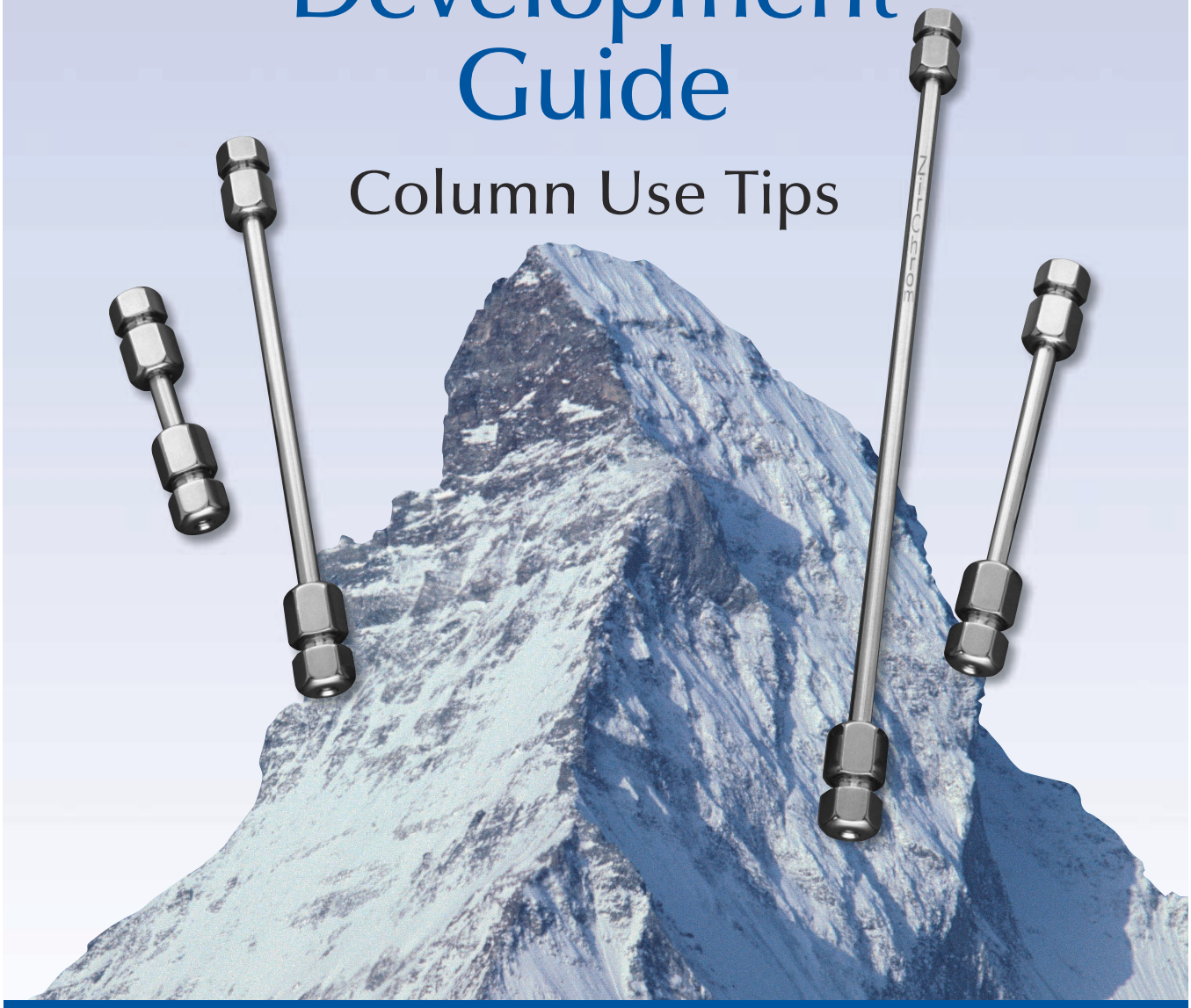
ZirChrom®

Zirconia-Based Columns for HPLC

Includes NEW Quick-Start Guide!

Reversed-Phase Method Development Guide

Column Use Tips



tel 1-866-STABLE-1 / fax 1-763-421-2319 / www.zirchrom.com

PART I / QUICK-START GUIDE

PART II

PART III

Please read this guide carefully before using your new column.

INTRODUCTION

Thank you for purchasing a ZirChrom® HPLC column. Due to its unique characteristics—namely that its packing material is zirconia-based rather than silica-based, we strongly recommend that you read this guide before using your column. Method development with zirconia-based columns involves different steps than those used with silica- or polymer-based columns. In this guide, we have outlined those steps and different chemistries. If at any time you have questions about your column or method development, please call our technical support line at 1-866-STABLE-1 (1-866-782-2531) to speak to one of our on-staff chemists, or email us at support@zirchrom.com.
We will be happy to help you.

PART I / QUICK-START GUIDE

Start-up Procedure

This section contains start-up guidelines to successfully install and equilibrate your ZirChrom zirconia-based column.

INSTALLING THE COLUMN

Connect the column with the direction of the flow arrow on the label pointing **toward** the detector. To maximize the life of your column, we recommend using a guard column. (Refer to your instrument's manual for information on the proper installation of columns.)

VALIDATING COLUMN PERFORMANCE

Upon receipt of your column, duplicate the results on the Column Evaluation document enclosed with this guide. You should be able to achieve a plate count consistent with the operating conditions listed for your column. Be sure to inject roughly the same amount of material indicated on the chromatogram. Repeat this test periodically to track column performance over time.

Slight variations may be obtained on two different HPLC systems due to system electronics, plumbing, operating environment, reagent quality, column condition and operator technique.

MOBILE PHASE PH

The pH range for the mobile phase is 1-14.

EQUILIBRATING THE COLUMN

1. Equilibrate your column when it is installed for the first time. Verify that your mobile phase is miscible in the shipping solvent, which is acetonitrile. Always re-equilibrate the column before using it again after it has been stored.
2. The column can be used with any common organic modifier (i.e., acetonitrile, methanol, tetrahydrofuran or isopropanol).

3. The column temperature may be set at any temperature **up to 200 °C (150 °C for PBD and PS)**. (Note that for the best results, operating at elevated temperatures often requires special hardware not supplied with most HPLC instruments.) Preferably, use the highest temperature consistent with the stability of your analyte. ZirChrom columns are stable **up to 200 °C (150 °C for PBD and PS**—refer to your column's Care and Use sheet for exact temperatures).
4. Purge the column with at least 10 column volumes of your mobile phase until you achieve a stable baseline.

USE AT HIGH TEMPERATURE OR LC/MS

We recommend running at least two 30-minute gradients of 10-90% acetonitrile/water to condition your column before LC/MS or high temperature use.

ZirChrom®-PBD/ZirChrom®-PS/ZirChrom®-CARB/ DiamondBond®-C18

General Use Tips

1. Upon receipt, we suggest you duplicate the results on the enclosed chromatogram. You should be able to achieve a plate count of at least 90,000/meter for toluene under the operating conditions given on the chromatogram (80,000/meter for ZirChrom®-CARB and DiamondBond®-C18 for ethylbenzene). Be sure to inject roughly the same amount of material as indicated in the chromatogram.
2. This column can be used with any common organic modifier (i.e., acetonitrile, methanol, tetrahydrofuran or isopropanol) but we find that acetonitrile typically gives better plate counts. ZirChrom®-PBD is less hydrophobic than common silica-based supports. For simple non-ionic compounds, we recommend that you use about 10-30% less organic modifier to obtain roughly the same retention as you would on a typical C8 or C18 silica-based column.
3. We very strongly advise that you use our columns at temperatures well above ambient. At a minimum, we urge you to set the column to 50 °C, but preferably to the highest temperature consistent with the stability of your analyte. We routinely use ZirChrom®-PBD columns at 75 °C and find them to be stable at 150 °C. We also recommend that you set the flow rate to 3 ml/min at these above-ambient temperatures. This will substantially increase the speed of analysis. The back pressure of zirconia-based columns is remarkably low at room temperature and decreases substantially at 50 °C and higher.
4. When running basic compounds on any stationary phase a buffer must be used (see ZirChrom Buffer Wizard at www.zirchrom.com). We strongly advise the use of phosphate, hydroxide, bicarbonate/carbonate or carboxylic acid (acetate, citrate) buffers instead of amine buffers used with silica columns. Our first choice for cationic analytes is 10-25 mM pH 7.0 ammonium phosphate buffers. However, this column is stable from pH 1 to pH 14 and you can use any buffer you like in this pH range.

5. When carboxylated molecules are chromatographed, we strongly advise the use of a ammonium phosphate buffer or above pH 6.0 an appropriate buffer (See Figure 12 for Buffering Ranges) plus 5mM ammonium fluoride. A good buffer for carboxylates is 10-25 mM ammonium phosphate at pH below 3.0. However, excellent results can be obtained at very low pH (pH < 1, *do not use fluoride* below pH 4.0) and up to quite high pH (> 11).
6. We recommend the following precautions regarding day-to-day operation of the column:
   - Always check the solubility of the buffer being used when mixing with organic mobile phases using an LC pump.
   - Avoid fluoride buffers below pH 4.
   - Avoid injecting metal chelating compounds or proteins.

