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Minimizing Carryover Using a Four Solvent Wash Station

Fred D. Foster, Edward A. Pfannkoch, John R. Stuff
Gerstel, Inc., 701 Digital Drive, Suite J,
Linthicum, MD 21090, USA

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Abstract
When analytes from a sample injection into an LC system are not completely flushed from the sample introduction hardware, valve and connections following the injection, a carryover effect can be seen in subsequent injections of blank samples. Minimizing this carryover effect during an analytical run is important for providing reproducible and reliable data. Typically, limiting carryover to less than 20 % of the lower limit of quantitation is desired. As today's detection systems become increasingly sensitive, reduction of carryover becomes increasingly important.

In this work, we present a wash station that allows the user access to up to four independent wash solvents that can be used following an LC injection to minimize the carryover effect for compounds with particularly strong retention properties. Using a GERSTEL MPS 3 autosampler with MAESTRO software enables the analyst to control the Four Solvent Active Wash Station, toggle the injection valve to clean the sample loop, and to control valve switching used for offline column conditioning when multiplexing analytical runs.

Techniques used to minimize the carryover effect are discussed and examples of HPLC injection cycle control options available within the MAESTRO software are illustrated. LC-MS/MS data are presented showing a reduction of the carryover effect when using a Four

Solvent Active Wash Station for a compound having particularly strong retention properties.

Introduction

In an analytical method, "carryover" is defined as the presence of analyte in a blank sample that follows the injection of a high concentration sample. Carryover is usually caused by residual analyte accumulating in the sample injection flow path and is typically specified as a percentage of the lower limit of quantiation (LLOQ) of the analytical method being used.

Possible locations of an injection system in which residual analyte can accumulate leading to carryover are:

- The injection syringe barrel and/or needle.
- "Dead volume" spaces present within the injection valve.
- The rotor of the injection valve.
- The tubing material used within the injection system.

Guidelines for carryover mitigation when using an MPS 3 multi-purpose sampler are:

- Following sample injection, wash the syringe and valve with the proper choice of solvents.
- Ensure the needle penetration depth is correct within the injection valve to avoid creating a "dead volume" space.
- Inspect the needle inlet sleeve for wear and replace as necessary.
- Inspect the valve's rotor for scoring or scratches that could be locations at which residual analyte could collect. Replace worn rotors as necessary.
- Ensure the syringe plunger is not worn or loose and that the plunger is seated at the bottom of the syringe barrel during an injection.
- Ensure that proper injection volumes are used during partial loop filling methods. The recommended maximum volume for a partial loop filling technique is one third of the sample loop volume.
- Ensure that all fittings associated with the injection valve have been properly installed.
- Increase the number of syringe and valve washes.
- Use the most appropriate wash solvents for the sample being injected to ensure that the wash solvents being used are miscible with each other and with the sample solution being injected.
- Toggle the injection valve during the washing protocol in order to effectively wash all ports of the injection valve and the sample loop.

Even when following the guidelines for carryover mitigation presented above, for compounds with particularly strong retention properties, the two wash solvents available on standard wash stations may not be enough. In this paper we present data showing that the use of additional wash solvents can help the user to minimize carryover for such compounds.

Using GERSTEL's novel and proprietary Four Solvent Active Wash Station, the additional wash solvent capability coupled with its active pumping feature and the ability of the MAESTRO control software to toggle the injection valve during the wash protocol were examined for an LC/MS/MS method in which the analyte of interest had particularly strong retention properties.

The measure of carryover used in this study followed the suggested definition outlined in the FDA Guidance for Industry Bioanalytical Method Validation [1]. As stated, carryover was calculated from the analyte peak area in a blank injection immediately following an injection at the analyte's upper limit of quantitation (ULOQ). The percentage of the blank peak area compared to the peak area from an injection made at the analyte's lower limit of quantitation (LLOQ) is defined as the analyte carryover. The analyte used in this study is a pharmaceutically relevant small molecule with known propensity for carryover in LC/MS/MS analysis.

Experimental

Materials. The analyte chosen to test the Four Solvent Active Wash Station was a proprietary small molecule within the molecular weight range of 200-800 amu with drug like properties. A stock solution of the analyte was prepared at 1 mg/mL in dimethyl sulfoxide (DMSO) and further diluted with acetonitrile (ACN) to prepare working solutions at several concentrations. To prepare plasma extracts, 10 mL of working solution was spiked into 90 mL of K_2 EDTA blank rat plasma (Bioreclamation Inc.) at various concentrations. The LLOQ and ULOQ samples were spiked with 0.01 µg/mL and 50 µg/mL working solution, respectively, to produce final concentrations of 1 ng/mL (LLOQ) and 5000 ng/mL (ULOQ). The spiked plasma was precipitated with 400 uL acetonitrile followed by 5 minutes of shaking and pelleted by 20 minutes centrifugation at 3000 rpm (Eppendorf 5804R).

. The resulting supernatant was transferred to shallow 96-well microtiter plates for injection. Blank plasma extracts were prepared using blank working solution and following the same procedure as used for the

LLOQ and ULOQ samples. Each sample was injected only once to prevent cross contamination.

Analysis conditions.

