Welcome Ladies & Gentlemen to our presentation Advancements in Separation Science. I hope to show you how to increase your HPLC capabilities and to help you understand how HPLC is advancing in at least one area.

My name is Bill Ciccone from MicroSolv and I am pleased to be here today.
Abstract of today’s Presentation

- Review Current HPLC Barriers & Limits
- Discuss Root Causes of these Limits
- Introduce You to Silica Hydride HPLC Columns
- What they do for you and how to you gain as a chromatographer
- Real Life Applications

Today I hope to show you this advancement in HPLC stationary phase technology and how you can do things you could not do with HPLC before. At least not easily.
Let’s start with a short review of HPLC and why it is still the number one technique for analysis of small molecules after 30 years.

Most of these features of HPLC are well know to all of us but so that we don’t miss any important points in this lecture, we have listed these here. And they include…. This is by no means a complete list.

Common Benefits and Features of HPLC

- Hydrophobic Compounds retain very well when using Reverse Phase
- Very reproducible
- Well known and easy to predict and develop methods
- Retain bases at high pH, Acids at low pH, or Neutral Compounds
- Rugged Methodologies is possible
- Can easily validate and automate HPLC
- Scale from microbore to preparative separations
- Works with Normal Phase or Reverse Phase
- Most common & successful analytical technique for quantifying analytes
Silica particles have always been the backbone of HPLC from its beginning. This simplified structure illustrates the surface chemistry of modern HPLC grade silica. In fact most of the today’s presentation shows mostly highly simplified structures.
Even today, the most popular HPLC columns are made with silica. Even your favorite columns are most likely a C18 or C8 that is a modified silica stationary phase.
Why is silica the preferred backbone of HPLC columns?

This begs the question: Why is silica the preferred backbone of HPLC columns!
Why is silica the preferred backbone of HPLC columns?

*Excellent Physical Properties!*

Well suited for high pressure & constant flow of liquids

The short and simple answer is that silica has physical and chemical properties are very well suited to the high pressure and constant flow of liquids.
Why is silica the preferred backbone of HPLC columns?

**Excellent Physical Properties!**

<table>
<thead>
<tr>
<th>Property</th>
<th>Advantage</th>
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</thead>
<tbody>
<tr>
<td>Easily modified by bonded phases</td>
<td>Allows for different selectivity</td>
</tr>
<tr>
<td>Mechanical strength</td>
<td>Allows for well packed beds-Efficiency</td>
</tr>
<tr>
<td>Rigidity</td>
<td>Low back pressure-stable beds</td>
</tr>
<tr>
<td>Porous, High Surface Area</td>
<td>Allows for small column length</td>
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Silica not only withstands high pressure in routine use but also its surface can be easily modified to allow retention of hydrophobic compounds.

Also, the mechanical strength and rigidity of silica that is made for HPLC allows us to use very high pressure to pack columns and therefore produce highly efficient and reproducible columns.

Lastly, silica has enormous surface area which allows columns to have small ID and short length and still have high theoretical plates.
Making HPLC Grade Silica

To gain some of these features, silica is “fired” or baked and is hydrated during manufacturing for HPLC to produce surface silanols and form rigidity to product features needed for HPLC.

May not be well known

What is perhaps not well known is that HPLC grade silica is “fired” or baked to 600C and then hydroxilated to produce as many surface silanols as possible. This is done so that the silica produces all the features of HPLC and can be chemically modified through the use or organo-silanes.
How does this surface structure impact the performance of the columns?

Important Property of Silica Impacts Performance positively & negatively.

One of the properties of silica that was until now very important and also at the same time a side effect. Is that... CLICK
Is that the silanols form a very strong association (hydrogen bonding, ionic interaction) with water. Of course, water does not exist in nature as H2O as shown here but as 6H2O and even larger molecular aggregates.
**HPLC Grade Silica**

Attracts and holds water and will even adsorb water from your mobile phase until it has reached its maximum permanent hydration shell

*Silica is used as a desiccant for non chromatographic purposes*

Since silica attracts and holds water so strongly, a hydration shell or water layer forms on all silica with silanols. Think in terms of silica loving water and is used as a desiccant and will take water out of your mobile phase until the “steady state” of this hydration shell is formed.

