

Application Note

# Improved Chlorhexidine Carryover Performance Using the Alliance iS HPLC System

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## Abstract

Carryover is an all-too-common problem for many users of High Performance Liquid Chromatography (HPLC) systems. There are multiple forms of carryover, including volumetric, or carryover as a result of void volumes in the flow path, and adsorptive carryover, where sample “sticks” or adsorbs to surfaces of the flow path. If a method suffers from carryover, it is important to know which form of carryover it is so that you can most effectively eliminate the source of the carryover. In many cases, methods may display both volumetric and

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adsorptive carryover. In this application note, carryover of chlorhexidine will be evaluated on a variety of HPLC systems across various vendors. In addition, mitigation strategies, namely implementation of needle wash and/or extending washing will be explored as well.

## Benefits

- Improved carryover performance using the Alliance iS HPLC System
- The Alliance iS system uses easy to use tool free fittings to eliminate volumetric carryover
- Improved wash function to significantly reduce adsorptive carryover

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## Introduction

Across the pharmaceutical and biopharmaceutical industry, there is a common need to develop and run methods on a variety of HPLC platforms. When migrating methods across different LC systems, often times manufactured by different vendors, it is critical to obtain consistent method performance, regardless of the LC system used. Because critical method performance typically includes attributes such as retention time and area precision, peak resolution, and signal to noise to name a few, one method performance parameter that can often be overlooked or not well investigated is sample carryover. Carryover occurs when analyte(s) from one injection is seen in subsequent injections, either due to adsorptive properties of analyte sticking somewhere in the flow path, or due to void volumes contained in the flow path where unswept volumes may contain analyte(s) of interest that will then show up in subsequent injections. In this application note, a scaled method based on the USP monograph for chlorhexidine hydrochloride organic impurities was used to assess carryover performance across several HPLC systems.

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## Experimental

### Sample Description

Chlorhexidine challenge solution was prepared by weighing chlorhexidine dihydrochloride (Sigma-Aldrich, St.

Louis, MO) and dissolving in mobile phase A to give a final concentration of 1.13 mg/mL chlorhexidine (1.4 mg/mL chlorhexidine dihydrochloride). A chlorhexidine standard solution was prepared by dilution of stock with mobile phase A to a final concentration of 11.3 µg/mL chlorhexidine (14 µg/mL chlorhexidine dihydrochloride). All blank injections (mobile phase A) were from separate vials.

## LC Conditions

LC system:	Alliance iS HPLC System, Vendor X HPLC Systems (HPLC Systems 1 & 2), Vendor Y HPLC/UHPLC System (HPLC System 3), Vendor Z HPLC System (System 4)
Detection:	UV for all systems
Vials:	LCGC Certified Clear Glass 12 x 32 mm Screw Neck Vial, Max Recovery, with Cap and Preslit PTFE/Silicone Septum (p/n: 186000327C)
Column(s):	XSelect HSS C <sub>18</sub> Column, 100 Å, 2.5 µm, 3 mm x 100, p/n: 186006143
Column temp.:	30 °C
Sample temp.:	8 °C
Injection volume:	5 µL
Flow rate:	0.6
Mobile phase A:	0.1% trifluoroacetic acid in 80:20 water:acetonitrile
Mobile phase B:	0.1% trifluoroacetic acid in 90:10 acetonitrile:water

Needle wash solvent:

50:50 acetonitrile:water

## Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.600	100	0	6
0.6	0.600	100	0	6
9.1	0.600	80	20	6
12.6	0.600	80	20	6
15.4	0.600	70	30	6
17.4	0.600	70	30	6
17.8	0.600	100	0	6
22.0	0.600	100	0	6

## Data Management

Chromatography software:

Empower 3.7.0

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## Results and Discussion

### Volumetric vs Adsorptive Carryover

When running any regulated HPLC method, the ability to obtain consistent and accurate quantitative

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measurements is paramount. As such, the performance of any LC system with respect to carryover is an important attribute. However, the differences in instrument design, fittings, and needle washing behavior vary significantly across HPLC systems. These differences may require the user to identify the appropriate parameters and/or wash solvents to realize the best performance from a system. Additional complexity in addressing carryover is that it can be typically classified as either being volumetric or adsorptive, though it is certainly possible to experience both forms of carryover within the same method. While both types of carryover may impact chromatographic results, the contributors are very different.

