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Application Note

Ion-Pairing Selection and Mixer Considerations in the Development of LC-MS Workflows

Robert E. Birdsall, David Dao, Brooke M. Koshel, Ying Qing Yu

Waters Corporation

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

Selection of an appropriate ion-pairing reagent is not always straightforward as a balance between chromatographic performance, and MS sensitivity is often required when optimizing LC-MS workflows. In this study we demonstrate how large volume mixers can be leveraged to reduce optical-baseline noise while maintaining MS sensitivity for hyphenated workflows. Specifically, UV-baseline noise was reduced up to 3-fold in FA-based assays when using Waters 340 µL mixer with comparable chromatographic performance to TFA-based assays. Furthermore, MS-sensitivity was maintained with a maximum peak intensity observed at 9×10^7 counts when using FA in conjunction with the large format mixer compared to a maximum peak intensity of 2×10^6 counts observed when using TFA. This study demonstrates large volume mixers provide an effective means to reduce baseline noise in UV chromatograms while maintaining MS sensitivity for RPLC-based methods that incorporate FA as an ion-pairing reagent.

Benefits

- The ACQUITY UPLC H-Class PLUS Bio System enables efficient development of robust and reproducible methods for peptide mapping assays
- High volume mixers reduce UV-baseline noise while maintaining high MS sensitivity in formic acid-based RPLC-UV/MS workflows.

Introduction

To ensure growth and sustainability, pharmaceutical companies continue to invest resources in the evaluation of new technology and its ability to deliver robust and efficient methods for improved productivity. As part of the method development process, end-use application and detector needs are often considered to determine the most appropriate instrument configuration and starting method conditions prior to optimization. For methods that incorporate ion-pairing reagents, the end-point detector, whether it be optical or MS-based, can determine the selection of ion-pairing reagent used in the assay. More specifically, formic acid (FA) and trifluoracetic acid (TFA) are common ion-pairing reagents used in reversed-phase liquid chromatography (RPLC) methods. As a “weak” ($pK_a = 3.75$) ion-pairing reagent, FA is generally preferred for MS-based detection to maximize MS detector response for increased assay sensitivity while delivering modest chromatographic performance. In contrast, as a “strong” ($pK_a = 0.6$) ion-pairing reagent known to

cause MS ion suppression, TFA is generally preferred in UV-based assays given its ability to minimize adsorption artifacts such as peak tailing for greater peak capacity. However, in LC-MS workflows that rely on UV data as well, selection of the most appropriate ion-pairing reagent may not be as straightforward, particularly when trying to maximize both assay sensitivity and chromatographic performance.[1] Recently it was demonstrated Waters large volume mixers effectively reduce baseline noise in UV-based assays to improve chromatographic performance in terms of assay sensitivity and quantitative accuracy with minimal impact to dwell volume.[2] Considering this, solvent mixers represent a hardware component that can be potentially leveraged in LC-MS workflows to improve chromatographic performance in terms of optical-baseline noise while maintaining MS sensitivity. The objective of this study is to evaluate Waters' large volume mixer as a configuration option to optimize detector response in LC-MS workflows that incorporate dual detectors.

