RECENT DEVELOPMENTS IN
HPLC AND UHPLC
Steroids represent a chemically distinct class of hormones with wide-ranging biological functions. Synthetic derivatives of endogenous steroid prototypes are used medically in birth control and in the treatment of asthma, arthritis, inflammation and osteoporosis.

Steroids share a characteristic, polycyclic structure and have varying degrees of lipophilicity (log P). In this study, a reversed phase HPLC method was developed for separation of five steroid hormones with partition coefficients ranging from 1.47 (cortisone) to 4.5 (pregnenolone) on a Hamilton PRP-C18 HPLC column.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Lipophilicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Estriol</td>
<td>(log P = 2.5)</td>
</tr>
<tr>
<td>2. Cortisone</td>
<td>(log P = 1.5)</td>
</tr>
<tr>
<td>3. Testosterone</td>
<td>(log P = 3.3)</td>
</tr>
<tr>
<td>4. Estrone</td>
<td>(log P = 3.1)</td>
</tr>
<tr>
<td>5. Pregnenolone</td>
<td>(log P = 4.5)</td>
</tr>
</tbody>
</table>

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An Assessment of Stationary Phase Selectivity in SFC
Tony Edge, Matt James, Colin Pipe, Sergey Bylikin, Jennifer Field, and Melvin Euerby
Sustainability concerns are renewing interest in SFC, often as a substitute for HPLC. With the broader application of SFC, we need a better understanding of selectivity in SFC, where stationary phase classification is not well established. This study assessed and measured the selectivity differences afforded by three prototype SFC phases.

Developing a Fast Purification Method for a Natural Product with a Preparative LC Column Packed with Superficially Porous Particles
Lakshmi Subbarao
Superficially porous particles (SPPs) are routinely used in analytical liquid chromatography (LC) columns, but they also provide benefits for preparative LC columns for use in pharmaceutical discovery laboratories and high-throughput environments. In this study, a preparative method using SPP-packed column had a 45% decrease in run time over a method using columns packed with fully porous particles, at standard flow.

Modifying the Metal Surfaces in HPLC Systems and Columns to Prevent Analyte Adsorption and Other Deleterious Effects
A novel surface modification technology has been developed to reduce interactions between analytes and metal surfaces in HPLC instruments and columns. We demonstrate the impact of this technology on peak symmetry, peak area, and injection-to-injection and column-to-column reproducibility for several metal-sensitive analytes.
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Protein 160 Å
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Welcome to the 2022 edition of Recent Developments in LC Column Technology.

In this issue, we feature fundamental and innovative efforts from within the walls of some the leading companies in the separation science industry. The theme was inspired by the continuous output of essential separation tools and the seemingly endless streams of pertinent information that these companies provide the scientific community.

In the first article featured, Tony Edge, Matt James, and their colleagues at Avantor discuss stationary phase selectivity in supercritical fluid chromatography (SFC), a technique that is orthogonal and complementary to high-performance liquid chromatography (HPLC). Using a modified Neue approach and contrasts against HPLC to investigate variables that impact selectivity in SFC, the stationary phase is found to be of vital importance, whereas mobile phase modifiers are more useful for adjusting retention.

It is hard to believe that superficially porous particle (SPP) technology has now been around now for over 15 years—and still having a significant impact. In a contribution from Agilent Technologies, Lakshmi Subbarao discusses the use of SPP technology for preparative work. Using the purification of withaferin from ashwagandha, Subbarao demonstrates that the reduced mass transfer term in the van Deemter, as compared to fully porous particle-based columns, can be utilized to run at faster flow rates without significant loss of resolution. This feature can substantially improve throughput and productivity.

Addressing the very hot topic of analyte interactions with metals in HPLC (1), Tom Walter and coworkers from Waters introduce a novel metal surface modification that mitigates interactions and inhibits potential on-column chemical reactions with analytes and extractables from metal surfaces. The organic/inorganic treatment is applied to both instrument and column hardware, proving impactful for traditional reversed-phase HPLC, as well as size-exclusion chromatography (SEC).

I may be a bit biased in this area, but I am sure what lies within these pages will convince you that the efforts put forth by these companies and their talented employees are essential for the continued progress of separation science.

Enjoy!

References
An Assessment of Stationary Phase Selectivity in SFC

Tony Edge, Matt James, Colin Pipe, Sergey Bylikin, Jennifer Field, and Melvin Euerby

Supercritical fluid chromatography (SFC) has seen a recent resurgence in interest following investment in the development of instrument technology by numerous instrument manufacturers. Increased focus on sustainability in chromatographic science, coupled with the orthogonality to reversed phase HPLC, is likely to further drive the uptake of SFC in many sectors. As with any form of chromatography, optimizing separation selectivity is a key variable in providing adequate resolution and accurate identification and quantification of target analytes. Stationary phase chemistry can be readily exploited to substantially alter the separation selectivity obtained. This article examines and characterizes the selectivity differences offered by three prototype SFC phases.

An Assessment of Stationary Phase Selectivity in SFC

Tony Edge, Matt James, Colin Pipe, Sergey Bylikin, Jennifer Field, and Melvin Euerby

Supercritical fluid chromatography (SFC) has seen a recent resurgence in interest following investment in the development of instrument technology by numerous instrument manufacturers. Increased focus on sustainability in chromatographic science, coupled with the orthogonality to reversed phase HPLC, is likely to further drive the uptake of SFC in many sectors. As with any form of chromatography, optimizing separation selectivity is a key variable in providing adequate resolution and accurate identification and quantification of target analytes. Stationary phase chemistry can be readily exploited to substantially alter the separation selectivity obtained. This article examines and characterizes the selectivity differences offered by three prototype SFC phases.

A supercritical fluid is a substance which exists above its critical temperature and critical pressure (Figure 1). It has a range of physical properties that are intermediate between gases and liquids; however, these properties can be varied in accordance with the pressure-temperature phase space. One of the major advantages of a supercritical fluid is that there are no phase transitions, as are observed between gaseous, liquid, and solid states. Phase transitions involve large enthalpy changes, and can have substantial physical impact on the column packed bed structure.

The history of supercritical fluid chromatography (SFC, see Table I) began in the 1800s when the supercritical state was characterized for CO₂ by Andrews (1). It was, however, James Lovelock who first suggested in 1958 the use of supercritical CO₂ as a chromatographic mobile phase at an international gas chromatography meeting. Just as the Nobel Prize-winning inventors of liquid-liquid partition chromatography, Martin and Synge, had proposed that the mobile phase could also be a gas, Lovelock realized the high liquid-like density, high gas-like diffusivity and low viscosity of fluids above the critical point would extend both gas chromatography (GC) and liquid chromatography (LC). In confirmation, packed-column SFC was demonstrated in 1962 (3), and then quickly developed throughout the 1960s with new detection and pressure programming methods.

It was the introduction of SFC capillary columns, invented by Novotny and Lee in 1981 (8), that led to an exponential growth in SFC applications. However, the practicalities of capillary SFC, similar to the challenges associated with capillary high-performance liquid chromatography (HPLC), reduced the applicability of the technology. The recent revival in SFC has been driven by the introduction of analytically scaled packed columns, together with improved instrumentation offering greater reliability and reproducibility.

SFC has found niche application areas, such as chiral chromatography, although the benefits associated with the technology have never been strong enough to displace HPLC. The uptake of SFC in the niche areas has persuaded mainstream manufacturers to develop the technology, and now many leading manufacturers have offerings. This has increased the visibility of the technology and of its utilization by end users, though the greatest challenge associated with the large-scale use of the technology is still the dominance of HPLC for the separation of small and large pharmaceutical molecules.

