High Sensitivity MS Determination of Carryover in a New Autosampler Design

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Introduction

Sample Carryover has become a significant issue in HPLC analyses for a number of reasons:

- Overall increased detection sensitivity of typical HPLC detectors (UV/VIS, Fluorescence, etc.).
- Increased use of Mass Spectrometry as the detector of choice in the Pharmaceutical and Biotechnology arena (LC-MS and LC-MS/MS)
- Compounds analyzed that have an affinity for the wetted surfaces of the HPLC system components and fluidics.

In general, sample carryover is a major problem causing the reduction in accuracy and precision of LC, LC-MS and LC-MS/MS analyses.
What is Sample Carryover?

- Previously injected sample that elutes upon subsequent analyses due to chemical/physical characteristics of the sample, analysis system or both. As a result, peaks attributed to the previously analyzed sample may be observed in the subsequent chromatogram(s) which may co-elute or interfere with desired analytes. Also the MS spectrum at the background area shows the profile of a sample compound.
Primary Causes of Carryover

- Sample interaction with fluidics:
  - Adsorption of elements due to ionic interaction with metallic materials (e.g.: ionic compounds or basic compounds)
  - Adsorption of elements due to hydrophobic interaction with resinous materials (e.g.: lipophilic compounds)

- Flow path and flow path components:
  - The surface of the autosampler needle, in particular the outer surface
  - Flow path (tubing type) and Injection port composition (rotor-stator)
  - Grooves on the rotor seal (or flow line switching valves)
  - Other: Residual sample in “dead space” volumes of the flow path
Preventative Methods to Reduce Sample Carryover

There are two ways to reduce carryover: 1) Remove it by rinsing or 2) Prevent it in the first place. Rinsing can be effective; however, with the need for increased throughput, taking the time needed for rinsing may not be the best option. Careful choice of materials used in the design and construction of an autosampler will go a long way to prevent carryover. To that end, consider the following situations:

For samples that have the propensity for ionic or coordination interaction with metallic materials (e.g.: ionic compounds or basic compounds), carryover can be reduced by:
- 1) Removing adsorbed sample from the system with rinsing solution – time penalty.
- 2) Controlling element adsorption by changing sample needle composition or by coating the needle with chemically inert materials.

For samples that are hydrophobic and interact with resinous materials (e.g.: lipophilic compounds), carryover can be reduced by:
- 1) Removing adsorbed sample from the rotor seal groove by rinsing or flushing the system with organic solvents – time penalty.
- 2) Controlling sample adsorption by changing the rotor seal material and geometry.
Sample Needle Rinsing Solution

Choosing a rinse solution is not a trivial matter. For example, if the analytes are soluble in an acidified AcN/buffer diluent, using a 50:50 mix of methanol and water will likely not remove carryover. Rinse solution chemistry can have a huge effect and should be considered carefully to best counteract carryover.

Sample adsorption of ionic or very basic compounds due to ionic or coordination interactions can be reduced:
- By acidification of the rinse solution
- By formation of ion pairs by introducing counter ions with large ionic radii to the diluent and rinse phase (steric interaction).
  
  (e.g.) 100mM Perchloric acid solution/ Methanol (or Acetonitrile)

Sample Adsorption due to hydrophobic interactions (lipophillic compounds) can be reduced by:
- utilizing suitable organic rinsing solvents to solubulize and wash away the residual sample
  
  (e.g.) 100% Methanol, Acetonitrile, THF etc.
Effects of Rinse Solution on Sample Carryover and Precision

Carryover occurs when ionic compounds are adsorbed onto the outer surface of the sample needle resulting in reduced precision.

<table>
<thead>
<tr>
<th>No. of Analyses</th>
<th>Rinse Solution (100mM Perchloric Acid Solution)</th>
<th>Without</th>
<th>With</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caffeine</td>
<td>Thiamine</td>
<td>Caffeine</td>
</tr>
<tr>
<td>1st</td>
<td>50487</td>
<td>43031</td>
<td>50327</td>
</tr>
<tr>
<td>2nd</td>
<td>50580</td>
<td>42935</td>
<td>50372</td>
</tr>
<tr>
<td>3rd</td>
<td>50499</td>
<td>43637</td>
<td>50479</td>
</tr>
<tr>
<td>4th</td>
<td>50681</td>
<td>43864</td>
<td>50259</td>
</tr>
<tr>
<td>5th</td>
<td>50518</td>
<td>43679</td>
<td>50224</td>
</tr>
<tr>
<td>average</td>
<td>50553</td>
<td>43429</td>
<td>50332</td>
</tr>
<tr>
<td>RSD(%)</td>
<td>0.16</td>
<td>0.96</td>
<td>0.20</td>
</tr>
</tbody>
</table>

One can see here that peak area is reduced and reproducibility is affected when the counter ion is not present. This is due to the Thiamine adsorbing to the active sites on the Stainless Steel needle. The larger Perchlorate ion masks the active sites of the stainless steel resulting in increased recovery and improved precision.

