Method Development on Next Generation RPLC Supports

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Specialists in High Efficiency, Ultra-Stable Phases for HPLC
ZirChrom Separations, Inc. is a company formed in 1995 located in Anoka, Minnesota, USA. ZirChrom manufactures a full line of zirconia-based high performance chromatographic materials for the analytical analysis of compounds primarily by high performance liquid chromatography (HPLC).

The extreme stability of zirconia and its unique chromatographic selectivity allows for the optimization of separation conditions, which are totally incompatible with other types of supports. ZirChrom columns will give you full access to all chromatography tools that have traditionally been limited when using silica (i.e. pH, high temperature, and buffers).

Zirconia-based supports promise to revolutionize the way that liquid chromatographic analyses are done. These supports will last longer, and allow for faster more selective separations resulting in significant cost savings per analysis.
Outline

- RPLC Columns Sold By ZirChrom
- Surface Chemistry Considerations
- Method Development on ZirChrom Columns – Where Do I Start
  - Column Choice
  - Mobile Phase
  - pH
  - Column Temperature
- Example Method Development of A Real Pharmaceutical Samples
Why Use ZirChrom Phases?

- Selectivity - similar, different, or very different to ODS-silica
- Stability - run the operating conditions needed to get the separation, don’t worry about the column
- Speed - get 3x - 5x faster analysis by modest increases in temperature.
- Cost - Longer column life, greater productivity
Analytical Diameter Porous Zirconia Particles

ZirChrom®
# Properties of Porous Analytical Zirconia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Property</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface area (m²/g)</td>
<td>22</td>
</tr>
<tr>
<td>Pore volume (cc/g)</td>
<td>0.13</td>
</tr>
<tr>
<td>Pore diameter (Å)</td>
<td>250-300</td>
</tr>
<tr>
<td>Porosity</td>
<td>0.45 (silica 0.48)</td>
</tr>
<tr>
<td>Density (gm/cc)</td>
<td>2.6 (2.5x silica)</td>
</tr>
<tr>
<td>Particle size (µm)</td>
<td>3.0 (130,000 p/m)</td>
</tr>
</tbody>
</table>

**Prep-scale particles also available**
# 4 RPLC Stationary Phases

<table>
<thead>
<tr>
<th>Column Name</th>
<th>Stationary Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZirChrom-PBD</td>
<td>Polybutadiene</td>
</tr>
<tr>
<td>ZirChrom-PS</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>ZirChrom-CARB</td>
<td>Carbon</td>
</tr>
<tr>
<td>DiamondBond-C18</td>
<td>Octadecylphenyl-Carbon</td>
</tr>
</tbody>
</table>
ZirChrom®-PBD

Zirconia Substrate
ZirChrom®-CARB

Zirconia Substrate
DiamondBond®-C18

This bond is chemically and thermally stable -- great for LC/MS
How do I Start?

- Sample – size, matrix and solubility?
- Column(s) Selection
- Mobile Phase Optimization
  - Organic modifier
  - pH
  - Buffer choice
- Temperature – Can I speed it up?
## General Starting Point

<table>
<thead>
<tr>
<th>Separation Variable</th>
<th>Preferred Initial Choice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimension</td>
<td>10 x 0.46 cm</td>
</tr>
<tr>
<td>Particle Size</td>
<td>3 $\mu$m</td>
</tr>
<tr>
<td>Stationary Phase</td>
<td>ZirChrom-PBD or DBC18</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Water/Acetonitrile</td>
</tr>
<tr>
<td>%B</td>
<td>Variable</td>
</tr>
<tr>
<td>Buffer</td>
<td>25 mM phosphate, pH 7.0</td>
</tr>
<tr>
<td>Additives</td>
<td>Variable</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1-2 mL/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>30-80°C</td>
</tr>
<tr>
<td>Sample Volume</td>
<td>5 microliters</td>
</tr>
<tr>
<td>Sample Mass</td>
<td>100 $\mu$g</td>
</tr>
</tbody>
</table>
Which Column Do I Use?

- Are the analytes acidic, basic or neutral?
- Are the analytes very polar?
- Are the analytes closely related? Chiral?
- Are the analytes highly aromatic?
- Are the analytes thermally stable?
- How complex is the sample?
### Reversed-Phase Column Selection Guide