Caution: Do not use PEEK tubing at temperatures above 100 °C, or with tetrahydrofuran-rich mobile phases.

Special Considerations for Carbon Columns (ZirChrom®-CARB/ DiamondBond®-C18)

- Routinely flush column with a strong solvent (i.e. 100% tetrahydrofuran) to elute any potentially retained materials off the phase. If necessary iso-propanol or toluene may also be used to elute nonpolar contaminants.
- Try operating at elevated temperature for optimal performance. Peak shape is often improved above 60 °C.
- A small (1-5%) amount of a very strong solvent (i.e. tetrahydrofuran, octanol, octanitrile) often improves peak shape.

Column Maintenance

To maximize the life of this ultra-durable column, we recommend the following:

- ☑ **Always use a guard column.**
- ☑ Clean up samples before injection (either filtering to remove particulates or solid phase extraction techniques).
- ☑ Use HPLC grade solvents and filter all solutions before use.
- ☑ Minimize pressure surges.
- ☑ Use an in-line filter (0.5 micron) in front of the column to catch large particles.
- ☑ Routinely flush the column with a strong solvent to elute retained materials off of the phase.
- ☑ Flush all buffers and salts from the column before storage.

Do not use PEEK tubing at temperatures above 100 °C or with mobile phases rich in tetrahydrofuran (> 10%).

Please refer to your column's Care and Use sheet for specific instructions.

Cleaning/Regeneration Procedure

Carboxylic acids, fluoride and phosphate all adsorb strongly to zirconia-based columns. To fully remove these from the zirconia surface, **or to remove substances that may have fouled the column**, use the following three-step cleaning protocol (**IMPORTANT*—During these steps, you should remove your detector from the flow path to protect it from aggressive cleaning conditions*):

1. Flush column with a mixture of 20/80 acetonitrile/0.1 M sodium hydroxide or tetramethyl ammonium hydroxide for 50 column volumes at ambient temperature. Follow base wash with 10 column volumes of water at ambient temperature.
2. Flush column with a mixture of 20/80 acetonitrile/0.1 M nitric acid for 50 column volumes at ambient temperature. Follow acid wash with 10 column volumes of water at ambient temperature.
3. Flush column with 100% organic solvent for 20 column volumes at ambient temperature. For ZirChrom®-PBD and ZirChrom®-PS, methanol, acetonitrile, isopropanol, and tetrahydrofuran are all adequate solvents. For ZirChrom®-CARB and DiamondBond®-C18, the same organic solvents can be used, however, the solvent should contain **at least** 20% tetrahydrofuran (do not use PEEK tubing).

Storage Conditions for ZirChrom®-PBD/ZirChrom®-PS

Do not store your column in phosphate buffer.

Refer to your column's Care and Use sheet, but in general, ZirChrom zirconia-based columns should be flushed with 50/50 organic modifier/water for 30 column volumes prior to storage overnight. For long-term storage, flush the column with 5/95 organic modifier/0.1 M ammonium hydroxide for 10 column volumes, followed by 50/50 organic modifier/water.

Storage Conditions for ZirChrom®-CARB/DiamondBond®-C18

Do not store your column in phosphate buffer.

We strongly suggest flushing the column with 50/50 organic modifier-water for 30 column volumes, followed by flushing with 100% tetrahydrofuran (do not use PEEK tubing) for 10 column volumes and re-equilibration with 50/50 organic modifier-water prior to storage overnight. For long-term storage, we recommend flushing the column with 5/95 organic modifier/0.1 M ammonium hydroxide first, followed by 50/50 organic modifier-water and 100% tetrahydrofuran (do not use PEEK tubing) for 10 column volumes each, followed by re-equilibration with 50/50 organic modifier-water.

PART II

High-Performance Reversed-Phase Chromatography

Reversed-phase separations employ a polar eluent and a nonpolar (hydrophobic) stationary phase. The hydrophobic layer (or phase) is bonded onto a rigid support that can withstand the high pressures commonly used in HPLC. Until recently, about 80% of all HPLC methods specified silica-based stationary phases. However, silica does have some limitations:

- Silica is readily soluble in aqueous solutions at pHs higher than 7.5.
- Bonded silicas may briefly resist chemical attack at higher pH ranges—but silica exposed through the coating is attacked and progressively broken down.
- Many silica-based bonding chemistries are not stable at high temperatures (> 40 °C).
- Hydrolysis of the siloxane bond at low pH, resulting in stationary phase "bleed", leads to retention time irreproducibility and high background noise for LC/MS applications.

Polymeric column packings frequently exhibit shrinking or swelling as mobile phase modifier composition changes. They are inherently less efficient than silica and zirconia based particles for most separations.

Zirconia-based columns are revolutionary HPLC phases. Zirconia particles are mechanically stable, and have a porous structure similar to that of silica. However, zirconia's main advantage over silica is that it is **very stable in a wide range of eluent pH**—and more importantly, at elevated temperatures—**up to 200 °C (150 °C for PBD and PS). This makes ZirChrom columns ideal for rugged, robust reversed-phase HPLC separations.**

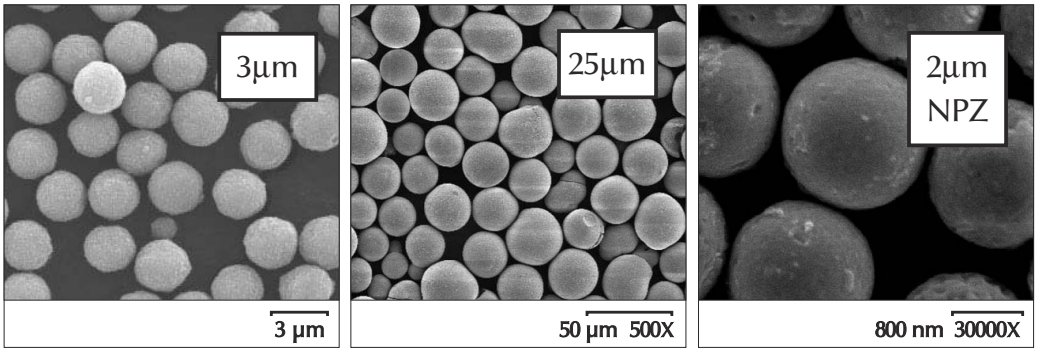


Figure 1. Porous and Nonporous Zirconia Particles

Why is Temperature Important?

For any HPLC system, raising the column temperature does the following:

- **Increases column efficiency**—for sharper peaks and better separation.
- **Reduces mobile phase viscosity**—so you can increase eluent flow rate with low back pressure, and achieve a much faster separation. ZirChrom's highly efficient, ultra-stable columns let you develop methods that run up to **5 or more times faster** with equal or better resolution at temperatures that degrade the typical silica-based column. You get faster analysis, better resolution and longer column life for a robust method.

ZirChrom columns differ from silica-based columns in the following areas:

- **Greater flexibility/for your method development**—Work at both high and low pH (from 1-14).
- **Longer column life**—Zirconia won't degrade in extreme conditions or at high temperatures.
- **Faster separations**—Higher temperatures enable faster analysis.
- **Tunable selectivity**—Zirconia surface chemistry creates mixed-mode possibilities (and affects buffer selection for ionic analytes).

NOW AVAILABLE - Short ZirChrom Columns for Ultra Fast High Temperature Liquid Chromatography (UFHTLC).

ZirChrom now offers short (2 cm) all-stainless steel columns for ultra-fast high temperature reversed-phase liquid chromatography. These columns are only sold in sets of two (mixed sets are available). Contact ZirChrom technical support at 1-866-STABLE-1 or support@zirchrom.com for more details.

General Method Development Guidelines for Zirconia-based Columns

Zirconia-based phases are much closer to silica-based phases in behavior than are polymeric and carbon-based phases, but yet significantly differ from silica in several important ways.

The method development differences between silica-based and zirconia-based reverse-phase method development can be illustrated as follows:

Operational *Similarities* for Silica and Zirconia RP Media

- k′ increases with molecular hydrophobicity (-CH_3, -CH_{2-}, phenyl, etc.).
- Similar elution sequence of non-electrolytes.
- k′ decreases 2-fold per 10% increase in volume % organic modifier.
- Log k′ versus % organic modifier is linear.
- k′ decreases as temperature is increased (3-fold/50 °C).
- Solvent strength: tetrahydrofuran > acetonitrile > methanol.
- ZirChrom®-PBD and ZirChrom®-PS reversed-phases are very efficient (N > 90,000 plates/meter) hydrophobic, inorganic-based, stationary phases.