One thing to keep in mind is the possible effect of a counter ion when dealing with a mass spectrometer as a detector. It is possible that it may have a suppression effect on ionization. Also, one must be careful not to introduce any non-volatile components to the MS detector.
Relationship Between Injection Volume and Degree of Carryover

- The relative degree of carryover increases with reduction in injection volume.

The amount of carryover is proportional to the area of the adsorption site and independent of the amount of sample injected. Therefore carryover becomes relatively greater as injection volume is decreased.
The Relationship Between Sample Concentration and Sample Carryover

- The relative degree of sample carryover increases with a reduction in sample concentration.

The amount of carryover is proportional to the area of the adsorption site and independent of sample concentration. Carryover becomes relatively greater with a reduction in sample concentration.
The Effects of Sample Needle Rinsing on Sample Carryover

<table>
<thead>
<tr>
<th></th>
<th>Relative Area after Blank Injection (mobile phase)</th>
<th>Percent Carryover</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Rinsing</td>
<td>0.000730</td>
<td>0.0730</td>
</tr>
<tr>
<td>Rinsing</td>
<td>0.000425</td>
<td>0.0425</td>
</tr>
</tbody>
</table>

Rinsing solution: same as the mobile phase. 3 Sec needle dip before and after sample aspiration. Needle material: Stainless Steel

<table>
<thead>
<tr>
<th></th>
<th>Relative Area after Blank Injection (mobile phase)</th>
<th>Percent Carryover</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immersion</td>
<td>0.000425</td>
<td>0.0425</td>
</tr>
<tr>
<td>Solvent delivery</td>
<td>0.000225</td>
<td>0.0225</td>
</tr>
</tbody>
</table>

Static dip versus active rinse solvent delivery around needle – 3 sec each.

An investigation into Rinsing dip times showed no appreciable difference in carryover reduction with dip times in excess of 3 seconds.
The Effects of Sample Needle Composition and Coatings on Sample Carryover

<table>
<thead>
<tr>
<th>Sample Needle Composition/Coating</th>
<th>Relative Area after Blank Mobile Phase Injection</th>
<th>Percent Carryover</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stainless Steel</td>
<td>0.000425</td>
<td>0.0425</td>
</tr>
<tr>
<td>Teflon coated</td>
<td>0.000023</td>
<td>0.0023</td>
</tr>
<tr>
<td>PEEK coated</td>
<td>0.000021</td>
<td>0.0021</td>
</tr>
<tr>
<td>Platinum Coating</td>
<td>0.000009</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

- **Teflon coating**
  - Mechanically weak: coating layer peels off after about 300 injections.

- **PEEK coating**
  - Thin-layer coating (of a few dozen microns) is technically difficult as the coating technology is still being developed.

- **Platinum coating**
  - Coatings of a few microns are possible via a special coating process.
  - Durable: Carryover is suppressed even after 20,000 injections.
Reducing Carryover of Hydrophobic Compounds

Rinsing of the rotor groves with a suitable strong organic solvent can remove adsorbed Hydrophobic compounds. However, reduction or elimination of sample adsorption to the rotor seal can be realized by careful choice of the rotor seal material:

- **Vespel (Material with excellent durability)**
  - Common material employed in the rotor seals.
  - Unfortunately is has a strong affinity for lipophilic samples.

- **Delrin**
  - Common material used with alkaline mobile phase.
  - Little adsorption of lipophilic compounds.

- **PEEK**
  - Can be used with mobile phase across the entire pH range.
  - Little adsorption of lipophilic compounds.
Shimadzu Autosampler Design Evolution

The concern about carryover was recognized by Shimadzu early in the stages of LCMS utilization. Shimadzu engineers have studied the carryover phenomenon very carefully and have grown to understand it thoroughly as evidenced above and in the design and performance of the Prominence series autosamplers. Many of the points mentioned previously are incorporated as standard features of the SIL-20 as well as several others including: fast valve rotation, unique rotor groove shape for better fluid flow, additional rinse solvents, needle/needle port geometry and a few others we are not ready to divulge. We have also not missed the importance of throughput in the design. The SIL-20 is capable of a 10 second injection cycle to reduce the time between injections. Addition of an optional sample rack changer increases the capacity to keep even the busiest labs operational for an extended time. The performance speaks for itself when demonstrated on the world’s most sensitive MS, the API-5000™.
Determining Autosampler Carryover using the API-5000™

Autosampler carryover was determined using the API-5000™ by injecting a standard of antipyrine (9 pg/uL) with a response close to the limit of the dynamic range of the API-5000™, followed immediately by a blank injection. Carryover was found to be less than the limit of detection for this method on the API-5000™, and is estimated as <0.01%.

