

LEAP Technologies

Minimizing and Eliminating Carryover in PAL Systems



A Primer for Reducing Carryover in PAL Autosampler Systems

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Two of the biggest problems in chromatography are reproducibility and carryover. This document is intended as a brief overview of the issue of carryover, and to provide some suggestions as to how to minimize or eliminate it as it relates to LEAP PAL systems.

What is Carryover?

"Carryover" is a detector response from a previously injected sample, detectable when a subsequent injection is made. The response is best seen when that subsequent injection is a "blank", i.e. an injection of mobile phase with none of the compound intentionally included. Whenever an injection system that comes into direct contact with sample solutions gets "re-used" for subsequent injections, carryover will occur to a certain extent. It now becomes a question of detector sensitivity. The improved detector sensitivity might be at the heart of the "rash" of carryover occurrences in recent years. The first question a user needs to answer is: Does the amount of carryover matter? How does it compare to the compound-specific detection limits and to error limits of the sample preparation and LC methods? Example: Even though visible, a peak below the detection limit for a specific compound is, by definition, not detected.

In the literature, carryover is usually quantified as a percentage value of either the "Upper Level of Quantitation" (ULOQ) or the "Lower Level of Quantitation" (LLOQ). This value is always compound specific and, therefore, meaningless either by itself or referring to a different compound than the one you are having problems with. Example: Using a LEAP CTC autosampler, the carryover for

Acetaminophene (Tylenol®) was determined to be 0.0004% ULOQ. This is a fantastic number but says nothing about the autosampler performance using your samples.

Due to these reasons no general value for carryover can be given nor guaranteed for a specific analytical problem/task.

What Causes Carryover?

Carryover is caused by a failure to completely eliminate residual compounds from a previous sample injection into the HPLC system. The lingering residual becomes mixed with a subsequent injection. This can result from inadequate or ineffective flushing after the previous injection, or by some or all of the sample components becoming temporarily trapped or bound to the surface material somewhere in the HPLC pathway.

Where the Residual Lingers

Possible sites for residual accumulation include anywhere and everything from the syringe to the detector, but the injection valve and the syringe are the most likely places for compounds to be unintentionally retained.

Any surface that is not completely inert has the potential to be an adsorption site. For example, certain compounds tend to adsorb onto active sites in Stainless Steel, even in the flow path, and require enough of the right wash solvent in the right concentration to be released. This can also be true for PEEK tubing, depending on the nature of the analytes and the matrix.

More insidious are "dead volume" spaces, where sample can remain trapped after an injection, sometimes even after routine rinsing. Two common examples of dead volume spaces are the

inside of the sample syringe needle and the valve injection port inlet including the valve rotor groove. See figure 1.

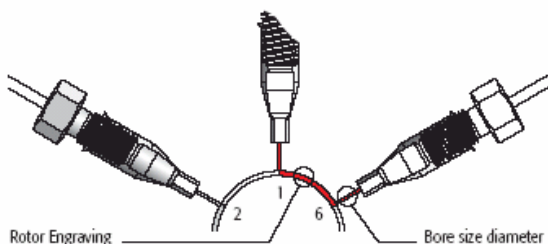


Figure 1: Cutout of an injection valve; An injection leaves the injection port inlet filled with sample. Additionally in case of a loop overflow injection, a rotor groove will also contain sample.

However, any space where sample is not completely displaced or purged by mobile phase or wash solvents is a potential problem. The biggest source of this type of carryover is from the fittings and how they are made. For example, PEEK fittings with PEEK ferrules can slip under high pressure creating a dead volume and thus allowing sample to be trapped.

What can be Done to Minimize Carryover?

The Basics

Obviously, flushing the syringe and valve is essential. The LEAP CTC PAL has two wash stations included as a standard accessory. The selection of solvents and flush volumes are of paramount importance and controlled by the user. Although it may seem obvious, it is worth stating that flushing with ineffective solvent(s) is useless. You need to consider the basic rule of solubility: "Like Dissolves Like". There are many factors that will influence the

effectiveness of the rinse, solvent strength and polarity among them, but there are no hard and fast rules that apply to every case. Consequently, the end-user chemist must be sure to choose solvents that will get the job done. Often, a one-two punch of solvents, e.g. an aqueous followed by an organic wash, can be more effective than just one. "Cocktails" of wash solvent modifiers like 0.1% to 1.0% of volatile acid, base or salts may produce the desired result. It is important to make sure that the choices of solvents are not immiscible with each other.