This hydration shell like all nature, is dependant on its environment and therefore is variable in the way it will adsorb or desorb water. This may or may not be obvious and may or may not effect your chromatography. This is the basis for the...CLICK..
Some separation mechanisms of silica based HPLC Columns

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Type of Interaction</th>
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</thead>
<tbody>
<tr>
<td>Hydrophobic Interaction</td>
<td>Bonded Phase Required</td>
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<tr>
<td>Silanophilic Effects</td>
<td>Silanols</td>
</tr>
<tr>
<td>Partitioning</td>
<td>Hydration Shell/Organic Layer</td>
</tr>
<tr>
<td>Adsorption</td>
<td>Silica Chemistry</td>
</tr>
</tbody>
</table>

Some of the mechanisms in HPLC

If you have a bonded phase like C18 or other organo silanes, you can retain hydrophobic compounds but the unmodified silanols have their own place in HPLC and can be desired or not.

Partitioning occurs between the hydration shell and the organic layer of your mobile phase. This is responsible for many compounds to retain in HPLC and not the bonded phase like C18.

Also, since bonded phases including end capping only covers a very small amount of the surface area, the silica chemistry can play a very big role in the retention of your analytes.

So you can see that there are many mechanism, and some that are not listed here, that play a role in HPLC. The more mechanisms working at one time, the more difficult it is to get good peak shapes for complex mixtures and molecules. But HPLC is not the preferred analytical technique for no reason. For many compounds, this type of stationary phase has been extremely successful.
Silica is not perfect.

Limiting Properties!

- Unstable at high pH: Upper limit is typically pH 9
- Surface Acidity: Problems for basic compounds
- Silanols-Surface Chemistry: Hydration Shell becomes virtually permanent
- Trapped Metals: Complex with Chelating solutes. Tailing

However, as you can see here, silica is not perfect as nothing in life is. Silica has some shortcomings as we all know and we have listed here some of the main properties of silica that put limits and what you have been able to do with HPLC until now.
Common Barriers/Limits of HPLC

Summary of what we already know.

- Hydrophilic Compounds do not retain in RP
- Loss of RT of very hydrophobic compounds using 100% Water with C18
- Need to dry and control moisture in your solvents in NP Chrom
- Hysteresis when changing chromatographic modes on the same column
- Long Run Times
- Long Equilibration times between gradients
- Low pH instability
- Short column life when using some MP additives & some analytes
- Polar & Non Polar Solvent Compatibility
- Cannot retain bases at low pH or acids at high pH
- Low MS sensitivity with MP needed for retention in RP or NP
- On-Column Degradation of analytes due to silanols or surface water layer
- Bleed of end capping and bonded phases

Again, just to summarize what we already know, some of the barriers of silica based HPLC put limits on what we as chromatographers have been able to do with our HPLC.

These barriers or limits as we say are not all of the barriers but this represents many of the limits we share with silica based HPLC columns.

One of the most common limits for HPLC has been polar or hydrophilic compounds. Using “work arounds” like high pH or low pH to neutralize compounds above or below their pKa to main them more solvophobic has always been a barrier. Other work arounds that have not been successful is to use a completely aqueous mobile phase but ordinary silica columns do not like this and it is difficult to use.

If anyone has ever done NP HPLC you know why it is not commonly used anymore. The difficulty in getting reproducible results from run to run is very time consuming and difficult. This is largely due to the silica taking moisture from your MP and chaning the hydration shell on the fly. This can be controlled but it is tedious.

Some of the other limits to HPLC have been…
What are some of the root causes of the Features & Limits of silica based columns?

Why has it not advanced or improved on the limits in the last 30 years?

What are some of the root causes of these limits? Why have we not improved on this in 35 years?
HPLC Grade Silica

The hydration shell is somewhat immiscible with organic solvents needed for HPLC and a bi-layer of liquids is created. The hydration shell is changeable and difficult to control between runs with changing mobile phases and or additives.

The short answer is that to bond ligands such as C18 and others you need silanols present. Also, without silanols, the surface is not amenable to traditional HPLC.

The result is that we have learned to live with limits and barriers and to work around and even use the hydration shell and organic layer of the mobile phase to get many good results.

