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Application Note

Peptide Mapping using Binary Biocompatible LC Systems: Evaluation of Retention Time Precision and Mixing Effects on Waters and Competitive LCs

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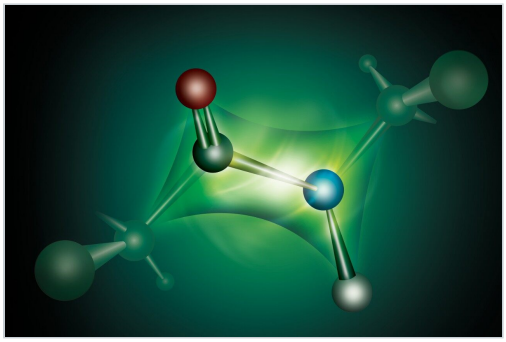

Abstract

A fully biocompatible LC system equipped with binary high-pressure mixing solvent delivery produces the highest precision results while maintaining flexibility to run biomolecule methods which require a corrosion-resistant system for high salt applications. This application note focuses on the impact of challenging method conditions, typical to peptide mapping applications, which include low flow rates and small percent change in organic composition over time. For many LC systems, this combination of method conditions can result in large variation in retention times and a noisy baseline due in part to inefficiencies in system mixing behavior. Using the binary based biocompatible ACQUITY UPLC H-Class PLUS System, the data produced demonstrates a high level of reproducibility, which is a critical attribute of reliable and accurate data. In addition, a mixer designed specifically for use with trifluoroacetic acid containing mobile phases shows a significant reduction in baseline noise, improving signal to noise for increased assay sensitivity. In addition to the ACQUITY UPLC H-Class PLUS Bio Binary System, two other competitive LC systems-both equipped with binary high-pressure mixing pumps-were evaluated for impact on retention time reproducibility and baseline noise.

Benefits

- Generate reproducible retention times for challenging methods
- System flexibility for many biomolecule applications with no decrease in performance
- Mixing options to reduce baseline noise and increase sensitivity

Introduction

A fully biocompatible binary-based LC system provides flexibility for running biomolecule methods which may require a corrosion-resistant flow path, while still providing the highest degree of gradient precision for challenging methods. Peptide mapping methods represent a class of methods that can be challenging in terms of gradient precision and reproducibility across injections and thus is the ideal application to explore the performance of the new ACQUITY UPLC H-Class PLUS Bio Binary System. This application note will focus on reproducibility of gradient delivery (measured by retention time precision over multiple injections) and mixing efficiency (measured by calculated noise and signal to noise values), two key aspects of pump performance.

The solvent delivery mechanism of high-pressure mixing binary pumps has been shown to provide superior gradient precision compared to their low-pressure mixing counterparts, such as quaternary and ternary blending pumps, under certain method conditions. These method conditions include low flow rates and small changes in gradient composition over time, commonly referred to as shallow gradients.[1] There are multiple application areas that require the use of these shallow gradients, the most common being peptide mapping. Enzymatic digests of a protein, which can generate complex mixtures of peptides, often require high resolving shallow gradients run with low flow rates to chromatographically separate individual peptides for identification and/or quantification purposes. Even for high-pressure binary mixing pumps, these challenging programmed methods can be difficult to deliver reproducibly. Because of the sensitivity of peptide elution to small changes in organic composition, inter and intra-run variability in gradient composition delivery is traditionally observed as retention time variation across injections.

To deliver a gradient reproducibly, it is also important, especially in high-pressure mixing systems where the independent flow paths are combined at the mixer, that the system mixing is adequate and reproducible. If the mixing is not reproducible and results in slightly different compositions delivered to the analytical column from injection to injection, retention time differences or variability may be observed. Furthermore, for peptide methods which require the use of additives with a low UV cutoff, such as trifluoroacetic acid, any heterogeneity of mobile phase composition due to inadequacy of mixing can manifest as a “wavy”, increased, or non-reproducible baseline. This is a function of both the additive and the wavelength needed for data collection (214 nm). For low level impurities especially, any “dip” or “bump” in the baseline near the retention time of your peak of interest can have a significant impact on peak integration and peak area, rendering quantitative results questionable. When a peak elutes in an unstable region of baseline, it is difficult to determine where the peak start and end occur. This means that a portion of the peak may be under the baseline, thus the integrated area may not be the true area of the peak, leading to quantitative inaccuracy and/or inconsistency. In addition, the effects of insufficient mixing are exacerbated for

smaller intensity peaks. In this application note the effects of gradient delivery precision and mixing efficiency across different LC systems are explored.