Recent industrial focus on sustainability has renewed interest in switching to SFC from HPLC. The use of organic solvents in HPLC has substantial detrimental environmental impact, and although the primary mobile phase used in SFC is CO₂, which, albeit a greenhouse gas, is still substantially preferable to organic solvents such as methanol, acetonitrile, and tetrahydrofuran. It is anticipated that the drive for a more sustainable chromatographic solution will provide an impetus to increase the use of SFC within industry. This renewed interest has, in turn, started to highlight some of the other benefits associated with the use of supercritical fluids. One of the major advantages is the ability to radically change the physical properties of the mobile phase without undergoing a phase transition. This can be either due to the large parameter space that a supercritical fluid occupies (10), or because moving through a super-
critical fluid state offers the opportunities to move from a gaseous state to a liquid without a phase transition. Supercritical CO\(_2\) is non-polar, therefore a co-solvent, typically methanol, is used (up to 40% v/v) to increase the solvating capacity of the mobile phase, which enables the analysis of compounds with logP values in the range of -1 to 10 (9). The low viscosity of such compositions and enhanced analyte diffusion means that elevated flow rates can be used compared to HPLC to achieve high resolution, high-throughput analyses (11). The use of higher percentages of the co-solvent (for example, >50%), along with additional polar components such as water, can further extend the use of SFC to more polar compounds including endogenous metabolites, plant extracts, water-soluble vitamins, pesticides, sugars, and peptides (9,12). Given the wide potential applicability of modern SFC, an understanding of how to optimize the separation selectivity using a variety of instrumental parameters is clearly important for addressing the plethora of complex applications that can now be addressed.

**Selectivity in Liquid Chromatography**

The fundamental aim of any form of chromatography is to resolve analyte peaks of interest, thus enabling their accurate determination and quantification. It is therefore necessary to understand how chromatographic parameters can be adjusted to maximize resolution. Resolution between two peaks is defined according to equation 1, where \(t_R\) is the analyte retention time and \(w\), the peak width at the peak base. From this, the Purcell equation (2) can be derived, which describes how resolution is impacted by a combination of three factors: the number of theoretical plates (\(N\)), retention factor (\(k\)) and selectivity (\(\alpha\)).

\[
R_s = \frac{t_R - t_{R_1}}{0.5 \left(\frac{w_1}{w_2} + \frac{w_2}{w_1}\right)} \quad [1]
\]

\[
R_s = \frac{\sqrt{\frac{N}{4}} \left(\frac{x-1}{x} \frac{k_2}{1+k_2}\right)} {\frac{k}{1+k_2}} \quad [2]
\]

\(N\) can be increased by either increasing the column length or decreasing the particle size; \(k\) is a measure of analyte retention, and is defined by equation 3, where \(t_R\) is the retention time of the analyte and \(t_d\) is the column dead time. Decreasing the mobile phase elution strength or providing stronger analyte interaction with the stationary phase will increase \(k\).

**TABLE I: History of the development of SFC**

<table>
<thead>
<tr>
<th>Author (Ref.)</th>
<th>Year</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrews (1)</td>
<td>1879</td>
<td>Characterisation of critical point</td>
</tr>
<tr>
<td>Lovelock (2)</td>
<td>1958</td>
<td>Suggestion of SFC</td>
</tr>
<tr>
<td>Klesper (3)</td>
<td>1962</td>
<td>Packed column SFC demonstrated</td>
</tr>
<tr>
<td>Sie and Rijnders (4)</td>
<td>1966</td>
<td>Use of FID in SFC</td>
</tr>
<tr>
<td>Sie and Rijnders (5)</td>
<td>1967</td>
<td>First use of term “SFC.” UV-visible detector in SFC</td>
</tr>
<tr>
<td>Jentoft and Gouw (6)</td>
<td>1970</td>
<td>Pressure programming in SFC</td>
</tr>
<tr>
<td>Randall and Wahrhaftig (7)</td>
<td>1978</td>
<td>Coupling of SFC to MS</td>
</tr>
<tr>
<td>Novotny and Lee (8)</td>
<td>1981</td>
<td>Invention of capillary SFC</td>
</tr>
<tr>
<td></td>
<td>1982 onwards</td>
<td>Commercial SFC chromatographs</td>
</tr>
</tbody>
</table>

![Phase diagram for supercritical CO\(_2\)](image)

**FIGURE 1:** Phase diagram for supercritical CO\(_2\), showing the critical temperatures and pressures, above which the phase of the CO\(_2\) is supercritical.

![Effect of efficiency (N), selectivity (\(\alpha\)) and retention factor (\(k\)) on resolution for fixed values of 5000 for N, 1.05 for \(\alpha\) and 5 for \(k\) (13,14).](image)

**FIGURE 2:** Effect of efficiency (\(N\)), selectivity (\(\alpha\)) and retention factor (\(k\)) on resolution for fixed values of 5000 for \(N\), 1.05 for \(\alpha\) and 5 for \(k\) (13,14).
The selectivity ($\alpha$) of the separation is the ratio of the retention factors of two adjacent peaks (equation 4) and a measure of how well the peaks are separated. Higher values of $\alpha$ indicate that the peaks are well separated and the value approaches 1 as the two peaks are coeluted. Separation selectivity can be adjusted in several ways, including (but not limited to) changing the stationary phase, using a different organic modifier, and changing the pH.

$$\alpha = \frac{k_2}{k_1} \tag{4}$$

The impact of each of these three variables on resolution can be assessed by fixing two parameters and varying the third, as demonstrated in Figure 2. In this case, fixed values of 5000 for column efficiency, 5 for retention factor and 1.05 for selectivity were used. In this case, selectivity is clearly the most powerful variable that affects resolution for this combination of values. This is often the case for typical LC separations and is the reason that it is critical to optimize separation selectivity during method development. Many chromatographic variables (mobile phase, temperature, instrument and column parameters) can affect selectivity, one of the most powerful is column stationary phase chemistry. Exploring column selectivity and identifying the optimal stationary phase chemistry early in method development often provides an efficient route for separating the analytes of interest.

Selectivity in SFC

One of the controlling elution parameters within SFC is the density of the mobile phase, which is affected by the pressure. Indeed, pressure, when pure CO$_2$ is used as the mobile phase, is a key parameter affecting both retention time and the separation selectivity. The relationship when dealing with a single mobile phase is straightforward, as increasing the pressure increases the density, resulting in the lowering of the retention factor. The amount of variation with respect to the density is quite large when dealing with carbon dioxide due to the compressibility of pure mobile phase, meaning that the changes in retention time can be significant. Thus, performing a pressure gradient when using pure carbon dioxide will drive a separation (15).

The situation becomes much more complicated when an organic modifier is incorporated in the mobile phase. The concentration of the organic modifier will determine how the compressibility is impacted. At higher modifier concentrations, the increase in density will not be as large as that observed at lower concentrations; therefore, the importance of pressure as a variable diminishes. If the...
Experimentally determined - Separation of 10 steroids under (a) SFC and (b) RPLC conditions. (a) SFC conditions: silica column (5 μm, 150 x 4.6 mm); mobile phase A: CO₂; B: methanol, gradient: 5-50% B in 5 min; flow rate: 4 mL/min; temperature: 40 °C; BPR pressure: 150 bar. (b) RPLC conditions: C18 column (3 μm, 50 x 2.1 mm); mobile phase A: water, B: acetonitrile, gradient: 20-60% B in 6 min; flow rate: 0.5 mL/min; temperature: 22 °C.

FIGURE 3: Separation of 10 steroids under (a) SFC and (b) RPLC conditions. (a) SFC conditions: silica column (5 μm, 150 x 4.6 mm); mobile phase A: CO₂; B: methanol, gradient: 5-50% B in 5 min; flow rate: 4 mL/min; temperature: 40 °C; BPR pressure: 150 bar. (b) RPLC conditions: C18 column (3 μm, 50 x 2.1 mm); mobile phase A: water, B: acetonitrile, gradient: 20-60% B in 6 min; flow rate: 0.5 mL/min; temperature: 22 °C.