<table>
<thead>
<tr>
<th>CURRENT PROBLEM/CONCERN</th>
<th>COLUMN</th>
<th>SUGGESTED CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Improve Selectivity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Need improved selectivity for nonelectrolytes, isomers, disastereomers. Currently using carbon, cyano, phenyl or fluoro phases</td>
<td>DiamondBond™ C18, ZirChrom®-CARB</td>
<td>Temp ≥50 °C, with acetonitrile or preferably THF eluent.</td>
</tr>
<tr>
<td>Need improved selectivity for bases.</td>
<td>ZirChrom®-PBD</td>
<td>Use with Lewis base buffer (e.g. phosphate, citrate, fluoride) at pH 7.0, 5-50 mM. If not effective, increase pH to 11.0 or higher - See Technical Bulletin #241.</td>
</tr>
<tr>
<td>Need improved selectivity for acids.</td>
<td>DiamondBond™ C18</td>
<td>Use with 10 mM phosphate and 2 mM fluoride at pH 7.0. If not effective, drop pH to 2.0 or lower.</td>
</tr>
<tr>
<td><strong>Change Retention</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Need more retention for very polar (hydrophilic) nonelectrolytes. Currently using nearly 100% water eluent or polar embedded phase</td>
<td>DiamondBond™ C18, ZirChrom®-CARB</td>
<td>Can use in high water mobile phase. Temperature can be lowered to room temperature if needed to increase retention.</td>
</tr>
<tr>
<td>Need more retention for very polar bases. Currently using nearly 100% water eluent or polar embedded phase or sulfonic acid paired ion reagent</td>
<td>ZirChrom®-PBD</td>
<td>Use at pH 7.0 with &lt; 5mM phosphate. Vary pH as needed. Can use in high water mobile phase.</td>
</tr>
<tr>
<td>Need more retention for very polar acids. Currently using nearly 100% water eluent or polar embedded phase or quaternary amine paired ion reagent</td>
<td>DiamondBond™ C18</td>
<td>Use low pH to protonate acid. Choose a pH &lt;&lt; pKa, even pH 0.5 is no problem.</td>
</tr>
<tr>
<td>Need less retention with any solute type.</td>
<td>ZirChrom®-PS</td>
<td>Least hydrophobic ZirChrom phase. Can easily achieve use of 100% water eluent.</td>
</tr>
<tr>
<td><strong>Improve Efficiency / Productivity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inadequate stability and selectivity. Having trouble with silica-based phases, changed to alumina or polymer column and problems were still not sufficiently resolved</td>
<td>DiamondBond™ C18, ZirChrom®-PBD</td>
<td>pH range: 0.5 - 13, Temp &lt; 200 °C. Any of the listed reversed phases will give higher efficiency and better peak shape than alumina or polymer columns.</td>
</tr>
<tr>
<td>Poor column stability. Experiencing retention drift at low or high pH, at above ambient temperature or when using phosphate or carbonate buffer.</td>
<td>DiamondBond™ C18, ZirChrom®-PBD</td>
<td>Our phases are the “Most Durable” phases on the market.</td>
</tr>
<tr>
<td>Separations taking too long.</td>
<td>DiamondBond™ C18, ZirChrom®-PBD</td>
<td>Increase temperature up to max. operating range for LC &amp;/or analyte. Increase flow rate. Easily improves speed 2-3 fold.</td>
</tr>
<tr>
<td>Column overloaded too easily with basic solutes.</td>
<td>ZirChrom®-PBD</td>
<td>Use phosphate buffer.</td>
</tr>
<tr>
<td><strong>Improve Detection Sensitivity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Need to go to shorter wavelength to enhance sensitivity in UV. Solute does not have long wavelength absorption or is very dilute</td>
<td>ZirChrom®-PS</td>
<td>Use a high water or pure water eluent and go deep into UV.</td>
</tr>
<tr>
<td>Need to decrease bleed in LC/MS.</td>
<td>DiamondBond™ C18</td>
<td>Recommend THF or acetonitrile as eluent modifier; can also enhance sensitivity.</td>
</tr>
</tbody>
</table>

See www.zirchrom.com literature articles numbers 1, 3, 4, 18, 27, 28, 30, 35, 38, 39, 41, 44, 46, 50, 51, 55, 59 Technical Bulletin #240
Part II.
Zirconia the “Un-Silica”. The difference is the surface chemistry.
Surface Chemistry of Zirconia

**Brönsted Acid:** $\text{ZrOH} + \cdot\text{OH} \rightleftharpoons \text{ZrO}^- + \text{H}_2\text{O}$

**Brönsted Base:** $\text{Zr}\overset{\text{O}}{\text{Zr}} + \text{H}^+ \rightleftharpoons \text{Zr}\overset{\text{O}}{\text{Zr}}$

**Lewis Acid:** $\text{Zr(OH}_2) + \cdot\text{OOC\text{–}}\text{R} \rightleftharpoons \text{ZrOOC\text{–}}\text{R} + \text{H}_2\text{O}$
Interaction Strength of Lewis Bases with Lewis Acid Sites on Zirconia

<table>
<thead>
<tr>
<th>Interaction Strength</th>
<th>Lewis Base (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strongest</td>
<td>Phosphate, Fluoride,</td>
</tr>
<tr>
<td></td>
<td>Citric acid, Sulfate,</td>
</tr>
<tr>
<td></td>
<td>Acetic acid, Formic</td>
</tr>
<tr>
<td></td>
<td>acid, Nitrate, Chloride</td>
</tr>
<tr>
<td>Weakest</td>
<td></td>
</tr>
</tbody>
</table>

- Lewis bases with higher electron density and lower polarizability interact more strongly with zirconia.
Resolution: The Importance of Selectivity

Selectivity ($\alpha$) has the greatest impact on improving resolution.

$$R = \frac{\sqrt{N}}{4} \cdot \frac{k'}{k'+1} \cdot \frac{\alpha-1}{\alpha}$$

$$\alpha = \frac{k_j'}{k_i'}$$
Effect of $\alpha$ and N on Resolution

In general it is better to optimize selectivity rather than column efficiency as resolution is directly related to selectivity changes, but only varies with the square root of N.