Experimental

Sample Description

This work uses the Waters mAb Tryptic Digestion Standard (part number 186009126). The standard was reconstituted with 100 µL of 0.1% trifluoroacetic acid in water (mobile phase A). Blank injections used the same 0.1% trifluoroacetic acid in water (mobile phase A).

Method Conditions

LC Conditions

LC system:	ACQUITY UPLC H-Class PLUS Bio System with Binary Solvent Management
Detection:	TUV with 10 mm analytical flow cell: 214 nm, 10 Hz
Column(s):	ACQUITY UPLC Peptide CSH C_{18} Column, 130 Å, 1.7 µm, 2.1 mm x 150 mm (part number: 186006938)
Column temp.:	60 °C
Sample temp.:	8 °C
Injection volume:	10 µL
Flow rate:	Method 1–0.5 mL/min Method 2–0.2 mL/min
Mobile phase A:	0.1% trifluoroacetic acid in water
Mobile phase B:	0.1% trifluoroacetic acid in acetonitrile

Gradient

Method 1

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.500	99	1	--
1.3	0.500	99	1	6
24.7	0.500	60	40	6
27.0	0.500	35	65	6
30.3	0.500	35	65	6
33.0	0.500	99	1	6
40.0	0.500	99	1	6

Method 2

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.200	99	1	--
3.3	0.200	99	1	6
96.7	0.200	60	40	6
102.5	0.200	35	65	6
110.8	0.200	35	65	6
117.5	0.200	99	1	6
135.0	0.200	99	1	6

Data Management

Chromatography software:	Empower 3, FR 3

Results and Discussion

Reproducibility of peptide methods is impacted by gradient compositional change per unit time or column volume. To showcase this effect, two methods were run. The first method used a flow rate of 0.5 mL/min and a gradient change of 99:1 to 40:60 over 23.4 min (equivalent to ~1% change in solvent B per column volume). The second method used a flow rate of 0.2 mL/min and a gradient change of 99:1 to 60:40 over 93.4 min (equivalent to ~0.5% change in solvent B per column volume).

Method 1 Results – Impact of Higher Flow and Shorter Gradient Time on Retention Time Precision

Method 1 (0.5 mL/min and 23.4 min gradient) is a relatively "easy" gradient for the pump to deliver reliably due to the higher flow rate and decreased gradient slope. This method requires the pump to deliver a compositional change of 39% over 23.4 minutes, or 1.7% change per minute. Binary pumps deliver a change in gradient composition by adjusting the relative flow rates of pump A and pump B. In this example, pump A decreased flow rate by 1.7% of the total flow, or about 8.5 µL over each minute. Conversely, pump B increases its flow rate by the same amount so that the resulting flow rate is always 0.5 mL/min. As stated above, an easy way to assess the precision of the flow delivery is to look at the resulting retention times over multiple injections. Figure 1 shows 6 replicate injections overlaid for a typical run acquired with method 1 using the new ACQUITY UPLC H-Class PLUS Bio Binary System.