Volumetric carryover occurs when there are void or unswept volumes within the flow path where sample can reside until subsequent injections. This is typically a measure of how well the system and connections are mechanically put together. For example, any tubing that is not bottomed out in the port when connections are made, or any mechanical tolerances in the manufacturing process of the valves, *etc*, can all be sources of unswept volumes. Clearly, the manufacturing and assembly process plays an important role here, but any user made connections are also extremely important. Because each vendor uses different fittings, each with slightly different instructions on how to make a connection properly, it can be easy for a user to do this incorrectly without even knowing it. The tool free fittings that come on the Alliance iS HPLC System are designed to be foolproof so that it is impossible to make an improper connection, thus eliminating a common source of volumetric carryover.

The other form of carryover encountered is adsorptive carryover, where the analyte(s) of interest adsorbs to parts of the LC system. Adsorptive carryover is dependent on several factors including the materials used in the LC flow path, the chemical structure of the analytes, and the method conditions used in analysis. Chlorhexidine is a compound that is commonly used to determine adsorptive carryover, as it is known to be “sticky”, and is often used in vendor carryover specification documents because of its adsorptive nature. Regarding the materials used in the LC flow path, all systems evaluated in this study use a flow path, including the sample needle, comprised mainly of stainless steel. The needle is the only component that directly contacts the sample, and thus is most likely to be affected by adsorption. However, in this study, all the HPLC systems have a flow through needle injection design, in which the interior of the needle is part of the flow path and is continuously washed by the programmed gradient. Thus, the washing mechanism of the sample needle and the wash solution will have the greatest impact on adsorptive carryover on the exterior of the needle. While each vendor and model of LC has a different mechanism of wash and recommendation for wash, the default or recommended wash was used initially for all systems. More extensive washing was done to determine the impact of additional washing on measured carryover. The wash solvent chosen for all systems was a mix of 50:50 water:acetonitrile, slightly

stronger than the elution conditions of chlorhexidine.

## Method Scaling and Optimization

To evaluate the carryover of chlorhexidine across various vendors and systems, the USP monograph for chlorhexidine hydrochloride<sup>1</sup> was used as the starting point, but modifications were required to observe the impact of needle wash settings and design on carryover performance. To increase throughput, the method was geometrically scaled from a 4.6 x 250 mm, 3.5 µm column to a 3 x 100 mm, 2.5 µm column, thereby reducing the run time from 65 minutes to 19 minutes. The injection volume was increased from the scaled value of 1.7 µL to 5 µL to increase the impact of various parameter changes on observed carryover. Finally, the scaled method included an extended hold at gradient step 4 (80:20 A:B) so that the chlorhexidine peak would elute in a flat portion of baseline, thus making integration less subjective and more consistent from injection to injection and across systems. It also ensured that the chlorhexidine peak eluted in a stable portion of the baseline regardless of differing dwell volumes across the various systems.