Results and Discussion

To investigate the impact of mixer volume on LC-MS workflows, the ACQUITY UPLC H-Class PLUS Bio System was selected as the LC platform because of its demonstrated ability to deliver exceptional compositional accuracy of mobile phases under gradient conditions.[2] The Waters 340 µL mixer (P/N 700011554) was selected for comparison to the stock 50 µL mixer given its precedence to improve chromatographic performance.[2] To evaluate mixer volume impact on MS response, the ACQUITY QDa Mass Detector, which can be readily configured with the ACQUITY LC product line and is compatible with both FA and TFA conditions, was plumbed in-line post UV detection to acquire mass data.[3] A peptide mapping assay using reversed phase chromatography was selected for this study as a commonly encountered technique in industry that uses gradients deployed with FA or TFA. Mobile phases (MP) were prepared as water (MP: A) and acetonitrile (MP: B) with 0.1% (v/v) of either FA or TFA. A 24-min peptide mapping assay was performed using a gradient of 0.6 %B/min at a flow rate of 0.500 mL/min using the Waters Tryptic Digest Standard (P/N 186009126 <https://www.waters.com/nextgen/in/en/shop/standards--reagents/186009126-mab-tryptic-digestion-standard.html>) separated on an ACQUITY UPLC Peptide CSH C_{18} Column (130 Å, 1.7 µm, 2.1 mm x 100 mm, P/N 186006937 <https://www.waters.com/nextgen/xg/en/shop/columns/186006937-acquity-uplc-peptide-csh-c18-column-130a-17--m-21-mm-x-100-mm-1k.html>). Optical (UV) detection was performed at a wavelength of 214 nm with mass data acquired using a full scan range of (50–1250 *m/z*). To ensure baseline response was stable after hardware changes, sample sets consisting of 4 blank injections were performed followed by 5 injections of the tryptic digest standard.

To benchmark performance, the peptide mapping assay was performed with both FA and TFA using the stock 50 µL mixer. As shown in Figure 1, both ion-pairing reagents performed well in the analysis of enzymatically treated proteins and are able to utilize the chromatographic space in conventional RPLC-based methods. As previously mentioned, both ion-pairing reagents exhibit unique characteristics which are often leveraged during method optimization. In this instance, TFA was observed to have marginally better peak shape overall as demonstrated by the higher peak capacity (FA P_c=446, TFA P_c=468) when calculated using a selection of peaks throughout the chromatogram. It should be noted that peak capacity is not exclusively determined by ion-paring reagent and results may vary based on selection of stationary phase. In addition, TFA exhibited increased retentivity to a degree based on the overall retention time shift of the chromatographic profile as well as selectivity differences which were confirmed using mass information acquired with the ACQUITY QDa (data not shown). In terms of reproducibility, both ion-pairing reagents exhibited exceptional assay repeatability over a 3-day period based on the minimal deviation observed in retention time (Table 1). Given this information one may conclude both ion-paring reagents performed at an acceptable level, where selectivity or retention factor may be the deciding factor. However, closer inspection of the individual chromatograms reveals some subtle differences between the ion-paring reagents. As shown in Figure 2A, formic acid exhibited ≥5-fold more noise (1.3 mAU) in the optical-baseline of a water blank when compared to TFA (0.23 mAU) when using the stock 50 µL mixer. As shown in Figure 2B, this translates to increased baseline noise in the UV chromatogram which can negatively impact quantitative results and assay reproducibility.[2] In contrast, TFA was observed to have better performance in terms of UV-baseline noise, but as previously mentioned, is not as well suited for MS detectors. This is demonstrated in Figure 3, where the MS-spectrum of the same data showed TFA exhibiting significant ion suppression with peaks not being observed between UV and MS chromatograms while FA provided significantly higher MS-response allowing for peaks to be readily discerned from baseline noise.