TABLE II: Experimentally determined S-values for five different stationary phase (SP) chemistries. S-values were derived from the retention times of 41 diverse analytes (acidic, basic, neutral and phenolic) run on a 5-min gradient (3-100% B) on 50 x 2.1 mm columns. Columns were from the same manufacturer with different stationary phase chemistries bonded to the same base silica. The mobile phases were A: 20 mM ammonium formate pH 3.0 (aq), B: 20 mM ammonium formate pH 3.0 in methanol:water (9:1 v/v); flow rate: 0.4 mL/min; temperature: 40 °C; injection volume: 0.6 μL and detection: UV, 214 or 254 nm.

<table>
<thead>
<tr>
<th>SP Chemistry</th>
<th>C18</th>
<th>C18-AR</th>
<th>C18-PFP</th>
<th>C18-Amide</th>
<th>CN-ES</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18</td>
<td>0</td>
<td>19</td>
<td>15</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>C18-AR</td>
<td>0</td>
<td>15</td>
<td>28</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>C18-PFP</td>
<td>0</td>
<td>19</td>
<td></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>C18-Amide</td>
<td>0</td>
<td></td>
<td>0</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>CN-ES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Organic modifier composition and the pressure are both varied during a chromatographic run, then a very complex relationship will exist between the selectivity and retention time. Overall, pressure can be used to fine tune a separation, but due to the complex relationship with the organic modifier, it is typically not recommended to be used as an experimental parameter.

Another factor to consider with the pressure is the pressure drop across the column and ensuring that:
- there is no phase transition occurring within the column;
- an understanding of the impact of reducing the pressure will have on the local temperature;
- when dealing with small particles (sub 2 μm), a consideration of the frictional heating effects.

The organic modifier typically has the greatest impact on the retention and selectivity of the chromatographic process. In this respect it has a similar mechanism to that observed with reversed-phase LC (RPLC), with an increasing amount of organic modifier resulting in an increase in the elution strength of the mobile phase. A range of different modifiers can be used, with the most popular ones being:
- Methanol
- Acetonitrile
- Ethanol
- Isopropanol (IPA)
- Butanol
- Water
- Tetrahydrofuran (THF)
- Dimethylsulfoxide (DMSO)

Of these, methanol is the most common modifier. It is important when choosing the modifier that the solubility between the bulk mobile phase (typically carbon dioxide) and the modifier are considered.

As well as modifiers, pH adjusters and buffers can also be added to the mobile phase such as:
- Trifluoroacetic acid (TFA)
- Diethylamine (DEA)
- Dimethylethanolamine (DMEA)
- Triethylamine (TEA)
- Ammonia (NH₃)
- Acetate buffers

The latter work in the same manner as when used in HPLC, adjusting the nature (acid/base) of either the stationary phase or the compound, or both.

Both packed and open-tubular capillary columns can be used in SFC. While manufacturers are moving away from open tubular, there is a possibility of increased efficiency when using this approach compared to packed columns. Less efficient, packed columns are used for less complex mixtures, as they allow shorter analysis times and much higher loadability. Typical column characteristics are 30 to 250 mm length, 2.0 to 4.6 mm I.D., and 5-6 μm particle size. Currently, packed columns are commonly used, since the driver for using SFC is the selectivity and not efficiency. SFC is also used substantially in the field of preparative chromatography.

A wide range of achiral and chiral stationary phases (CSP) can be used in SFC, most of which are silica-based, though polysaccharide, zirconia, polystyrene, divinylbenzene, and porous graphitic carbon-based packings have been used. Most columns used in SFC were first designed for HPLC.
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Silica - D S - 51 2-Ethylpyridine 77 - 2 Cyano 0 - 59 - R 0 0 -

is almost entirely inverted. Such analytes, but the retention order is very different selectivity for these SFC and RPLC not only provide separations generated using these two techniques shown in Figure 3.

mobile phase. As such, SFC provides orthogonal and complementary selectivity to RPLC. This is visually demonstrated by the steroid selectivity to RPLC. This is visually demonstrated by the steroid selectivity to RPLC. This is visually demonstrated by the steroid selectivity to RPLC. This is visually demonstrated by the steroid selectivity to RPLC.

TABLE II: Selectivity values generated using 0.1% ammonium hydroxide in methanol (v/v) as the organic modifier.

<table>
<thead>
<tr>
<th>SFC SP</th>
<th>Silica</th>
<th>Cyano</th>
<th>2-Ethylpyridine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica</td>
<td>0</td>
<td>51</td>
<td>77</td>
</tr>
<tr>
<td>Cyano</td>
<td>0</td>
<td></td>
<td>59</td>
</tr>
<tr>
<td>2-Ethylpyridine</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SFC is often considered a normal-phase technique, involving a polar stationary phase and a non-polar mobile phase. As such, SFC provides orthogonal and complementary selectivity to RPLC. This is visually demonstrated by the steroid selectivity to RPLC. This is visually demonstrated by the steroid selectivity to RPLC.

SFC and RPLC not only provide very different selectivity for these analytes, but the retention order is almost entirely inverted. Such dramatic changes in retention and selectivity between the two techniques are highly desirable, and the observed complementary is valuable in areas such as drug discovery. The choice of stationary phases is huge, and listing all of them is not practical. Common examples of achiral polar stationary phases include bare silica, diol, cyanopropyl (CN), aminopropyl, 2-pyridylpropyl urea and one that has been specifically developed for SFC: 2-ethylpyridine (2-EP). Non-polar HPLC stationary phases such as the very popular octadecylsilane packing (C18) have also been used in SFC.

Quantifying Stationary Phase Selectivity

Various empirical approaches for characterizing LC stationary phases have been developed over the years (LSER, Product Quality Research Institute (PQRI), NIST 870, Uni Leuven, Tanaka). All approaches assess column selectivity by comparing the retention of well-defined analytes under specific conditions. For example, the well-established Tanaka approach (16) and extended modifications (17–19), assess hydrophobic, aromatic, phenolic, and shape and steric selectivity, along with ion-exchange capacity, hydrogen bonding capacity, and dipole-dipole interactions. In the linear solvation energy relationships (LSER) approach, retention factors for selected analytes are correlated with specific molecular properties (molar refraction, dipolarity, polarizability, hydrogen bonding acidity and basicity, and McGowan characteristic volume) to derive a characteristic set of stationary phase specific coefficients (20). These approaches have been thoroughly evaluated and applied to develop databases of stationary phases for column selection purposes.

A convenient assessment of the selectivity difference between two stationary phases can be derived from selectivity correlations, according to the Neue selectivity approach (21), where gradient retention times of a set of compounds run on different columns or mobile phase conditions are correlated.

The coefficient of determination ($R^2$) is used to define a selectivity correlation value (S-value), according to equation 5. Table II shows S-values generated using retention data for an in-house set of 41 analytes for five LC stationary phases bonded onto the same base silica. A value of 0 indicates identical selectivity, while low S-values of

FIGURE 4: Separation of an eight-component test mixture on the three stationary phases under identical gradient conditions: (a) silica, (b) cyano, (c) 2-ethylpyridine. Columns: 150 x 4.6 mm, 5 µm, mobile phases: A: CO₂, B: methanol, gradient: 10 to 55% B in 5 min, flow rate: 4 mL/min, BPR pressure: 150 bar, injection volume: 5 µL, temperature: 40 °C, detection: UV, 254 nm. Sample: 1. theophylline, 2. caffeine, 3. cortisone, 4. prednisone, 5. hydrocortisone, 6. prednisolone, 7. sulfaquinoxaline, 8. sulfamerazine.

FIGURE 5: PCA Score plot acquired for all test compounds (logD_{o/w}, pH 3.4). The spread and lack of clumping of the data demonstrates the applicability of the test compounds.

TABLE III: Selectivity values determined for the three prototype SFC stationary phases (SP) using 0.1% ammonium hydroxide in methanol (v/v) as the organic modifier.