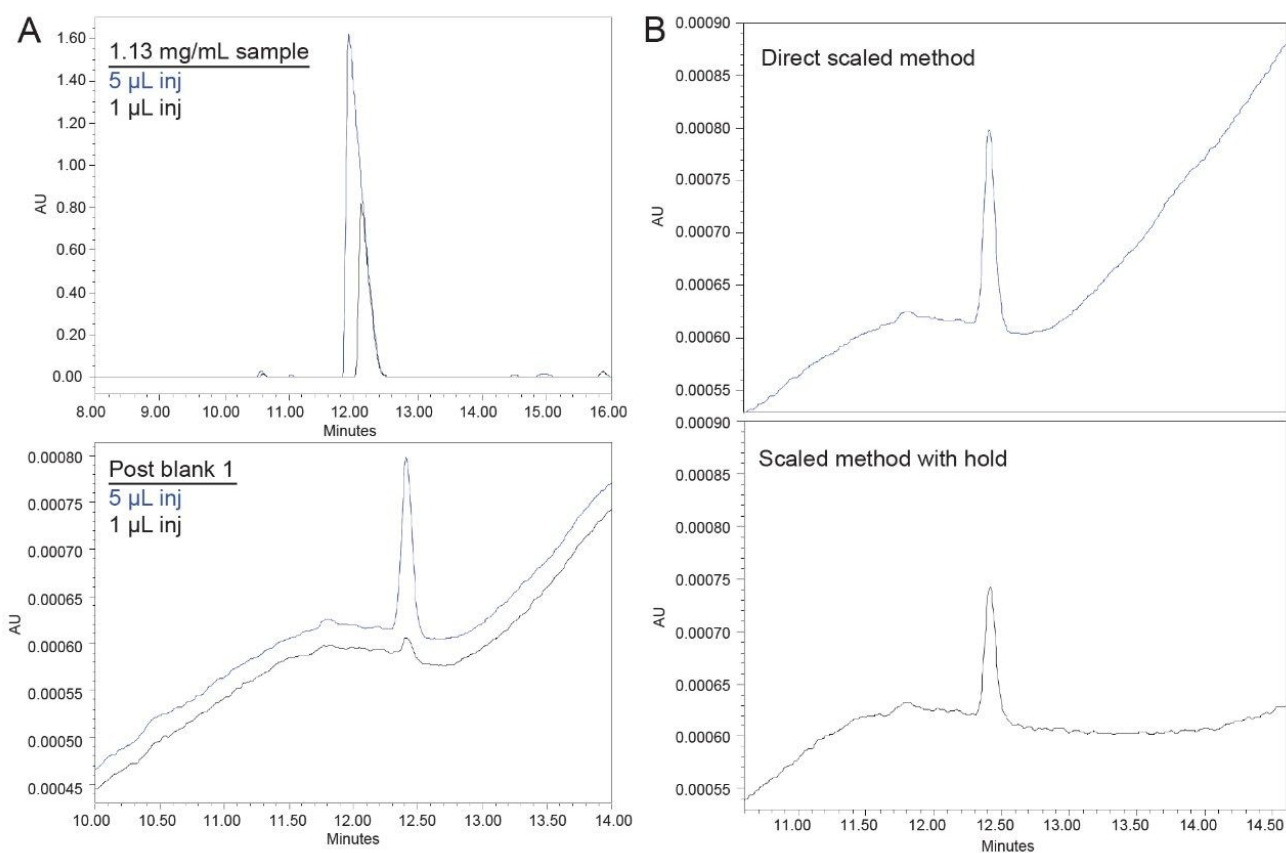


Figure 1. A) challenge sample (top) and post blank 1 (bottom) demonstrating measured carryover vs injection volume and B) extended gradient step to provide flat baseline in region of chlorhexidine peak elution.

## Experimental Design

Each carryover measurement sequence was set up as: pre blank x 3, chlorhexidine standard solution (11.4 μg/mL) x 3, chlorhexidine challenge solution (1.14 mg/mL) x 3, post blank 1 (unique vial 1), post blank 2 (unique vial 2), post blank 3 (unique vial 3). Multiple pre blank injections were used to confirm the system was “clean” prior to initiation of each carryover measurement. Mobile phases were used for a maximum of three days, then new batches were prepared.