Autosampler:	MPS 3 multi-purpose sampler with Four Solvent Active Washstation
LC Pump:	Agilent 1100 series
Flowrate:	800 µL/min
Mobile Phase:	A - 10 mM ammonium acetate at pH 4.5 B – Acetonitrile Linear gradient, from 5 % to 95 % B
Column:	2 mm x 30 mm, 3 mm, C-18 analytical column
Inj. valve:	6 port (0.40 mm) Cheminert C2V with 50 µL stainless steel sample loop
Inj. volume:	5 µL
Detector:	Applied Biosystems, API-4000, SRM mode, APCI (+) ionization

All analytes tested were manually tuned to identify SRM transitions and source conditions optimized for sensitivity. Peak area integration was performed using the IntelliQuan function in Analyst 1.4.1 software. To calculate carryover, peak areas of 3 LLOQ injections were averaged and compared to the peak area of a single blank plasma extract injection following a single ULOQ injection. This injection sequence was performed in triplicate (n=3) for each condition tested and all carryover reported as a % average of the LLOQ.

Figure 1 shows the fluidics diagram of the Four Solvent Active Wash Station. The MAESTRO software is used to access the different wash solvents to clean the injection system. An example of a PrepSequence which defines the wash protocol used during a typical sample injection using the GERSTEL MPS 3 is shown in Figure 2.

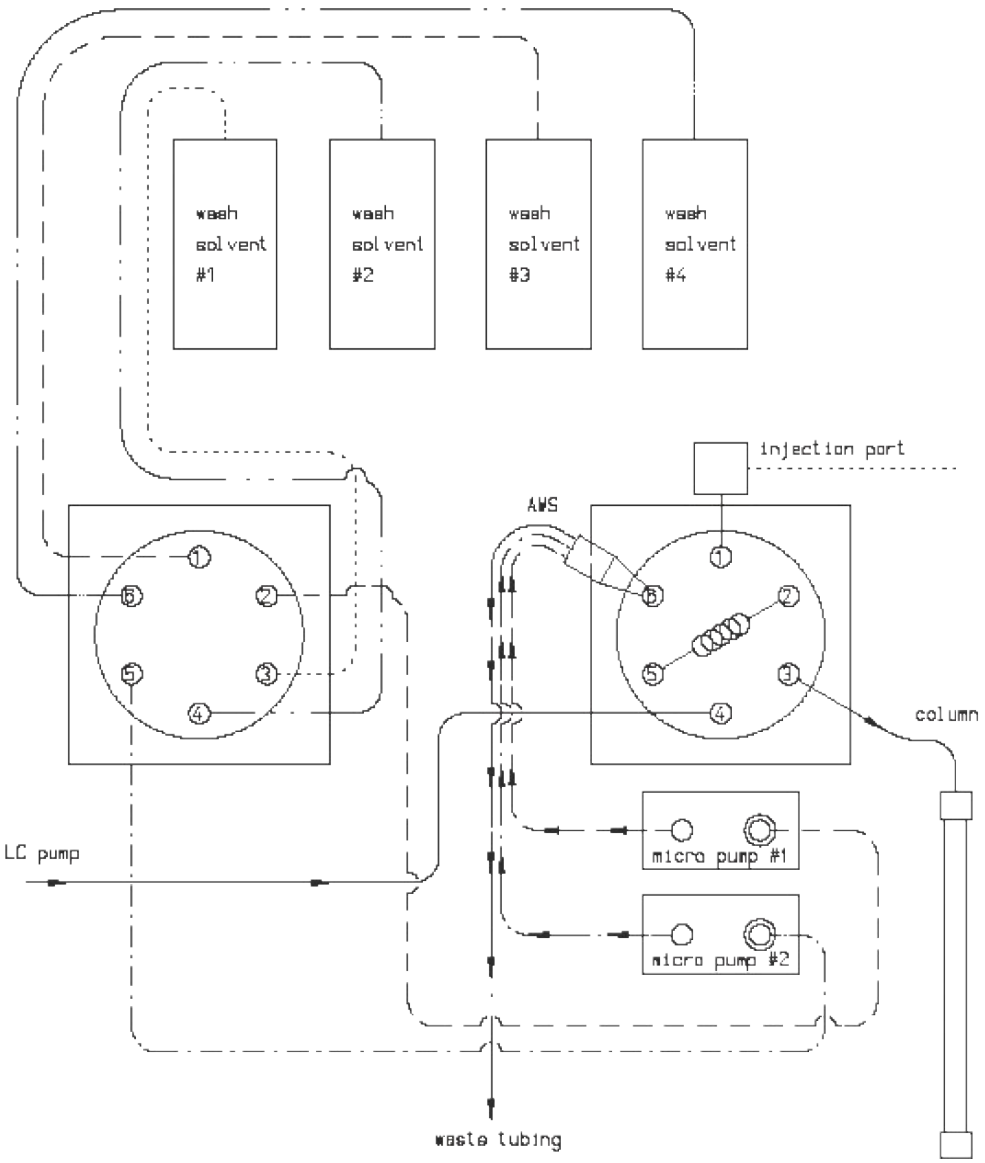

Figure 1. Fluidics diagram of the GERSTEL MPS 3 with Four Solvent Active Wash Station.