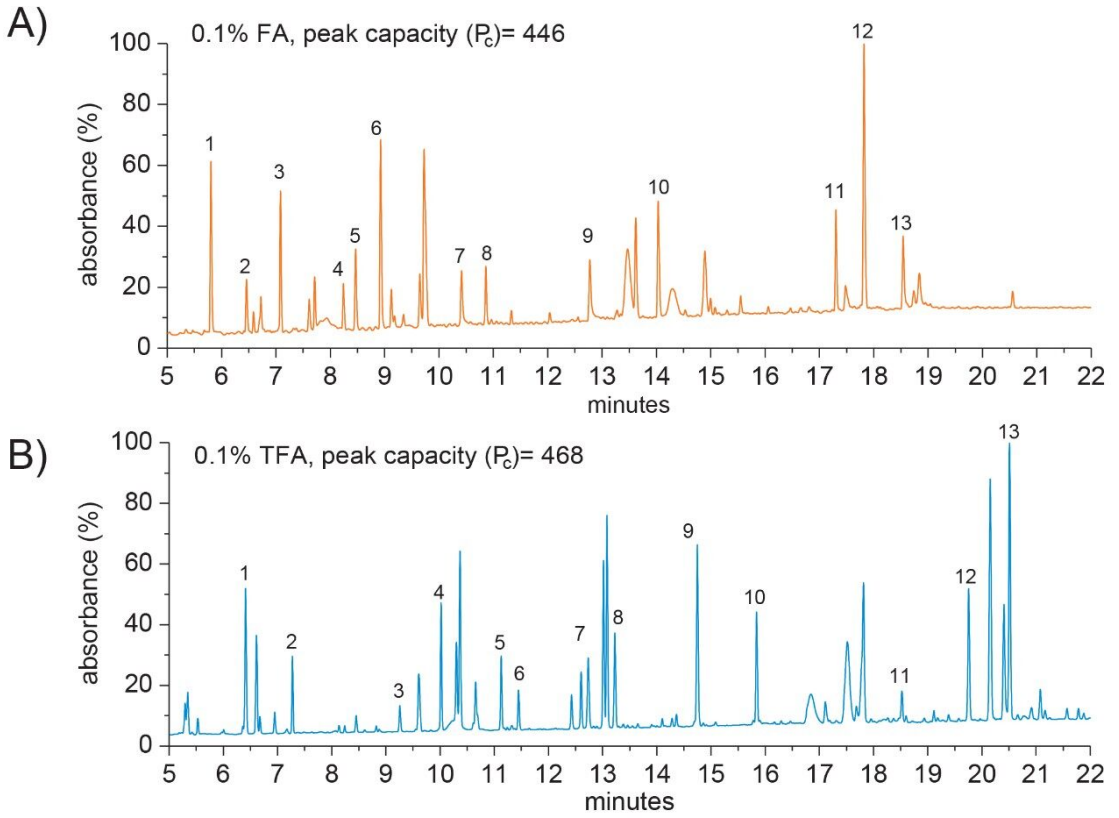

Figure 1. Profile Comparison. Reversed-phase peptide mapping profiles of Waters Tryptic Digest Standard using A) FA or B) TFA as an ion-pairing. Annotated peaks represent a random selection of peaks throughout the individual chromatograms and are not related to each other.

0.1% FA - NIST mAb RM peptide map

Peak	Retention time	Standard deviation		
		Day 1	Day 2	Day 3
1	5.80	0.0006	0.0025	0.0015
2	6.46	0.0020	0.0021	0.0020
3	7.08	0.0015	0.0021	0.0015
4	8.24	0.0015	0.0020	0.0021
5	8.46	0.0010	0.0020	0.0015
6	8.92	0.0010	0.0025	0.0021
7	10.41	0.0015	0.0026	0.0020
8	10.86	0.0012	0.0021	0.0015
9	12.77	0.0015	0.0038	0.0015
10	14.03	0.0015	0.0038	0.0020
11	17.30	0.0025	0.0044	0.0023
12	17.81	0.0031	0.0042	0.0026
13	18.54	0.0020	0.0026	0.0021

0.1% TFA - NIST mAb RM peptide map

Peak	Retention time	Standard deviation		
		Day 1	Day 2	Day 3
1	6.41	0.0012	0.0006	0.0007
2	7.28	0.0002	0.0005	0.0012
3	9.26	0.0005	0.0004	0.0016
4	10.02	0.0006	0.0005	0.0012
5	11.13	0.0003	0.0008	0.0006
6	11.45	0.0003	0.0003	0.0005
7	12.73	0.0001	0.0004	0.0012
8	13.23	0.0005	0.0006	0.0016
9	14.76	0.0001	0.0004	0.0013
10	15.84	0.0003	0.0006	0.0019
11	18.53	0.0006	0.0008	0.0015
12	19.76	0.0007	0.0006	0.0016
13	20.51	0.0015	0.0007	0.0012

Table 1. Assay Repeatability. Standard deviation of 13 peptide peaks (un-related) monitored over a 3-day time period from a RPLC-based peptide map using either FA or TFA as an ion-pairing reagent at a concentration of 0.1% v/v.