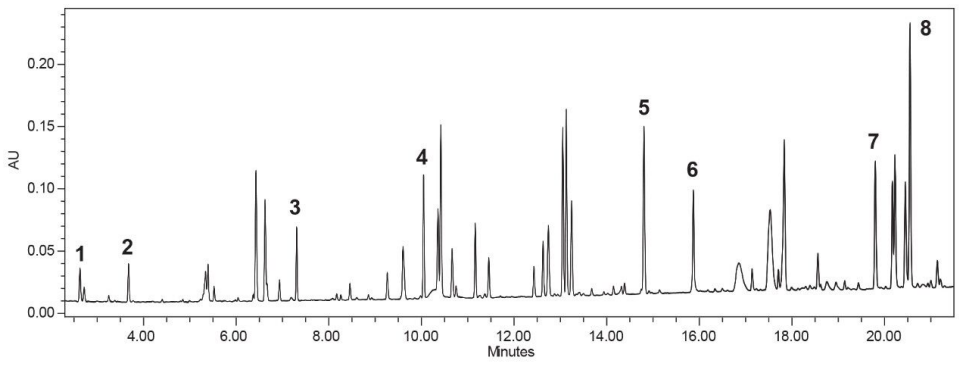

Figure 1. Overlay of six replicate injections acquired on the ACQUITY UPLC H-Class PLUS Bio Binary System using Method 1 conditions of 0.5 mL/min and a gradient change of 99:1 to 60:40 over 23.4 minutes.

Visually, it appears that the retention times are very consistent. To delve deeper into the data, eight peaks were chosen spanning the entire gradient to monitor the precision of gradient delivery. The

average retention time variation for the eight labeled peaks is only 0.16 seconds across the six replicate injections. Comparing the results acquired on the biocompatible binary Waters system with the results of the same method run on two competitive LC systems (which also use a binary pump for gradient delivery) reveals that they all perform similarly (Table 1).

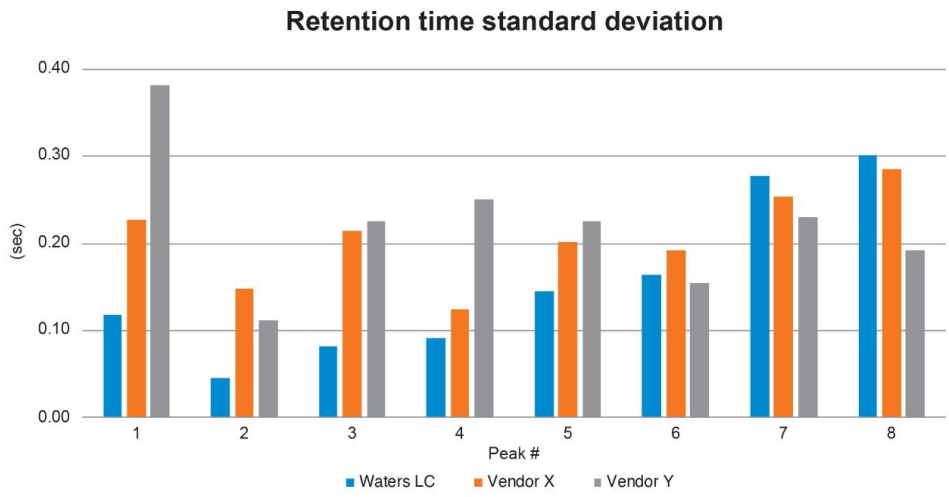

Table 1. Retention time standard deviation results obtained on the Waters LC and two competitive systems using Method 1 conditions of 0.5 mL/min and a gradient change of 99:1 to 60:40 over 23.4 minutes.

Of all the systems analyzed, the largest variation in peak retention time observed was still less than 0.4 seconds. While the performance of the systems was largely the same, there were slight differences in selectivity based on instrument differences such as mixing, system volume, and other design factors (data not shown). Again, for the method with a typical analytical flow rate and the steeper gradient, all systems perform similarly.

Method 2 Results – Impact of Lower Flow and Extended Gradient Time on Retention Time Precision

Method 2 represents more challenging method conditions with a decreased flow rate of 0.2 mL/min and a gradient of 99:1 to 60:40 over ~93 minutes. Long gradients can be challenging for a pump to deliver reproducibly, even for high-pressure binary mixing systems. The programmed gradient delivers a compositional change of 39% over 93 minutes, which is roughly equivalent to 2.4% change in composition per minute. A 2.4% change is equivalent to just 4.8 µL change in flow rate for each pump head per minute, this is approximately ½ the µL change as shown in the first example. This requires an exceptional level of flow precision to deliver the same results over multiple injections, multiple days, etc. Figure 2 shows 6 replicate injections obtained on the Waters Bio Binary LC system using method 2.