<table>
<thead>
<tr>
<th>SFC SP</th>
<th>Silica</th>
<th>Cyano</th>
<th>2-Ethylpyridine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica</td>
<td>0</td>
<td>51</td>
<td>77</td>
</tr>
<tr>
<td>Cyano</td>
<td>0</td>
<td></td>
<td>59</td>
</tr>
<tr>
<td>2-Ethylpyridine</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
approximately 6–8 indicate the two columns are similar with small observable selectivity differences. A value of 100 would denote complete orthogonality, while intermediate values demonstrate that the stationary phases are complementary, with substantially different selectivity. The S-values show that the five stationary phases offer different, yet complementary, selectivity to one another. For analytes with different structural properties, significantly different separation selectivity can be expected on the different phases.

\[ S = 100 \times (1-R^2) \]  

Assessing the Selectivity of SFC Stationary Phases

The aim of this study was to assess and measure the selectivity differences afforded by three prototype SFC phases, prepared in-house, including bare silica, CN, and 2-EP chemistries. Column stationary phase classification is not as well established in SFC as it is in RPLC. An LSER-based approach has however been extensively used for characterization by West and Lesellier and associates (22). According to these authors, these three phases are all characterized as polar SFC phases. Unbonded silica phases can establish dipole-dipole interactions with analytes, and show both hydrogen bond donor and acceptor characteristics, while dispersive interactions are not favorable (23). Comparison to bare silica phases suggests that CN phase hydrogen bonding characteristics are lower, with the ligand being a better acceptor than donor, and showing greater dipole character (22). The 2-EP phase shows enhanced \( \pi-\pi \) and dipole-induced dipole interactions and enhanced hydrogen bond acceptor characteristics, while dispersive interactions are slightly higher than those for the other phases (24). Interpreting analyte retention and selectivity in SFC can be challenging. The exact nature of retention mechanisms and the impact of additives is complex, involving a multitude of molecular interactions that can make deconvoluting retention behavior difficult (25). Work by several authors (26–28) has suggested that organic modifiers and additives, including water if present, can be adsorbed on the stationary phase surface, in an analogous manner to HILIC, to create a pseudostationary phase (29). The adsorbed mobile phase components will effectively modify the stationary phase surface, impacting analyte retention (9). Analyte retention, therefore, may be a combination of partitioning into the adsorbed layer and adsorption onto the stationary phase itself, and it may be somewhat ambiguous as to whether the stationary phase surface itself, or the nature of the adsorbed pseudo-layer, drives analyte retention.

Figure 4 illustrates an initial assessment of selectivity differences offered by these stationary phases, provided by injecting a standard test mixture containing eight analytes, including four corticosteroids (sample analytes 3–6), two xanthines (sample analytes 1–2) and two sulfonamides (sample analytes 7–8) on three phases, using identical gradient conditions. The corticosteroids contain complementary pairs of analytes, the cortisone/hydrocortisone and prednisone/prednisolone pairs differing by reduction of the \( C_{11} \) carbonyl to a hydroxyl group. Additionally, prednisone/cortisone and prednisolone/hydrocortisone differ by a carbon-carbon double bond at \( C_1 \).

The xanthines are polar neutral hydrogen bond acceptors, although theophylline contains an additional hydrogen bond donor group. The two sulfonamides are very similar structurally, with sulfaquinoxaline containing an additional aromatic ring.

The 2-EP phase generally showed stronger retention than the other two phases, and provided full separation of the eight components, except for prednisone and cortisone. The silica and CN phases produced somewhat similar retention, although, notably, the silica phase provided \( >80\% \) increase in retention of the xanthines. Increased xanthine retention is likely due to increased interaction of these hydrogen bond acceptors with surface silanols or adsorbed methanol on the silica surface, which could be somewhat reduced on the bonded CN phase. The 2-EP phase provided excellent separation of caffeine and theophylline, whereas the silica and CN phases did not resolve these components.

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2-EP phases show greater hydrogen bond acceptor characteristics than silica or CN phases (22,24,30), and are therefore likely to show affinity towards the hydrogen bond donor group of theophylline, which drives this improved separation.

For the corticosteroids, the silica phase could resolve cortisol and prednisone. These two components differ by a single C-C double bond, although the reason why a similar increase in resolution between peaks 5 and 6, which differ by the identical substitution, is not observed remains unclear.

The CN phase showed a reversal in elution order of peaks 7 and 8, presumably due to enhanced dipole interaction between the CN phase and sulfaquinoxaline. The 2-EP phase appears to provide extra π-π aromatic interaction and enhanced retention for these two components, with the additional aromatic ring in sulfaquinoxaline giving further increased interaction and a much superior separation. This application demonstrates that the stationary phase selected can impart significantly different separation selectivity.

To further assess the selectivity of these stationary phases, the Neue approach to characterization was adapted. The same compounds used to characterize reversed-phase columns could not be utilized, due to low analyte solubility in SFC compatible diluents; therefore, an alternative set of analytes was assessed. A total of 48 compounds, comprising of 11 neutrals of varying polarity, 11 heterocyclics (purines, nucleosides, and nucleotides), 7 acids, 9 bases, 3 diuretics, 2 sulfonamides, and 5 steroids were chromatographed using a 5-min gradient.

Principal component analysis (PCA) is a statistical manipulation of a set of data to allow identification of key variables. This is achieved by initially standardizing the range of each variable, so that a comparable scale is used for all and then developing a covariance matrix, which allows for an understanding of how the variables of the data input set deviate from the mean with respect to each other. For the latter part, if two variables are highly related, then only one may be needed...
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to explain the output data set. Finally, the eigenvectors and eigenvalues are calculated to determine the principal components.

Initially, this approach was used to test the validity of the compounds used to test the columns, ensuring that there was no redundancy in the choice of test compounds. Analyte molecular descriptors (Abraham descriptors, logD<sub>ow</sub>, pH 3.4 and 10.8, acid, and basic pK<sub>a</sub>) were calculated using ACD/ Labs Percepta software and PCA analysis performed using Sartorius SIMCA software. Figure 5 shows a plot of the resulting PCA. It can be clearly seen that the individual components are well segregated, and cover a large relative domain of the available parameter space, meaning that, in this scenario, the choice of compounds for profiling the compounds is a good one.

Initially, a binary CO<sub>2</sub>-methanol eluent system was used; however, poor peak shapes and excessive retention for basic analytes on the polar silica and CN stationary phases were observed. Additives (typically acids, bases, or salts) are often introduced into the organic modifier at typical concentrations of 0.1-1% to improve solubility and limit secondary interactions with the stationary phase surface (9,25,31,32). The addition of 10 mM ammonium formate was found to be highly beneficial, providing good peak shape and retention of bases. Neutral analytes showed poor retention on all three phases, while the polar neutral components showed only marginally better retention. The acids, bases, diuretics, and heterocycles were generally well retained on all three phases, with a broad spread of retention times. Although the steroids are neutral and moderately hydrophobic, they showed good interaction with the stationary phases to provide reasonable retention under SFC conditions, demonstrating the applicability of SFC for such compounds (33,34).

A few general points can be made regarding the absolute retention of the various compound classes. First, the 2-EP phase was overall the most retentive for most analytes. This phase showed a notably stronger affinity for acids than either the CN or silica phases, presumably due to partial negative charge on acidic components and a partial positive charge on the pyridine moiety of the stationary phase ligand. The silica and CN phases showed stronger retention of bases compared to the 2-EP, indicating a degree of silanol acidity on the surface of these phases, or electrostatic repulsion and reduced retention on the 2-EP phase. Comparing the silica and CN phases, heterocyclic and basic analytes showed greater retention on the silica phase, while the remainder showed somewhat similar retention on both phases.

From this data, retention time plots were generated for each column pair (Figure 6) and, in turn, used to calculate the corresponding correlation coefficients and S-values. The values obtained on these three stationary phases were strikingly higher than those observed for the reversed-phase stationary phases in Table II, indicating a large degree of complementary selectivity between the phases for this set of compounds. While the silica and CN phases gave the lowest overall retention, they still clearly demonstrated different selectivity to one another (S = 51). Correlations that include the 2-EP phase produced the highest S-values, indicating the unique selectivity of this SFC phase. This data overall confirms that in SFC, the stationary phase chemistry can impart a powerful effect on separation selectivity.