<table>
<thead>
<tr>
<th>$\alpha$</th>
<th>Column</th>
<th>N</th>
<th>Run Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.10</td>
<td>4 cm, 3 $\mu$m</td>
<td>6,000</td>
<td>2-5 min</td>
</tr>
<tr>
<td>1.05</td>
<td>30 cm, 5 $\mu$m</td>
<td>25,000</td>
<td>30-60 min</td>
</tr>
<tr>
<td>1.02</td>
<td>5 m, 10 $\mu$m</td>
<td>160,000</td>
<td>8-15 h</td>
</tr>
</tbody>
</table>
Method Development Knobs on Zirconia RP Phases

<table>
<thead>
<tr>
<th>Stationary Phase</th>
<th>pH (1-14)</th>
<th>Organic Modifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Types</td>
<td></td>
<td>Type (many)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Percent Organic Modifier</th>
<th>Buffer - Type and Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>up to 200°C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Method Development Knobs on Silica RP Phases

- Stationary Phase: Many Types
- pH (2-9)
- Organic Modifier: Type (many)
- Temperature: up to 50°C
- Percent Organic Modifier
Why Choose Zirconia?

Silica    Zirconia

Which tool set would you rather have to keep your method development vehicle going?
When Do We Use the Stationary Phase Knob?

*Before we try anything else . . .*

- To change *selectivity*
- To change *retention*
22 Non-electrolyte Solutes

Nonpolar

- Benzene
- Toluene
- Ethylbenzene
- p-xylene
- Propylbenzene
- Butylbenzene

Polar

- Bromobenzene
- p-Dichlorobenzene
- Anisole
- Methylbenzoate
- Naphthalene
- Acetonphenone
- Benzonitrile
- Nitrobenzene
- p-Nitrotoluene
- p-Nitrobenzyl Chloride
- Benzophenone

HB Donor

- Benzylalcohol
- 3-Phenyl Propanol
- N-Benzyl Formamide
- Phenol
- p-Chlorophenol
## Selectivity Matrix*

<table>
<thead>
<tr>
<th></th>
<th>CARB</th>
<th>DB-C18</th>
<th>PBD</th>
<th>RP18</th>
<th>C18 (2)</th>
<th>PLRP-S</th>
<th>RP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZirChrom-CARB</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DiamondBond-C18</td>
<td>0.80</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZirChrom-PBD</td>
<td>0.51</td>
<td>0.90</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xterra RP18</td>
<td>0.53</td>
<td>0.85</td>
<td>0.90</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luna C18 (2)</td>
<td>0.53</td>
<td>0.86</td>
<td>0.93</td>
<td>0.97</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLRP-S</td>
<td>0.60</td>
<td>0.90</td>
<td>0.93</td>
<td>0.92</td>
<td>0.96</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gammabond RP-1</td>
<td>0.52</td>
<td>0.88</td>
<td>0.96</td>
<td>0.97</td>
<td>0.98</td>
<td>0.95</td>
<td>1</td>
</tr>
</tbody>
</table>

* Column names are the trademarks of their respective manufacturers.

- **ZirChrom-PBD** is the most similar to ODS for non-ionic analytes
- **ZirChrom-CARB** is the most different to ODS
- **DiamondBond-C18** is ODS-like but with some CARB selectivity (generally better peak shapes than CARB)
Selectivity and Shape: Isomeric Analytes

<table>
<thead>
<tr>
<th>Compound</th>
<th>ODS</th>
<th>Carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>di(phenethyl)amide</td>
<td>1.19</td>
<td>1.20</td>
</tr>
<tr>
<td>cis-/trans-stilbene</td>
<td>1.02</td>
<td>22</td>
</tr>
</tbody>
</table>

- **di(phenethyl)amide**
- **cis-/trans-stilbene**
Dinitroaniline Herbicide Separation (EPA Method 627)
DiamondBond-C18 Selectivity*

**a)** 150 x 4.6 mm ODS

**b)** 100 x 4.6 mm DiamondBond-C18

---

**Ethylbenzene** ➔

**p-xylene** ➔

---

**LC Conditions:**

**a)** Column, 150 x 4.6 Zorbax Eclipse XDB-C8 S/N: USRK010769; Mobile phase, 65/35 ACN/Water; Temperature, 30 °C; Flow rate, 1.0 ml/min.; Injection volume, 5 ul; Detection at 254 nm; Solutes: 1=Ethylbenzene, 2=p-xylene.

**b)** Column, 100 x 4.6 DiamondBond-C18, OD082401A; Mobile phase, 37.5/5/57.5 ACN/THF/Water; Temperature, 60 °C; all other conditions the same as a).

* Column names are the trademarks of their respective manufacturers.
When Do We Use the pH Knob?

- For ionizable compounds
- To improve *peak shape* of acidic or basic compounds
- To change *selectivity* of acidic or basic compounds
- To change *retention* of acidic or basic compounds
Silica after aging with Base*

Aging conditions: 0.1g of Extend C18 was placed in 50mls of 40:60 - Methanol:1M Potassium hydroxide solution. It was aged for 24 hours at room temperature in a shaking bath.

* Column names are the trademarks of their respective manufacturers.
Why Use pH Extremes?

- pH Stability
  - pH < 1
    - Cleaning with Conc. Acid
  - pH > 13
    - Ion Supression for Acids
    - Ion Supression for Amines
    - Sanitation/Depyrogenation
Correct buffering can eliminate unwanted residual surface interactions (or enhance desirable residual surface interactions)
Exposure Conditions: Mobile phase, ACN/50mM Potassium phosphate buffer at indicated pH; Temperature, 30 °C.