What we are here to learn about today is different silica surface manufactured without silanols that allows for bonded phases to be used. This surface is populated with silica hydrides instead of silanols…
A Recent Advancement in HPLC!

Silica Hydride Based HPLC Columns

For the purposes of this presentation and in HPLC terms, what is silica hydride? Click.
What is “Silica Hydride”? 

Silica Hydride is a highly purified silica particle that has been manufactured to have a surface that is populated with silica hydride instead of silanols.

All physical features of HPLC silica are the same for silica hydride but the surface chemistry and resulting capabilities are different.

Basically, silica hydride is a high purity silica particle that would normally be used in HPLC that retains all the features of HPLC grade silica but has a somewhat different chemical structure on the surface.
Ordinary is a term we will use to differentiate silica hydride from silica that is populated with silanols.

You can see here that the surface and structure of silica between both silica hydride and ordinary silica is essentially the same in that the siloxane bonds that makes for the rigidity and other physical characteristics are the same.

What is different is the terminal silanols are gone and replaced with silicon hydrides or Si-H.
This difference may seem small and subtle but think in terms of the apple shown here that has a different chemical structure instead. It will be different. So we like to say that structure impacts the performance even when the difference seems small.
How does this structural difference impact the performance of the columns?

Here is the comparison again, keep in mind the red boxes and how they are different on the surface and how it impacts HPLC.
How Does this structural difference impact the performance of the columns?

This slide is really the key to why these two different substrates are structurally similar but the performance is very different. As we have previously shown, ordinary silica retains water and does not desorb it unless it is baked at 600C and kept under non aqueous conditions or you use a lot of non aqueous mobile phase for a long long time.

The silica hydride particle however is slightly hydrophobic and will desorb any liquid very differently. Don't misunderstand this weak association with water, the surface can be “wetted” so to speak but it does not build up any hydration shell. I think the operative word here is shell.

To sum this slide, the silica hydride particle has all the physical characteristics of ordinary silica but has different adsorption character.
We now need to challenge our bias about HPLC column limits to benefit from this advanced and completely different column technology.

Enjoy the benefits of silica hydride without limits that exist with hybrid silica and polymeric columns.

What about bonding? Don’t you need silanols?

What this presentation is about is that we are showing an advance in HPLC column chemistry that breaks many of the barriers and limits due to what seems to be a subtle difference from ordinary silica when in fact, the difference is new limits need to be set in HPLC and what it can do for you and your lab.

One question that may seem obvious so far is that I mentioned previously that ordinary silica is made for HPLC and that silanols are created for one use and that is to use organo silation to created needed bonded phase. This was true.

Professor Joe Pesek at San Jose State University working under a grant from Nasa, developed the silica hydride surface and developed a new bonding process that uses terminal alkenes or alkynes and bonds directly to the silica hydride.
Using hydrosilation on silica hydride, direct silicon carbon bonds (with 2 points of attachment) are produced when derivatizing the hydride surface.

Some more of the benefits of this new route to making HPLC columns is that you create silicon-carbon bonds between the silica and the bonded phase. Unlike organo silation which results in silicon-oxygen-silicon-carbon bonds. Direct silicon carbon bonds are extremely stable and when using an aklyne, two points of attachment result.

Where as the ordinary bonded phases are susceptive to hydrolysis at low pH, these bonds are not. The bottom line here is that extremely stable HPLC stationary phases result. Take a better look at this.
And you can see that the amount of bonded phase is not 100% like ordinary silica and the surface remains a silica hydride with all these properties and no hydration shell. The bonded phase is extremely stable and there is no end capping.

This column with a dual point of attachment and a C18 chain bonded to it is more retentive for hydrophobic compounds than virtually all ordinary C18 and is not limited to use of less than 100% aqueous mobile phase.

So you can use this column for compounds that you want to analyze in 100% water, not lose retention and if you need PIC reagents for your method, it will not destroy this column chemistry.

This series of columns using silica hydride is commercially available as
Cogent TYPE-C Silica. This is a trademark for HPLC columns based on the silica hydride and the Pesek method of bonding to silica hydride particles. At this point in time, unmodified silica hydride for normal phase HPLC is available along with 4 bonded phases for different uses. We will review each of these phases and how they can be used to break the barriers and limits of traditional HPLC during the remainder of this presentation.
What do we gain from silica hydride HPLC columns?