In RPLC, large differences in S-values (>80) may be observed for a single stationary phase when comparing acidic and basic mobile phases, especially when ionizable analytes are present (35). Mobile phase pH determines the ionization state (and hence polarity) of such analytes, and therefore can substantially affect analyte retention and selectivity. The same set of compounds were run using 0.1% (9 mM) ammonium hydroxide as the additive, to a) establish whether the interphase selectivity difference is still apparent when using different additives; and (b) to assess whether this additive can impart different separation selectivity. High, almost identical S-values were obtained (Table III), indicating a similar degree of complementary selectivity between the stationary phase chemistries is maintained with the basic additive. On the silica phase, a general increase in retention was observed for most analytes, except for the neutral and polar neutral compounds, which showed minimal change. The basic and heterocyclic analytes showed retention increases of 20–30%, while the steroids showed more modest increases of around 15%. Notably, acidic analytes showed a large increase in retention, in many cases between 45–50%. If ammonium hydroxide increased the apparent pH of the mobile phase, it may be anticipated that increased ionization of surface silanol groups should result in electrostatic repulsion of negatively charged acidic analytes, therefore decreasing their retention. The fact that the opposite was observed suggests that a more favorable partitioning of polar analytes into an adsorbed polar surface layer of mobile phase or additive is occurring. In recent work, West proposed that the presence of salt and water as additives increase the thickness of the adsorbed layer of mobile phase components on the stationary phase surface for a silica phase (29). In the current study, aqueous ammonium hydroxide solution was used to prepare the mobile phase, therefore introducing a small fraction of water into the mobile phase. This may potentially be providing a thicker adsorbed layer, leading to increased retention of these polar analytes, via an enhanced capacity for analyte partitioning into this pseudo-layer.

Similar trends were observed on the CN phase, although the magnitude of the retention increase was substantially reduced. Interestingly, the basic compounds showed
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no retention increase on the CN phase, indeed many showed a small decrease. In contrast, the 2-EP phase showed minimal changes in retention time for all analytes under the two sets of conditions, with all but adenine, salicylic acid and 4-hydroxyphenylacetic acid showing less than +/- 5% change.

The selectivity data can also be used to examine the selectivity difference between the two mobile phase additives on each stationary phase (Figure 7). Substantially lower selectivity values were obtained compared to those correlating the different stationary phases in Figure 6 and Table III. As discussed, the 2-EP phase showed little difference in retention time for any analytes between the two additives; therefore, it is unsurprising that the S-value for this comparison is just 6, indicating little change in selectivity when changing the additive. On the silica and CN phases, higher S-values were determined between the two additives, indicating that some selectivity difference is apparent, although the magnitude is far less significant than that obtained between stationary phase chemistries.

The low S-values obtained on all three stationary phases when comparing the two additives implies that under the selected SFC conditions, analyte ionization state was not affected in the same way as in RP conditions upon moving to a more basic mobile phase additive. This agrees with recently published studies by West and associates, regarding the apparent pH of CO$_2$-methanol mobile phase systems (29,32). The authors estimated an apparent pH of around aqueous pH 5, with increasing methanol composition shifting this perhaps lower, due to methoxycarboxonic acid formation. Notably, they concluded that the addition of salts or basic additives seemed to have little effect on the apparent pH of the mobile phase system, possibly due to titration with methoxycarboxonic acid. As a result, changes in retention for ionizable analytes, due to changes in ionization state, were not observed here. Additionally, Ovchinnikov and associates observed close similarity when using both diethylamine and ammonium acetate as additives. They proposed that titration of the amine additive in the acidic CO$_2$:methanol media results in formation of the corresponding methylcarbamate salt, hence both additives are present in the mobile phase as ammonium salts (25). The data for the 2-EP phase is also interesting, as the predicted pK$_a$ of the pyridine nitrogen atom of the stationary phase ligand is 5.55. Therefore, assuming the apparent pH of the SFC conditions is approximately 5, the pyridine ring should be partially ionized, and the overall ionization state highly sensitive to any change in apparent pH. The fact that the retention times obtained using both additives were almost identical on this stationary phase therefore seems to add further weight to the argument that salt and basic additives appear to have little impact on the apparent pH of the mobile phase in CO$_2$:methanol systems. The effect on the silica and CN phases are perhaps more difficult to interpret. Clearly, particularly for the silica phase, switching to ammonium hydroxide does appear to alter the stationary phase surface, primarily from a retention perspective, rather than substantially altering the selectivity. At the pH values discussed above, residual silanols on the stationary phase surface could be partially anionic in character, and again potentially sensitive to changes in apparent pH (31). It is worth noting that, although highly acidic mobile phases were not assessed during the current study, acidification of ammonium formate with formic acid did not yield appreciably different S-values when tested on the CN phase. The use of a pure acidic additive (such as formic, acetic, or trifluoroacetic acid) to affect selectivity may warrant investigation, as such additives have been suggested to provide acidic conditions close to aqueous pH 1 (32).

Overall, the data presented in this study demonstrate that substantial changes in separation selectivity can be expected with different SFC stationary phases. An assessment of different stationary phase chemistries during method development, an approach which is widely used for other chromatographic modes, would therefore be valuable. Additionally, it appears that the selectivity difference afforded by the two different additives tested was substantially lower on the silica and CN phases, and insignificant on the 2-EP phase.

Conclusions

This study aimed to assess the degree of selectivity difference offered by three prototype SFC phase materials, namely silica, CN, and 2-EP. Clear selectivity differences were observed on the three phases. A modified NeNe selectivity approach was used to quantify the selectivity differences observed. Selectivity plots generated from the retention times of 48 analytes, with differing physicochemical properties using methanol and 10 mM ammonium formate as the organic modifier and additive, produced selectivity values substantially higher than typically observed between reversed-phase stationary phases. Replacing ammonium formate with a basic additive (ammonium hydroxide), provided similar selectivity differences between the three phases. However, comparisons between data for ammonium formate and ammonium hydroxide on the same stationary phase revealed much lower selectivity differences on the silica and CN and insignificant differences on the 2-EP phase. Stationary phase chemistry, therefore, appears to be a powerful tool for optimizing the selectivity of SFC separations. From the current study, the choice of additive has significant impact on retention, but a less substantial impact on selectivity.
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Developing a Fast Purification Method for a Natural Product with a Preparative LC Column Packed with Superficially Porous Particles

Lakshmi Subbarao

A fast and simple purification method for withaferin A (WFA) in ashwagandha extract was successfully developed using a preparative column packed with superficially porous particles (SPPs). The SPP column provided better resolution and a 45% decrease in run time over a traditional preparative column packed with totally porous particles. The performance benefits of SPP preparative columns, especially at high flow rates, align well with the needs of pharmaceutical discovery laboratories and other high-throughput environments.

Superficially porous particle (SPP) chromatography columns are a popular choice for analytical method development. Columns packed with SPPs have higher efficiency and lower back pressure than their totally porous particle (TPP) counterparts. Figure 1 compares the two particle types. A SPP consists of a solid silica core surrounded by a porous silica outer layer. The solid core prevents the analyte from traveling too far into the particle, shortening its diffusion path and resulting in a narrower chromatographic peak.

Using a van Deemter curve provides a simple way to compare the efficiency of SPP and TPP columns, where an isocratic separation of a void volume marker and analyte mixture is run across a wide range of flow rates. The efficiency, or plate number \( N \), of the analyte is calculated using equation 1, where \( t_r \) is the analyte retention time and \( w_{1/2} \) is the peak width at half height.

\[
N = 5.54 \left( \frac{t_r}{w_{1/2}} \right)^2 \quad [1]
\]

From \( N \), the plate height \( H \) can be calculated using equation 2, where \( L \) is the length of the column.

\[
H = \frac{L}{N} \quad [2]
\]

\( H \) is plotted against linear velocity \( (u) \), which is calculated from the retention time of the void volume marker \( (t_0) \) using equation 3.

\[
u = L/t_0 \quad [3]
\]

The van Deemter curve for a 4 µm SPP column and a 5 µm TPP column of the same column dimension are shown in Figure 2.