Syringes

The choice of syringe can significantly affect carryover. Needle, plunger and barrel characteristics can all contribute to or reduce problems.

For LC, the needle should always be a 22 gauge, point style 3, i.e. with a blunt tip, to ensure a good seal at the bottom of the valve inlet (assuming proper penetration). Use of any other tip will result in an unswept "dead volume" area around the tip, a primary cause of carryover. The inner diameter (i.d.) of the needle determines the inner surface area exposed to sample and the residual left in the dead volume of the needle after the plunger has fully expelled the contents of the barrel. Obviously, a smaller i.d. reduces both. A "gauge 22s" needle has a smaller i.d. than a "gauge 22" by a factor of approximately 10. See figure 2. It can potentially reduce carryover by reducing the surface area and needle volume, albeit with some sacrifice of injection speed due to the greater restriction and a higher occurrence of needle clogging due to the smaller inner diameter.

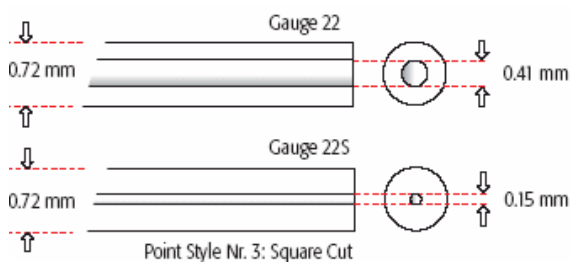


Figure 2: Dimensional comparison of commonly used syringe needle sizes for LC injections.

However, additional rinses can somewhat reduce the practical difference between the two. As with the rest of the system, in reducing carryover from the syringe, the importance of determining the most effective solvents and performing adequate washes cannot be overstated. Also, though more expensive, it is possible to find needles with Teflon® (PTFE) or glass coatings to reduce adhesion.

Buildup of compounds on the inner surface of the glass barrel has been clearly established as a problem, both in potential leeching into subsequent samples and even more notably in causing premature wear of plunger tips. Worn plunger tips have channels and pockets which are able to retain sample solution. Better syringes have polished and/or treated surfaces to reduce this phenomenon, and the proper selection of wash solvents and their application can substantially reduce this buildup and its effects.

Various compounds are used as plunger tip material, though most are either PTFE or Polyethylene (PE). There is little evidence that the plunger tip contributes significantly to carryover and the difference in the materials primarily

influences durability. However, there is one way in which the plunger can contribute to the carryover problem: if the plunger fails to reach the bottom of the barrel, especially during rinsing, a dead volume area will exist between the tip and the barrel bottom. It is recommended that plungers with a variable plunger stop are very carefully adjusted, or avoided altogether.

Valves and Tubing

Regularly check that the valve inlet sleeve fits snugly around the syringe needle when inserted. Otherwise, replace the inlet sleeve. This can easily be determined by inserting the needle into the valve inlet by hand. Typically the needle sleeve should be changed every 15 to 60 days depending on the number of injections made and if salts are present in sample and/or wash solutions. Be sure that the inlet sleeve is correctly dimensioned. There should be approximately 2.5mm of tubing below the ferrule. If the tubing below is either longer or shorter, a dead volume will result in the valve injection port. See figure 3. Use only LEAP-approved inlet sleeves to ensure conformity to specifications.

When connecting tubing or loops to the valve, be sure that the ends are cleanly cut and perpendicular, and take care to fully insert the tubing ends into each port and push the tubing as you tighten the fittings (to eliminate any gap between the end of the tubing and the bottom of the port).

Avoid "Fingertight" fittings and use real crimp-style ferrule fittings. Fingertight fittings can loosen enough under high pressure to cause a dead volume without leaking.