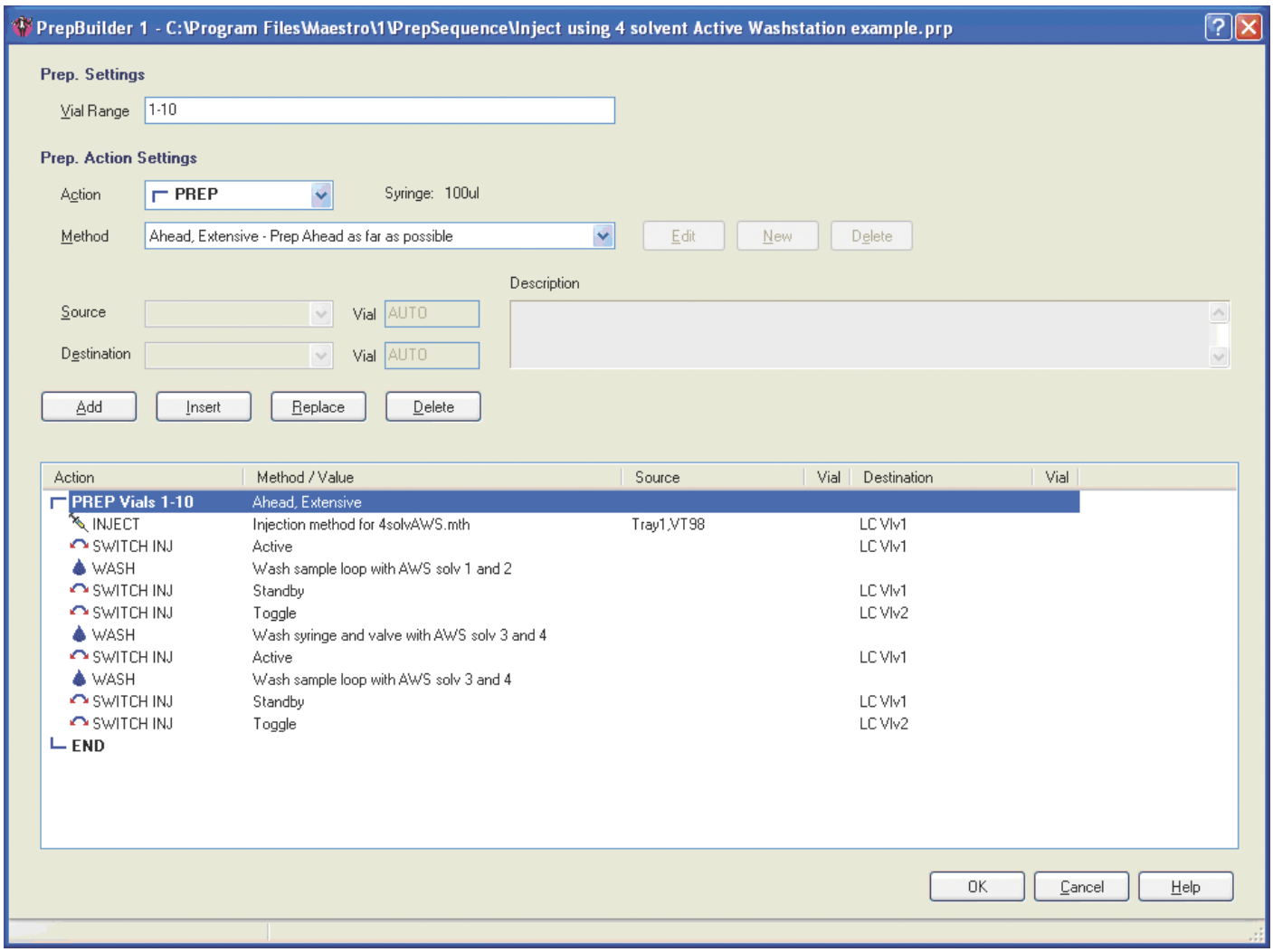

Figure 2. Example of a MAESTRO Prep Sequence used for control of the Four Solvent Active Wash Station.

Results and Discussion

To compare carryover results obtained using the Active Wash Station, a baseline measure of the carryover was first conducted using the standard conditions employed in the lab using the existing Fast Wash Station. The Fast Wash Station consists of two gravity filled solvent reservoirs that allow the injection syringe access to wash solvent during the cleaning procedure. Using the Fast Wash Station, the injection valve is cleaned by transferring wash solvent from a reservoir to the injection valve using the injection syringe. The Active Wash Station consists of two self priming micro pumps mounted underneath the valve drive, a special needle guide assembly and tubing set including check valve. Once activated, these micro pumps allow cleaning solvent to flow through the valve groove, enabling the entire sample flow path to be cleaned in a back flush direction and rinses valve engravings, needle seal and syringe at the same time.

A representative chromatogram of the LLOQ is shown in Figure 3 A for comparison. The standard wash protocol using the Fast Wash Station consisted of syringe and injection valve flushing using three steps of 100 µL 90/10 ACN/H_2O 0.1% formic acid (ACN wash) followed by three steps of 100 µL 90/10 H_2O/ACN 0.1% formic acid (H_2O wash). The initial results of carryover using the Fast Wash Station were found to be over 5 x the response at the LLOQ (Figure 3 B). The same column/gradient/wash protocol was then used to measure carryover using the GERSTEL Active Wash Station. The addition of the GERSTEL Active Wash Station to the autosampler was found to reduce the carryover by over 50 % compared to the results using the existing Fast Wash Station, as shown in Figure 3 C.

