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Note d'application

Improved Sensitivity for Trifluoroacetic Acid Gradients on the Alliance™ iS HPLC Systems

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Abstract

Given the nature of biomolecules, such as amino acids and peptides, many HPLC reversed phase bio-separations often require ion-pairing reagents, such as Trifluoroacetic acid (TFA), in combination with low wavelengths (<250 nm) for analysis. However, due to the strong retention of TFA on the stationary phase and continual displacement with acetonitrile/water gradients, baseline ripples are often observed at low UV wavelengths. For methods that require the combination of conditions, baseline ripples can impact sensitivity and reproducible quantitation. One such method, the USP Monograph for Tryptophan, Organic Impurities (Procedure 1) specifies these conditions including 0.1% TFA in water/acetonitrile gradient and 220 nm wavelength for analysis.

In this work, we will test the monograph on the Alliance™ iS Bio HPLC System and Alliance iS HPLC System and evaluate the performance of the method with systems in the default configurations, including the stroke volumes. Both systems will be tested with the standard mixer and an optional diffusion bonded mixer. The latter optional mixer has a minimal impact on dwell volume but is designed to improve mixing efficiency. Results will demonstrate the improved sensitivity observed with the diffusion bonded mixer, as well as the impact of instrument settings.

Benefits

- Diffusion bonded mixer reduces baseline fluctuations for TFA gradients on the Alliance iS HPLC System
- Improved sensitivity achieved for challenging TFA gradients at low wavelengths
- Reduced baseline noise with diffusion bonded mixer allows for more stable baseline and improved peak area precision for USP Monograph of Tryptophan

Introduction

Trifluoroacetic acid (TFA) as a mobile phase additive is typically used as an ion pairing reagent for UV based separation of biomolecules, including proteins and peptides. However, TFA has a strong UV absorption band at wavelengths typically used for bioseparations (<220 nm). In addition, TFA may be slightly retained by reversed-phase columns in water-rich mobile phases, resulting in short-term variations in the mobile phase. At low wavelength, these variations can appear as perturbations in the baseline which produces an increase in noise. For analyses this can result in a decrease in sensitivity and less reproducible peak integration, impacting peak area precision. To address these affects, trifluoroacetic acid (TFA) mixers have evolved to reduce baseline noise in TFA gradients by improving mixing performance.[1]

In this work, the impact of mixer design was tested for a regulated method, USP monograph for Tryptophan Organic Impurities, which requires both a TFA acetonitrile gradient method and a low UV wavelength for analysis.[2] The monograph was evaluated on both the Alliance iS HPLC System and Alliance iS Bio HPLC System, to assess differences in system and mixer design as well as solvent manager settings (***e.g.*** stroke volume). The standard (default) mixers and the titanium (Ti) diffusion bonded mixer were tested on both systems as described. [3] The impact of stoke volume was assessed by setting two different stroke volumes and comparing signal to noise.

Experimental

System Suitability Preparation

The system suitability solution was prepared by transferring 1 mL of tryptophan related compound B stock solution to a 100 mL volumetric flask and then diluting to volume with water at a concentration of 1 mg/L.

LC Conditions

LC systems:	Alliance iS HPLC System and Alliance iS Bio HPLC System
Detection:	TUV Detector
Wavelength:	220 nm
Sampling rate:	5 points/sec
Vials:	TruView™ pH Control LCMS Certified Clear Glass, 12x32 mm, Screw Neck Vial, with Cap and preslit PTFE/Silicone Septum, 2 mL Volume, 100/pk (p/n: 186005666CV)
Column(s):	XBridge™ C_{18} Column, 5µm 4.6 x 250 mm (p/n: 186003117)
Column temperature:	30 °C
Sample temperature:	15 °C
Injection volume:	20 µL
Flow rate:	1.0 mL/min
Mobile phase A:	0.1% TFA in Water
Mobile phase B:	0.1% TFA in 80:20 Acetonitrile:Water
Mobile phase C:	90:10 Water:Acetonitrile
Sample manager wash:	90:10 Water:Acetonitrile

Sample manager purge: 0.1% TFA in Water

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	1.000	95.0	5.0	0.0	0.0	Initial
2.00	1.000	95.0	5.0	0.0	0.0	6
37.00	1.000	35.0	65.0	0.0	0.0	6
42.00	1.000	0.0	100.0	0.0	0.0	6
47.00	1.000	0.0	100.0	0.0	0.0	6
50.00	1.000	95.0	5.0	0.0	0.0	6
60.00	1.000	95.0	5.0	0.0	0.0	6

Data Mangement

Chromatography data system: Empower™ 3, FR 3.8.0.1

Results and Discussion

System and Method Considerations

To test the HPLC analysis, the Alliance iS HPLC System were selected. Both systems have quaternary solvent managers, and were designed for HPLC separations, including dwell volume range typically found in HPLC systems, column accommodation of HPLC columns and HPLC flow rate range (up to 10 mL/min). The quaternary solvent manager allows for blending of up to 4 solvents and employs a gradient proportioning valve to deliver aliquots or packets of mobile phase. Low pressure mixing then occurs throughout the pump heads followed by the in-line mixer.

