
A: 0.1% TFA in water
B: 0.08% TFA in MeCN
Flow Rate: 1.5 mL/min.
Temperature: 60 °C
Reversed Phase HPLC

Fast LC/MS Applications

- 2.1 mm 5 µm and 2.5 µm columns
- length: 5 cm and 3 cm
- flow rates 0.2 and 0.6 mL/min
- Conditions:
  - HPLC:
    - 65/35 0.1% formic acid / MeCN
    - 1 µL injection of 200 ng/mL of samples
  - MS:
    - ESI+; SR 4 channels
    - HV: 3.15 kV, Cone 25 V
    - Drying Gas: 380 L/h
    - Source Temp: 175°C

Fast LC-MS Analysis

XTerra™ MS C18, 2.1 x 50 mm (5 µm)

- 10 µL injection of 200 ng/mL sample (in 40% MeOH), 1 = Propranolol, 2 = Doxepin, 3 = Nortriptyline, 4 = Trimipramine
- 65/35 0.1% Formic Acid / MeCN
- 0.2 mL/min

Masschromatograms of Std. 10ppm (APci +/- )

- Tinuvin 770DF
- Chmazorb 944LD
- Irganox 1076

Fast LC-MS Analysis

XTerra™ MS C18: 5 µm vs. 2.5 µm

- HPLC: 65/35 0.1% Formic Acid / MeCN
- 1 µL injection of 200 ng/mL of samples
- MS: ESI+
- SR 4 channels
- HV: 3.15 kV
- Cone 25 V
- Drying Gas: 380 L/h
- Source Temp: 175°C

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
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Stationary Phase Properties

**CHEMISTRY:**
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  - C-18, C-8, C-4, C-1, CN, phenyl
- % COVERAGE
- TYPE OF SILICA GEL

**GEOMETRY**
- SPHERE- IRREGULAR
- PARTICLE DIAMETER
- POROSITY

**Pore size, shape and distribution**

Pore size defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface. This is especially important because the ratio of the outer particle surface to its inner one is about 1:1000. The surface molecular interaction mainly occurs on the inner particle surface.

![Macroporous spherical silica particle.](K.K.Unger, Porous silica, Elsevier, 1979)

**Silica Gel Pore Structure**

- Silica is Porous
- Pore Size, or nm -- distribution
- Specific Pore Volume, mL/g
  - Range: 0.3 – 1.3 mL/g
  - SV: Particle Strength
  - Analyte MW: Pore Size Recommendation
    - < 3,000: 60 - 130 (6 - 13 nm)
    - 3,000 - 10,000: 100 (10 nm)
    - >10,000: 300 - 1,000 (30 - 100 nm)
    - Very Large: None

**Pore Size**

- Most silica gel packings are porous
  - >99% of the surface area is contained within the particle (not on the surface) - "Where the chromatography happens."

- Rules of Thumb
  - "The smaller the pore size, the greater the surface area."
    - (100 Å approx. 300 m²/gram)
    - (300 Å approx. 100 m²/gram)
  - "The greater the surface area, the greater the retention."

- A typical 15 cm column holds a surface area of ~100-300 square meters

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
Exclusion - Inclusion Effects

Polarity/Aqueous Columns:

- Low ligand density
- High pore volume

Pore Size Effects on Resolution

Conditions
- Sample: Tryptic digests of cytochrome (bovine)
- Injection: 20 µL
- Mobile Phase:
  - Solvent A: 0.1% TFA in water
  - Solvent B: 0.1% TFA in acetonitrile
- Temperature: 25 °C
- Flow Rate: 0.75 mL/min.
- Detection: 214 nm

Mechanism of Retention of Polar Compounds on Aqueous Columns

Polar analytes are not able to “Fit” between ligands – can’t interact with surface

- High Coverage – High Ligand Density
- Low Coverage – Low Ligand Density

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il

Reversed Phase HPLC
Reversed Phase HPLC

Polar Compounds - Aqua Columns

**Conditions**
- Columns: 4.6 x 150 mm, 5 µm
- Mobile Phase A: H₂O
- Mobile Phase B: ACN
- Mobile Phase C: 100 mM NH₄COOH, pH 3.0
- Flow Rate: 2.0 mL/min
- Gradient: Time Profile

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
<th>%C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>80</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10.0</td>
<td>0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>15.0</td>
<td>0</td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

- Injection Volume: 5.0 µL
- Detection: UV @ 254 nm
- Vₜ = 0.98 min

**Polar and Non-Polar Compounds Test Mix**

1. Uracil 1.04
2. Acetanilide 0.93
3. Triamcinolone 1.02
4. Hydrocortisone 1.03
5. 2-Amino-7-chloro-5-oxo-5H-[1]-benzopyran-3,3-diyllinecarboxitile 1.01
6. 6a-Methyl-17a-hydroxyprogesterone 1.01
7. 3-Aminofluoranthene 0.97
8. 2-Bromofluorene 1.00
9. Perylene 0.99
10. Naphth[2,3-d]pyrene 0.96

Dr. Shulamit Levin, Medtechnica
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Chromolith Packing

By utilising an innovative new "Gel-Sol" technology, a silica gel polymer is formed, which after ageing, is dried into the required form of a straight rod of highly porous silica with a bimodal pore structure.

Batch-to-Batch Reproducibility of Columns

Columns: Symmetry™ C₈ 3.9 mm X 150 mm with Sentry™ Guard
Manager: 3.9 mm X 20 mm
Sample: Barbiturate Standard
Mobile Phase: 100 mM potassium phosphate, pH 6.9/acetonitrile/water
20:30:50

Dr. Shulamit Levin, Medtechnica
levins@medtechnica.co.il
Reversed Phase HPLC

Chromatogram of lifetime test

1: Sulfanilamide
2: Sulfadiazine
3: Sulfathiazole
4: Sulfamerazine
5: Sulfamethazine
6: Succinylsulfathiazole

Columns: Symmetry™ C8 3.9 mm X 150 mm with Sentry™ Guard Column 3.9 mm X 20 mm

Mobile Phase: water/methanol/glacial acetic acid 79:20:1

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levins@medtechnica.co.il