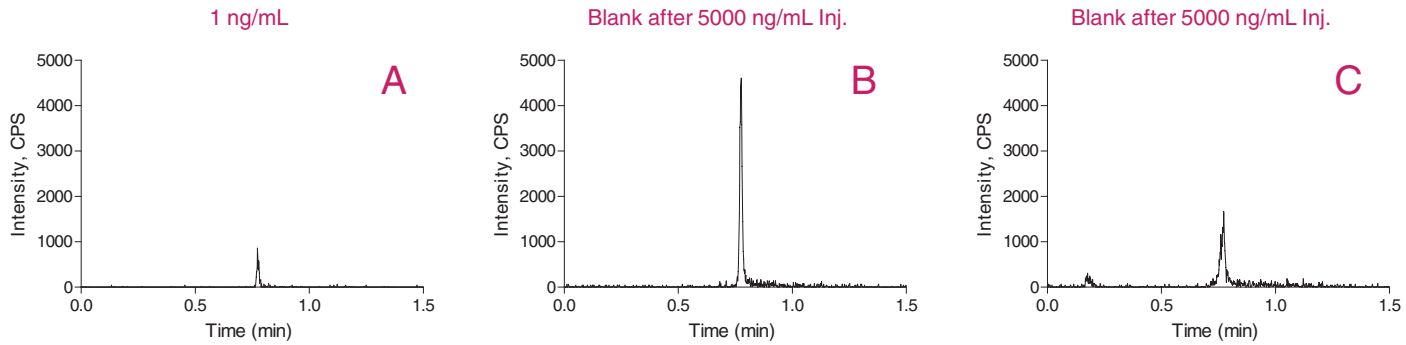

Figure 3. Resulting carryover when using Fast Wash Station (Fig. 3B) or Active Wash Station (Fig. 3C).

Further studies were performed which focused on the optimization of wash solvents, columns, gradients, and valve toggles using the GERSTEL Four Solvent Active Wash Station to achieve the desired reduction in carryover. Although the injection valve was found to be a significant source of carryover for the method used, smaller contributions of the analytical column were found as well.

Table 1 provides an overview of the results obtained during the optimization of the wash protocol using the GERSTEL Four Solvent Active Wash Station.

An additional reduction in carryover was obtained by adding a third solvent to the wash protocol. In Figure 4, dimethyl sulfoxide was used as the initial wash solvent, followed by the ACN and H_2O wash protocol employed in Figure 3. Figure 4 C demonstrates that using a 3 x DMSO wash reduces carryover to 172 % LLOQ (compared to the initial carryover results of 263 % of LLOQ shown in Figure 3 C). Increasing the number of initial DMSO washes from 3 x to 6 x reduced carryover even further to 156 % of LLOQ (Figure 4 D).

Table 1. Optimization of wash protocol.

Associated Figure	Comments	Carryover [% LLOQ]
Figure 3	Initial results using Fast Wash Station	516%
Figure 3	Initial results using Active Wash Station	263%
Figure 4	Results after additional wash solvent (6x DMSO)	156%
Figure 5	Results after optimization of additional wash solvent (50/50 DMSO/Methanol)	100%
Figure 7	Results after optimization of wash protocol with Four Solvent Active Wash Station	15%

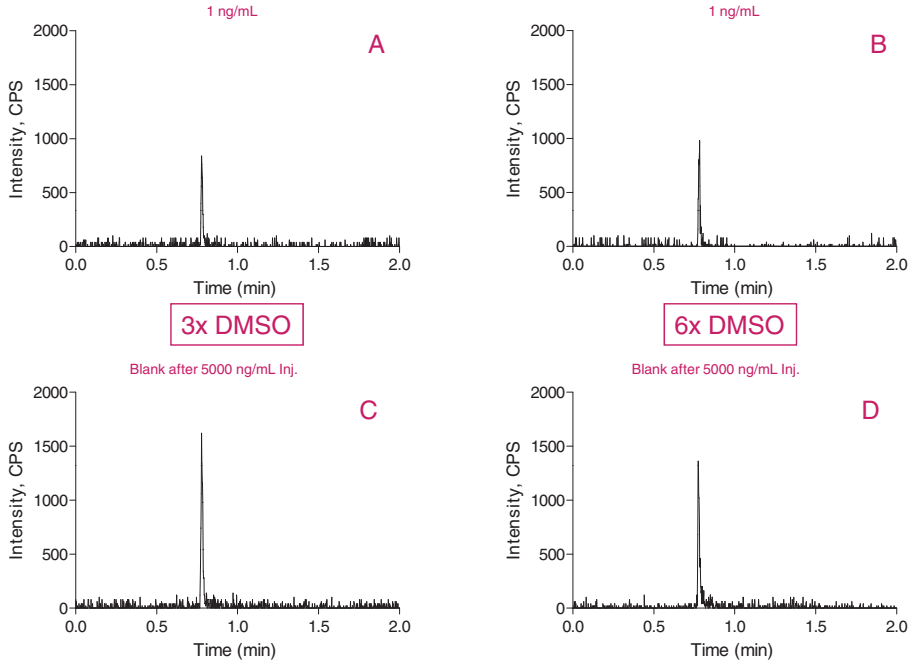

Figure 4. Resulting carryover when using Four Solvent Active Wash Station and three or six additional wash solvent replicates.