Operational *Differences* in Silica and Zirconia RP Media

- Cations are typically more retained and *sometimes much more retained* on zirconia in buffered (PO_4) eluents than on silica phases.
- Elution sequence of anions and cations can be very different on zirconia and silica at neutral pH.
- Must use Lewis base eluent for carboxylated analytes. Strongly advise use of 5 mM or more phosphate (or tetramethyl ammonium hydroxide) for all electrolytes.
- Lewis base modifier at mid-range pH creates mixed-mode reverse-phase/ion-exchange possibilities.
- Use 10-30% less organic modifier (depending on phase and analyte) .
- k′ of bases DECREASES at pH above pKa!

note: k′ = retention factor

1. Phase Selection

ZirChrom offers the following types of reverse-phase columns:

- **ZirChrom®-PBD**
  ZirChrom®-PBD (polybutadiene) is a general-purpose column intended for basic compounds, similar to ODS for non-electrolytes. The zirconia is coated with a thin polymer layer. Phase selectivity is most similar to conventional ODS silica bonded phases.
- **ZirChrom®-PS**
  ZirChrom®-PS (polystyrene), an alternative to ODS selectivity, is ideal for highly aqueous mobile phases. It also features a thin polymer coating.
- **ZirChrom®-CARB**
  ZirChrom®-CARB is made by coating zirconia with a very thin layer of elemental carbon. This column is particularly ideal for diastereomers/geometric isomers. The chemistry of carbon-based reversed-phases are very different from typical RPLC phases. When a different reversed-phase selectivity is required, ZirChrom®-CARB offers a high efficiency alternative to most silica-based stationary phases.
- **DiamondBond®-C18**
  DiamondBond®-C18 uses a proprietary covalent bonding technology to graft C18 groups to carbon's surface, giving it outstanding stability for LC/MS applications. DiamondBond®-C18 has selectivity that is intermediate between ODS silica and unbonded carbon supports.

Please keep in mind throughout the method development process that ZirChrom®-PS and ZirChrom®-PBD phases have different selectivities, especially for highly aromatic compounds.

ZirChrom®-PS is significantly less retentive than ZirChrom®-PBD, just as Phenyl-Silica is less retentive than C18-silica.

The selectivity and retentivity of ZirChrom®-PBD is more like a C8/C18-silica phase, where as that of the ZirChrom®-PS is more like a Phenyl-silica phase.

ZirChrom®-CARB will have very different selectivity and more retention, especially for polar compounds and highly aromatic compounds.

DiamondBond®-C18 selectivity and retentivity will be intermediate between ZirChrom®-PBD and ZirChrom®-CARB.

Reversed-Phase Column Selection Guide

Current Problem/Concern	*Column*	*Suggested Conditions*
Improved Selectivity		
Need improved selectivity for nonelectrolytes, isomers, diastereomers. Currently using carbon, cyano, phenyl or fluoro phases.	DiamondBond®-C18, ZirChrom®-CARB	Use an acetonitrile and/or tetrahydrofuran eluent. Set column temperature ≥ 50 °C. Add octylamine to improve peak shape.
Need improved selectivity for bases.	ZirChrom®-MS, ZirChrom®-PBD	Use buffer of your choice in a pH range of 1 - 10. If required, 5 - 25 mM PO_4 may improve peak shape. If using ZirChrom®-PBD, increase pH above pKa (stable up to pH 14).
Need improved selectivity for acids.	DiamondBond®-C18, ZirChrom®-EZ, ZirChrom®-MS	Use 5 - 25 mM phosphate, pH 7.0. If not effective, drop pH to 2 or lower.
Change Retention		
Need more retention for very polar (hydrophilic) nonelectrolytes. Currently using nearly 100% water eluent or polar embedded phase.	DiamondBond®-C18, ZirChrom®-CARB	Can use in high water mobile phase.
Need more retention for very polar bases. Currently using nearly 100%water eluent or polar embedded phase or *sulfonic acid paired ion reagent.*	ZirChrom®-MS	Use buffer of your choice in a pH range of 1 - 10. If required, 5 - 25 mM PO_4 may improve peak shape. High water mobile phases are no problem.
Need more retention for very polar acids. Currently using nearly 100%water eluent or polar embedded phase or *quaternary amine paired ion reagent.*	DiamondBond®-C18, ZirChrom®-EZ, ZirChrom®-MS	Use buffer of your choice in a pH range of 1 - 10. Try low pH first. 5 - 25 mM PO_4 may improve peak shape.
Need less retention with any solute type.	ZirChrom®-PS	Least hydrophobic phase. Can be used with 100% water eluent.

Current Problem/Concern	*Column*	*Suggested Conditions*
Improve Efficiency/Productivity		
Inadequate stability and selectivity. Having trouble with silica-based phases, changed to alumina or polymer column and problems were *still not* sufficiently resolved.	All ZirChrom® Reversed-Phase (RP) Columns	Zirconia phases exhibit excellent pH and temperature stability. ZirChrom® RP columns give higher efficiency and better peak shape than alumina or polymer columns.
Poor column reproducibility. Experiencing retention changes at extreme pH, at elevated temperature or when using phosphate or carbonate buffer.	All ZirChrom® RP Columns	Zirconia phases are very reproducible from batch-to-batch, column-to-column, and run-to-run. Every column is QCed.
Separations taking too long.	All ZirChrom® RP Columns	Increase temperature up to max. operating range for LC and/or analyte. Increase flow rate. Easily improves speed 2-3 fold.
Column overloaded too easily with basic solutes.	ZirChrom®-MS, ZirChrom®-PBD	The mixed-mode (reversed-phase / cation exchange) retention mechanism enables enhanced column loadability.
Improve Detection Sensitivity		
Need to go to shorter wavelength to enhance sensitivity in UV.	ZirChrom®-PS	Use a high water or pure water eluent and go deep into UV.
Need to decrease bleed in LC/MS.	All ZirChrom® RP Columns	All ZirChrom® columns are extremely low bleed. The ZirChrom®-MS column was designed for LC/MS.

For help, call our technical support group at 1-866-STABLE-1 or support@zirchrom.com.

2. Mobile Phase Composition

ZirChrom columns can be used with many common organic modifiers, including acetonitrile, methanol, tetrahydrofuran and isopropanol. For mobile phase optimization, follow these steps:

- Define optimum solvent strength so that 1< k' < 20.
- Do stepwise isocratic study in 20% steps starting at 100% organic.
- Do gradient determination of % organic.
- Define the best type of modifier: acetonitrile, methanol or tetrahydrofuran.
- Keep in mind that the order of eluent strength is tetrahydrofuran > acetonitrile > methanol.

Acetonitrile is usually the eluent of choice due to its superior UV absorbance and low viscosity.

Special Considerations when Choosing an Organic Modifier for Carbon Columns (ZirChrom®-CARB / DiamondBond®-C18)

Special consideration must be taken when choosing an organic modifier additive for use with carbon-clad reversed phase materials (i.e. ZirChrom-CARB and DiamondBond-C18), particularly for separations involving strongly retained analytes such as highly conjugated aromatic or planar flat molecules. Method development issues such as peak tailing, low column efficiency, and irreversible adsorption can often be overcome with the inclusion of a relatively small amount (1 -10%) of a very strong solvent additive in the organic portion of the mobile phase. Widely employed "strong" HPLC solvents such as tetrahydrofuran (THF) are extremely effective mobile phase additives for carbon phases, however, they also have some limitations including: high absorbance at low UV wavelengths, incompatibility with PEEK tubing, peroxide formation, and general toxicity. In an effort to discover alternatives, we systematically studied the use of small amounts (0.1-10% (v/v)) of aliphatic alcohols, nitriles, and amines of increasing chain length as mobile phase additives to improve the chromatographic performance of strongly retained compounds (in this case steroidal compounds) on our carbon phases. To study the effects of these additives, a base mobile phase of 70/30 ACN/water was used with a 100 mm x 4.6 mm i.d. ZirChrom-CARB column at 80 °C, and the retention, peak

shape, and efficiency of three anabolic steroids were measured as a function of the amount and type of additive used. As an example, to test the effect of 1% 1-octanol, a fraction of the ACN was replaced with the 1% 1-octanol such that the total volume fraction of organic modifier remained the same (i.e. 69/1/30 ACN/1-octanol/water).

Figure 2 shows the effect of adding only 1% of several different 8-carbon additives (octanol, octanenitrile, octanedinitrile, and octylamine) on the retention of the three steroids, in comparison to the 70/30 ACN/water mobile phase alone, and the use of 10% THF (i.e. 60/10/30 ACN/THF/water). These data show that even small amounts of these 8-carbon additives have a profound effect on the retention of highly retained compounds.