The two systems assessed for this study, Alliance iS HPLC System and Alliance iS Bio System, are comparable with modifications on the Alliance iS Bio System to reduce non-specific adsorption. The Alliance iS HPLC System is a stainless-steel system, with a 675 µL stainless steel bead mixer in the standard configuration. Given typical

HPLC conditions including flow rate, the pump stroke volume is full stroke or 132 µL. In contrast the Alliance iS Bio System contains an exceedingly bio-inert flow path that incorporates the MaxPeak™ High Performance Surfaces Technology (HPS) to address the unique needs of bioseparations. The Alliance iS Bio HPLC System is also biocompatible, comprised of MP35N® and titanium to resist corrosion under high salt conditions. Additional components to improve bioseparations performance include a 680 µL bead titanium standard mixer and a default stroke volume of half stroke or 66 µL, with the option to increase to 132 µL. The half stroke is designed to reduce noise from the stroke of each pump cycle.

Both systems are also configurable with an optional 690 µL Ti diffusion bonded mixer, in place of the standard mixer. This diffusion bonded mixer was specifically designed for the Alliance iS HPLC Solvent Managers to provide improved mixing performance, specifically for UV absorbing mobile phases at low UV wavelengths.

System	Default stroke volume (µL)	Standard bead mixer	Ti diffusion bonded mixer
Alliance iS HPLC System	132 µL	675 µL Stainless steel	690 µL Ti diffusion bonded
Alliance iS Bio HPLC System	66 µL	680 µL Titanium	690 µL Ti diffusion bonded

Table 1. Description of System Configurations.

Table 1 shows the system configuration differences between the two systems.

To assess impact of mixer configuration, the USP Monograph for Tryptophan Organic Impurities Method (Procedure 1) was tested. The monograph was chosen as it requires 1 mL/L (0.1%) TFA in both mobile phases with a UV detection wavelength of 220 nm, where Solution A is TFA in water and Solution B is TFA in 80:20 acetonitrile/water. Method conditions specify a L1 column, and a gradient with 1.7% B/min at 1 mL/min. The system suitability solution criteria for the system suitability solution (1 mg/L), Relative Compound B, specifies relative standard deviation of Not More Than (NMT), 5% for both peak area and retention, based on six replicate injections.

Analysis of USP Monograph for Tryptophan, Organic Impurities System Suitability Standard on Alliance iS HPLC System

Analysis of the system suitability solution (Tryptophan Related Compound B) at 1 mg/L and a 20 µL injection on the Alliance iS HPLC System produced relative standard deviations well within the acceptance criteria. In

addition to acceptance criteria, the results were monitored for signal-to-noise given the low concentration of the sample. In the standard configuration with beaded mixers both systems showed comparable signal-to-noise values. However, it should be noted that inspection of the baseline for both systems showed typical baseline noise indicative of inadequate mixing of TFA packets that can be retained on the column. The Alliance iS Bio HPLC System did appear to produce slightly lower fluctuations in the baseline, likely due to the lower stroke volume setting of the pump.

	System suitability criteria (n=6)			
System	Peak area % RSD	Peak retention time % RSD	Acceptance criteria	Signal-to-noise
Alliance iS HPLC System	2.1	0.031	NMT 5%	313
Alliance iS Bio HPLC System	1.0	0.007	NMT 5%	316

Table 2. System suitability and signal-to-noise results comparison with the standard mixer for the system suitability solution (n=6) in Procedure 1 of the USP Tryptophan Impurities Method on the Alliance iS HPLC System and the Alliance iS Bio HPLC System.

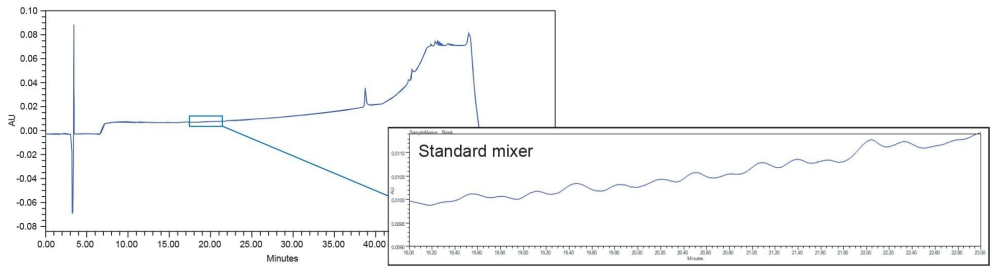

Figure 1. Baseline ripple on Alliance iS HPLC System effect showing a representative blank using the standard mixer.