A modification of the initial wash solvent from 6 x 100 % DMSO to 6 x 50/50 DMSO/Methanol resulted in a further reduction of the carryover from 156 % of LLOQ (as shown in Figure 5 D) to 100 % of LLOQ shown in Figure 5 B. This helps to illustrate the importance of proper wash solvent selection in reducing carryover.

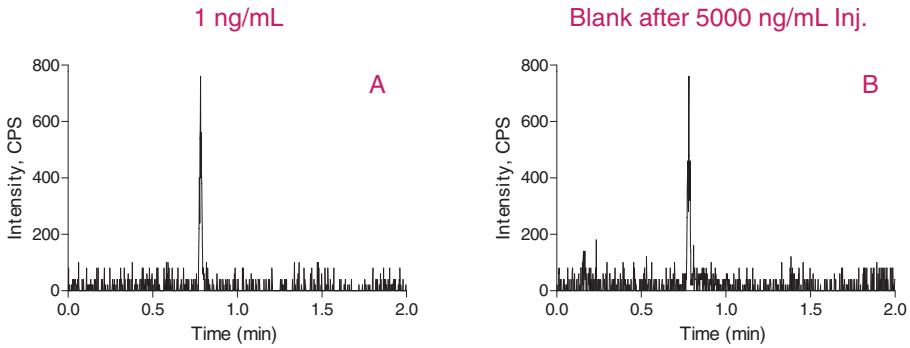

Figure 5. Resulting carryover after optimization of the additional wash solvent.

The initial 1.5 minute runtime was extended to 3 minutes to allow sufficient time for completing the wash protocol. Using a 6 x DMSO/MeOH, 6 x ACN, 3 x H_2O wash protocol as reference (Figure 6 E), several additional solvent wash combinations were compared. Figure 6 G shows the resulting carryover using a 50-30-20 mixture of isopropyl alcohol (IPA)-ACN-H_2O as the initial wash step (x 6). This wash protocol was used with several different gradient profiles in which the total runtime was modified (Figures 6 F and 6 H). Further reduction in the resulting carryover was not found using the gradient profiles tested that employed the 50-30-20 IPA-ACN-H_2O wash protocol. The original 50-50 DMSO-MeOH protocol shown in Figure 6 E resulted in the best reduction in carryover (35 % of LLOQ).

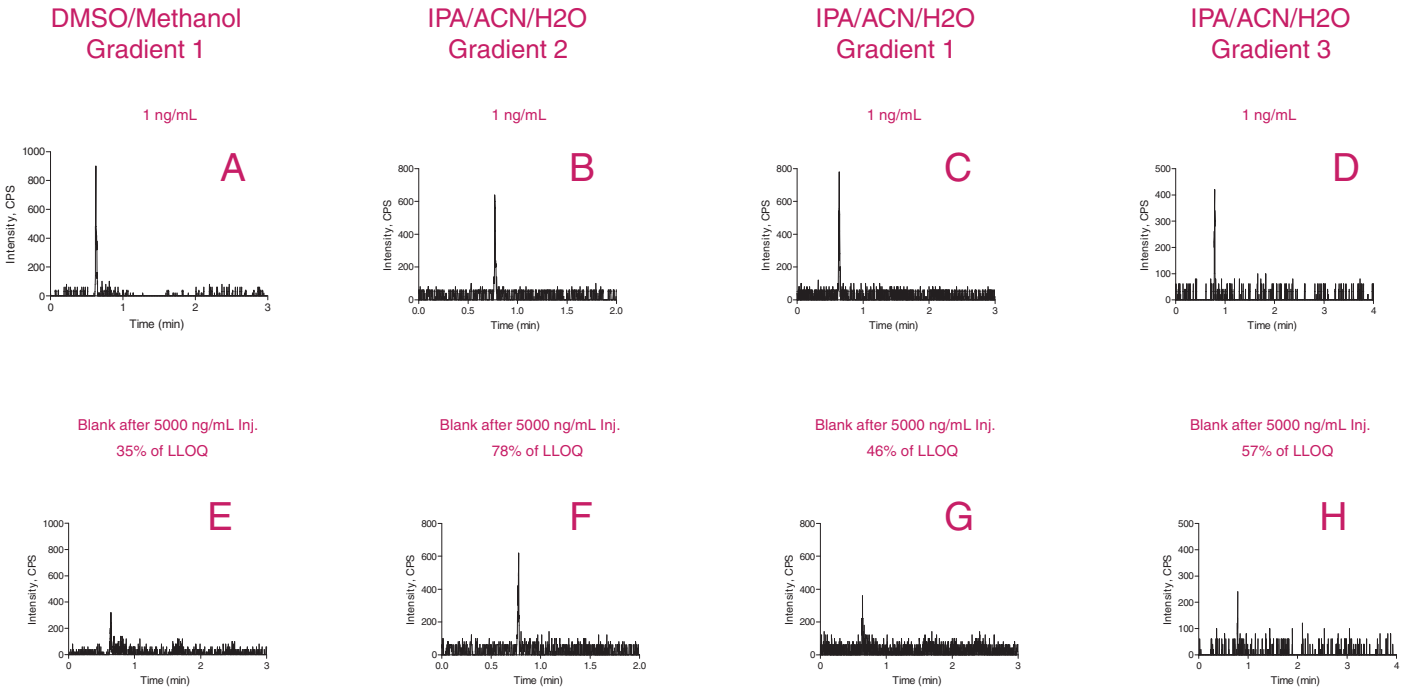

Figure 6. Resulting carryover when using DMSO/Methanol or IPA/ACN/H_2O as additional wash solvent.