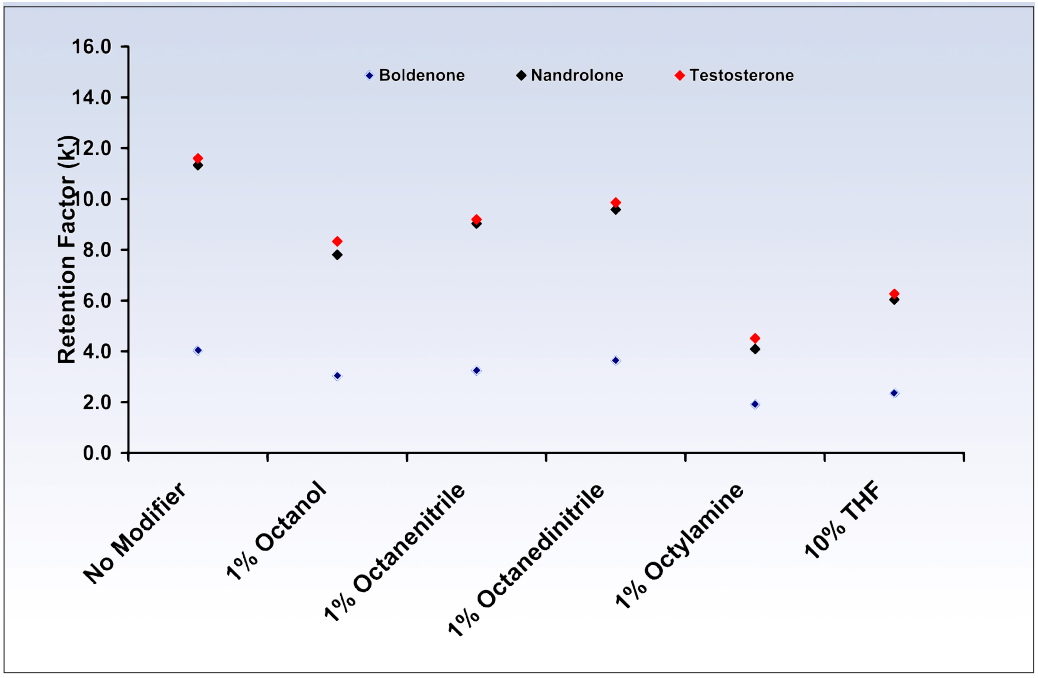


Figure 2

Figures 3-5 show that as the length of the alcohol, nitrile, or amine modifier that is used is increased, the retention of the steroids decreases. Also shown in Figures 3-5 is that a broad range of solvent strength is available through the use of relatively innocuous solvents such as octanol, significantly reducing the need for more unfriendly solvents such as THF.

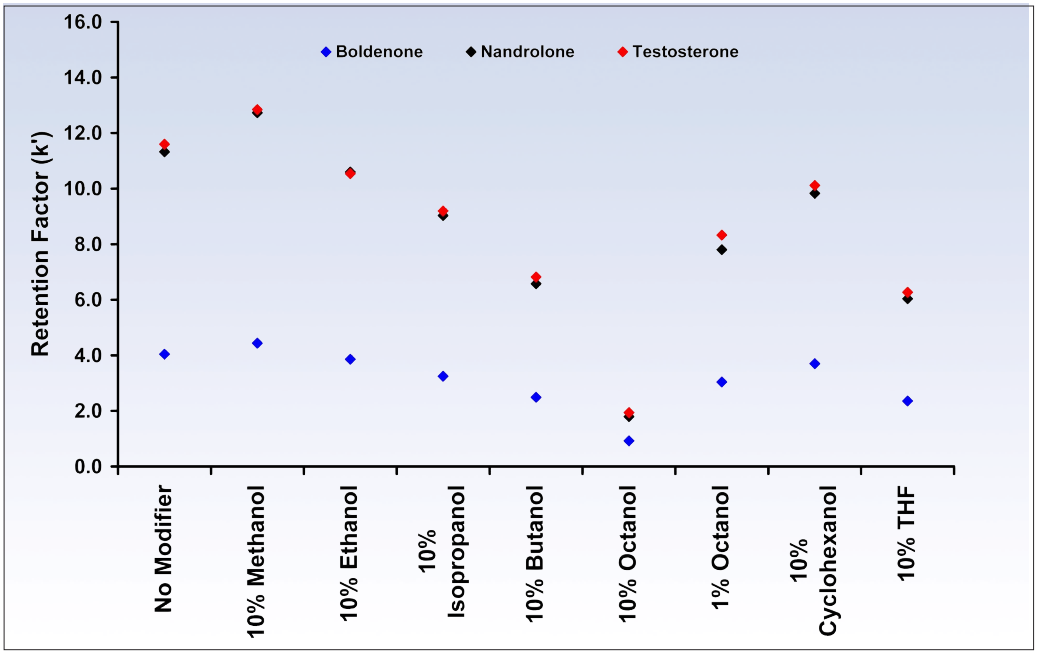


Figure 3

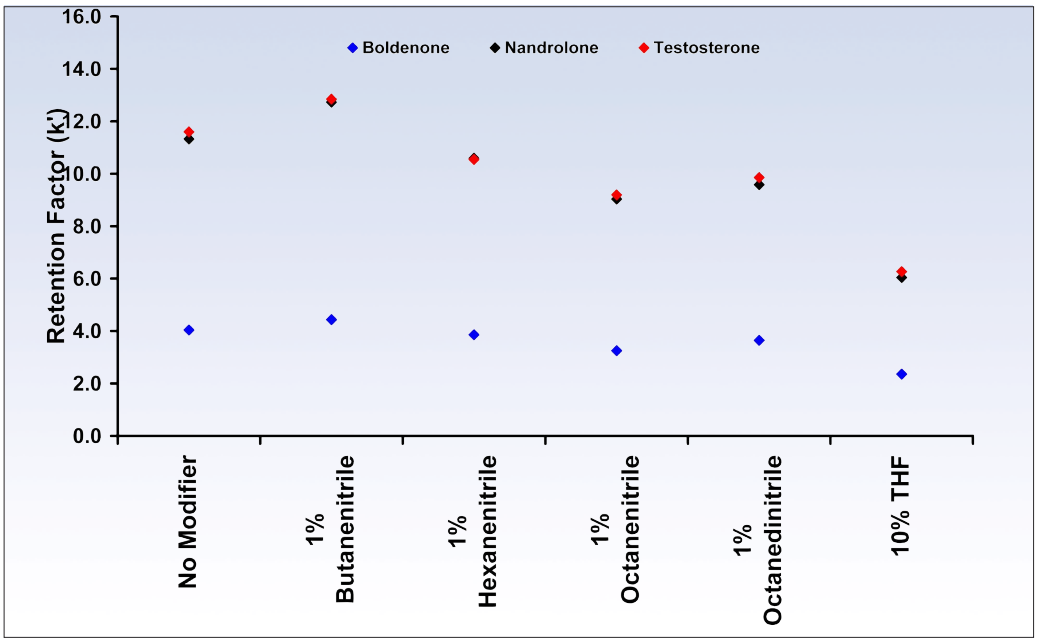


Figure 4

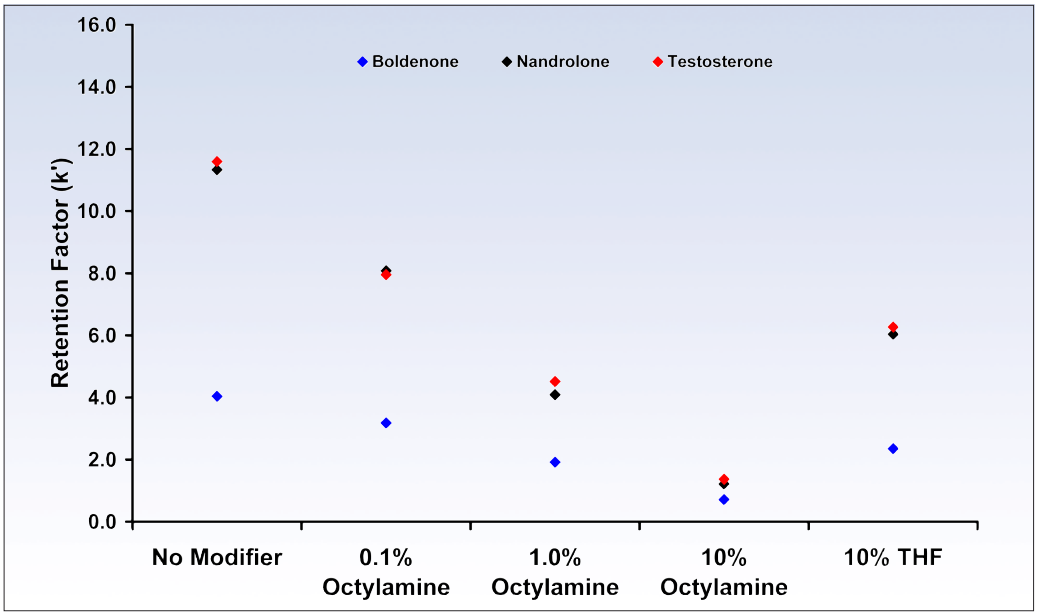


Figure 5

Compared to the large dependence of the retention on the type of additive used, the effects on the peak shape and efficiency were much less dramatic, however the peak shape and efficiency did improve with decreasing retention. When working with UV detection, it is important to be mindful of the background absorbance of the mobile phase, particularly when working with low UV wavelengths. Figures 6 and 7 show UV spectra in the wavelength range of 200-300 nm for mixtures of all of the additives compared in this work, where each mixture consisted of 65% ACN, 30% water, and 5% (v/v) of the indicated additive. Several of the additives were not available as

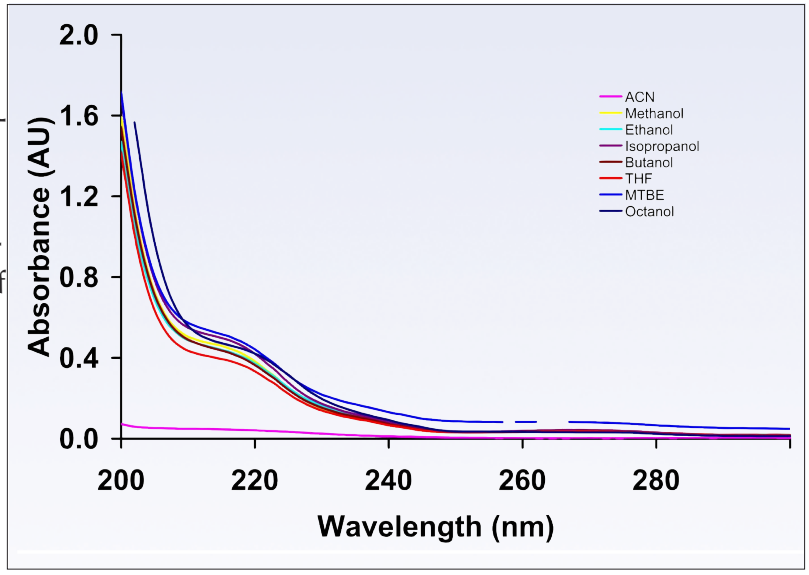


Figure 6

HPLC grade solvents which is reflected in some of the spectra shown in Figure 7.