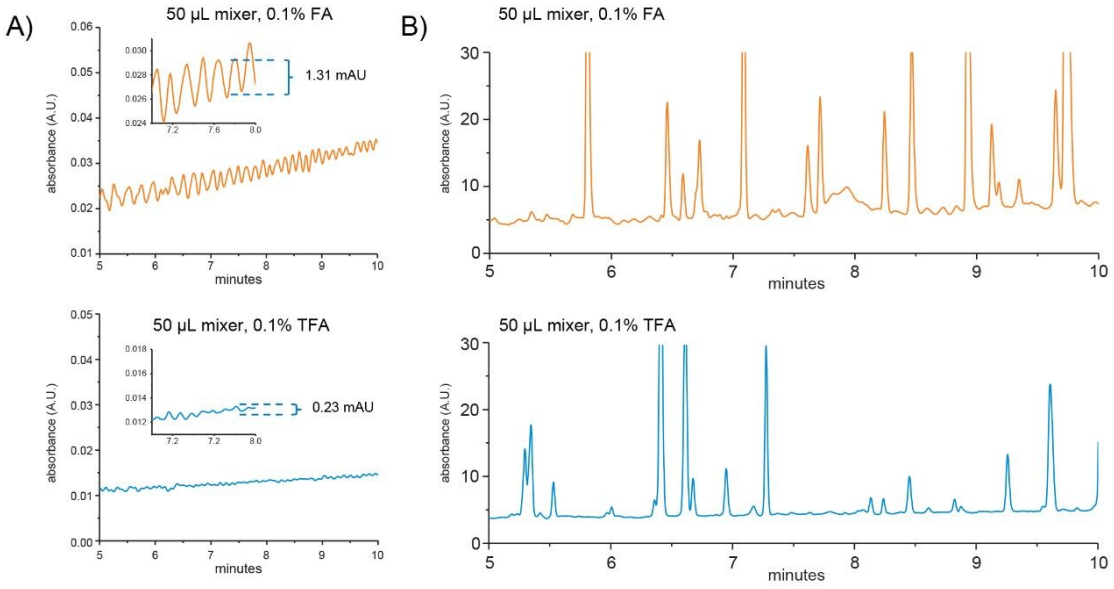

Figure 2. Baseline Noise. A) baseline noise of a water blank calculated as standard deviation of detector response over a 10-minute segment using a stock 50 µL mixer with FA (top panel) or TFA (bottom panel) as an ion-pairing reagent. B) A close-up of accompanying chromatograms of a peptide map using the same configuration and mobile phase.