LC Conditions: Mobile phase, ACN (or THF)/50mM Potassium phosphate buffer at indicated pH; Flow Rate, 1.0 mL/min.; Temperature, 30 °C; Injection Volume, 5 uL; Detection, 254nm.

* Column names are the trademarks of their respective manufacturers.
Effect of pH on Bases

RNH₂ + H⁺ ⇌ RNH₃⁺

RP

RP + CEX

5 and 6 switch elution order
Fast Separations of NSAIDs on DiamondBond™-C18

pH = 1.75

LC Conditions: Column, 100 x 4.6 DiamondBond™-C18; LC Conditions: Mobile phase, 50/50 ACN/50mM Phosphoric acid, pH 1.75; Flow rate, 1.0 ml/min.; Temperature, 65 °C; Injection volume, 1.0 ul; Detection at 254nm; Solutes: 1=Acetominaphen, 2=Ketoprofen, 3=Ibuprofen, 4=Naproxen
Why Work at High pH?

- To adjust retention
- To improve peak shape by deprotonating amines
- To adjust selectivity

Why tie your hands with columns with limited lifetime, limited buffer types and limited pH range?

ZirChrom RPLC phases are stable: $1 < \text{pH} < 14$
When Do We Use the Mobile Phase Knobs?

- To change **selectivity**
- To change **retention**
Molecular Interactions in LC

**Dispersion**

**Dipole**

\[
\text{CH}_3 - \overset{+}{\text{C}} \equiv \overset{-}{\text{N}} \leftrightarrow \text{CH}_3 - \overset{-}{\text{C}} \equiv \overset{+}{\text{N}}
\]

**Dielectric Interactions**

**Hydrogen Bonding**

\[
\text{Cl}_3 \text{C} - \overset{+}{\text{H}} \leftrightarrow :\overset{-}{\text{N}} - (\text{CH}_3)_3
\]
Overview of Mobile Phase Optimization

- Define optimum solvent strength so that
  \[ 1 < k' < 20 \]
  a. Do stepwise isocratic study in 20% steps starting at 100% organic.
  b. Do gradient determination of % organic.
- Define the best type of modifier; MeOH, ACN or THF - TRIANGLE OPTIMIZATION.
- Keep in mind that the relative “strengths of MeOH, ACN and THF are related.
Separation Optimization

EASY SAMPLES

Initial run with ACN/water → Define $k'$ range vs % ACN → Best % ACN for $k'$ range and band spacing

AVERAGE SAMPLES

Initial run with MeOH/water → Define $k'$ range vs % MeOH → Best % MeOH for $k'$ range and band spacing

Initial run with THF/water → Define $k'$ range vs % THF → Best % THF for $k'$ range and band spacing
Triangle Optimization

HARD SAMPLES

Use $k'$ range from earlier runs

MeOH

H$_2$O

ACN

THF
Define % solvent for $1 < k' < 20$ in MeOH (Solvent 1).

If band spacing is not OK then use equieluotropic mixtures for ACN and THF. (Solvents 2 and 3)

If 2 or 3 are not OK then do mid-points as shown. (Solvents 4, 5, 6).

If a significant improvement is seen then look for intermediate ternary mixture.

If no significant improvement is seen then do mixture for all three solvents (Solvent 7).
Solvent Properties Triangle

[Image of a solvent properties triangle diagram]
When Do We Use the Buffer Knob?

- To improve *peak shape* of acidic or basic compounds
- To change *selectivity* of acidic or basic compounds
- To change *retention* of acidic or basic compounds
- To modify *band spacing* or elution order
Effect of Different Lewis Base Additives

1. Lidocaine
2. Norpseudoephedrine
3. Tryptamine
4. Quinidine
5. Amitriptyline
6. Nortriptyline

Acetate
Fluoride
Phosphate

20% ACN, 20 mM Lewis base additive, pH 7.5; 0.8 mL/min; 30 °C

(5) pKa 9.4
(6) pKa 9.7
Effect of Additive Concentration on Bases

- **20 mM NH₄H₂PO₄**
  - pH 7.5

- **100 mM NH₄H₂PO₄**
  - pH 7.5

- CEX is adjustable by ionic strength of mobile phase
Effect of Buffer Concentration on Retention

Plot of log $k'$ for antihistamines versus logarithm of phosphate buffer concentration in milimole on (A), ZirChrom-PBD column and (B), ODS column. Experimental conditions: mobile phase, 40/60 acetonitrile/potassium phosphate buffer at pH 7.0; temperature, 30°C; solutes: (●), pheniramine; (○), thenyldiamine; (▼), chlorpheniramine; (▽), brompheniramine; (■), cyclizine; (□), thonzylamine; (◆), meclizine; (◇), chlorcyclizine; (▲), pyrrobutamine.
Separation of Antiarrythmic Drugs

1. Chlorpropamide
2. Tolbutamide
3. Procainamide
4. Acetylprocainamide
5. Propionylprocainamide
6. Lidocaine
7. Quinidine

(1) $pK_a = 4.9$

(2) $pK_a = 4.9$

Gradient elution; 30mM Additive, 15 mM TRIZMA, pH 7.5; 0.8 mL/min; 40 °C
Separation of Alkoxybenzoic Acids on ZirChrom®-PBD

40 mM Acetate pH 4

Problem

\[ N = 260 \]
\[ A_s = 3.35 \]

50 x 4.6 mm

40 mM Phosphate pH 2.15

No Problem

\[ N = 3250 \]
\[ A_s = 1.07 \]

50 x 4.6 mm

25% ACN, 40 mM above additive, 5 mM NH₄F; 0.6 mL/min; 30 °C; 254 nm.
Carboxylate Problem - Solved!