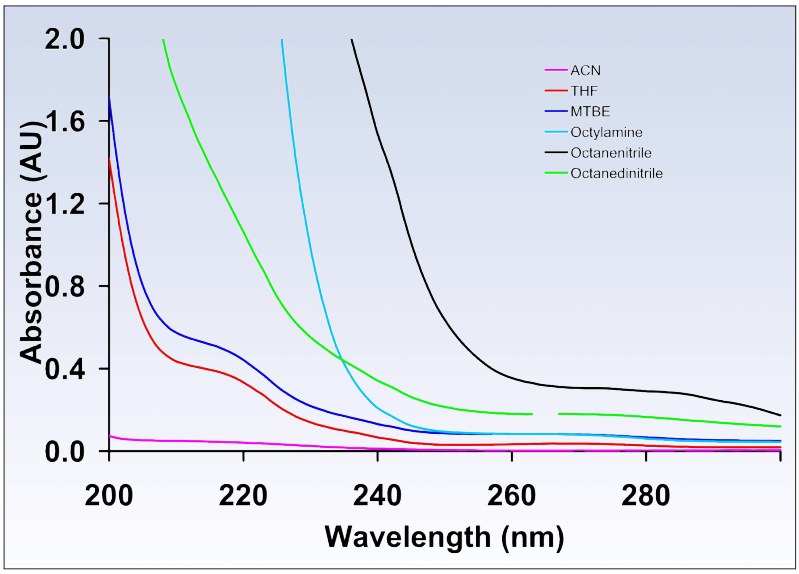


Figure 7

The similarity of the spectra for all of the mixtures containing HPLC grade additives shown in Figure 6 suggests that the strongest solvent additive available should be used so that the least amount of additive will be required to achieve the desired retention, thereby allowing the smallest background absorbance due to the mobile phase. Of course if one is working with a mass spectrometer as a detector, the issue of additive purity is not one of UV absorbance, but rather mass spectral interference.

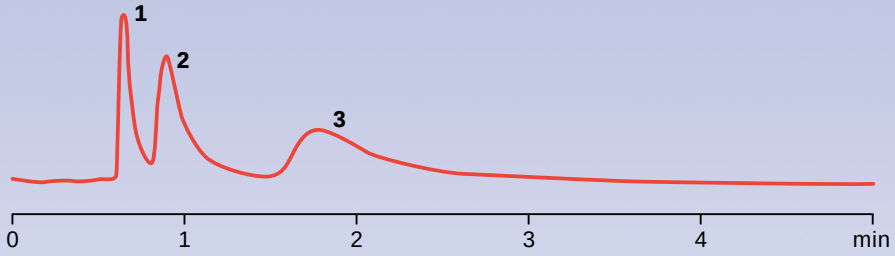
3. Buffer Composition

Obtaining satisfactory peak shape for your analytes requires the proper choice of a Lewis base buffer. Buffers improve peak shape of acidic compounds and can help modify the band spacing (or selectivity) and retention of acidic or basic compounds. Furthermore, if you are separating carboxylic analytes, you must use a Lewis base eluent. We strongly recommend 5 - 25 mM phosphate (or tetramethyl ammonium hydroxide for LC/MS compatibility) for electrolytes (see Figure 8).

A Difficult Separation: Alkyl Benzoic Acids Solved on ZirChrom®-PBD

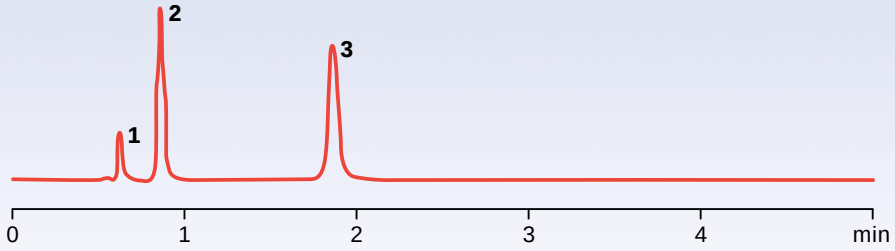
SOLUTES
1 - Benzoic acid
2 - Ethylbenzoic acid
3 - Butylbenzoic acid

Problem?



LC CONDITIONS
Mobile phase: 30/70 ACN/25mM Ammonium acetate, 5mM Ammonium fluoride, pH 4.0
Flow rate: 1.0 ml/min Temperature: 35 °C 50 mm x 4.6 mm
Injection volume: 1 ml Detection: 254 nm

…No Problem—Just Add Phosphate Buffer!



LC CONDITIONS
Mobile phase: 30/70 ACN/25mM Phosphoric acid, pH 2.2
Flow rate: 1.0 ml/min Temperature: 35 °C 50 mm x 4.6 mm
Injection volume: 1 ml Detection: 254 nm

Figure 8

4. pH Selection

Because ZirChrom columns are ultra-stable at low or high pH, they are ideal for the following:

- Ionizable compounds
- Improving peak shape of acidic or basic compounds
- Changing selectivity or retention of acidic or basic compounds

For more information on how pH can affect method development on basic analytes (see page 25).

5. Temperature

Although ZirChrom columns are excellent at room or slightly elevated temperatures, to speed any analysis, raise the temperature! ZirChrom columns reach cruising speed at 80 °C and withstand temperatures **up to 200 °C (150 °C for PBD and PS)**—without degrading.

Set your flow rate to 3 ml/min (for a 4.6 mm i.d. column) at these super-ambient temperatures. **This will substantially increase the speed of analysis.** The back pressure of zirconia-based columns is remarkably low at room temperature and decreases substantially at 50 °C and higher.