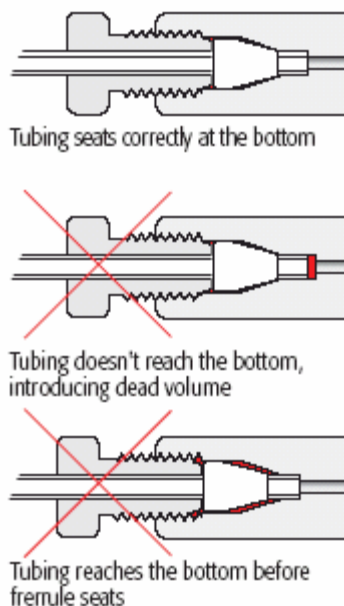
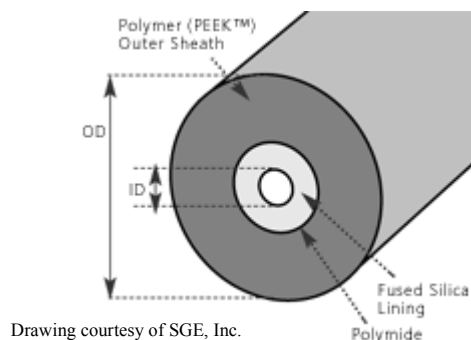


Figure 3: The importance of a correct tubing length below the ferrule. Tubing being too short or long can both produce dead volume which in turn can cause carryover.

Typically PEEK tubing produces less carryover than Stainless Steel (SS) tubing, but Stainless Steel ferrules seem to outperform PEEK ferrules. Care must be used when using a SS ferrule on PEEK tubing to prevent closing off the i.d. by excess crimping and, thus, causing a blockage in the flow path.

In recent years a third alternative tubing material became practical for use in LC applications. Fused silica in form of PEEKsil™ tubing offers unique and different material properties compared to PEEK and SS. PEEKsil™ comes in pre-cut tubing lengths with polished and perpendicular cut tubing ends and should be used without further cutting or shortening. Cutting PEEKsil™ without proper tools will result in a jagged edge of the fused silica inner tubing ends which then results in carryover and blockages downstream. See figure 4.



Drawing courtesy of SGE, Inc.

Figure 4: Cross section of PEEKsil™ tubing. Pre-cut tubing lengths come with polished perpendicular tubing ends and should not be cut or shortened by the end user.

Consider using a valve that will be inert with your samples. Valco valves come in several material choices, Nitronic 60 Stainless Steel, Hastelloy C, PAEK polymeric, and either the standard “rotor” design or the Cheminert design. The Valcon “H” rotor, which is a proprietary polymer of carbon fiber reinforced PTFE is the standard rotor material. The Valcon “E” material is a mixture of PAEK and PTFE and is more inert than Valcon H, however, it does not withstand higher pressures very well and wears out more quickly as a result. The “H” version is good up to 10,000 PSI and the E material is good up to 3,500 PSI. The rotor needs to be checked for scratches or wear. Replace a scratched or worn valve rotor.

Cheminert valves with PAEK stator and Valcon “E” rotor are more inert than stainless steel valves. (Note: you can consult the VICI (Valco) catalog for additional specific material descriptions and compatibility issues.)

Component & Material Choices

- Choose the proper valve design and material based on the potential for carryover.

- Choose the proper syringe design and size for your application and washing needs.
- Choose the proper needle format, e.g., “gauge 22” vs. “gauge 22s”.
- Choose the proper tubing material for the sample loop and other tubing connections.
- Choose the appropriate solvents for washing/rinsing purposes.
- Determine if ancillary washing devices will be required i.e. LEAP Valve Self Wash System, or the Dilutor module.
- Order additional control software to run custom macros or cycles with special wash procedures.

Steps to Minimize or Eliminate Carryover in PAL Systems

Hardware

1. Adjust Needle penetration in the valve inlet to 0.2 mm from the bottom of the inlet port. Using the handheld controller, move the needle slowly down until a clear audible noise (“clunk”) occurs. Move the needle 0.2 mm upwards and check the position, again.
2. Replace injection valve inlet sleeves every 15 to 60 days or sooner depending on the number of injections.
3. Check the rotor and stator of the valve for scratches - these are convenient places for compounds to be retained. The stator can be cleaned ultrasonically and the rotor should be replaced as needed.
4. Check the syringe for discoloration on the plunger, plunger tip wear and tightness of the plunger in the syringe barrel.