Keeping in mind that \( H \) is inversely proportional to \( N \), the curve with smaller \( H \) values has better performance. Looking at the curves, it is obvious that the 4 µm column performs better than its 5 µm counterpart. The linear velocity at the minimum of each curve \( (u_{\text{opt}}) \) corresponds to the flow rate at which the column will have the highest efficiency. The SPP column had a \( u_{\text{opt}} \) is 150% faster than the TPP column, which means that the SPP column can provide a better separation in less time than the TPP column.

Next, the data can be integrated in the van Deemter equation to calculate the \( A, B, \) and \( C \) terms (equation 4).

\[
H = A + B/u + Cu \quad [4]
\]

These constants represent the main contributions to band broadening. The impacts of the \( A \) term (eddy diffusion) and the \( B \) term (longitudinal diffusion) are significant at low linear velocities (<0.5 mm/s) and are discussed in the literature (2). At normal or above normal chromatographic linear velocities (>1.5 mm/s), the \( C \) term (resistance to mass transfer) is the main contributor to band broadening. For a TPP column, the long diffusion path of an analyte within the particle causes band broadening. The resistance to mass transfer only gets worse as the velocity increases. In contrast, the porous layer in an SPP column is much shorter. As a result, \( H \) is considerably lower for the SPP column, and the slope of the van Deemter curve at high flow rates (>3 mm/s) is shallower than it is for the TPP column. A lower \( H \) value allows the user to run SPP columns at higher flow rates without experiencing a significant decrease in performance.

Pharmaceutical discovery laboratories and other high-throughput environments regularly utilize preparative chromatography to purify large batches of samples. They require small amounts (10–100 mg) of high purity fractions for downstream workup and characterization. Given the fast-paced nature of these environments, minimal time can be spent on method development. Therefore, these customers could benefit from SPP columns that provide high resolution and throughput.
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SPP columns have not been widely adopted in preparative LC for several reasons. Most analytical instrumentation has been designed to minimize system volume and exceed pressures of 400 bar, whereas most preparative instrumentation has large system volume to prevent pump overpressure. Using smaller-sized SPPs in a preparative column could cause excessive pressure, and a large system volume could negate the chromatographic benefits. These concerns can be mitigated by using preparative columns packed with 4 µm SPPs. This particle size is large enough to operate well within the pressure range of traditional preparative instruments without significant band broadening while still performing better than traditional TPP preparative columns, especially at higher flow rates.

The purification of bioactive components in natural products represents a similar challenge to that of drug candidates in crude mixtures—both have complicated matrices. Withania somnifera (L.) Dunal, well known as ashwagandha, is a plant that contains many withanolides, which are natural steroids. Withaferin A (WFA) is the most bioactive withanolide in ashwagandha. This study focuses on the efficacy of a generic purification method for WFA on both a 4 µm SPP and traditional 5 µm TPP column at standard and elevated flow rates.

**Experimental Instrumentation**

All work was performed on an Agilent 1290 Infinity II autoscale preparative liquid chromatography (LC) system.

**Columns and Supplies**

Purification methods were developed on two columns. The SPP column was an Agilent InfinityLab Poroshell 120 SB-C18, 21.2 × 150 mm, 4 µm preparative LC column, acquired from Agilent Technologies. The TPP column was a traditional C18, 19 × 150 mm, 5 µm preparative LC column acquired from Neta Scientific. The ashwagandha extract in 2:1 ethanol:water (100 mg/mL) was purchased from Banyan Botanicals. The WFA standard was purchased from Sigma Aldrich. LC grade solvents were acquired from Burdick and Jackson. The extract was filtered using Agilent Captiva premium syringe filter (0.2 µm).

**Gradient Separation at Optimal Flow Rate**

The optimal flow rate for each column was determined by its respective van Deemter curve (not shown). A previously published analytical method was used to select the mobile phase (3). A generic gradient was run on both columns, adjusting the gradient time to keep the number of column volumes in the gradient time to keep the number of column volumes in the gradient.

**TABLE I: Summary of data acquisition parameters at optimal flow rate**

<table>
<thead>
<tr>
<th>Column</th>
<th>Flow Rate</th>
<th>Mobile Phase</th>
<th>Gradient</th>
<th>Injection Volume</th>
<th>Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPP C18, 21.2 × 150 mm, 4 µm</td>
<td>25 mL/min</td>
<td>A: Acetonitrile + 0.1% formic acid</td>
<td>5–95% B in 15 min</td>
<td>1,000 µL</td>
<td>220 nm</td>
</tr>
<tr>
<td>TPP C18, 19 × 150 mm, 5 µm</td>
<td>17 mL/min</td>
<td>B: Water + 0.1% formic acid</td>
<td>5–95% B in 18 min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE II: Summary of data acquisition parameters at elevated flow rate**

<table>
<thead>
<tr>
<th>Column</th>
<th>Flow Rate</th>
<th>Mobile Phase</th>
<th>Gradient</th>
<th>Injection Volume</th>
<th>Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPP C18, 21.2 × 150 mm, 4 µm</td>
<td>37.5 mL/min</td>
<td>A: Acetonitrile + 0.1% formic acid</td>
<td>5–95% B in 10 min</td>
<td>1,000 µL</td>
<td>220 nm</td>
</tr>
<tr>
<td>TPP C18, 19 × 150 mm, 5 µm</td>
<td>25.5 mL/min</td>
<td>B: Water + 0.1% formic acid</td>
<td>5–95% B in 12 min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 1:** Analyte diffusion path for a totally porous particle (TPP) versus a superficially porous particle (SPP).

**FIGURE 2:** van Deemter plots of 4 µm SPP and 5 µm TPP columns.
Gradient Separation at Elevated Flow Rate

Each column was run at 50% above its optimal flow rate. The gradient time was adjusted to keep the number of column volumes in the gradient consistent. A summary of run conditions is shown in Table II.

Results and Discussion

Figure 3 shows a comparison of both columns run at their respective optimal flows. The total mass on each column was 100 mg. The WFA peak eluted at 8.59 min on the SPP column and at 9.66 min on the TPP column. Both columns provided separation between the WFA peak and the adjacent impurities. The impurities had baseline separation with WFA on the SPP column. However, on the TPP column, one of the impurities appeared as a shoulder of the WFA peak.

When the flow was increased by 50% (Figure 4), the SPP column maintained separation between WFA ($t_R = 5.77$ min) and its impurities. On the TPP column, the leading impurity partially coeluted with WFA ($t_R = 6.47$ min), and the trailing impurity completely coeluted with the target compound.

The TPP column was only able to separate impurities at its optimal flow rate, which was an 18-min gradient. Meanwhile, the faster gradient on the SPP column was able to separate all peaks in 10 min, reducing the run time by 45%.

Conclusion

The SPP preparative LC column successfully separated WFA from adjacent impurities with minimal method development. When run at 1.5 times the optimal flow, the SPP column maintained the separation, whereas the TPP column had significant coelution. The faster SPP method resulted in a 45% decrease in run time over the TPP method at standard flow. Therefore, this study shows that the speed and loading ability of the 4 µm SPP preparative LC columns are well suited to the needs of high-throughput discovery laboratories.