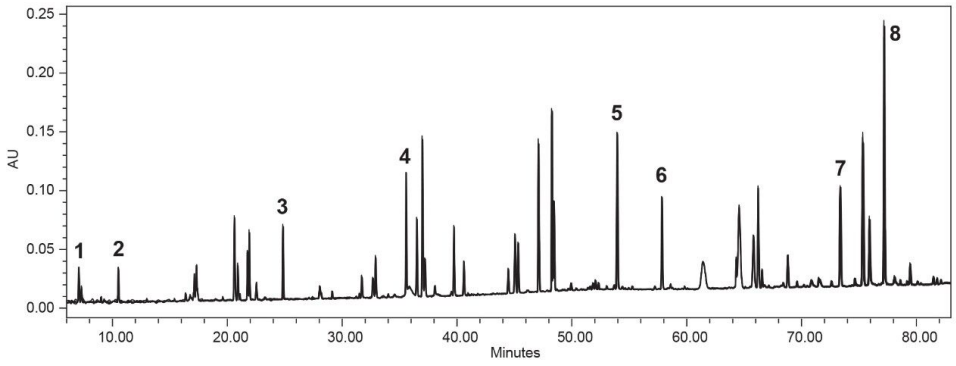

Figure 2. Overlay of six replicate injections acquired on the ACQUITY UPLC H-Class PLUS Bio Binary System using Method 2 conditions of 0.2 mL/min and a gradient change of 99:1 to 60:40 over 93.4 minutes.

As with Method 1, the results visually look reproducible from the resulting chromatograms. For the eight highlighted peaks, the average retention time standard deviation was determined to be 1.5 seconds. To reiterate, this increase in deviation is not unexpected due to the more challenging method conditions required by Method 2. When compared to the results obtained on the two competitive systems for the same method, the Waters system clearly provides a higher degree of reproducibility of gradient delivery throughout the entire gradient (Table 2).

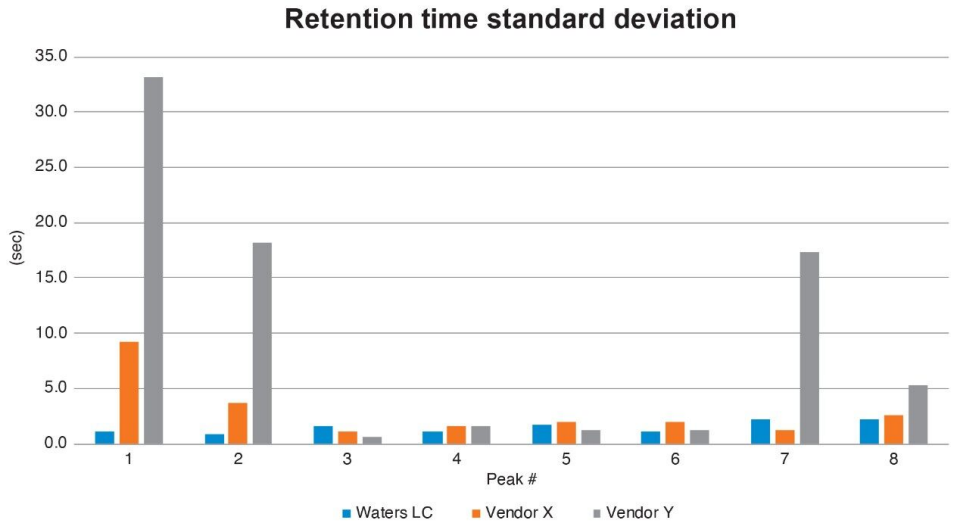

Table 2. Retention Time Standard deviation results obtained on the Waters LC and two competitive systems using Method 2 conditions of 0.2 mL/min and a gradient change of 99:1 to 60:40 over 93.4 minutes.