As shown in Figure 7, comparison between the 6 x 50/50 DMSO/Methanol, 6 x ACN, 3 x H_2O wash protocol (Figure 7 C) to one in which the initial wash solvent was 6 x 50/30/20 DMSO/IPA/ACN (Figure 7 D) confirms that the DMSO/Methanol wash solvent resulted in the most reduction in carryover. A resulting carryover of 15 % of LLOQ was shown for the DMSO/Methanol wash protocol compared to a resulting carryover of 92 % of LLOQ shown for the DMSO/IPA/ACN wash protocol.

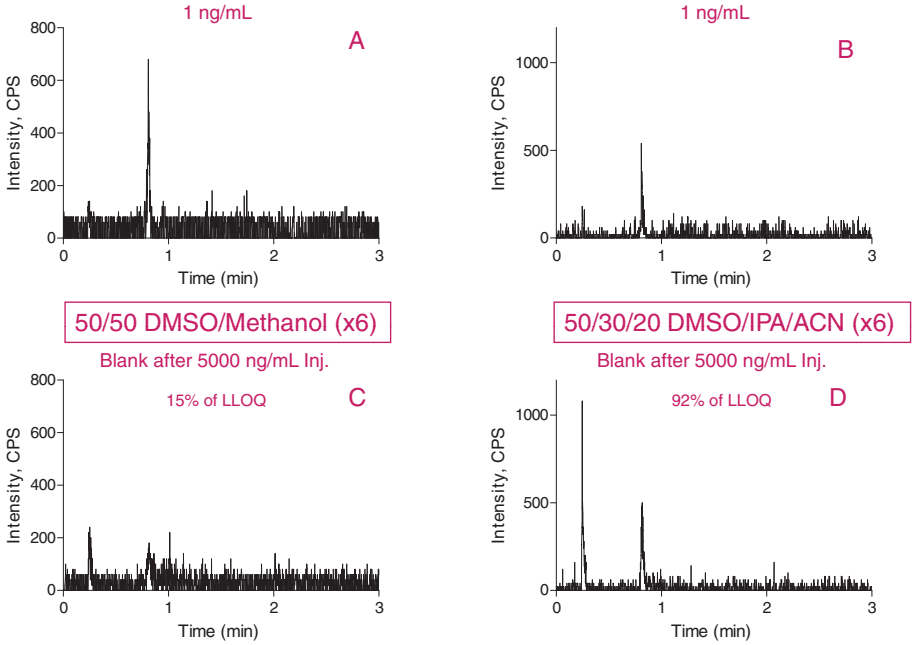

Figure 7. Resulting carryover when using DMSO/Methanol or IPA/ACN/H_2O as additional wash solvent.

As shown in Figure 8, comparison between the 6 x 50/50 DMSO/Methanol, 6 x ACN, 3 x H_2O wash protocol (Figure 8 C) to one in which the initial wash solvent was 100 % THF (Figure 8 D) confirms that the DMSO/Methanol wash solvent resulted in the biggest reduction in carryover. The resulting carryover shown for the 100 %THF was twice that of the 50/50 DMSO/Methanol wash protocol.

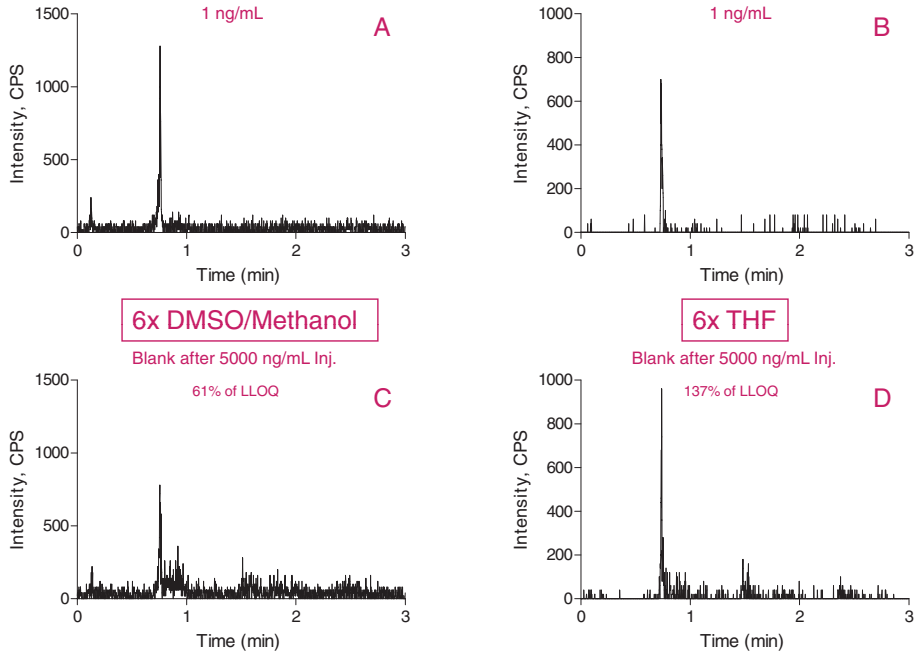

Figure 8. Resulting carryover when using DMSO/Methanol or THF as additional wash solvent.