References


Lakshmi Subbarao has over 15 years of experience in the chromatography industry, including analysis and purification of pharmaceutical and natural products. She has provided training and consultation services to Fortune 100 pharmaceutical companies and her work has been published in many industry magazines. She received a Bachelor of Science degree in Chemistry from the University of Delaware and a Master’s degree in Analytical Chemistry from Illinois Institute of Technology. Lakshmi is currently a Senior Applications Scientist at Agilent Technologies. Direct correspondence to: lakshmi.subbarao@agilent.com
The need for high pressure tolerance in high performance liquid chromatography (HPLC) instruments and columns has led to the widespread use of metal components, most commonly made from stainless steel. However, some analytes interact with the oxide layer present on the metal surfaces in the flow path of HPLC systems, resulting in a range of deleterious effects from peak broadening and tailing, low peak areas, and the formation of new peaks due to chemical reactions. To mitigate these effects, we have developed a novel surface modification technology in which a hybrid organic/inorganic surface based on an ethylene-bridged siloxane chemistry is applied to the metal components in HPLC instruments and columns. We demonstrate the impact of this technology on peak symmetry, peak area, and injection-to-injection and column-to-column reproducibility for several metal-sensitive analytes. We also show an example of the mitigation of an on-column oxidation reaction. A variant of this technology has recently been developed for size-exclusion chromatography of proteins. An example is shown demonstrating the use of this variant applied to size-exclusion columns for the separation of a monoclonal antibody monomer and higher molecular weight species. Together, these results highlight the importance of preventing interactions of analytes with metal surfaces in HPLC in order to achieve accurate and precise results.

Interactions of certain analytes with metal surfaces in high performance liquid chromatography (HPLC) instruments and columns cause a range of deleterious effects, including peak broadening and tailing, low peak areas, and the formation of new peaks due to chemical reactions. To mitigate these effects, we have developed a novel surface modification technology in which a hybrid organic/inorganic surface based on an ethylene-bridged siloxane chemistry is applied to the metal components in HPLC instruments and columns. We demonstrate the impact of this technology on peak symmetry, peak area, and injection-to-injection and column-to-column reproducibility for several metal-sensitive analytes. We also show an example of the mitigation of an on-column oxidation reaction. A variant of this technology has recently been developed for size-exclusion chromatography of proteins. An example is shown demonstrating the use of this variant applied to size-exclusion columns for the separation of a monoclonal antibody monomer and higher molecular weight species. Together, these results highlight the importance of preventing interactions of analytes with metal surfaces in HPLC in order to achieve accurate and precise results.

Modifying the Metal Surfaces in HPLC Systems and Columns to Prevent Analyte Adsorption and Other Deleterious Effects


Polyether ether ketone (PEEK) is another material that has been used in place of stainless steel in HPLC.
columns and systems. While this engineering plastic has been used with pressures below about 5000 psi, it lacks the mechanical strength to tolerate higher pressures unless cladded with steel. In addition, PEEK is not recommended for use with some solvents, notably tetrahydrofuran, dimethyl sulfoxide, chloroform, and methylene chloride. PEEK is also relatively hydrophobic, and may cause analyte adsorption due to hydrophobic interactions (25,26). The internal diameter of PEEK tubing is also more variable than that of metal tubing, which leads to greater column-to-column retention time variability for PEEK-lined steel columns (27).

An alternative approach to mitigating these interactions is to add reagents to the mobile phase or the sample that block the binding sites. Examples include phosphate buffers and chelators, such as ethylenediaminetetraacetic acid (EDTA), citrate, acetylacetone, and medronic acid (1,5,7,10,28–31). However, phosphate buffers and EDTA are not volatile, making them incompatible with mass spectrometry detection. Even when using volatile additives with MS detection, ion suppression may occur (30,31). In addition, long equilibration times are often associated with the use of these types of additives (32). It is also known that chelators such as EDTA and citric acid increase the rate of corrosion of stainless steel (14).

In 2020, a new solution to the problems caused by metal surfaces was introduced, obviating the need for chelators in the mobile phase (33). Using a proprietary process, the metal components of the HPLC flow path and column were treated to form a hybrid organic/inorganic barrier covering the oxide layer on the metal surfaces (16). This process was termed hybrid surface technology (HST). A composition that is similar to that of the particles used in ethylene-bridged hybrid inorganic–organic stationary phases (34) was shown to be stable over a wide pH range (from 1–12) (33). This composition is well-suited for reversed-phase chromatography, as demonstrated for separations of oligonucleotides (16,23,35–37), acidic and phosphorylated peptides (38,39), certain small molecule pharmaceuticals (23,40,41), veterinary drugs (42), vitamins (43), and phospholipids (44). This composition has also been shown to be beneficial for mixed-mode reversed-phase/anion-exchange separations of tricarboxylic acid cycle metabolites (17) and acidic glycans (45), as well as hydrophilic-interaction chromatography (HILIC) separations of nucleotides and other cellular metabolites (46–48). Most recently, a variant of this composition termed hydrophilic hybrid surface technology (h-HST) was introduced for use in size-exclusion and ion-exchange columns for separations of proteins and nucleic acids (49). Here, we demonstrate the performance of HPLC systems...
and columns that incorporate these technologies for analytes ranging from small molecules to proteins.

**Materials and Methods**

Conventional ultrahigh-pressure liquid chromatography (UHPLC) instruments and columns were compared to versions employing hybrid surface technology (HST), designated as Acquity Premier Systems and MaxPeak Premier Columns, respectively (Waters).

**Comparison of HST vs. Conventional Systems and Columns for a Nucleotide Separation**

Separations of adenosine monophosphate (AMP) and adenosine triphosphate (ATP) were carried out using both conventional and HST Acquity UPLC Systems with Acquity UPLC BEH Amide 1.7 μm particles packed into either conventional stainless steel or HST column hardware, both 2.1 x 50 mm. The mobile phase was 65:35 v/v acetonitrile:aqueous 60 mM pH 6.8 ammonium acetate and the flow rate was 0.5 mL/min. The sample contained 20 μg/mL each of adenosine monophosphate disodium salt and adenosine triphosphate disodium salt hydrate dissolved in 95:5 v/v acetonitrile:water and the injection volume was 1.0 μL. The columns were maintained at 30 °C and ultraviolet (UV) detection was used (λ = 260 nm).

**Comparison of HST vs. Conventional Columns for the Separation of a Mixture of Twelve Nucleotides**

Separations of 12 nucleotides were carried out using an Acquity UPLC I-Class System with a TUV detector, with the analyte detected at a wavelength of 290 nm. Conventional stainless steel and HST 2.1 x 50 mm columns packed with Acquity UPLC BEH C18 1.7 μm particles were used with a linear 25–80% acetonitrile gradient in 10.31 min employing an aqueous mobile phase containing 0.05% (w/v) ammonium hydroxide. The sample contained 6 mg/mL clozapine in 20:80:0.08 acetonitrile:water:acetic acid, and the injection volume was 0.25 μL. The sample was maintained at 4 °C, and the columns at 30 °C.

**Investigation of On-Column Oxidation for HST and Conventional Columns**

Separations were carried out using a conventional Acquity UPLC I-Class System with a TUV detector, with the analyte detected at a wavelength of 290 nm. Conventional stainless steel and HST 2.1 x 50 mm columns packed with Acquity UPLC BEH C18 1.7 μm particles were used with a linear 25–80% acetonitrile gradient in 10.31 min employing an aqueous mobile phase containing 0.05% (w/v) ammonium hydroxide. The sample contained 6 mg/mL clozapine in 20:80:0.08 acetonitrile:water:acetic acid, and the injection volume was 0.25 μL. The sample was maintained at 4 °C, and the columns at 30 °C.

**Comparison of h-HST vs. Conventional Columns for SEC Separations of a Monoclonal Antibody**

Size-exclusion separations were carried out using an Acquity UPLC I-Class Bio System with a TUV detector equipped with a 1500 nL titanium flow cell. Conventional stainless steel and h-HST 4.6 x 150 mm columns packed with XBridge Protein SEC 250 Å 2.5 μm particles were used with aqueous mobile phases containing 100 mM pH 6.8 sodium phosphate and varying concentrations of NaCl (0–200 mM). The flow rate was 0.35 mL/min. The sample contained 2 mg/mL NISTmAb (NIST reference material 8671), and the injection volume was 1 μL. The samples were maintained at 8 °C, and the columns at 30 °C.