## Carryover Results

Following the method conditions and sample sequence described above, carryover was determined for each

system. Percent carryover was calculated as:

$$\% \text{ Carryover} = \frac{(\text{area in post blank 1})}{(\text{average area of standard solution})} \times \frac{(\text{conc. Standard solution})}{(\text{conc. Challenge solution})} \times 100$$

Equation 1

As stated previously, each vendor has their own recommended or default wash modes. More advanced settings in an instrument method, such as needle washing, are not typically changed from the default and therefore initial carryover evaluation was done using the vendor defaults. Figure 2 shows the chlorhexidine carryover results obtained for each system that was evaluated using the vendor recommended/default wash mode.

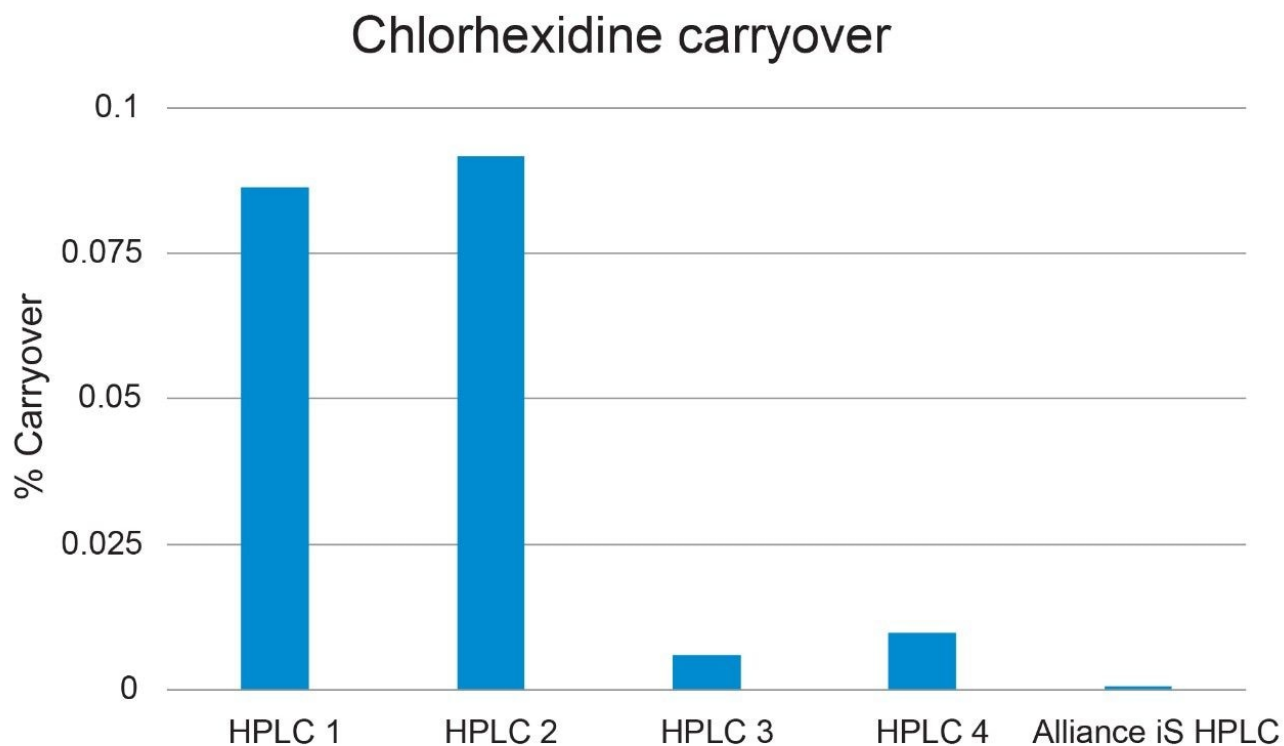


Figure 2. Chlorhexidine carryover results obtained for each LC system using the vendor recommended or default wash mode.