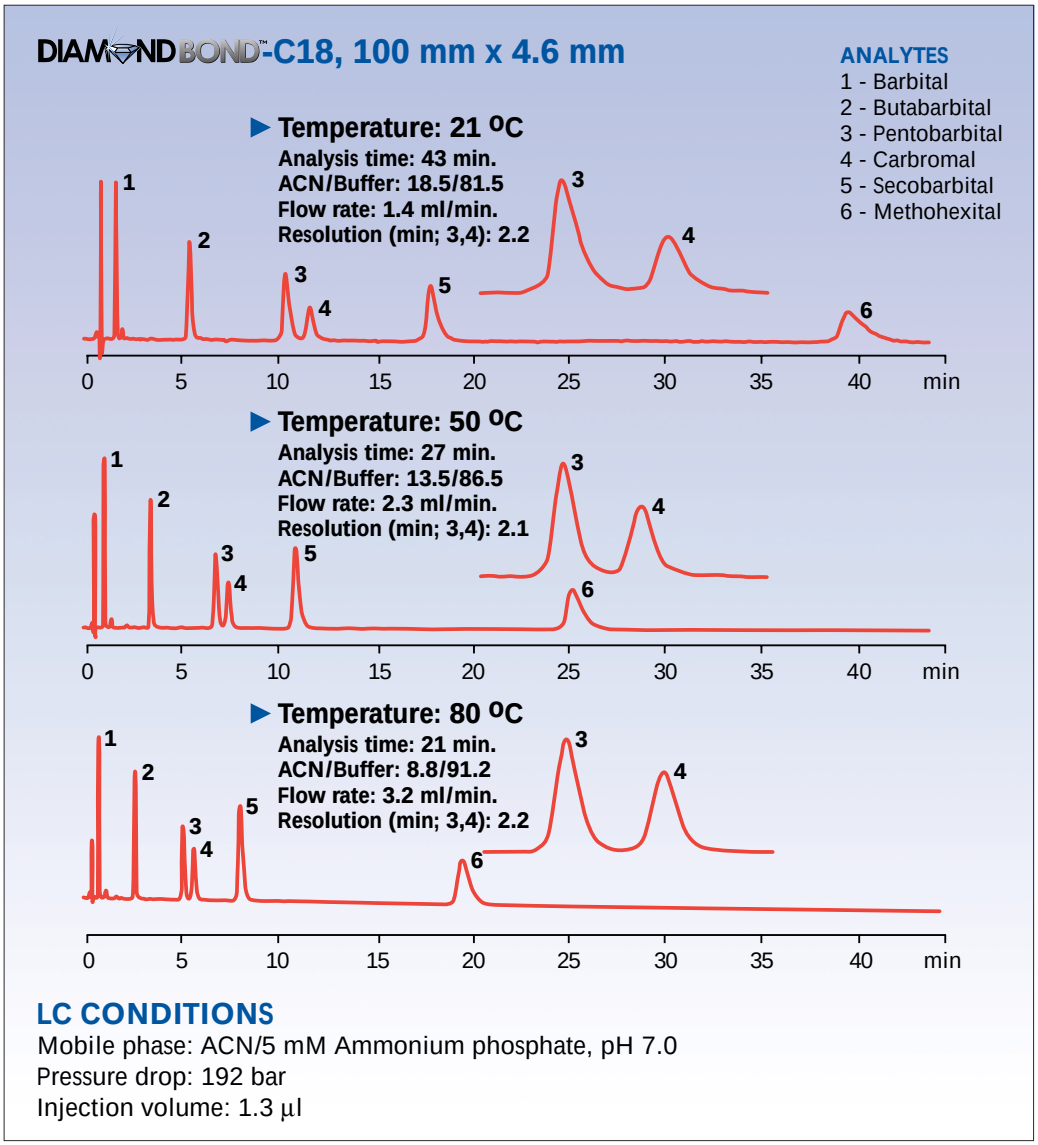


Figure 9

Analyte-Specific Guidelines

Method Development for Nonelectrolytes

For any nonelectrolyte analyte at neutral pH (e.g. ester, ether, alkane, alkene, alkyne, amides, alcohols, etc.) which is not a carboxylic acid or amine you should choose the eluent just as you would for a Type A or B silica-based phase. However, *we suggest that you use about 10-30% less organic modifier* than you would with a silica-based phase. If you would normally start method development with 80% acetonitrile on a typical ODS phase we suggest you use 60-70% acetonitrile on our ZirChrom®-PBD, ZirChrom®-CARB and DiamondBond®-C18 phases and indeed even a lower percentage of organic (say 50-60%) on our ZirChrom®-PS phase. This will put the k′ in the same range as you would observe on a silica-based phase. If in normal method development you would change the strong solvent by 5% to either increase or decrease retention we suggest you make the same approximate change in composition using zirconia-based phases.

The chromatographic selectivity of ZirChrom®-PBD and ZirChrom®-PS zirconia columns will be similar (but not exactly the same) as ODS and phenyl type bonded phases on silica respectively (J. Zhao and P.W. Carr, "Synthesis and Evaluation of an Aromatic Polymer-Coated Zirconia for Reversed Phase Liquid Chromatography," *Anal. Chem.*, 71, 5217-24 (1999)). Substituents which cause an increase in retention on silica phases cause an increase in retention of zirconia, and uncharged substituents that decrease retention (e.g. OH, NO_2, CHO, etc.) on silica cause a decrease in retention on zirconia phases.

ZirChrom®-CARB has RPLC-like properties, but has radically different selectivity than ODS or ZirChrom®-PBD, which is great for steroids and geometric isomers. Much more hydrophobic than ODS, it is great for polar analytes. DiamondBond®-C18's retention properties are intermediate between ZirChrom®-CARB and ODS silica columns. DiamondBond®-C18 and ZirChrom®-CARB are very stable, and can be used at low and high pH, as well as at high temperatures. Lewis acid-base properties are still important on all zirconia-based phases. Peak shapes are best with tetrahydrofuran (10%) and/or elevated temperature.

Method Development for Ionic (Cations and Anions) Analytes

In the method development process for ionic analytes on zirconia-based phases, one must understand that zirconia's surface contributes significantly to the total retention of the analyte through electrostatic interactions between the analyte and the surface. These electrostatic interactions are in addition to the reversed-phase interactions that take place in RPLC between the analyte and the hydrophobic stationary phase. The extent to which the zirconia surface contributes to the retention of the analyte is determined by three principal factors:

1. The *pH* of the mobile phase affects both zirconia's surface charge and the charge state of the analyte.
2. The *type of buffer* used in the eluent.
3. The pK_a of the analyte.

The effects of the pH of the mobile phase on the surface charge on zirconia and silica are best illustrated in Figure 10a and 10c. The zirconia surface is populated by several species of Lewis, and Brönsted acid sites, as well as Brönsted base sites, which have charge states that are highly dependent on the mobile phase pH.

Understanding the interactions previously outlined is critical to successful method development for ionic analytes. Please note that the behavior of zirconia indicated in Figure 10 and the previous discussion is *dramatically different* from the behavior observed with traditional silica-based stationary phases. The zirconia surface inherently contains more charged sites than does silica due to ionization of surface species and adsorption of hard Lewis bases (See Figure 10b). Consequently, electrostatic interactions between the analyte and the zirconia surface are potentially much more significant than in the analogous case encountered with silica-based phases. The information presented so far has been very general, a more thorough consideration of conditions for both acidic and basic analytes is given below.

The retention (k′) and selectivity (α , band spacing) of zirconia-based phases for positively charged species is frequently, and indeed almost always, very different than for any silica-based phase and at this point it is definitely best to think of zirconia as the "*un-silica*".

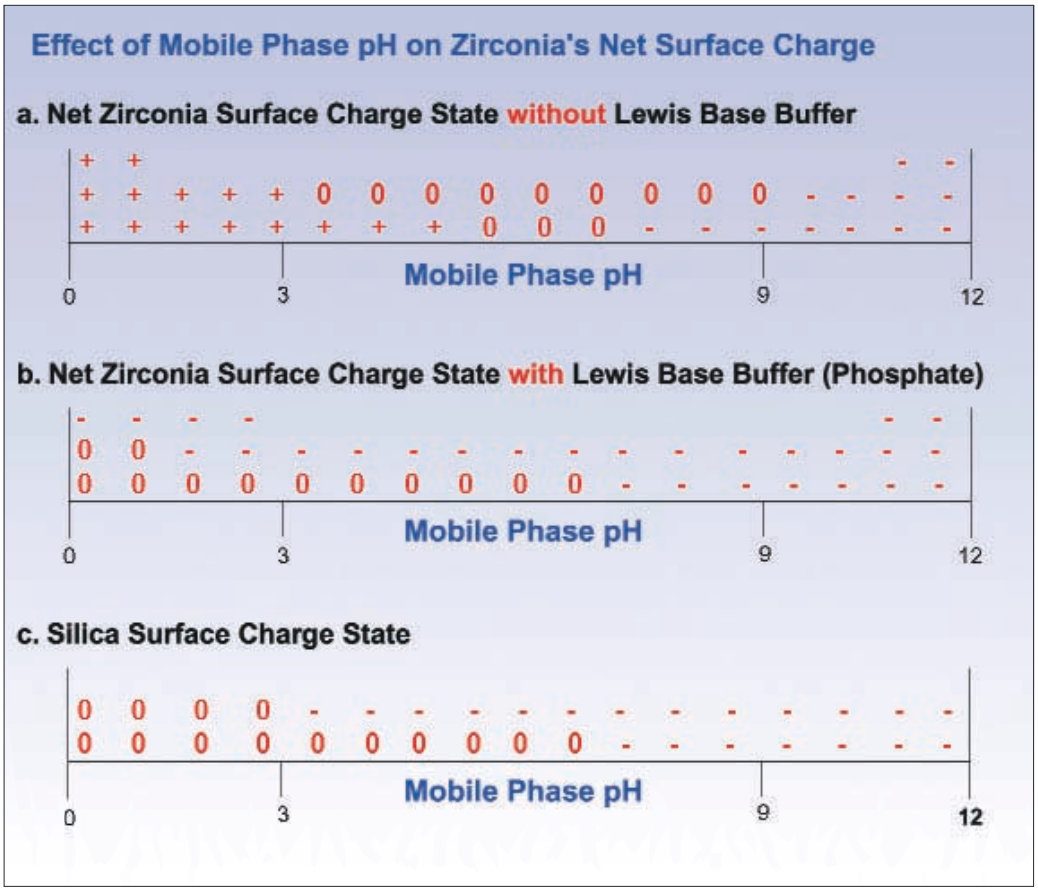


Figure 10

Method Development for Carboxylic Acid (Anionic) Analytes

Without question carboxylic acid containing analytes are the most challenging species in terms of method development with zirconia-based phases. It is absolutely essential that the eluent contain a fairly high concentration (> 20 mM) of a stronger, harder Lewis base (Figure 11) to obtain acceptable plate counts and peak shapes. Fortunately for low molecular weight monocarboxylate analytes, this problem has been solved. If the sample is a simple carboxylic acid as exemplified by benzoic acid (as are ibuprofen, naproxen, etc.), it is easy to obtain good peak shape. **We strongly suggest the use of dilute phosphoric acid (pH 2-2.5, 20-50 mM), or fluoride plus a suitable buffer (pH > 4, 20-50 mM) as the aqueous component of the mobile phase, a column temperature of 40 °C or higher, and acetonitrile as the organic component of the eluent.**

Because all proteins have multiple carboxylate groups they act as chelating agents and they adsorb irreversibly to reversed phase zirconia supports in aqueous-organic media. *We strongly advise against doing RPLC of proteins with zirconia-based reversed-phases*, but rather recommend ion-exchange chromatography of proteins using any of our four zirconia-based ion-exchange materials. We must point out that polycarboxylate compounds such as EDTA and other compounds that are able to chelate metals by having two or more functional groups (COOH, aromatic OH) positioned in the analyte so as to be able to form 5 or 6 membered rings and thus chelate surface Zr(IV) sites can still give problems on zirconia-based columns.