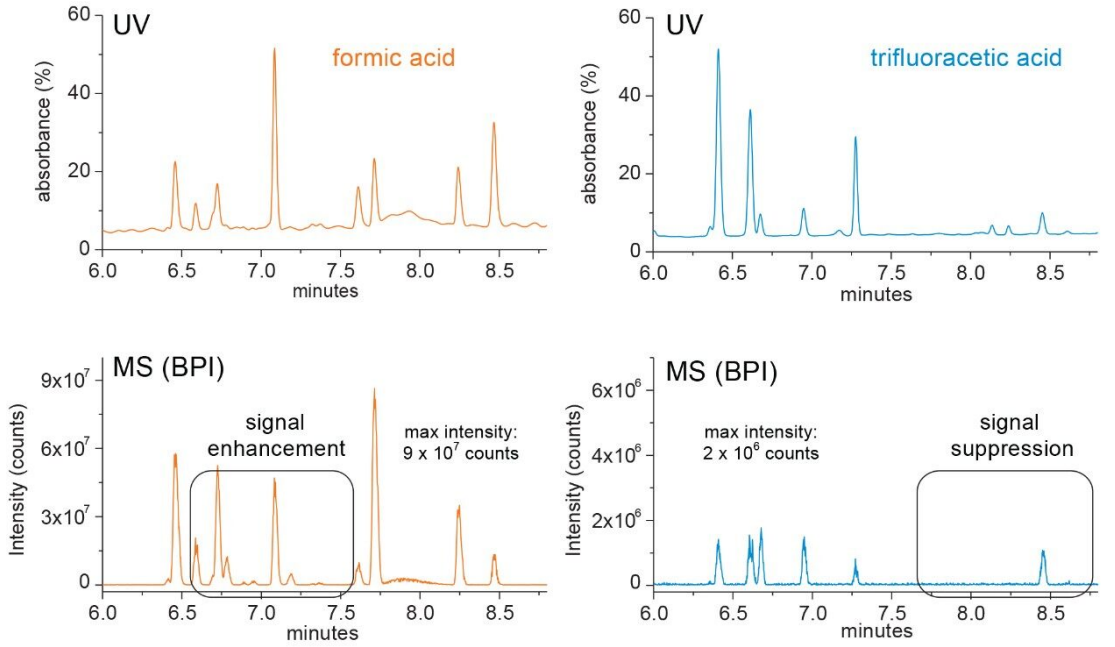

Figure 3. Ion-pairing Impact. UV and accompanying MS response for a RPLC-UV/MS peptide mapping assay performed using a stock 50 µL mixer with A) FA or B) TFA as the ion pairing reagent. ACQUITY QDa settings: Sampling rate=10 Hz, Probe temp.=600 °C, Capillary voltage=0.8 kV, Cone voltage=15 V.

To test the applicability of the larger volume mixer to improve chromatographic performance in hyphenated workflows, the same separation was performed using the Waters 340 µL mixer (P/N 700011554). As shown in Figure 4A, Waters 340 µL mixer was able to effectively reduce the UV-baseline noise up to 3-fold (1.31 mAU vs. 0.46 mAU) when using formic acid (inset) compared to the 50 µL mixer results. Interestingly, the large volume mixer exhibited marginal improvement when using TFA with only a 1.2-fold reduction in baseline noise (0.19 mAU vs. 0.23 mAU) compared to the 50 µL mixer results (data not shown) indicating the chromatographic performance of FA is approaching that of TFA-based assay in terms of baseline noise. The reduced noise in the UV chromatogram allows for low abundant peaks to be readily discerned from the baseline noise with minimal impact to the retention time (Δ RT $\cong$ +0.30 min) as shown in Figure 4A.
More importantly, the use of the large volume mixer facilitated the ability to retain MS-sensitivity in the LC-MS workflow. As shown in Figure 4B a comparable MS-response (MS intensity = 9 x 10^7) was observed when using FA with the 340 µL mixer compared to the FA results using the 50 µL mixer. These results demonstrate large volume mixers can be deployed in FA-based assays to improve UV detector response while maintain MS sensitivity in hyphenated LC-MS workflows.