Reduce Baseline Noise for Challenging TFA Gradients

As described earlier, TFA gradients can produce baseline disturbances at low wavelengths, impacting chromatographic results. To address these method challenges, the Alliance iS HPLC System have a diffusion

bonded mixer to improve baseline noise. This optional mixer is slightly larger in volume than the standard bead mixer. It contains microfluidic channels, which are machined into titanium plates.[3] The mixer is tuned for the stroke volume of the Alliance iS HPLC System quaternary pump and provides more efficient mixing and was designed for the challenging TFA gradients.

To assess the impact of mixer selection, the previously described analysis was tested on the Alliance iS HPLC System and Alliance iS Bio HPLC System with the diffusion bonded mixer in place of the default beaded mixer. On both systems, the diffusion bonded mixer produced lower baseline noise. Furthermore, both systems met system suitability for the solution (Table 3).

	System suitability criteria (n=6)			
System	Peak area % RSD	Peak retention time % RSD	Acceptance criteria	Signal-to-noise
Alliance iS HPLC System w/diffusion bonded mixer	0.46	0.004	NMT 5%	2496
Alliance iS Bio HPLC System w/diffusion bonded mixer	0.20	0.007	NMT 5%	5739

Table 3. Peak Area %RSD and Peak Retention Time %RSD results comparison with the diffusion bonded mixer using Procedure 1 of the USP Tryptophan Impurities Method on the Alliance iS HPLC System and the Alliance iS Bio HPLC System.

Investigation of the system suitability results demonstrate the impact of baseline noise on peak area integration. While system suitability criteria were met with both mixers, the peak area precision (n=6) showed significant improvement when analysis was performed with the diffusion bonded mixer on both systems. On both Alliance iS HPLC systems, peak area %RSD decreased from 1–2% with the beaded mixer to less than 0.5% with the diffusion bonded mixer. The improvement in peak area %RSD can be attributed to the reduced baseline noise, as the perturbations observed in the standard mixer were significant enough to impact integration reproducibility, specifically due to baseline disturbances around the peak of interest (Figure 3).

While system suitability criteria were met, another measure of baseline noise, the sensitivity of the method, also improved. As mentioned previously the system suitability solution of Tryptophan Related Compound B is at 1 mg/L or 1 ng/µL Given the low concentration any reduction in baseline noise can have a dramatic impact on sensitivity. Thus, when the analysis was performed on both Alliance iS HPLC System, 8–18 times improvement in

sensitivity was observed, demonstrating the ability of the diffusion bonded mixer to improve mixing efficiency and reduce baseline disturbances due to TFA absorbance bands.

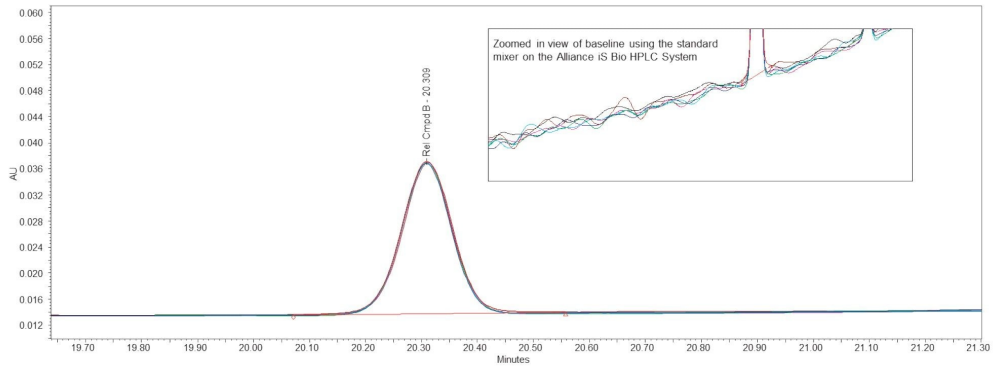

Figure 2. Overlay of six replicate injections using the standard mixer on the Alliance iS Bio HPLC System.