- Phosphate as buffer ([PO₄] > 20-30 mM).
- Small amount of fluoride (5mM).
- Low pH (< 3).
- High temperature (40 °C).
- ACN as organic modifier.
Separation of Carboxylic Acid NSAIDs on ZirChrom®-PBD

A, 40 mM $\text{H}_3\text{PO}_4$, 5 mM $\text{NH}_4\text{F}$, pH 2.1; B, 50% ACN + A; 10-60% B, 0-2 min; 60% B, 2-10 min; 0.8 mL/min; 40 °C.

Absorbance (mAU) vs. Time (min)

1. Acetaminophen
2. Aspirin
3. Ketoprofen
4. Fenoprofen
5. Ibuprofen
6. Indomethacin

No Problem
Solve Any Tailing Problem
Use High pH on Zirconia Phases

ZirChrom-PBD Gives Excellent Peak Shapes For Basic Drugs at High pH

...In LC/MS-friendly amine buffer

**ANALYTES**
1 - Labetalol
2 - Acebutolol
3 - Oxprenolol
4 - Lidocaine
5 - Alprenolol
6 - Propranolol

**LC CONDITIONS**
Mobile phase: 35/65 ACN/10mM Triethylamine, 50mM Tetramethylammonium hydroxide, pH 12.2
Flow rate: 1.0 ml/min  Injection volume: 5 μl
Temperature: 30 °C  Detection: 254 nm

...Or in phosphate buffer

**LC CONDITIONS**
Mobile phase: 35/65 ACN/20mM Potassium Phosphate, pH 12.2
Flow rate: 1.0 ml/min  Injection volume: 5 μl
Temperature: 30 °C  Detection: 254 nm
Combination of ODS and PBD-ZrO$_2$ for the separation of basic drugs

Partition Mechanism

Adsorption Mechanism

Phosphate Buffer
pH=7

Temperature 1
C18-SiO$_2$

Temperature 2
Carbon-ZrO$_2$

Reversed Phase Mode

+ Cation-Exchange Mode

Cation-Exchange Mode

+ Reversed Phase Mode
Separation of Anti-Histamine Drugs by T3C

C18-SiO2
30 °C

PBD-ZrO2
30 °C

T3C
40 °C +35 °C

Resolution

R^2=0.147
When Do We Use the Temperature Knob?

- To *speed up* analyses
- To modify *band spacing*

Temperature up to 200°C
Temperature
The Third Dimension in HPLC
Why Use Temperature?

Thermal Stability

- Lower Pressure Drop
- Less Organic Solvent
- Thermally Optimize Selectivity

- Less Wear and Tear
- Higher Flow Rate: Fast Analysis
- More Robust Analysis
- Easier Method Development
Antihistamine Example: Speed it up!

**LC Conditions: (A) Mobile Phase, 29/71 ACN/50mM Tetramethylammonium hydroxide, pH 12.2; Flow Rate, 1.35 mL/min.; Injection volume, 0.5 μl; 254 nm detection; Column Temperature, 21°C; Pressure drop = 195 bar; Solutes: 1=Doxylamine, 2=Methapyrilene, 3=Chlorpheniramine, 4=Triprolidine, 5=Meclizine**

100 x 4.6 ZirChrom-PBD (B) same as A, except Mobile Phase, 26.5/73.5 ACN/50mM Tetramethylammonium hydroxide, pH 12.2; Flow Rate, 3.00 mL/min.; Column Temperature, 80°C; Pressure drop = 195 bar.
Fast $\beta$-Blockers Separation

Column Temperature = 150$^\circ$C, pH = 11

LC Conditions: Column, 50 x 4.6 Diamondbond-C18, OD0121601A; Mobile phase, 45/55 ACN/20mM Ammonium Phosphate pH11.0; Flow rate, 3.0 ml/min; Temperature, 150 °C; Injection volume, 1.0 ul; Detection at 210 nm; Solutes, 1=Labetalol, 2=Metoprolol, 3=Alprenolol

Separation in 0.4 minute!
Summary
Causes of Bad Peak Shape

- “Bad” Column (poorly packed).
- Build up of “GARBAGE” on the column.
- Too much sample per injection.
- Wrong solvent for sample.
- Extra-column effects.
- Chemical or secondary effects (Lewis acid site interactions).
- Poor buffering.
Operational Similarities for SiO₂ and ZrO₂ RP Media

- $k'$ increases with molecular hydrophobicity (CH₃, CH₂, phenyl, etc.).
- Elution sequence of non-electrolytes similar.
- $k'$ decreases 2 fold per 10% increase in volume % modifier.
- log $k'$ linear with % organic modifier.
- $k'$ decreases as temperature is increased (3 fold/50 °C).
- Solvent strength: THF > ACN > MeOH.
Operational Differences in SiO₂ and ZrO₂ RP Media

- Lewis base modifier at mid-range pH creates mixed-mode RPC/IEC possibilities.