- Think in terms of 3 modes of HPLC:
  - Normal Phase
  - Reverse Phase
  - Aqueous Normal Phase

You are probably sitting there thinking, okay, this all fine and looks nice but how do I use these columns. What does a chromatographer gain from them and what do we do with this new technology.

We are here to present these new columns that are most likely to become a standard of all silica based HPLC columns one day because you can use them for 3 modes of HPLC and break many if not all the barriers of silica based HPLC columns we are discussing today.
What do we gain from silica hydride HPLC columns?

- **Normal Phase**
  - Easier to do
  - More Reproducible

**Prep Chromatography**
**Water Free**

For example, for normal phase HPLC which is very heavily used for preparative methods to avoid water and getting the analytes ready for use in production. Now the barrier of drying solvents and the water poisoning the columns after extended use is eliminated. Remember, it does not remove and therefore change your mobile phase concentration during use.
What do we gain from silica hydride HPLC columns?

- Reverse Phase Limits are Broken
  - 100% Water Mobile Phase
  - pH stability
  - More retentive for hydrophobic compounds
  - Resistant to most additives like PIC reagents
  - No bleed of bonded phases or end capping
  - No on-column degradation of analytes due to acidity
  - Fast equilibration even when using ballistic gradients
  - Excellent for LCMS

For reverse phase, there are many limits and barriers that are broken that we all previously lived by. I already mentioned about retention of hydrophobic compounds with a C18 but you can see that even in our everyday life with C18, using a silica hydride column offers precision and methods that work with shorter run times and longer column life.
What do we gain from silica hydride HPLC columns?

- **Aqueous Normal Phase (ANP)**
  - Normal Phase with Reverse Phase Solvents
  - Retain polar compounds with precision
  - No hysteresis when changing from RP to ANP
  - Retain bases at low pH
  - Retain acids at higher pH
  - Excellent for LCMS
  - Extremely fast equilibration between runs
  - Very precise from run to run, day to day.

Aqueous Normal Phase is a lesser known chromatographic mode that is useful for hydrophilic or polar compounds that normally do not chromatograph well in RP. With ANP you use an inverse mobile phase content from RP since this mode is truly normal phase chromatography but you use RP type of solvents such as Water/Acetonitrile. Since there are no polar groups or the hydration on the surface and therefore equilibrates very fast. Because there are no polar groups on the surface like in an ion exchange or HILIC column, salts and polar compounds do not “poison” the column.

This mode is becoming very popular with metabolomics, drug discovery and analytical methods people that are challenged with polar compounds.
Using Silica Hydride stationary phases breaks the limits and barriers of traditional HPLC which results in easier and more productive HPLC labs

So, you can see that using a silica hydride stationary phase not only provides for a longer column life and more precise runs but also provides selectivity that until now has been very challenging.
How do we use the Silica Hydride Phases?

Normal Phase:

_Normal method development with standard MP to begin with followed by different methods to optimize method objectives by taking advantage of silica hydride column capabilities._

So, how does one use the silica hydride based columns? They are very easy but again, you have think in terms of new columns with different barriers or limits. Kind of expands the normal skill set of HPLC.

If you want to use normal phase for reasons outside of the scope of this presentation, you would use the unmodified silica hydride commercially called the Silica-C.

You would use a standard mobile phase that you would use in normal phase HPLC but you would not have to consider the moisture control steps.

What you might find is that you have different selectivity with the silica hydride surface from an ordinary silica surface and you may need to adjust the mobile phase accordingly. One of the costs involved is learning how to optimize your method and think outside the limits. With normal phase on silica hydride columns not only do you not worry about the moisture control but when you change from one mobile phase to another (assuming these are miscible) one column volume equilibration is most likely all you will need. So if you go from Hexane/Ethyl Acetate 90/10 to Hexane/IPA 60-40 the time to equilibrate will be much faster. This really comes in handy when scouting for methods that are optimal not just for results but also for solubility compatibility for greater sample load on the columns.
How do we use the Silica Hydride Phases?

Reverse Phase or ANP?