5. Avoid wetting of the lower needle guide on the injection head. If the lower needle guide is getting wet, try to find the source of the liquid and correct the problem. It may be coming from a splashing effect in the Wash Station, from a blocked Wash Station drain line or from sampling out of open vials/wells.

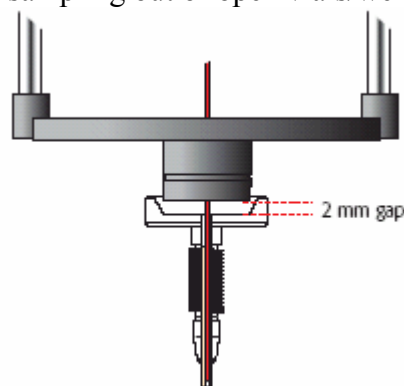


Figure 5: Positioning of the injection head needle guide relative to the injection valve inlet. A 2mm gap prevents liquid possibly present on the needle guide underside from contaminating the injection valve inlet port.

6. Run the Wash Station utility using the handheld controller and verify actuation of the Wash Station solenoid valves and the gravity-fed flow of wash solvent.
7. Verify that the drain line of the Wash Station is not blocked which can prevent fresh solvent from getting into the Wash Station.
8. Be sure that the caps on the wash solvent supply bottles of the Wash Station are loose to provide ventilation. If the caps are tight, a relative vacuum may develop in the bottles as the solvents are consumed. This diminished pressure will result in diminished solvent flow and, in turn, reduces the efficacy of syringe needle washing

at the Wash Station. (Note: Normally there is a small hole in the caps provided from CTC especially for this reason. However, the hole is small and on the side of the cap and in some cases may not provide adequate ventilation. Thus, a loosened cap will help to ensure that air can enter the bottle.)

Wash Procedures & Solvents

9. Use two different wash solvents, e.g., polar vs. non-polar. Make sure that wash solvents are compatible/miscible with each other and with the sample solution. A solvent miscibility table is provided in the appendix to offer help in the wash solvent selection process.
10. Add a volatile solvent modifier to the wash solutions, e.g., acids, bases, salts, etc. In addition to wash solvent miscibility, the wash solvent modifiers themselves need to be miscible/soluble in all wash solvents and sample solution.
11. Use the most appropriate wash solvents. Example: When the sample contains proteins, first rinse with an acidified aqueous solvent (e.g., 90:10 water/Acetonitrile with 0.5% Formic acid), followed by a more aggressive organic solvent (e.g., 30:70 water/Acetonitrile with 0.5% Formic acid). The acidified aqueous solvent will keep the proteins soluble and minimize the probability of denaturing, in turn reducing the chances of the proteins sticking to the inner surfaces of the valve or syringe. The organic wash solvent will wash away the small molecule analytes. This example illustrates that the order of wash solvents is important. Perform a syringe wash with the aqueous

solvent prior to the next injection to remove any high organic concentrations.

12. Increase the number of syringe rinses and the number of valve rinses. Use a 2-to-1 ratio of valve washes to syringe washes.
13. Lengthen rinses, possibly including a "dwell" time to promote a "leeching" effect.
14. Use a Valve Self Washing System or the PAL Dilutor module to increase the volume of valve washes.

Dilutor as Wash Station: -Adding a CTC dilutor can provide greatly enhanced flushing of the syringe and valve. A large volume of solvent is passed directly into and through the syringe and has been shown to significantly improve rinsing results. (Note: This is limited to either a 20uL or 80uL sample syringe size.) This accessory permits the flushing of the valve while the needle remains in the valve immediately after injection. You will have to do at least one syringe needle wash in wash solvent 1 and/or 2 prior to the next injection to remove any analytes from the needle's external surface.

Valve Self-Washing System: - Macros have been developed to control the LEAP Valve Self Washing System to provide specific wash times for the valve. Higher volumes of solvent can be used for cleaning the valve while the syringe is being washed separately. Syringe life is greatly enhanced with this technique. The syringe needle should be washed at least once in wash solvent 1 and/or 2 prior to the next injection.