- Cations are typically more retained and sometimes much more retained on ZrO₂ in buffered (PO₄) eluents than on SiO₂ phases.

- Elution sequence of anions and cations can be very different on ZrO₂ & SiO₂ at neutral pH.

- Must use Lewis base eluent for carboxylic analytes. Strongly advise use of 5mM or more phosphate (or TMAH) for all electrolytes.
Conclusions

- Silica-based HPLC columns work well between pH 3 and 8; outside these limits, these columns can have short lifetimes.
- Zirconia is stable at pH 1-14 and at elevated temperature.
- Zirconia can be functionalized by coating / cross-linking polymers, for very stable supports.
- ZirChrom’s zirconia columns reproducibly combine silica’s efficiency with polymer stability.
Thanks *very much* for listening!

ZirChrom Separations & Cabot Corporation
Partners in Chromatography

For more information and web access to the free **Buffer Wizard**: www.zirchrom.com
Additional Information
Efficiency Comparison of Leading HPLC Columns*

LC Conditions: Mobile Phase, 65/35 Acetonitrile/50mM Potassium phosphate buffer, pH 3.2; Flow Rate, 1.0 mL/min.; Injection volume, 1 ul; 254 nm detection; Column Temperature, 21°C. Solutes: uracil, phenol, pyridine, 4-butylbenzoic acid, N,N – dimethylaniline, toluene.

† Mobile Phase: 45/5/50 Acetonitrile/THF/50mM Potassium phosphate buffer, pH 7.0; Column Temperature: 30°C; all other conditions are identical.

* Column names are the trademarks of their respective manufacturers.
Reproducibility Data for 50 ZirChrom®-PBD Columns

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Average</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k'$ (Toluene)</td>
<td>2.32</td>
<td>2.4%</td>
</tr>
<tr>
<td>$\alpha$ (Methylbenzoate/Benzonitrile)</td>
<td>1.80</td>
<td>1.1%</td>
</tr>
<tr>
<td>N (plates/m)</td>
<td>151,000</td>
<td>6.8%</td>
</tr>
<tr>
<td>Symmetry</td>
<td>0.93</td>
<td>7.2%</td>
</tr>
</tbody>
</table>

LC Conditions: Mobile phase, 35/65 ACN/Water; Flow rate, 1.0 ml/min.; Temperature, 30 °C; Injection volume, 5 µl; Detection at 254 nm
Testing of DiamondBond Stability

**LC Conditions:**

- **Base Stability—DiamondBond™ Phase A,** 30 x 4.6 mm id; Mobile phase, 50/50 ACN/Water; Flow rate, 1.0 ml/min.; Temperature, 30 °C; Injection volume, 5ul; Detection at 254nm.
- **Acid Stability—DiamondBond™ Phase A,** 50 x 4.6 mm id; Mobile phase, 50/50 ACN/Water; Flow rate, 1.0 ml/min.; Temperature, 30 °C; Injection volume, 5ul; Detection at 254nm.
- **Temperature Stability—DiamondBond™ Phase B,** 50 x 4.6 mm id; Mobile phase, 50/50 ACN/Water; Flow rate, 1.0 ml/min.; Temperature, 30 °C; Injection volume, 5ul; Detection at 254nm.
Synthesis of ZirChrom-PBD (Polybutadiene)

\[
\begin{align*}
\text{Dicumyl Peroxide} \quad \text{Vacuum} \quad 160 \, ^\circ \text{C}
\end{align*}
\]
ZirChrom-PS Synthesis

**Step 1. Synthesis of Copolymer (CMS/VMS)**

**Step 2. Adsorption of Copolymer**

**Step 3. Thermal Crosslinking**

$\text{CMS/VMS-ZrO}_2 \parallel \text{PS-ZrO}_2$
Synthesis of ZirChrom-CARB

Bulk Zirconia

Lewis Acid Site
Synthesis of DiamondBond-C18

- General approach - Cabot Corporation (Billerica, MA):
  - functionalizing agent X-R-Y
  - X reacts with surface
  - Y = functional group
- X is typically a diazonium salt

\[
\text{NH}_2 - \bigcirc - Y + 2 \text{ HA } + \text{ NaNO}_2 = \text{ AN} \equiv \text{N} - \bigcirc - Y + 2 \text{ H}_2\text{O } + \text{ NaA}
\]

Carbon Clad Zirconia + Diazonium Salt → Modified Carbon Clad Zirconia
ZirChrom-CARB

- ZirChrom-CARB has RPLC like properties but has radically different selectivity than ODS or ZirChrom®-PBD -- great for steroids, and for geometric isomers.
- Much more hydrophobic & retentive than ODS -- great for polar analytes.

- ZirChrom®-CARB is very stable. Can be used at low and high pH. Can be used at high temperatures.
- Lewis acid-base properties are still important on all zirconia-based phases.
- Peak shapes best with THF (10%) and/or elevated temperature.