For HPLC systems 1–3, the default or recommended wash is No Wash. Unsurprisingly, when No Wash is performed, there is significant carryover seen for chlorhexidine, specifically on HPLC systems 1 and 2. Conversely, systems 4 & 5, which both employ needle washing by default, produced significantly lower carryover. The Alliance iS HPLC System, which utilizes a default needle wash of 1000  $\mu$ L, performed in two segments (pre inject and post inject) showed the lowest overall carryover of the systems tested, with only 0.00055% carryover (Figure 3).

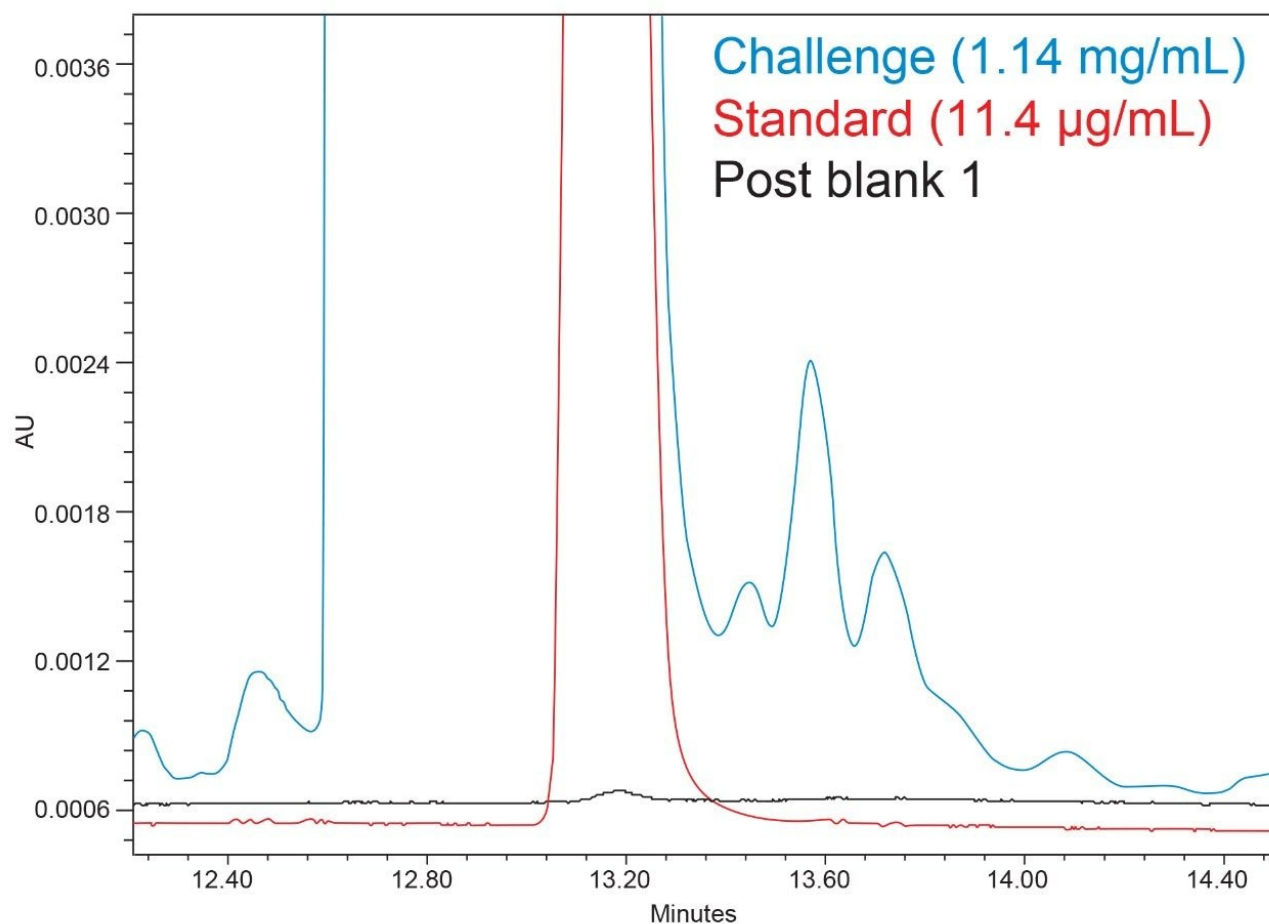


Figure 3. Measured chlorhexidine carryover (0.00055%) acquired on the Alliance iS HPLC System.