All washes occur with the valve in the “inject position”. The MAESTRO software allows the valve to be toggled between the “inject” position and “load” position. The effect of toggling the injection valve was also investigated. The valve toggling schemes used during the course of the wash protocol consisted of:

“During” wash protocol	“Post” wash protocol
3 x 50/50 DMSO/Methanol	6 x 50/50 DMSO/Methanol
Valve toggle	6 x ACN wash solvent
3 x 50/50 DMSO/Methanol	3 x H_2O wash solvent
Valve toggle	5 x Valve toggles
3 x ACN wash solvent	
Valve toggle	
3 x ACN wash solvent	
3 x H_2O wash solvent	
2 x Valve toggles	

Figure 9 F shows that alternating washing and valve toggling was more effective in reducing carryover than either no toggle (Figure 9 D) or 5 valve toggles after the wash protocol had finished (Figure 9 E).

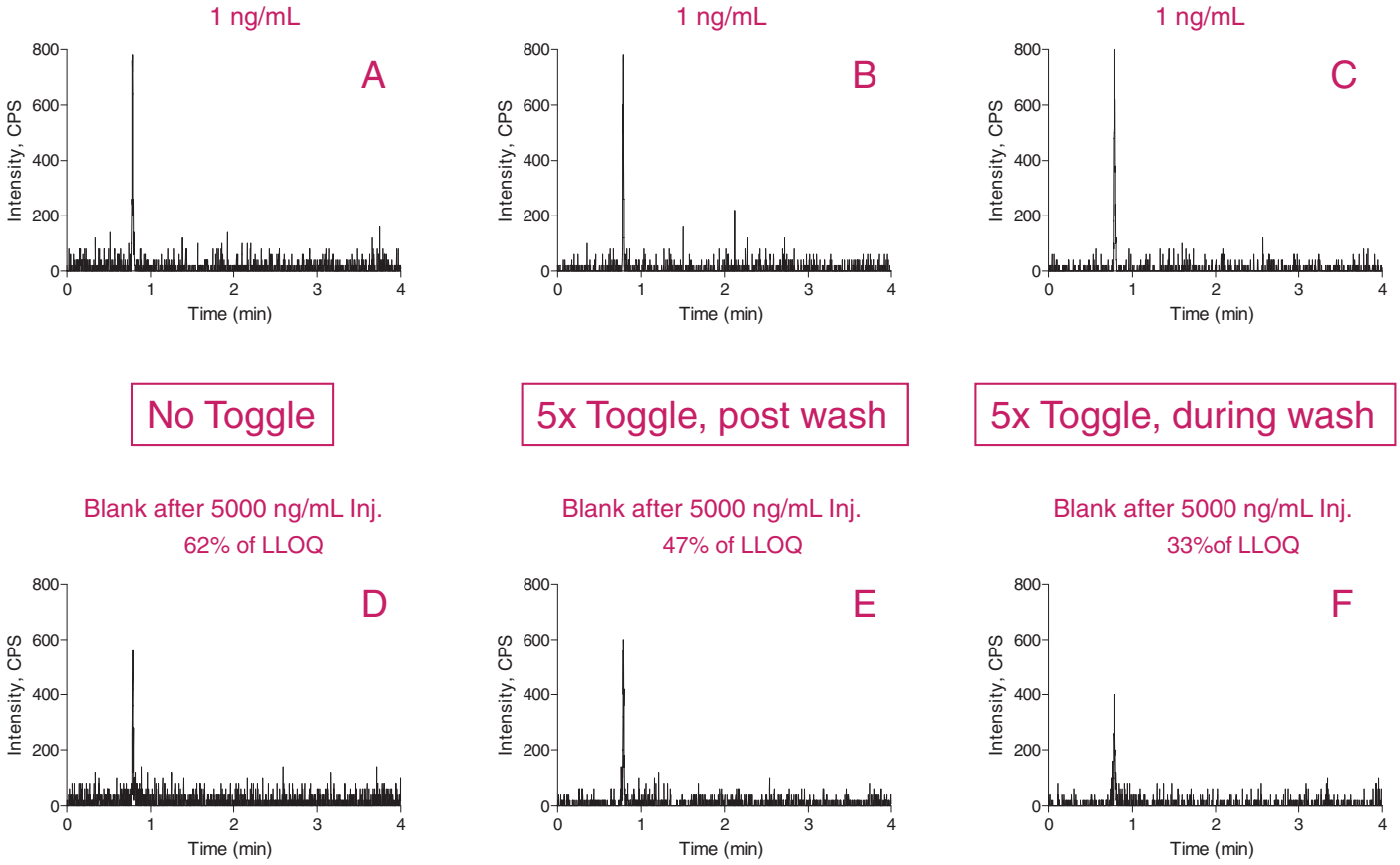

Figure 9. Carryover mitigation effect of valve toggling.

Toggling the injection valve required extra time, which necessitated the use of a longer (4 minute) method. Since the resulting carryover shown in the final analytical method without valve toggling met acceptance criteria, to conserve total runtime, the final method did not employ the use of valve toggling.