Many compounds are no longer in the domain of Reverse Phase HPLC

Since ANP or aqueous normal phase is so effective for polar compounds, they may no longer be in the domain of reverse phase HPLC as the first method of choice. So you have to evaluate the compounds and the method objectives and decide between these two equally effective, precise and robust methods.

If anything breaks the limits or barriers of traditional HPLC it is this opportunity.

The good news is that many of the bonded phases, especially the C8 phase can do both. And therefore it is great for complex mixtures such as polar impurities or degradation products of hydrophobic (non polar) analytes.
How do we decide between?

Rule of Thumb or Quick Start Method to determine best mode for unknown analytes.

1. Gradient 100% A to 40% B
2. Equilibrate for one column volume
3. Gradient from 95% B to 40% A with acid or base added

There are a few really good ways to determine which of the modes would be best for your compounds. If the chemical structures are known, you can determine by a simple rule of thumb:

Polar Compounds, use ANP
Non Polar Compounds use RP

But if they are not known and you are looking for a host of possible analytes from your sample, you can use a simple method on the Diamond Hydride, C8, Cholesterol or the C18 column.

This methodology utilizes one column, two injections and one set of solvents for you mobile phase. Basically, you inject your compounds with a gradient from 100% water (A) to 40% Acetonitrile (B). Then one column volume to equilibrate the column and the re inject the sample. This time run the Inverse Gradient from 95% Acetonitrile (B) to 40% Water (A). This gradient will retain and you will see your polar compounds. In both solvents, you would add 0.5% formic acid or 0.5% ammonium formate during the second run.

Total run time depending on your column length is typically about 30 minutes for each gradient.

From this point you then optimize your run with either a gradient or an isocratic method.
The mode called ANP or aqueous normal phase is not new. For years, you probably did ANP when you used a cyano or amino columns. The problem is that when you use ordinary silica, the mode is not reproducible When you use silica hydride columns, ANP is extremely precise and accurate. Some mass spec companies are building metabolite data bases using the Diamond Hydride column because according to them, it is the only column on the market that can retain polar compounds precisely enough to plot Retention time v. Accurate Mass and use this plot to identify the identity of the metabolite.
More about ANP

What is it?

- Normal Phase
- Adsorption
- Ion Interaction (Not Ion Exchange)
- Use RP Solvents
- Not HILIC

ANP is a type of ion interaction NOT ION EXCHANGE where the retention is due to the adsorptive properites of silica without the water shell. The elution order and the predictability of analytes is truly normal phase. Except you use Reverse Phase solvents instead of non polar solvents.
Real Life Applications of using the silica hydride columns and how you can benefit from the new technology in RP, ANP & NP

At this point, I would like to show you some applications that have resulted from laboratories using the silica hydride columns. For the sake of time, I will limit these to a well rounded sampling but if you have further interest, I would be happy to show you many others.
Real Life Applications of using the silica hydride columns and how you can benefit from the new technology

- Nucleotide Bases
- Glyphosate (Highly polar compound)
- Melamine and Cyanuric Acid
- Amino Acids
- ATP from AMP
- Succinic Acid
- Generic Pharmaceuticals
- Metabolites from human urine
- Isobaric Compounds for LCMS
- Organic Acids
- Cytochrome-C
- Guanidine
- Citric Acid from IsoCitric Acid
- Leucine from Iso-Leucine
- Biogenic Amines
- Purine Bases & Nucleosides

Some of the areas I will show today are shown here. I will not spend too much time on these but if you would like me to stop and discuss any of these methods, let me know.


You can use a silica hydride column for the unique selectivity shown compared to other C18 columns as well for the column life. Here you see a method that was developed for impurity testing.

Furazolidone is an antibiotic. The detection was 365nm UV and was done at 30C with a 20ul injection.
Here you can see that using the silica hydride C8, you can use a mobile phase with additives such as SDS that does not effect a silica hydride column. Ordinary C8 columns experience very short shelf life when using SDS. Also, the micelles in this case are very compatible with a silica hydride surface and offer good efficiency.

220 nm UV detection, 20ul injection at 25C.
Application-Reversed Phase


Column: Cogent Bidentate C18™, 4um, 100 Å
Dimensions: 4.6mm i.d. x 75mm
Mobile phase: 25% acetonitrile/75% DI water + 0.1% formic acid
Flow rate: 1.0 mL/min. Isocratic 5 minute separation

This is a quick method that not only shows the value of the silica hydride C18 column for speed and ease but this shows the precision. This is 8 chromatograms laid on top of each other.