Method details:
Mobile phase: 90/10 ACN/H2O 0.1% formic acid; 0.4 mL/min
Column: PolyLC 50 mm x 2 mm
Rinse solvent: 90/10 ACN/H2O 0.1% formic acid
Injection: 10 uL of 9 pg/uL Antipyrine
MRM transition: 189/56 positive mode Turbolon Spray
Determining Autosampler Carryover using the API-5000TM

XIC of +MRM (3 pairs): 189.1/56.0 amu from Sample 38 (9 pg/µL Antipyrine 1 pg/µL IS) of Antipyrine Plasma 4.wiff ...

Max. 2.4e6 cps.

2.4 x 10^6 cps

XIC of +MRM (3 pairs): 189.1/56.0 amu from Sample 39 (Blank) of Antipyrine Plasma 4.wiff (Turbo Spray)

Max. 1760.0 cps.

Blank
The blank level is low enough after the highest standard that the lowest concentration in this assay would not be affected by carryover. The ultimate method LOQ is determined by the API-5000™ sensitivity, and is not limited by autosampler carryover.
Calibration standards covering 9 fg/uL to 9 pg/uL were analyzed. Linear regression with a forced zero intercept and no weighting was used. Linearity was excellent with an r^2 of 1.0000. The excellent linearity with a forced zero intercept shows again that the system does not suffer from any carryover, and samples can be quantified down to the LOQ determined by the API-5000™.
Results Table Statistics

Overall system performance is excellent, with a highly precise and accurate assay. Precision is better than 1% above the lowest standard. Method LOQs are often defined as the concentration at which the reproducibility is 15%. This precision is determined by the combination of the autosampler and the mass spectrometer. In this case, the precision of better than 1% for the higher standards suggests that the LOQ will be determined by the ultimate sensitivity of the API-5000™ and not by the autosampler. For the lowest concentration of 9 fg/uL, the precision is ~6%, suggesting that the LOQ is certainly below 9 fg/uL.
<table>
<thead>
<tr>
<th>Expected Conc.</th>
<th>9 fg/uL</th>
<th>90 fg/uL</th>
<th>900 fg/uL</th>
<th>9 pg/uL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number Of Values Used</td>
<td>5 of 5</td>
<td>5 of 5</td>
<td>5 of 5</td>
<td>4 of 4</td>
</tr>
<tr>
<td>Data Point #1</td>
<td>0.009495</td>
<td>0.093831</td>
<td>0.919252</td>
<td>9.023516</td>
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<tr>
<td>Data Point #2</td>
<td>0.009375</td>
<td>0.09452</td>
<td>0.92484</td>
<td>8.973414</td>
</tr>
<tr>
<td>Data Point #3</td>
<td>0.009559</td>
<td>0.093911</td>
<td>0.911123</td>
<td>8.932331</td>
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<tr>
<td>Data Point #4</td>
<td>0.008159</td>
<td>0.093846</td>
<td>0.91726</td>
<td>9.062129</td>
</tr>
<tr>
<td>Data Point #5</td>
<td>0.00917</td>
<td>0.095806</td>
<td>0.91142</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.009152</td>
<td>0.094383</td>
<td>0.916779</td>
<td>8.997848</td>
</tr>
<tr>
<td>Low</td>
<td>0.008159</td>
<td>0.093831</td>
<td>0.911123</td>
<td>8.932331</td>
</tr>
<tr>
<td>High</td>
<td>0.009559</td>
<td>0.095806</td>
<td>0.92484</td>
<td>9.062129</td>
</tr>
<tr>
<td>Standard Dev.</td>
<td>0.000574</td>
<td>0.000846</td>
<td>0.005745</td>
<td>0.056805</td>
</tr>
<tr>
<td>%CV</td>
<td>6.27</td>
<td>0.90</td>
<td>0.63</td>
<td>0.63</td>
</tr>
<tr>
<td>Accuracy</td>
<td>101.69</td>
<td>104.87</td>
<td>101.86</td>
<td>99.98</td>
</tr>
</tbody>
</table>
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