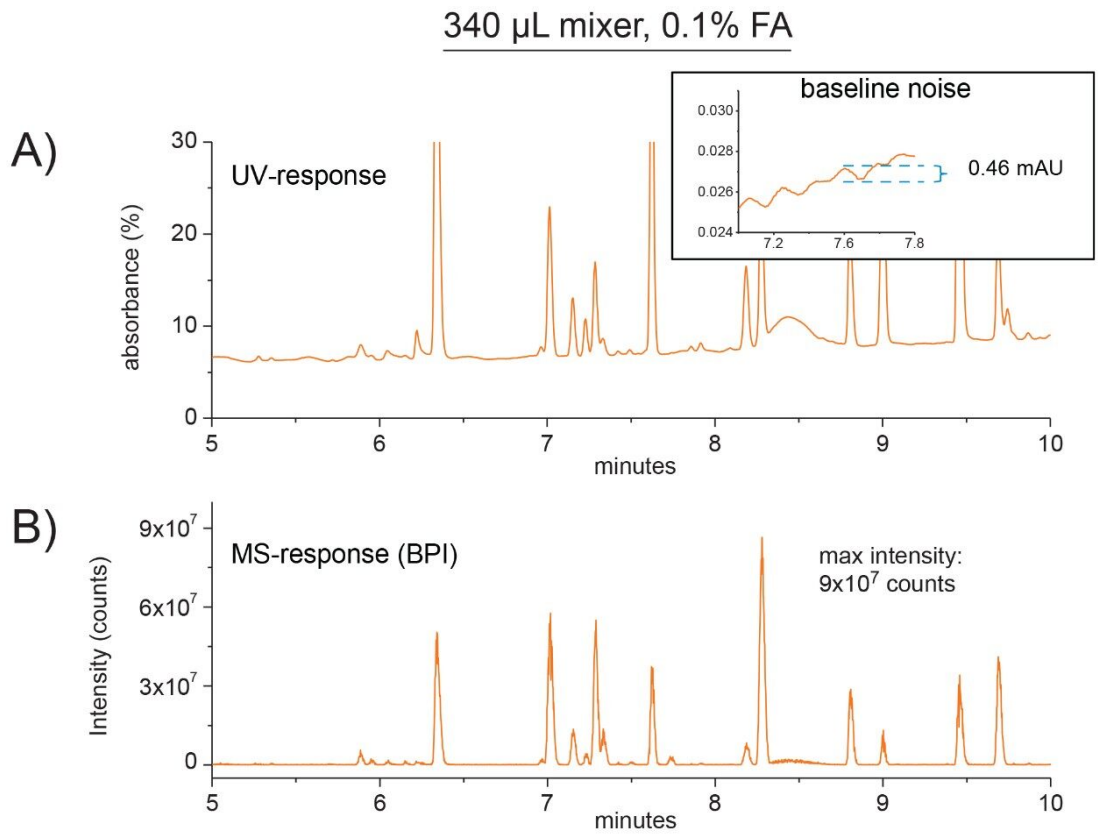

Figure 4. Mixer Impact. A) UV and B) MS response for a RPLC-UV/MS peptide mapping assay performed using a 340 µL large volume mixer with FA as an ion-pairing reagent. ACQUITY QDa settings: Sampling rate=10 Hz, Probe temp.=600 °C, Capillary voltage=0.8 kV, Cone voltage=15V.

Conclusion

As part of method development, optimizing system performance is necessary to obtain reliable and consistent results. Evaluation of ion-pairing reagents is often necessary, as their impact to chromatographic performance is not always readily apparent. This is particularly true in LC-MS workflows that incorporate UV detectors where a balance is often sought out in terms of UV-baseline noise versus MS sensitivity. This study demonstrates Waters large volume mixers provides an effective means to reduce baseline noise in UV chromatograms while maintaining MS sensitivity with minimal impact to retention time for RPLC-based methods that incorporate FA as an ion-pairing reagent.

References

1. 1. Chakraborty AB, Berger SJ. Optimization of Reversed-phase Peptide Liquid Chromatography Ultraviolet Mass Spectrometry Analyses using an Automated Blending Methodology. *J Biomol Tech.* 2005;16(4):327–335.

2. Dao, D., Birdsall, R., Yu, YQ. Instrument Mixer Considerations to Improve Assay Reproducibility and Sensitivity. Waters Application Note 720007011EN < https://www.waters.com/nextgen/us/en/library/application-notes/2020/instrument-mixer-considerations-to-improve-assay-reproducibility-and-sensitivity.html> , September 2020.

3. Birdsall, R., McCarthy S. Adding Mass Detection to Routine peptide-Level Biotherapeutic Analyses with the ACQUITY QDa Detector. Waters Application Note 720005266EN < https://www.waters.com/nextgen/us/en/library/application-notes/2015/adding-mass-detection-to-routine-peptide-level-biotherapeutic-analyses-with-acquity-qda.html> , Jan 2015.

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