Note, for competitor B, there were two problematic injections out of the 6 replicates acquired that skewed the data. One injection showed high variability early in the gradient (affecting peaks 1 and 2)

and one injection showed high variability at the end of the gradient (affecting peaks 7 and 8). This inconsistency also effects the chromatography in terms of selectivity and resolution of peaks and can pose challenges to proper identification and quantification of peaks (data not shown). To generate reliable sample data, it is important to have a system which can deliver method conditions in a reliable and precise manner for every injection.

Impact of Mixer Volume on Baseline Noise

Briefly mentioned above, solvent mixing also plays an important role in the generation of high-quality results. Inefficient or incomplete mixing of solvents prior to delivery to the analytical column can also be part of the cause of retention time variations across injections. The use of TFA increases the ability to see any mixing inefficiencies which is typically seen in the baseline. Regarding the mixing behavior of each system, for Method 1, the smaller volume mixers shipped with the systems were used since the faster flow rate and steeper gradient lessen the effect of baseline perturbations from inadequate mixing. Table 3 shows the measured peak to peak noise for three regions of the gradient from a blank injection for the 3 systems evaluated.

Noise Interval	4–8 min	12–16 min	20–24 min
Waters LC	1.3	0.7	0.3
Vendor X	1.5	1.1	0.3
Vendor Y	1.4	0.9	0.5

Table 3. Calculated peak to peak noise (mAU) for a blank injection using Method 1 (0.5 mL/min and 23.4 min gradient from 99:1 to 60:40).

The data shows two key trends; First, higher noise values are seen earlier in the gradient for all systems and second, all systems showed very similar results for calculated noise using the method conditions outlined.

To address baseline concerns, it is common for LC vendors to manufacture and recommend the use of larger mixers for applications which require the use of TFA in order to flatten out the baseline and provide consistent chromatography which ultimately may improve integration and quantification reproducibility and accuracy as well as increasing signal to noise. Additionally, homogenous mixing becomes more challenging as flow rates are decreased and/or the slope of the gradient is decreased.

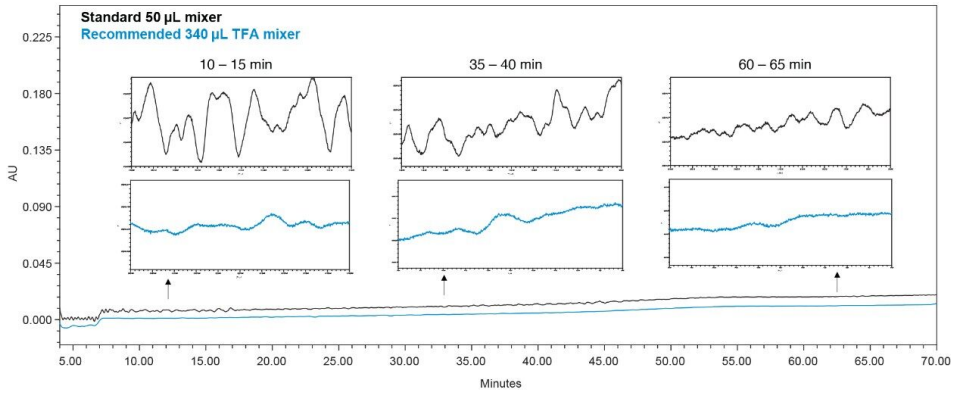

Figure 3. Overlay of blank injections using the standard and larger volume mixer (recommended when using TFA) using Method 2 conditions of 0.2 mL/min and a gradient change of 99:1 to 60:40 over 93.4 minutes. Zoomed in portions of the baseline at various time intervals throughout the gradient demonstrate the improvement in baseline with the larger mixing volume. For the zoomed in portions, the y-axis scale is set to 0.0022 AU for all chromatograms.
Note, the black trace is offset by 1.5 minutes to account for the increase in system delay volume when the larger mixer is installed.

The chromatograms clearly show why larger mixers are recommended for applications which use TFA since it provides a significantly smoother baseline. When using the smaller default mixer, the baseline noise becomes significant and has the potential to negatively impact peak integration and quantification, as well as decrease the overall signal to noise for peaks of interest. In addition to visually seeing the improvement, Table 4 shows the measured noise values were significantly reduced (over 3-fold) with the use of the larger TFA mixer, especially early in the gradient where mixing effects are most readily seen.