Base line separations were not the goal of this method. Just to ID them was sufficient.

This is not a fuzzy chromatogram. 😊
Application-Reverse Phase


Column: Cogent Bidentate C8™, 5μm, 300 Å
Dimensions: 4.6mm i.d. x 75mm
Mobile phase: A: DI Water + 0.1% TFA
B: Acetonitrile + 0.1% TFA
Flow rate: 1.0 mL/min. Fast 20 minute Gradient

Gradient
<table>
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<tr>
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<td>20.0</td>
<td>27</td>
</tr>
<tr>
<td>21.0</td>
<td>9</td>
</tr>
</tbody>
</table>

This slide shows how using a silica hydride based column with wide pores (300 Å) and modified with C8, you can achieve baseline separation.

This gradient was done with 214nm UV. RP gradient takes 21 minutes starting with 9% B for 5 minutes to 21% B.

This is not a fuzzy chromatogram. 😊
Using the same silica hydride column with C8 with 300A pores results in a great separation of cytochrome c.

This was done with a UV detector at 214nm

Gradient

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<tr>
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<td>40</td>
</tr>
<tr>
<td>18.1</td>
<td>20</td>
</tr>
</tbody>
</table>
Retention of sulfonamides can be challenging for ordinary C18 columns since they are somewhat limited to use with UV detectors. But using a silica hydride column you can see that good retention is very possible and very compatible with an LCMS over coming the detection limits normally seen for ordinary C18/UV. In this case APCI was used and is superior to ESI ionization method.

**Gradient**

<table>
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<tr>
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<tr>
<td>5.0100</td>
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<tr>
<td>10.0</td>
<td>30</td>
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<tr>
<td>15.0</td>
<td>30</td>
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</tbody>
</table>
This example shows a very nitrogenous compound as you can see. HPLC with ordinary silica based columns have hard time retaining a compound like this. Here you can use a silica based C18 to retain this compound at low pH with good efficiency and capable of good sensitivity. The other peaks shown are excipients.

UV 280nm, 10ul injection at 40C.
**Application-Reverse Phase**  
Diphenhydramine HCL on a Diamond Hydride

Using a silica based diamond hydride column, this antihistaminic drug will stay on the column at 80% B. When you change the MP to one shown here, the compound elutes. This opens a wide range of possibilities for this including on column SPE. This can be very beneficial for isolation of Dph from complex mixtures or for preparative scale use for compounds having similar properties.
Application-Reverse Phase
100% Aqueous Mobile Phase on a C18 Column

Column: Cogent Bidentate C18™, 4 mm, 100 Å
Dimensions: 4.6mm i.d. x 75mm
Mobile phase: 100% 0.05% v/v H3PO4
Flow rate: 1.0 mL/min. Isocratic

This is an example of breaking the barrier of using 100% water as a mobile phase on a C18 column. Using a silica hydride column that has been modified with a C18 phase, you can do 1,000’s of injections like this one with no change of RT. If you have compounds that require extremely high water diluents, you can match it to the mobile phase.

215 nm UV detection ws used for this.
Application- ANP
Melamine & Cyanuric Acid on a Diamond Hydride Column

Column: Cogent Diamond Hydride™, 4 mm, 100 Å
Dimensions: 2.1 mm i.d. x 150 mm
Mobile phase: A: DI Water 0.1% Acetic Acid
            B: Acetonitrile + 0.1% Acetic Acid
Flow rate: 0.4 mL/min. Fast 15 minute Inverse Gradient

Melamine and other metabolites of Cryamazine has been investigated by many using many different HPLC or ion chrom methods. You can see here that with a silica hydride based Diamond Hydride column, the separation of two very polar compounds is easy. Great peak shapes for such difficult compounds is another barrier of HPLC that can be broken.
Application- ANP
Xanthine, Uric Acid & Hypoxanthine on a Diamond Hydride Column

Samples from human urine, these metabolites are easily identified using the Diamond Hydride silica hydride column. In this case, we added 100ul of Acetonitrile to human urine and centrifuged. 20ul of the supernatant was mixed with 10ul of B component of the MP.