Figure 10 demonstrates the improved performance of the GERSTEL Four Solvent Active Wash Station over the standard Fast Wash Station protocol. The test compound was analyzed using the optimized column and gradient conditions established during the course of the investigation. The resulting carryover using the GERSTEL Four Solvent Active Wash Station (Figure 10 C) was reduced by 80 % compared to the carryover observed following the Fast Wash Station protocol (Figure 10 B) and met acceptance criteria for carryover of < 20 % LLOQ. This experiment directly compares the effect of actively pumping the wash solvents in combination with an optimized wash protocol to the static rinsing utilizing a more typical wash protocol.

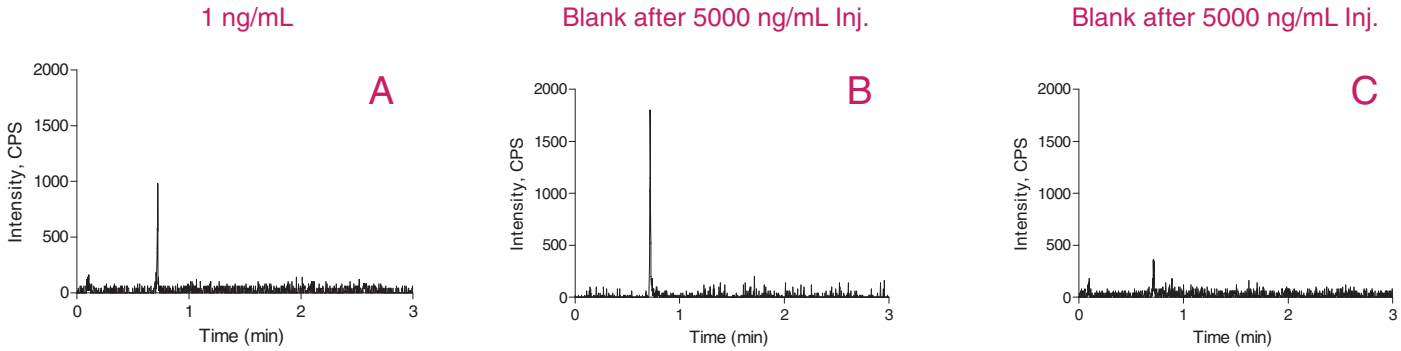

Figure 10. Resulting carryover when using Fast Washstation (B) or optimized Four Solvent Active Washstation (C).

Conclusions

- Several wash parameters were optimized in order to minimize carryover, including the wash solvent protocol and valve toggling.
- When compared directly to the Fast Wash Station, the Active Wash Station showed a 51 % reduction of carryover.
- MAESTRO software control of the entire injection protocol allowed for the easy development of an optimized injection protocol that included three separate wash solvents and lead to an 80 % reduction in carryover compared to original sample injection parameters.
- The availability of additional wash solvents when using the Four Solvent Active Wash Station was found to be beneficial in the mitigation of carryover.
- The GERSTEL MPS 3 Multi-purpose sampler with the Four Solvent Active Wash Station was found to achieve acceptable carryover for an analyte with particularly strong retention properties.

References

[1] FDA Guidance for Industry Bioanalytical Method Validation, Drug Information Branch (HFD-210), Center for Drug Evaluation and Research (CDER), available at: http://www.fda.gov/cder/guidance/index.htm

G L O B A L A N A L Y T I C A L S O L U T I O N S

GERSTEL GmbH & Co. KG
Eberhard-Gerstel-Platz 1
D-45473 Mülheim an der Ruhr
+49 (0) 208 - 7 65 03-0
+49 (0) 208 - 7 65 03 33
gerstel@gerstel.com
www.gerstel.com

GERSTEL Inc.
701 Digital Drive, Suite J
Linthicum, MD 21090
+1 (410) 247 5885
+1 (410) 247 5887
info@gerstelus.com
www.gerstelus.com

GERSTEL GmbH & Co. KG
Technisches Büro Berlin
Marburger Straße 3
10789 Berlin
(0 30) 21 90 98 28
(0 30) 21 90 98 27
tb_berlin@gerstel.de

GERSTEL AG
Enterprise
Surentalstrasse 10
CH-6210 Sursee
+41 (41) 9 21 97 23
+41 (41) 9 21 97 25
gerstel@ch.gerstel.com
www.gerstel.de

GERSTEL GmbH & Co. KG
Technisches Büro Bremen
Parkallee 117
28209 Bremen
(04 21) 3 47 56 24
(04 21) 3 47 56 42
tb_bremen@gerstel.de

GERSTEL K. K.
2-13-18 Nakane, Meguro-ku
Dai-Hyaku Seimei Toritsudai Ekimae Bldg 2F
152-0031 Tokyo
+81 3 5731 5321
+41 3 5731 5322
info@gerstel.co.jp
www.gerstel.co.jp

GERSTEL GmbH & Co. KG
Technisches Büro Karlsruhe
Greschbachstraße 6a
76229 Karlsruhe
(07 21) 9 63 92 10
(07 21) 9 63 92 22
tb_karlsruhe@gerstel.de

GERSTEL GmbH & Co. KG
Technisches Büro München
Stefan-George-Ring 29
81929 München
(04 21) 3 47 56 24
(04 21) 3 47 56 42
tb_bremen@gerstel.de