“Superheated Water: A New Look at a Chromatographic Eluent for Reversed-Phase Liquid Chromatography”,  
Thermally Tuned Tandem Columns (T³C)

A Mechanism to *Continuously Adjust* the Stationary Phase

A diagram shows the setup with Pump, Injector, Column 1, Column 2, and Detector. Column 1 uses an example stationary phase of C18-SiO₂, and Column 2 uses C-ZrO₂. The diagram illustrates the optimization of T³C.
Analogy between Column Temperature and Column Length

Increasing temperature and decreasing column length both decrease retention time

T increases 50 °C  \rightarrow  k’ decreases 3-fold
Effect of Temperature on T³C Selectivity

Temperature continuously changes the T³C selectivity between $\alpha_1$ and $\alpha_2$. 

\[ \alpha_{net} = f_1 \alpha_1 + f_2 \alpha_2 \]

\[ f_1 = \frac{k'_1}{k'_1 + k'_2} \]

\[ f_2 = \frac{k'_2}{k'_1 + k'_2} \]
Requirements for T^3C

1. Two columns with different (ideally orthogonal) selectivities

2. One column must be thermally stable
   - Zirconia based phase are thermally stable up to 200 °C
   - Polybutadiene-coated zirconia (PBD-ZrO_2)
   - Carbon-coated zirconia (C-ZrO_2)

3. Method development must be easy
   - Theory for T^3C
   - Guideline for method development
Method Development for T³C

\[ t_{R1} = t_{01}(1 + k_1') \]

\[ \log k_1' = A_1 + B_1/T \]

2 trial runs

\[ t_{R2} = t_{02}(1 + k_2') \]

\[ \log k_2' = A_2 + B_2/T \]

2 trial runs

\[ t_{R_{net}}(T_1, T_2) = t_{R1}(T_1) + t_{R2}(T_2) \]
Guidelines for Optimizing T³C

- Choose Two Stationary Phases
- Choose Mobile Phase (1 < k’ < 20)

Window Diagram Optimization

Calculating T³C Retentions
\[ \ln k’ = A + B/T \]
\[ t_{net} = t_{r1} + t_{r2} \]

No

Different Selectivity?

Yes

Two More Runs at Higher Temperatures
Thermally Tuned Tandem Column (T³C) Concept

**Optimized Single Column**

- **C18-SiO₂**
  - Absorbance (mAU)
  - Time (min)

- **C-ZrO₂**
  - Absorbance (mAU)
  - Time (min)

**Optimized T³C**

- Absorbance (mAU)
- Time (min)

**T³C Works!**
Separation of Ten Triazine Herbicides by T³C

Solutes:
1. Simazine
2. Cyanazine
3. Simetryn
4. Atrazine
5. Prometon
6. Ametryn
7. Propazine
8. Terbutylazine
9. Prometryn
10. Terbutryn

Other conditions:
- 30/70 ACN/water
- 1ml/min; 254 nm detection

T³C can improve separation without increasing analysis time.
Steps in T³C Optimization of Triazine Herbicides

- \( R^2 = 0.107, \text{sd} = 0.342 \)

- Temperature on ODS Column: 30 °C
- Temperature on C-ZrO₂ Column: 125 °C
- Rs = 3.30

- T³C, 3ml/min \( R = 2.02 \)
Compare T³C with Mobile Phase Optimization

- **40% ACN**
  - Time (min)
  - Percentage of organic modifier

- **50% MeOH**
  - Minimum Rs

- **30% THF**
  - THF, R<0.5

❖ T³C is more powerful than mobile phase optimization on ODS
More Applications of T³C Concept

Urea and Carbamate Pesticides

**C18-SiO₂** 30°C

**C-ZrO₂** 90°C

**T³C** 39°C+89°C
Introducing the Metalox™ 200-C High Temperature Column Heater

- Designed from the ground up for chromatographers.
- Overcomes issues such as thermal mismatch and inaccurate column temperature reporting.
Conclusion: Regardless of flow rate, the column inlet and outlet temperature are within 3 °C of each other.

Reducing thermal mismatch to a minimum at any flow rate solves broadening and peak splitting!

"Influence of Thermal Conditions on the Efficiency of High-Performance Liquid Chromatography."
Metalox™ 200-C Features
Advantages, and Benefits

<table>
<thead>
<tr>
<th>Feature</th>
<th>Advantage</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiabatic type oven (Adiabatic: Occuring without loss or gain of heat), <em>Patent Pending</em></td>
<td>All critical components (column insulation and mobile phase) are at a stable and defined temperature</td>
<td>Greatly reduces retention time shifts and band broadening commonly found in other column heater designs and thus improves overall data quality</td>
</tr>
<tr>
<td>Advanced oven design includes reduced internal dead volume by using laser welded components in the heat exchanger</td>
<td>Reduces band broadening normally associated with adding a &quot;modular column heater&quot;</td>
<td>Allows use of narrow bore columns and/or gradient elution without excessive increase in run time or loss of column efficiency</td>
</tr>
<tr>
<td>Close coupled heater element and temperature sensors <em>Patent Pending</em></td>
<td>Provides for more efficient heat transfer to mobile phase over air bath only type</td>
<td>Allows for faster and more responsive heat transfer with smaller (less expensive) instrument design.</td>
</tr>
</tbody>
</table>
Eliminates the problem caused by temp. variability inside large air bath ovens and thus removes another point of instrumental error and lowered chromatographic performance.

When column is changed the positioning geometry will be the same even with different operators.