To evaluate the impact of adjusting needle wash settings, in addition to evaluation of the default wash modes, additional wash modes and/or wash duration (depending on wash mechanism) were also examined on each system. The goal was to compare observed carryover for all systems, after optimization of wash settings, to

provide the lowest levels of carryover on each system. The results for the systems tested using various wash modes are displayed in Figure 4.

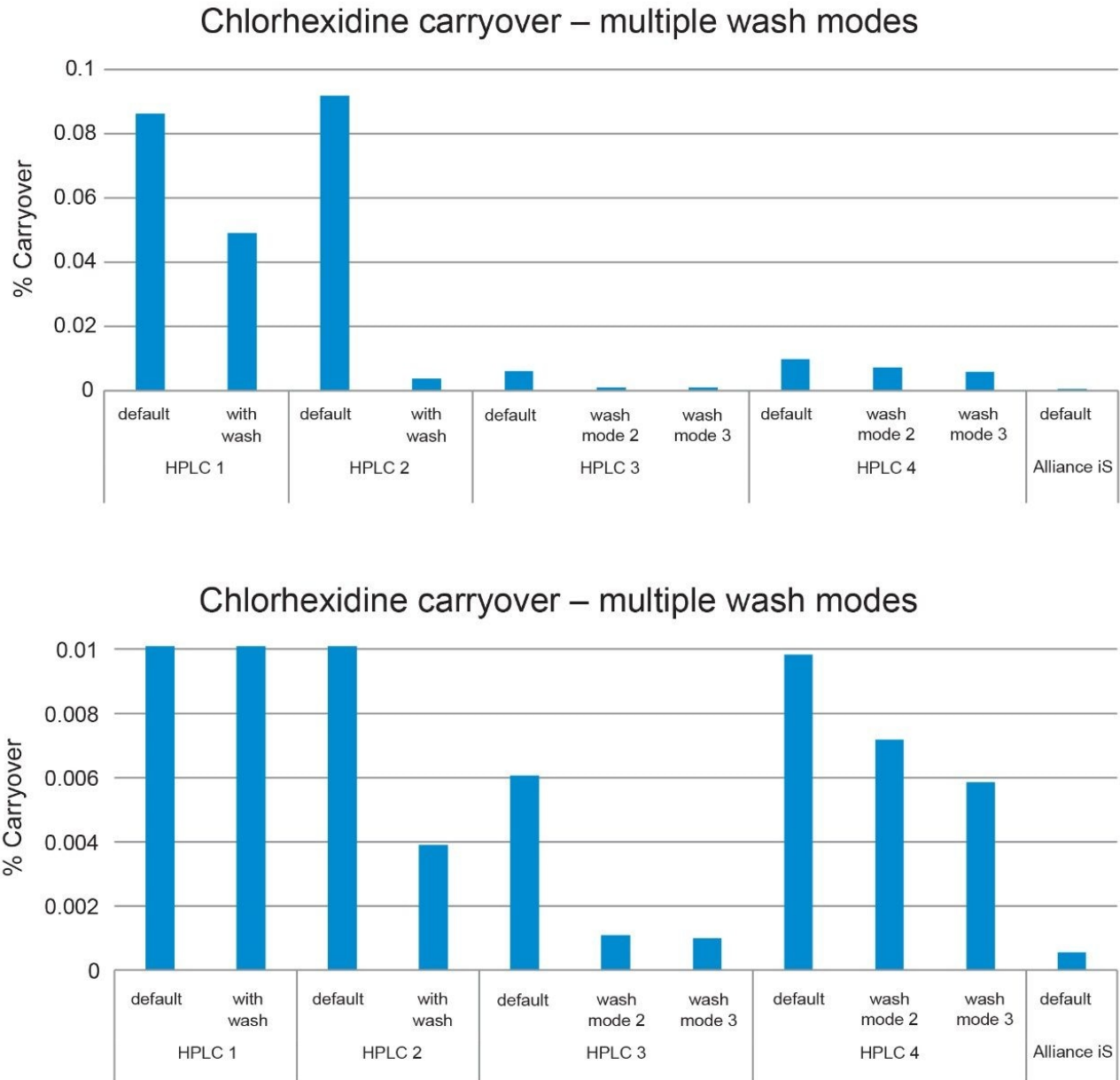


Figure 4. A) Full scale view of chlorhexidine carryover results obtained for each LC system using the vendor recommended/default wash mode compared to optional additional washing.

B) % Carryover scale zoomed in (0 to 0.01%).

As anticipated, adding a needle washing step for those systems where the recommendation is No Wash did show a reduction in carryover measured. Depending on the mechanism and design of the wash, the improvement in carryover varied significantly. In addition to impacting carryover, the exact washing mechanism can also impact the injection cycle time. For example, systems which have a separate washing station and injection port will suffer from increased cycle time because of the time it takes for the needle to move between the two stations. In contrast, the Alliance iS HPLC System performs the needle wash and the injection from a single port, which is a more time efficient design. Furthermore, the default needle wash settings and needle wash design have been optimized to obtain exceptional carryover performance from the Alliance iS HPLC System.

For HPLC systems 1 and 2, the wash mode consisted of dipping the needle into a vial of wash solution after the sample was drawn up in the needle. For HPLC system 3, the washing mechanism was performed by moving the needle to a wash station (at some point either before or after sample aspiration) and used actively flowing solvent supplied by a low-pressure syringe to wash the needle. The wash mechanism of HPLC system 4 involves washing the exterior surface of the needle while the needle is seated in the seal pack of the injector assembly. Finally, the Alliance iS HPLC System performs the needle washing in two stages as the needle is lowered into the injection port and needle wash solvent is actively flowed over the exterior of the needle. Because the washing happens in the same location as the injection, the increase in cycle time due to washing is negligible. Figure 5 shows the relative cycle times for each system which uses a default of No Wash vs adding an advanced needle washing step.