Interaction Strength of Several Commonly-Used Lewis Base Additives

Interaction Strength	Lewis Base
Strongest ↓ Weakest	Hydroxide
	Phosphate
	Fluoride
	Citrate
	Sulfate
	Acetate
	Formate
	Nitrate
	Chloride

Figure 11

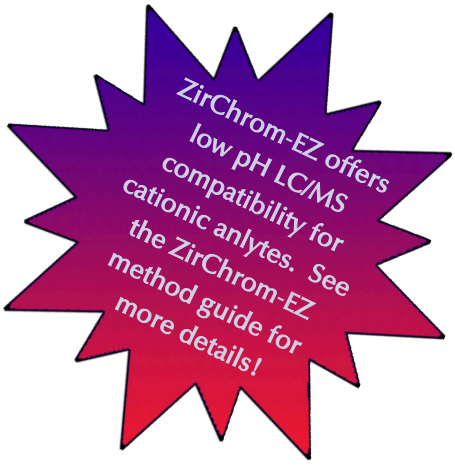
Method Development for Basic (Cationic) Analytes

pH < 2

In this pH range our favorite buffers are strong acids such as 25mM phosphoric acid (excellent UV transparency and relatively noncorrosive). *Don't be surprised if the elution orders and band sequences are very different at low pH on zirconia-based phases relative to those on silica-based phases.* You must keep in mind that in the phosphoric acid the surface of zirconia has a net negative charge, whereas silica is uncharged at pH < 2. These very acidic conditions are very good for chromatography of amines, as almost all amines will be positively charged at these very low pHs, allowing for unique mixed mode (ion-exchange and reversed-phase) selectivity.

The postive counterion concentration when using a phosphoric acid mobile phase is quite low and may need to be increased in order to obtain satisfactory peak shape for amines in this pH range. However the best peak shapes for amines are often obtained by using mobile phase pH ranges at least 1.5 pH units above the pK of the positively charged species (see page 28).

We strongly recommend that you consult with your instrument manufacturer to determine the compatibility of your instrument with these very acidic conditions. The use of fluoride buffers below pH 4 is not advised as HF will cause damage to any HPLC column and your HPLC instrument. Also keep in mind that there are significant differences in retentivity and selectivity of our zirconia based reversed-phases so if the selectivity is not adequate on one phase it may be adequate on another. All phases can be used at very high water content (> 95%).



2 < pH < 6

It is frequently the case that the retention of more hydrophilic (polar) bases is very low in strongly acidic media on silica and zirconia-based phases. If analyte retention is too low (k′ < 1) in highly acidic media (pH < 2) and you still want to work at low pH we suggest that you use a dilute (2-5 mM) but highly acidic phosphoric acid/phosphate buffer at pH 1.8 to 3.0, where phosphate has a high buffer capacity. Phosphate and citrate buffers and formate to a lesser extent are comprised of very strong hard Lewis bases, which chemically adsorb on the surface of zirconia and impart a negative charge. This negative charge does three things:

1. It increases retention (k′) of positively charged bases.
2. It substantially increases their loading capacity.
3. It has a tremendous effect on band spacing (selectivity) and elution order.

The first task is always to find conditions where retention is adequate (k′ > 0.5-1 for the least retained analytes) and only then should one attempt to optimize the selectivity. *Keep in mind that on zirconia-based phases, even at constant pH, retention will vary with the concentration of the buffer and any added "inert" salts such as ammonium chloride.*

If the separation is still not adequate due to low retention or inadequate band spacing then we suggest exploring the use of other buffer types and higher pHs. Citrate buffers can be used from pH 2.1-6.4 and fluoride buffers are very useful on zirconia since they are hard Lewis bases and adsorb on the surface. A more complete listing of buffers to use at a given pH is shown in Figure 12. We also encourage you to try out our fully automated Buffer Wizard on our website at www.zirchrom.com.

If you really need to increase retention we advise the use of a dilute (2-5 mM) ammonium phosphate or ammonium fluoride buffer at pH 4-8. Our number one choice for a starting buffer in this case is 2-5 mM ammonium fluoride, plus 10-50 mM of either formic acid/formate or acetic acid/acetate to buffer the pH. Because phosphate and fluoride adsorb very strongly on zirconia's surface at neutral pH, and because all aliphatic amines are positively charged at pH < 8 you will generally see stronger retention of bases on zirconia phases than on silica phases in neutral phosphate and fluoride buffers. Thus use of neutral phosphate buffers

will cause *a significant increase* in retention of bases on zirconia phases relative to use of an acidic eluent. If you then need to adjust selectivity we advise addition of an inert salt such as ammonium chloride or to adjust the electrostatic (charge-charge, or ion-exchange) interaction contribution to retention and selectivity.

You can and should also adjust the volume fraction of organic modifier to adjust retention and type of organic to alter chromatographic selectivity. However, our experience indicates that the salt concentration and pH are highly influential variables in terms of adjusting selectivity when dealing with charged analytes. Keep in mind that aromatic amines (aniline and pyridine functionalities) are much less basic than aliphatic amines and they tend to be deprotonated at pH > 5. Because the anionic form of the buffer will adsorb on zirconia, *the type of buffer (acetate, citrate, phosphate, etc.)* you use to achieve the desired pH will have a big effect on elution order, band spacing, retention and peak shape. This can be used very advantageously to adjust selectivity.

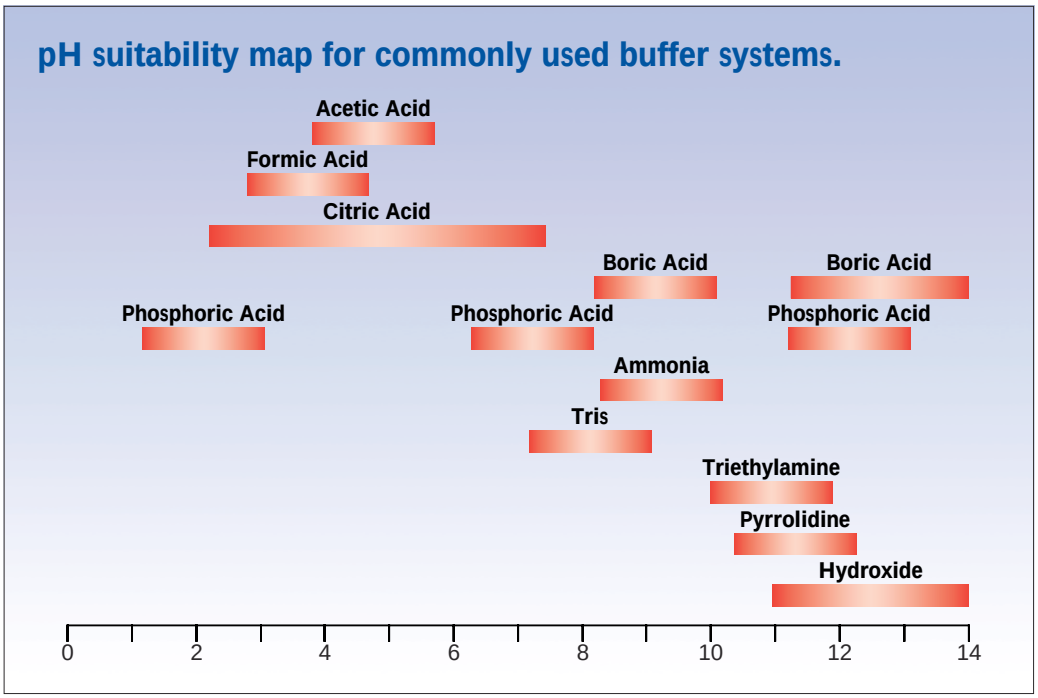
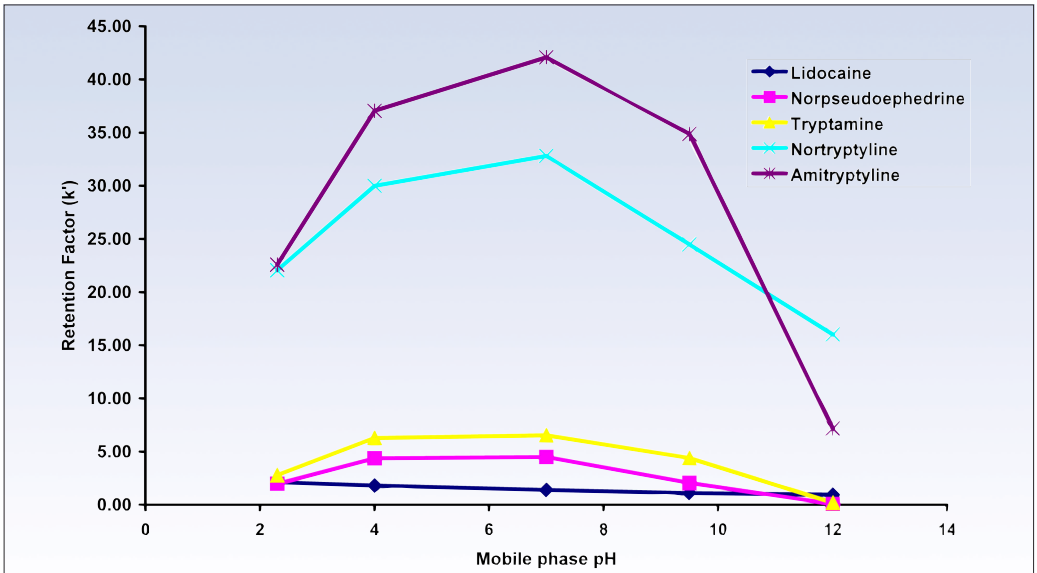