Techniques

15. Partial loop fill injections isolate the sample solution with a volume of solvent that was left in the loop. This procedure can minimize concentrated sample coming into contact with valve rotor grooves, the waste port outlet and fittings. Make sure that the valve is plumbed in such a way that the sample leaves the loop the same way it came in, e.g., “last in first out”. See figure 6. The upper sample volume limit for partial loop fill injections is 1/3 of the sample loop volume. (Note: In case of loop overflow injections, the injection volume needs to be at least 3 times the loop volume.)

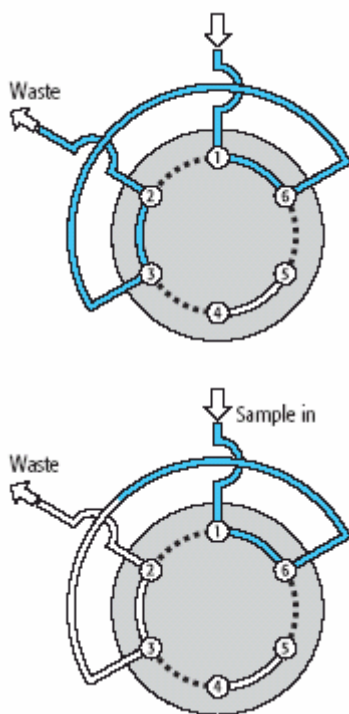


Figure 6: Loop overflow vs. partial loop fill injections; During partial loop fill injections, the number of rotor and valve surfaces including fittings that come in contact with concentrated sample is reduced.

Special Macros/Cycles

Create macros/cycles for the following special methods to help minimize/eliminate carryover. This will require the use of Cycle Composer software macros or Cycle Editor software cycles. Macro/cycle operation requires either the native software running concurrently or LC data system software controlling the PAL based on macros/cycles. Examples include:

Needle Dip: -Dip the syringe tip into wash solvent(s), after sample has been aspirated into the syringe, just before the injection to clean the outside of the needle before it is inserted into the injection port. This precludes matrix and/or analytes from contaminating the needle inlet sleeve and injection port.

Valve Toggle and Loop Rinse: - When overflowing the loop, both the inlet and outlet of the loop may contaminate the respective crossover ports. Toggling the valve after the injection subjects both crossover ports to clean mobile phase, which can reduce the concentration of analytes in the valve. A syringe wash of the injection loop can be accomplished as well. The toggling of the valve should be done when the gradient strength is at its highest point to dissolve any remaining analytes in the crossover port. Care must be taken not to leave a strong solvent in the loop so that the next injection is not subjected to the higher solvent strength. The loop may be washed with lower strength solvent so that the chromatography is not compromised.

Dispense Wash Solutions to Waste: - Wash solvent can be drawn from the capillary tube of the Fast Wash Station and ejected into the waste position,

instead of ejecting the contaminated solution back into the capillary tube. Note that this procedure should be followed by at least one more regular wash step including wash solvent ejection into the capillary tube to rinse the outside of the needle. Under normal operation, the solenoid valves close before a wash solvent ejection, thus, forcing the expelled solvent around the outside of the needle.)

Sandwich Injection: -A solvent plug may be picked up before the sample is aspirated in order to “isolate” the syringe plunger tip from contact with analytes. The same solvent plug will also come to rest in the injection port inlet and adjacent rotor groove after the sample injection is made. Note that this procedure is successful only if mixing strokes are not required and during partial loop fill injections. It can be done with or without an air gap between the solvent and sample. The technique minimizes the contact made between the concentrated analytes and the crossover port on the valve.

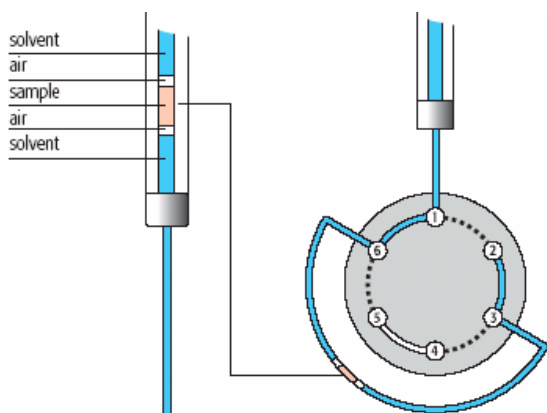


Figure 7: Sandwiche sample pickup and injection; The sample solution is “embedded” in a sandwich of solvent and separated by small air gaps. After injection, the sample will end up in the middle of the injection loop. Only solvents with a lower elution strength than the starting gradient mobile phase composition should be used to bracket the sample.