Peak to peak noise (mAU)			
Noise interval (min)	10–15	35–40	60–65
Standard mixer	2.10	1.47	0.37
TFA recommended mixer	0.64	0.49	0.29
Noise reduction	3.3x	3.0x	1.3x

Table 4. Measured peak to peak noise values for a blank injection at various time segments which span the gradient using Method 2 conditions of 0.2 mL/min and a gradient change of 99:1 to 60:40 over 93.4 minutes to demonstrate decrease in noise when a larger volume mixer is used.

Highlighting the improvement in signal to noise when baseline noise is decreased, a small peak that elutes in the early part of the gradient was examined. The average signal to noise across five replicate injections was increased from s/n = 1.9 to s/n = 5.1 when the mixer was changed from the standard 50 µL volume to the recommended 340 µL volume (Figure 4). This will improve the ability to not only to see low level peaks, but also to integrate and quantitate in a more reliable and reproducible manner. It should be noted that changing the volume of the mixer will result in a change of system volume and thus peak retention time (Figure 4).

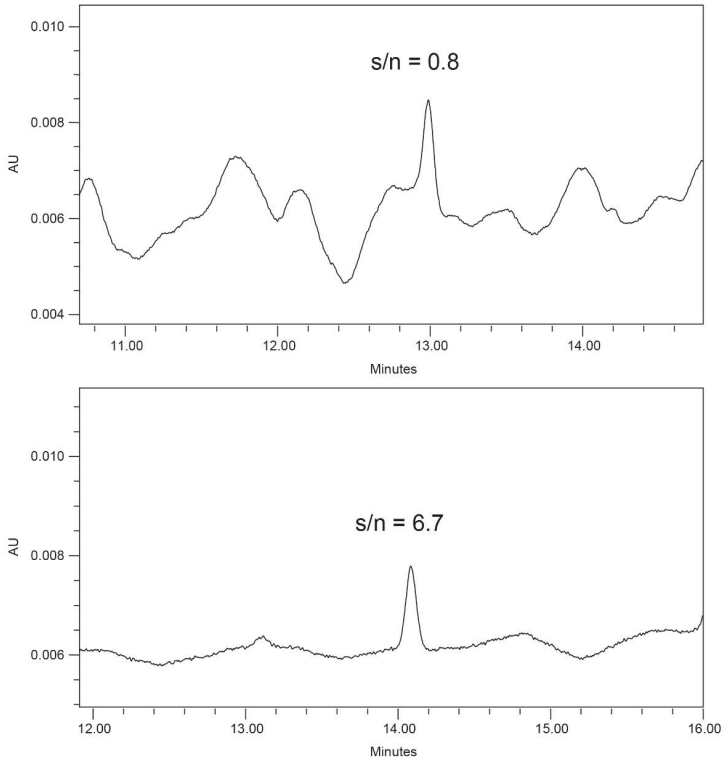

Figure 4. Example showing the increase in signal to noise by changing from the default 50 µL mixer to the recommended TFA 340 µL mixer on the ACQUITY UPLC H-Class PLUS Bio Binary System. Note: The peak retention time is shifted in the bottom chromatogram due to the increase in overall system volume when the larger mixer is installed.

Conclusion

Customers increasingly look for biocompatible systems that provide reliable and accurate data, regardless of the method conditions used. The new fully biocompatible binary based system provides the flexibility of a corrosion-resistant flow path, while still providing the highest degree of gradient precision for challenging peptide mapping methods. In a comparison including two other binary based UHPLC systems, the ACQUITY UPLC H-Class PLUS Bio Binary System gave the most reproducible retention times for a challenging peptide method. Additionally, the implementation of a larger mixer for TFA applications can significantly improve the baseline, resulting in improved ability to identify, integrate, and quantitate peaks.

References

1. Simeone J, Hong P, McConville P. Performance of the ACQUITY UPLC I-Class PLUS System for Methods which Employ Long, Shallow Gradients. Waters Application Note, 2007 Nov, 720002393en.

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