Figure 12. The lighter region of the red buffer range indicates the region of highest buffering capacity. Please do not use buffers outside the indicated ranges

pH > 6

If selectivity is still not adequate at weakly acidic or neutral pH, it is possible and advisable to go to still higher pH without causing any phase decomposition of your zirconia-based column. As pH is increased from 6 to 12 you can use any buffer you like that provides adequate buffer capacity. You are not restricted to the use of organic buffers (e.g. Tris, pyrrolidones, or triethylamine) or low operating temperatures as you are on silica-based phases. You can use phosphate, fluoride, borate, or carbonate buffers. They will not cause phase decomposition. This is a definite advantage of *Type-Z* zirconia-based phases over *Type-A* and *Type-B* silica-based materials. Additionally, 0.05 M tetramethyl ammonium hydroxide has proven to be an extremely useful buffer for cationic molecules at high pH and is compatible with LC/MS detection (however MS ionization and sensitivity of amines will be low at high pH).

It is extremely important to understand that as the pH is raised above the pKa of the basic analyte, retention will decrease relative to retention in a phosphate or other hard Lewis base type buffer at neutral pH (4-9). This is because the electrostatic interaction is "turned-off" at high pH. Solutes elute in order of their hydrophobicity. Retention of a set of basic drugs is nearly identical at pH 12 in both phosphate and ammonium hydroxide buffers of very different ionic strength proving that there is no electrostatic contribution to retention and selectivity (See Figure 13 &14). At high pH (>



LC Conditions: Mobile phase, premixed 30/70 ACN/20mM phosphate at indicated pH; Flow rate, 1.0 ml/min.; Temperature, 30 °C; Injection volume, 5 ul; Detection at 254 nm; Column, ZirChrom-PBD; Column dimension, 50 x 4.6.

Figure 13

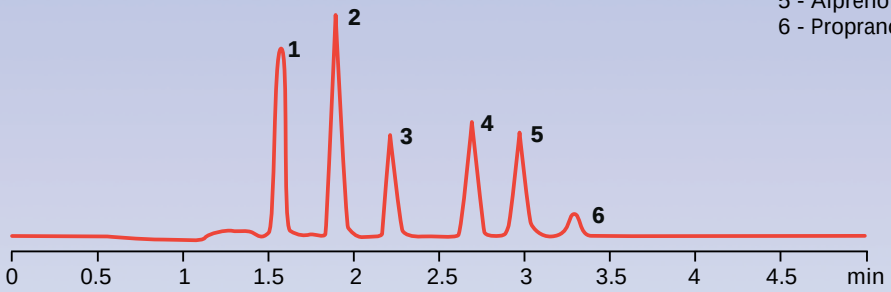
11.5) buffer type, concentration and inert salt concentration have very little effect. We advise the use of the organic type (i.e., acetonitrile, methanol, tetrahydrofuran or isopropanol) of the mobile phase to adjust selectivity and retention at high pH.

TIP: Use ZirChrom®-PBD to solve those basic compound problems requiring high pH.

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

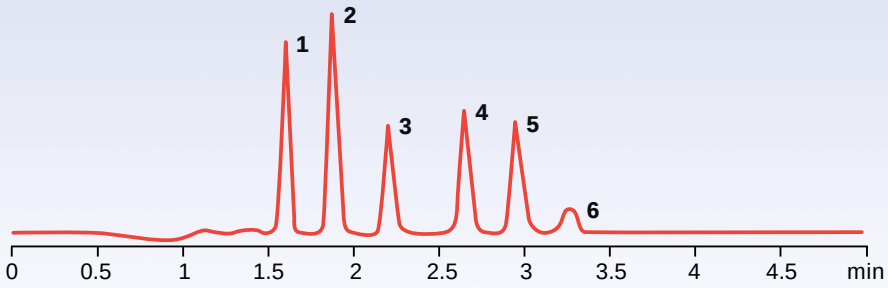
ANALYTES
1 - Labetalol
2 - Acebutolol
3 - Oxprenolol
4 - Lidocaine
5 - Alprenolol
6 - Propranolol

...In LC/MS-friendly amine buffer



LC CONDITIONS
Mobile phase: 35/65 ACN/10 mM Triethylamine,
50 mM Tetramethylammonium hydroxide, pH 12.2
Flow rate: 1.0 ml/min Temperature: 30 °C 150 mm x 4.6 mm
Injection volume: 5 µl Detection: 254 nm

...Or in phosphate buffer



LC CONDITIONS
Mobile phase: 35/65 ACN/20 mM Potassium Phosphate, pH 12.2
Flow rate: 1.0 ml/min Temperature: 30 °C 150 mm x 4.6 mm
Injection volume: 5 µl Detection: 254 nm

Figure 14

PART III

Other Products & Services

Method Development Kits

Not sure which column is best for your application? Try one of ZirChrom's method development kits. Each kit contains 3 columns with different selectivity for faster screening (columns are 4.6 mm x 50 mm; other sizes upon request).

MK01 **Ion Exchange Method Development Kit #1**
(contains 1 each of ZirChrom®-SAX, ZirChrom®-SHAX, and ZirChrom®-WAX)

MK02 **Ion Exchange Method Development Kit #2**
(contains 1 each of ZirChrom®-SAX, ZirChrom®-WCX, and ZirChrom®-PEZ)

MK03 **Reversed Phase Method Development Kit**
(contains 1 each of ZirChrom®-PBD, ZirChrom®-CARB, and DiamondBond®-C18)

Particle Sizes

ZirChrom's analytical columns are packed with 3 or 5 micron particles. On request, we can pack columns with 7, 10, or 25 micron particles, or larger, with identical chemistry, making scale-up using ZirChrom's products fast and easy. All particles have 300 angstrom pores.

Non-Porous Zirconia

ZirChrom has available non-porous particles for ultra-fast chromatography. These particles are available in standard sizes of 1.0, 2.0 & 3.0 micron and in all of our normal phase and reversed phase chemistries. Ion exchange versions can be custom made. Custom particle sizes up to 3.0 micron are available.

Prep Formats

All of our phases are available in semi-prep and prep formats. See our Technical Bulletin #196 for more information on easy scale-up to prep formats.

Microbore and LC/MS Formats

All of our phases are available in microbore and LC/MS formats upon request. Our reversed-phase columns have been shown to have significantly lower bleed than the leading silica phases in LC/MS, giving higher sensitivity.

Normal Phase LC /ZirChrom®-PHASE

Unmodified zirconia particles make an excellent support for normal phase LC. ZirChrom-PHASE is packed into the same column formats as our reversed phase and ion exchange supports. Specify ZR02 when ordering.

Technical Support

ZirChrom's products are often used in cutting edge separations where silica supports fail. Our technical support group has extensive experience, particularly in pharmaceutical and environmental HPLC. We are happy to assist you with your difficult separations.

Buffer Wizard

The ZirChrom BUFFER WIZARD is a web-based laboratory consultant designed to do the calculations needed to prepare buffers specifically for use in HPLC. In addition to doing the calculations it provides many helpful hints as to the proper choice of buffer and issues messages when the buffer capacity is too low to do the job or when too high or too low pH might damage conventional stationary phases.

To access the Buffer Wizard and other HPLC relevant data, visit the ZirChrom Home Page (www.zirchrom.com).

An advanced version of the Buffer Wizard with 50 buffer systems is available for sale (part# BW01; $100.00 list price).

International Inquiries /Orders

International customers can reach ZirChrom directly at +1 (763) 421-5264.

Our Fax number is +1 (763) 421-2319.

Call us at 1-866-STABLE-1, (763) 421-5264 or e-mail our technical support group at support@zirchrom.com.

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Technical Support

Our technical support group has extensive experience, particularly in pharmaceutical and environmental HPLC. We are happy to assist you with your difficult separations. Please contact us at 1-866-STABLE-1 or support@zirchrom.com.

For Peak Performance

ZirChrom manufactures a full line of zirconia-based high performance products used for the analysis of compounds by HPLC.

ZirChrom®

Specialists in Ultra-Durable, High Efficiency HPLC Phases

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