Gradient

<table>
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<tr>
<td>10.0</td>
<td>50</td>
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<tr>
<td>12.0</td>
<td>50</td>
</tr>
</tbody>
</table>
Application- ANP
Monitoring Patient Levels of Methotrexate on a C18

Column: Cogent Bidentate C18™, 4 mm, 100 Å
Dimensions: 2.1mm i.d. x 150mm
Mobile phase: A: DI Water 0.5% Formic Acid
B: Acetonitrile
Flow rate: 0.4 mL/min. Fast 12minute Inverse Gradient

Methotrexate is a chemotherapy drug that is very difficult to retain on most ordinary C18 columns with good peak shapes. This antimetabolite is used for cancers such as leukemia, osteosarcoma and breast cancer. This was an LCMS method in the positive mode with both specificity & sensitivity.

Gradient

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<tr>
<td>10.01</td>
<td>90</td>
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<tr>
<td>12.0</td>
<td>90</td>
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</table>
This was another clinical example of using silica hydride columns. This C18 was injected with only 200ng dissolved in 1ml of DI Water from actual patient samples in this LCMS method.
Application - ANP

Choline from Acetylcholine on a UDC Cholesterol

Column: Cogent UDC-Cholesterol™, 4 mm, 100 Å
Dimensions: 4.6mm i.d. x 75mm
Mobile phase: 90:10 Acetonitrile/DI Water w/ 0.5% FA
Flow rate: 0.5 mL/min. Fast isocratic runs

Here is another limit or barrier that is broken using silica hydride columns in HPLC. These challenging Quaternary amines are easy to retain and separate now. This is an example of LCMS of acetylcholine and choline. Normally these compounds are really hard to separate but in 90% Acetonitrile and Water on the cholesterol phase, these compounds separate.
Amino acid analysis is normally done with pre column and post column derivatization kits. With silica hydride columns, it is possible to retain and separate all amino acids without derivatizing them. This is an example of a very fast method that is consolidated so you can see how these acids retain on the Diamond Hydride column. For baseline separation of specific amino acids, some changes to the method will be required but the method remains simple and easy.

It is possible to separate the most difficult amino acids such as leucine from isoleucine.
Citric acid and isocitric acid are very important metabolites that have the same empirical formula. They differ chemically by the position of a hydroxyl group. A slight modification of solvent gradient A to gradient B resulted in near baseline separation of the two isomers. As can be seen in Fig. each compound has trace amounts of the other isomer in it.

This is not a fuzzy chromatogram. ☺
San Jose State University, Agilent Technologies and MicroSolv are involved in a joint program to develop a generic method for positive and negative ions with either the Diamond Hydride column or the Bidentate C18. With this generic method, a database of metabolites will be generated by plotting RT v. Accurate Mass. Using a quaternary system or two binary pumps and a TOF mass spectrometer, this gradient is used for compounds like these.

This method is fast and easy. Another barrier that is broken is using HPLC for metabolite identification. Since the silica hydride columns are easy to equilibrate and therefore precise, it is possible to use RT as a parameter of identification. These amino acids are analyzed with positive mode ESI.
Another class of compounds that are normally difficult for HPLC are small organic acids. Using the same generic method, you can analyze these compounds in the negative mode.
To analyze most carbohydrates by HPLC, amino columns or other special columns are used that usually do not have long useful lifetimes. Here you can see that using a silica hydride based Diamond Hydride, you can retain and separate and identify these small carbohydrates.

Again, this is the same generic method that was used for the amino acids and the organic acids. These sugars were monitored in the positive mode.
Glyphosate is an extremely difficult compound to retain and normally requires pre-
column derivatization to get it to retain. Another barrier is broken, retain compounds
like this without derivatizing them. Figure one is the first injection, figure 5 is the 5th
injection. Notice the RT is almost identical.