### Metalox™ 200-C Features

<table>
<thead>
<tr>
<th>Feature</th>
<th>Advantage</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat exchanger at mobile phase inlet</td>
<td>Pre-heats incoming mobile phase closer to desired temperature as well as reducing exiting mobile phase temperature as it enters the detector</td>
<td>Reduces needed heater size which lowers instrument size and cost. Lower detector temp. means less thermal noise and drift from detector cell.</td>
</tr>
<tr>
<td>Four monitored temperature zones</td>
<td>Monitors temperature at critical points in flow path</td>
<td>Ensures that column is at or near uniform temp., reducing thermal band broadening. Provides built-in capabilities for instrument validation. Column is at constant temp. during gradient elution and/or flow programming.</td>
</tr>
<tr>
<td>Column resides in defined location</td>
<td>When column is changed the positioning geometry will be the same even with different operators</td>
<td>Eliminates the problem caused by temp. variability inside large air bath ovens and thus removes another point of instrumental error and lowered chromatographic performance.</td>
</tr>
</tbody>
</table>
**Metalox™ 200-C Features, Advantages, and Benefits**

<table>
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<tr>
<th>Feature</th>
<th>Advantage</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small footprint and stand alone operation.</td>
<td>Easy to attach to existing systems and/or move between systems for added flexibility</td>
<td>User get top chromatographic performance in modular format</td>
</tr>
<tr>
<td>Front panel displays all four temperature controlled zones as well as column inlet and outlet differential temperature</td>
<td>Operator can easily tell when oven and column are truly ready for use - instead of just a single readout of an air temperature or block sensor</td>
<td>Avoids making reruns because column was not truly at desired operating temperature</td>
</tr>
<tr>
<td>RS-232 and analog output of all four temperature zones</td>
<td>Provides two convenient monitoring modes to log data to external devices</td>
<td>Very useful when setting up validation protocols, no need to attach extra sensor and recording devices</td>
</tr>
</tbody>
</table>
## Metalox™ 200-C Features

Advantages, and Benefits

<table>
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<th>Feature</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Bonded heaters are powered by isolated 24V DC</td>
<td>No line voltage in close proximity to chromatographic flow path or operator</td>
<td>Provides extra safety to both instrument and personnel in case of shorted heater component</td>
</tr>
<tr>
<td>Back pressure regulator down stream of detector</td>
<td>Keep constant back pressure on entire chromatographic system</td>
<td>Prevents mobile phase form boiling in detector and prevents mobile phase boiling in column upon loss of mobile phase supply</td>
</tr>
<tr>
<td>Two thermal cutout sensors inside of oven</td>
<td>Shuts off heater power in case of thermal runaway</td>
<td>Provides extra measure of safety for unattended operation</td>
</tr>
</tbody>
</table>
The Metalox™ 200-C
High Temperature Column Heater

- Introductory Offer
  - The new Metalox 200-C High Temperature Column Heater
  - Back Pressure Regulator
  - A set of three ZirChrom revolutionary RP HPLC Columns.
    - ZirChrom-PBD
    - ZirChrom-CARB
    - DiamondBond C18
Metalox™ 200-C Preliminary Specifications

✦ Operational Capabilities
  – Max. Column Operating Temp: 200° C (6 ml/min with water)
  – Min. Temperature: 7° C above ambient
  – Max. Flow Rate: 6ml/min (200° C, water mobile phase)
  – Max. Cal./s: 17.9
  – Temperature Reproducibility: ± 0.5° C
  – Accuracy of Temp. Reading: ± 1%
  – Display Resolution: 1° C

✦ Physical Specifications
  – Weight: 15 lbs
  – Footprint: 6”x10”x14”
  – Power Requirements: 115-230V 47-440 Hz
  – Internal Transfer Volumes:
    ♦ Pre-Column: 10.5 uL
    ♦ Post-Column: 4.0 uL

Note: Metalox 200-C design and specifications are pending patent
## Quantitative Value Assessment

### Steroids Separation

**Customer:** Contract Lab XYZ  
**Date:** 1/31/02

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Leading ODS</th>
<th>ZirChrom-PBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column Cost</td>
<td>$395.00</td>
<td>$595.00</td>
</tr>
<tr>
<td>Analysis Time (min)</td>
<td>10.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Average Time per Successful Cycle (min)</td>
<td>12.3</td>
<td>7.2</td>
</tr>
<tr>
<td>Possible Cycles per Instrument per Year</td>
<td>42,705</td>
<td>73,474</td>
</tr>
</tbody>
</table>

### Cost Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Leading ODS</th>
<th>ZirChrom-PBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column Cost per Successful Cycle</td>
<td>$1.01</td>
<td>$1.30</td>
</tr>
<tr>
<td>Solvent Cost per Successful Cycle</td>
<td>$0.02</td>
<td>$0.04</td>
</tr>
<tr>
<td>Waste Disposal Cost per Successful Cycle</td>
<td>$0.01</td>
<td>$0.01</td>
</tr>
<tr>
<td>Total Fixed Cost per Successful Cycle</td>
<td>$2.37</td>
<td>$1.38</td>
</tr>
<tr>
<td>Total Operator Cost per Successful Cycle</td>
<td>$0.26</td>
<td>$0.15</td>
</tr>
<tr>
<td>Total Cost per Analysis*</td>
<td>$3.67</td>
<td>$2.88</td>
</tr>
</tbody>
</table>