## Injection cycle times

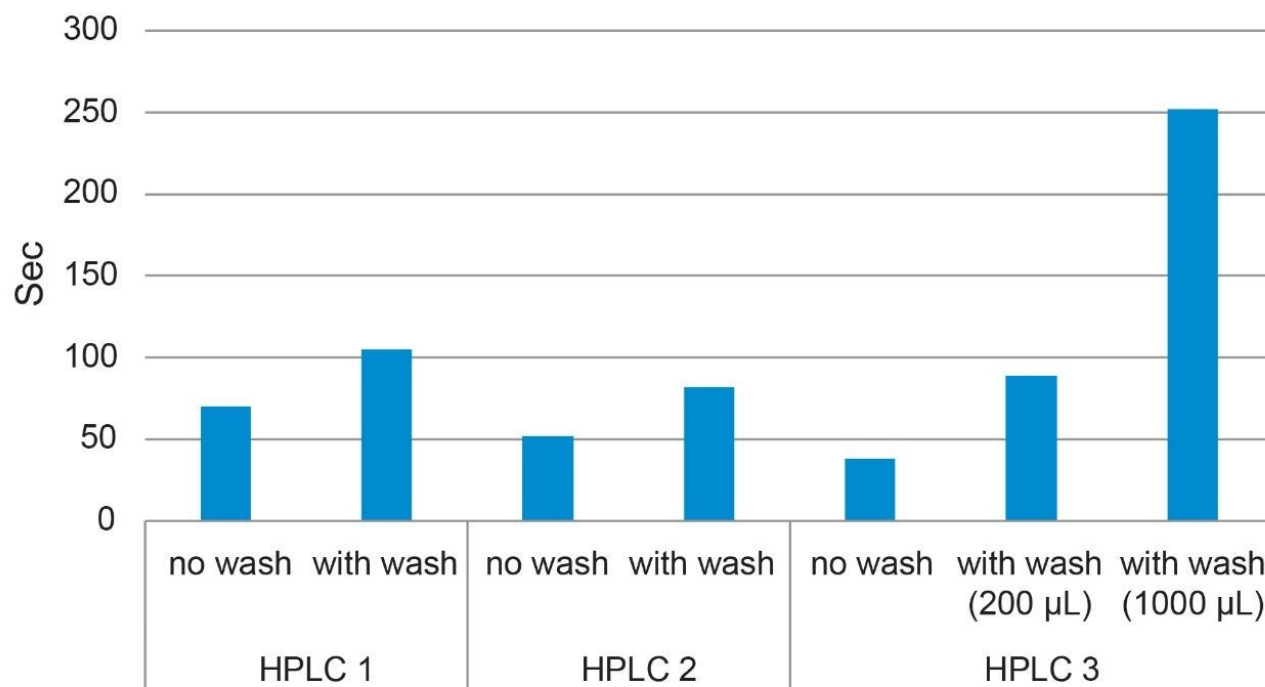


Figure 5. Cycle times observed for various HPLC systems that use a default of No Wash vs adding a washing step.

For the two HPLC systems (HPLC 1 and HPLC 2) which use a wash mechanism of dipping the needle into a vial containing wash solution, the injection cycle time was increased by 30–35 seconds on average. For the HPLC system which uses a discrete wash station and flows solvent over the exterior of the needle, the injection cycle time increase was highly dependent on the volume of needle wash used. A needle wash of 200 µL increased the injection cycle time by approximately 50 seconds, while a more extensive needle wash of 1000 µL increased the cycle time by a whopping 214 seconds. For reference, the Alliance iS HPLC system cycle time, which includes use of default wash conditions that ultimately accounted for the lowest amount of measured carryover, was shorter than all 3 HPLC systems which use a default of No Wash. Therefore, exceptionally low carryover performance does not require advanced washing mechanisms that result in significant increases to cycle time, but rather is more dependent on the design of the system itself.

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## Conclusion

Running HPLC methods on a variety of LC systems, often from different manufacturers, is a common occurrence in the pharmaceutical and biopharmaceutical industry. Additionally, method carryover is an important performance parameter that should be considered and monitored when migrating methods across different LC systems. In this application note, a scaled method based on the USP monograph for chlorhexidine hydrochloride organic impurities was used to assess carryover performance across several LC systems, with the Alliance iS HPLC System giving the lowest overall carryover of all the systems tested. The Alliance iS HPLC System utilizes tool free fittings for all frequently used touch points including column connections. These fittings are designed to be zero dead volume, meaning there is low risk of improper connections leading to voids in the flow path. Furthermore, the improved needle washing mechanism of the Alliance iS system results in superior carryover performance, even for challenging compounds such as chlorhexidine. The carryover performance obtained on the Alliance iS HPLC System did not require time consuming method optimization, nor did it require extensive washing mechanisms which can significantly increase injection cycle time.

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## References

1. Monograph: USP. Chlorhexidine Hydrochloride. In: USP–NF. Rockville, MD: USP; May 1, 2022.  
DOI: [https://doi.org/10.31003/USPNF\\_M15650\\_03\\_01](https://doi.org/10.31003/USPNF_M15650_03_01) <[https://doi.org/10.31003/USPNF\\_M15650\\_03\\_01](https://doi.org/10.31003/USPNF_M15650_03_01)> .

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[Empower Chromatography Data System <https://www.waters.com/10190669>](https://www.waters.com/10190669)

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