**Results and Discussion**

A sample injected into an HPLC system interacts with a number of metal components between the injector and the detector. This includes the...
injector needle, the seat port, the pre-heater, connection tubing, and the column. For a typical Acquity UPLC System with a UV detector and a 2.1 x 50 mm column, it has been estimated that the column contributes 71% of the total metal surface area contacted by the sample, while the system accounts for the remaining 29% (23). The column frits alone contribute 52% of the total metal surface area. Indeed, column frits have often been identified as the cause of many of the deleterious effects associated with metal surfaces in HPLC. However, to obtain the best results, all of these surfaces need to be modified. This approach is enabled by HST, since the technology has been found to effectively treat all of the metal components mentioned above (16).

An example demonstrating the importance of modifying the surfaces in both the system and the column is shown in Figure 1. A sample containing adenosine monophosphate (AMP) and adenosine triphosphate (ATP) was separated using (a) a conventional system and conventional column; (b) a conventional system with an HST column; and (c) an HST system with an HST column. With the conventional system and conventional column, no peak was seen for ATP, which is known to adsorb strongly on metal surfaces (6,10,16,23,31,32,46,48). While a peak was observed for AMP, it showed significant tailing. When an HST column was used on the same conventional system, ATP was detected as a severely tailed peak with a low peak area. Using the HST column on an HST system, ATP was observed with the expected peak area, and only slight tailing. This demonstrates the importance of modifying the surfaces in both the column and the system for analytes that exhibit strong adsorption on metal surfaces.

The ability to obtain narrow peaks and accurate peak areas for analytes that interact with metal surfaces is important in a number of applications, including studies of polar cellular metabolites (28,30). As demonstrated in Figure 1, nucleotides are problematic when using conventional UHPLC systems and columns. In Figure 2, we show the separation of a mixture of twelve nucleotides using an HST system with either a conventional column or an HST column. The results show dramatic improvements in peak height, peak width, and peak shape when using the HST column, particularly for the nucleotide di- and triphosphates.

While the largest increases in peak area observed for HST versus conventional systems and columns have been reported for low analyte mass loads (16,41), significant improvements may be seen over a wide range of loads. An example is shown in Figure 3 for the steroid prodrug hydrocortisone sodium phosphate. As in our first example, we compared the results using (a) a conventional system and conventional column; (b) a conventional system with an HST column; and (c) an HST system with an HST column. The masses injected ranged from 2–200 ng. The peaks were detected by absorbance at 260 nm.
and an HST column, while the lowest were seen for the conventional system with a conventional column. The latter results also showed the lowest correlation coefficient (0.992 vs 0.998 for the other two linear correlations), indicating a greater deviation from a linear dependence of peak area on injected mass (Figure 3a). As evident in the log-log plot (Figure 3b), the relative differences in peak area were greatest for the lowest injected masses. For the 2 ng mass load, the average peak area for the HST system was 25% greater than that for the conventional system with an HST column, and 58% greater than that for the conventional system with a conventional column.

Adsorption on metal surfaces in the column hardware is a source of column-to-column variability for some analytes. This is demonstrated by the results shown in Figure 4, which compares peak areas for fructose 1,6-bisphosphate obtained using a mixed-mode reversed-phase anion-exchange stationary phase packed into either conventional or HST column hardware. For each column, a series of ten consecutive injections was made of 200 ng of fructose 1,6-bisphosphate. The error bars in Figure 4 indicate ± one standard deviation for six columns of each type. The relative standard deviations (RSD) for peak areas in the tenth injections were 3.9% for the HST columns, versus 17.7% for the conventional columns. Comparing the variability of the average peak areas across the ten injections, the RSD for the HST columns was 2.9% versus 36.5% for the conventional columns. These results show dramatic reductions in both column-to-column and injection-to-injection variability when using HST column hardware.

In addition to adsorption, metal surfaces or metal ions released from them have been reported to catalyze chemical reactions of certain analytes. We investigated the amount of on-column oxidation for conventional and HST columns for the pharmaceutical clozapine, using a mobile phase containing ammonium hydroxide and acetonitrile. As shown in Figure 5a and 5b, when using the conventional column a new peak appears, which has been shown by mass spectrometry to be due to an oxidized form of clozapine (50). The peak area of the oxidation product relative to the parent drug increased over a series of consecutive injections, from 0.89% in the second injection to 2.05% in the thirteenth. Similar behavior was reported by Myers and associates (18) for the on-column formation of a nitrosation impurity. It was hypothesized that the protective chromium oxide layer on the stainless steel frits is gradually lost over a series of chromatographic runs, exposing a reactive surface. At the same time, metal ions eluted from the frit may adsorb on the stationary phase and catalyze the nitrosation reaction. When using

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**FIGURE 3:** (a) Plot of peak area vs. injected mass for hydrocortisone sodium phosphate obtained using a conventional system with a conventional column (orange circles), a conventional system with an HST column (green triangles), and an HST system with an HST column (purple diamonds). The lines are linear least squares fits of the data; (b) Log-log plot of the same results. Columns (2.1 x 50 mm) packed with Acquity UPLC BEH C<sub>18</sub> 1.7 μm particles were used with an acetonitrile gradient and a 10 mM pH 3.0 ammonium formate aqueous mobile phase. The peaks were detected by absorbance at 246 nm.

**FIGURE 4:** Plot of average peak area vs. injection number for fructose 1,6-bisphosphate using conventional (orange circles) and HST columns (purple diamonds) packed with 1.7 μm Atlantis BEH C<sub>18</sub> AX particles. The mobile phase was an aqueous solution of 10 mM pH 3.0 ammonium formate. The peaks were detected by negative ion electrospray ionization mass spectrometry (m/z = 339).
an HST column we did not observe the clozapine oxidation product (Figure 5c and 5d). This shows that HST is effective at mitigating an on-column reaction catalyzed by stainless steel columns or metal ions released from them.

An important feature of HST is that different chemistries may be used to match the surface properties to the needs of the chromatographic mode and the intended analytes. A new variant of HST was recently developed for use in size exclusion chromatography (SEC) columns for proteins (49). This variant was termed hydrophilic HST (h-HST). An example demonstrating the benefit of this technology is shown in Figure 6. The same batch of stationary phase was packed into a conventional column and column hardware modified with h-HST. SEC separations of a monoclonal antibody monomer, fragments, and higher molecular weight species (HMWS) were carried out using mobile phases containing different concentrations of sodium chloride, which is used to mitigate ionic interactions (51). When using the conventional column, we observed a strong dependence of peak width and tailing on the salt concentration, with lower concentrations resulting in peaks that were broader and had larger tailing factors. The peak from the HMWS, which elute before the monomer, showed a greater sensitivity to the salt concentration than did the monomer peak. Only at the highest salt concentration (200 mM) was accurate quantification of the HMWS achievable. In contrast, the h-HST column showed very similar peak widths and tailing factors across the entire range of salt concentrations, as well as a consistent relative peak area for the HMWS. This is important, because it allows SEC separations to be carried out using mobile phases that are less likely to change the concentration of reversible aggregates, as has been reported to occur for high ionic strength mobile phases (52).

**Conclusion**

Preventing interactions of analytes with metal surfaces in HPLC systems and columns is important for obtaining accurate and precise results. Modifying the metal surfaces using hybrid surface technology has been shown to be highly effective at mitigating a range of issues, providing improved peak areas and peak shapes as well as reduced injection-to-injection and column-to-column variability. We also demonstrated mitigation of an oxidation reaction catalyzed by metal surfaces or metal ions released from them. Improvements were demonstrated for chromatographic modes including reversed-phase, HILIC and SEC. This technology has proven to be valuable for a wide range of applications, including analyses of both small and large molecule pharmaceuticals, as well as cellular metabolites, veterinary drugs and vitamins.

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