Troubleshooting Summary

Check the easiest things first.

- Wash the syringe and valve with the proper choice of solvents
- Check needle penetration in the injection valve
- Check Needle inlet sleeve for wear
- Check Rotor for scratches and replace as needed
- Check syringe to be sure the plunger is going all the way to the bottom of the barrel
- Change your injection procedure going from low concentrations to higher concentrations if possible
- Use a partial Loop filling technique
- Use Valcon E instead of Valcon H rotor material
- Check fittings between the sample loop and valve for proper installation
- Use custom Macros or cycles to enhance rinsing

List of References

Dolan, J.W. “Autosampler Carryover” LCGC **19** (2) February 2001, 164-168

Dolan, J.W. “Attacking Carryover Problems” LCGC **19** (10) October 2001, 1050-1054

Appendix Wash Solvent Miscibility Table

| Solvent | Polarity Index | Refractive Index @20°C | UV(m) Cutoff @1AU | Boiling Point(°C) | Solubility In water (%w/w) |
|-----------------------------------|----------------|------------------------|-------------------|-------------------|----------------------------|
| Acetic Acid | 6.2 | 1.372 | 230 | 118 | 100 |
| Acetone | 5.1 | 1.359 | 330 | 56 | 100 |
| Acetonitrile | 5.8 | 1.344 | 190 | 82 | 100 |
| Benzene | 2.7 | 1.501 | 280 | 80 | 0.18 |
| n-Butanol | 4.0 | 1.394 | 254 | 125 | 0.43 |
| Butyl Acetate | 3.9 | 1.399 | 215 | 118 | 7.81 |
| Carbon Tetrachloride | 1.6 | 1.466 | 263 | 77 | 0.08 |
| Chloroform | 4.1 | 1.446 | 245 | 61 | 0.815 |
| Cyclohexane | 0.2 | 1.426 | 200 | 81 | 0.01 |
| 1,2-Dichloroethane ¹ | 3.5 | 1.444 | 225 | 84 | 0.81 |
| Dichloromethane ² | 3.1 | 1.424 | 235 | 41 | 1.6 |
| Dimethylformamide | 6.4 | 1.431 | 288 | 155 | 100 |
| Dimethyl Sulfoxide ³ | 7.2 | 1.478 | 268 | 189 | 100 |
| Dioxane | 4.8 | 1.422 | 215 | 101 | 100 |
| Ethanol | 5.2 | 1.360 | 210 | 78 | 100 |
| Ethyl Acetate | 4.4 | 1.372 | 260 | 77 | 8.7 |
| Di-Ethyl Ether | 2.8 | 1.353 | 220 | 35 | 6.89 |
| Heptane | 0.0 | 1.387 | 200 | 98 | 0.0003 |
| Hexane | 0.0 | 1.375 | 200 | 69 | 0.001 |
| Methanol | 5.1 | 1.329 | 205 | 65 | 100 |
| Methyl-t-Butyl Ether ⁴ | 2.5 | 1.369 | 210 | 55 | 4.8 |
| Methyl Ethyl Ketone ⁵ | 4.7 | 1.379 | 329 | 80 | 24 |
| Pentane | 0.0 | 1.358 | 200 | 36 | 0.004 |
| n-Propanol | 4.0 | 1.384 | 210 | 97 | 100 |
| iso-Propanol ⁶ | 3.9 | 1.377 | 210 | 82 | 100 |
| Di-Iso-Propyl Ether | 2.2 | 1.368 | 220 | 68 | |
| Tetrahydrofuran | 4.0 | 1.407 | 215 | 65 | 100 |
| Toluene | 2.4 | 1.496 | 285 | 111 | 0.051 |
| Water | 1.0 | 1.477 | 273 | 87 | 0.11 |
| Tichloroethylene | 9.0 | 1.333 | 200 | 100 | 100 |
| Xylene | 2.5 | 1.500 | 290 | 139 | 0.018 |

Miscible
 Immiscible
 Immiscible means that in some proportions two phases will be produced