Gradient

<table>
<thead>
<tr>
<th>Time</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>80</td>
</tr>
<tr>
<td>1.0</td>
<td>80</td>
</tr>
<tr>
<td>1.1</td>
<td>5</td>
</tr>
<tr>
<td>5.0</td>
<td>5</td>
</tr>
<tr>
<td>5.0</td>
<td>80</td>
</tr>
</tbody>
</table>
Nucleotides such as ATP, AMP and Cyclic AMP are other examples of limits of HPLC. However, using silica hydride stationary phases, you can use retain and separate these compounds.

Gradient

<table>
<thead>
<tr>
<th>Time</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.095</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>70</td>
</tr>
<tr>
<td>15.0</td>
<td>70</td>
</tr>
</tbody>
</table>
These two chemicals are listed on the World Health Organizations list of hazardous chemicals and are used as plant growth regulators. Monitoring their presence in food is very important. HPLC is unable to analyze these and the current accepted method is to use ion chromatography. Using a silica hydride column, Diamond Hydride, you can retain and separate with good peak shape and efficiency both compounds. Repeatability and reliability are extremely high with silica hydride columns. Now you can use HPLC for these compounds.
Application-ANP
Organic Acids on a Diamond Hydride Column

Column: Cogent Diamond Hydride™ HPLC Column, 4 mm, 100 Å
Dimensions: 2.1 mm i.d. x 150 mm
Mobile phase: A: DI Water 0.1% Am Formate
B: 90% ACN/10% DI Water/0.1%Am Formate
Flow rate: 0.4 mL/min. Fast 8 minute Inverse Gradient

1- MALEIC ACID
2- trans ACONITIC ACID
3- cis ACONITIC ACID
4- IMPURITY
5- FUMRAIC ACID
6- CITRIC ACID
7- OXALOACETIC ACID

Another example of organic acids by HPLC using a silica hydride column. Notice this is very Mass Spec friendly.
Application-Normal Phase
Pro Drugs in NP on a C18 Column

Column: Cogent Bidentate C18™ HPLC Column, 4 mm, 100 Å
Dimensions: 4.6mm i.d. x 75mm
Mobile phase: 95:5 Hexane/Ethyl Acetate
Flow rate: 1.0 mL/min. Isocratic

There are many typical methods in Normal phase that you can transfer to a silica hydride column such as the silica-C and take advantage of no need to control solvent moisture and the column is not poisoned by the water.

Here is one example of using a silica hydride column that is modified with a C18 and you can still do Normal Phase with it. This is the same column that you can use for RP (100% Water or typical RP solvents) or ANP (Inverse gradient) and you can switch from non polar solvents to polar solvents and back. Due to the low hysteresis or even lack of it, these columns can be changed back and forth if desired. Another barrier that is broken is that you can use non polar solvents to wash a column that might have been contaminated by a sample. Of course, you can also wash with polar solvents. You just have to pass a miscible solvent such as THF or IPA through the column each time you convert it.

In this case, the customer used a C18 with non polar solvents due to the fact that water would degrade or activate these compounds and they wanted to monitor them during manufacturing and still have hydrophobic retention ability.

Another barrier in HPLC. You can use solvents that your compounds are most likely to be stable in or maximize the solubility and still do HPLC and match the diluent to the mobile phase.
Review of Previous Barriers/Limits of HPLC

Summary of what we already knew.

- Hydrophilic Compounds do retain in RP
- No Loss of RT of very hydrophobic compounds using 100% Water with C18
- No Need to dry and control moisture in your solvents in NP Chrom
- No Hysteresis when changing chromatographic modes on the same column
- Shorter Run Times then before
- Very Short Equilibration times between gradients
- Low pH Stability
- Long column life even when using some MP additives & some analytes
- Polar & Non Polar Solvent Compatibility
- Retain bases at low pH & acids at high pH
- Increased MS sensitivity
- Minimal On-Column Degradation of analytes due to silanols or surface water layer
- No Bleed of end capping and bonded phases

Here is a review of the advancements in HPLC column technology we discussed today. Are there any questions?
Change your bias about HPLC Limits

- The possibilities for new methods are limitless
- Questions?
About MicroSolv

- MicroSolv started in 1992
- Formed to work with technology transfer departments at universities
- Role MicroSolv plays in product development
  - Tech Transfer
  - Product Development and Marketing R&D
  - Production Design
  - QC
  - Marketing and Sales Distribution of Wide Array of Products
  - Education and Technical Support
  - Product Line Extension