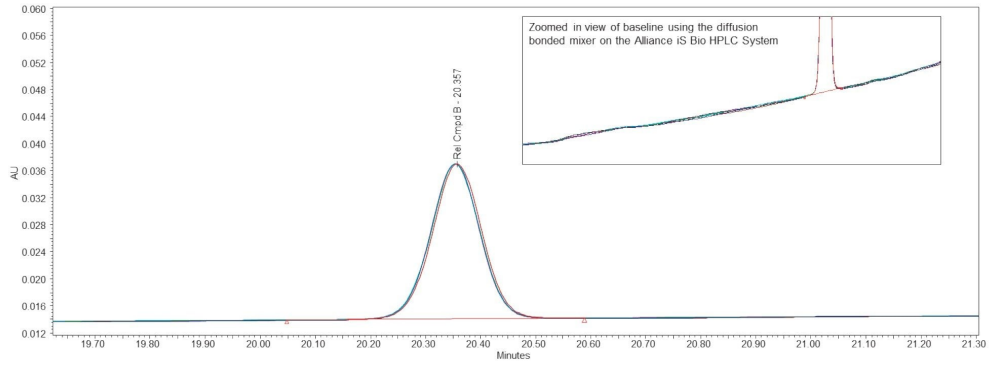

Figure 3. Overlay of 6 replicate injections using the diffusion bonded mixer on the Alliance iS Bio HPLC System.

Comparison of the sensitivity results on the Alliance iS HPLC System demonstrates the improved performance of the Alliance iS Bio HPLC System with the diffusion boned mixer (Figure 4). As described earlier, the default configurations produced similar signal-to-noise results on both systems. However, significantly higher signal-to-noise was observed with the diffusion bonded mixer on the Alliance iS HPLC Bio System, an almost two times

increase over the Alliance iS HPLC System. Both systems are comparable in design with the Alliance iS Bio System designed to reduce non-specific adsorption. While these changes would have minimal impact on the TFA gradient separation, one change that can impact baseline noise is the stroke volume setting. As describe earlier the Alliance iS Bio HPLC System has a default stroke volume of half that of the Alliance iS HPLC System. The more frequent stroke setting provides improved mixing, and, in combination with the diffusion bonded mixer, delivers the highest performance sensitivity for the TFA gradient tested.

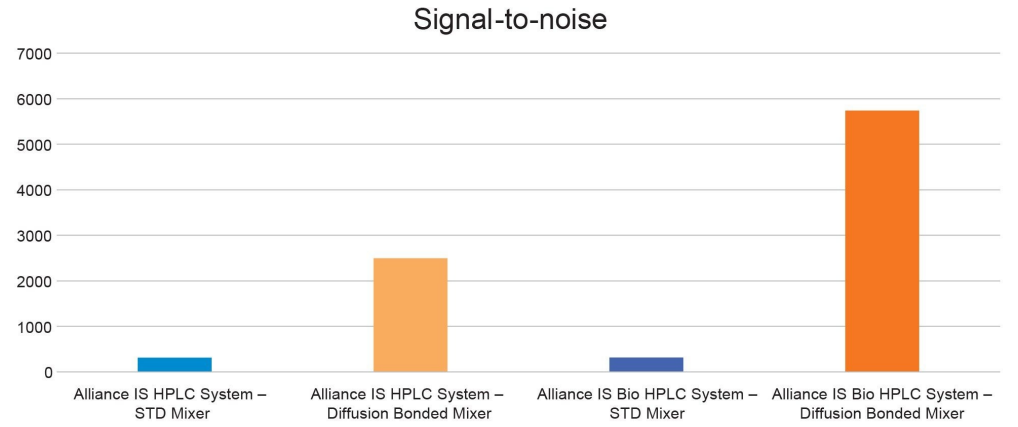

Figure 4. Impact of system and mixer on signal-to-noise for system suitability solution on Alliance iS HPLC System and Alliance iS Bio HPLC System.

Conclusion

In regulated labs, there is a need to ensure reproducibility of validated methods including meeting method criteria, such as sensitivity and/or area precision. This can become difficult with TFA mobile phases at low wavelengths, as TFA can cause fluctuations in chromatographic baselines, impacting reproducibility of peak integration or causing increased noise. However, the use of mixers designed specifically for these mobile phases can provide improved mixing and reduced baseline noise.

In this work, baseline noise was reduced through the use of diffusion bonded mixers on the Alliance iS HPLC System, for the USP Monograph of Tryptophan Organic Impurities. The diffusion bonded mixer showed improved

signal-to-noise and reduced baseline fluctuations with TFA gradient at 220 nm wavelength on both the Alliance iS HPLC System and Alliance iS Bio HPLC System. Furthermore, the Alliance iS Bio HPLC System demonstrated the highest sensitivity, showing the benefit of both the mixer and stroke volume setting. Benefits of improved mixing also provided improvements in peak area precision through more reproducible integration. The diffusion bonded mixer in conjunction with the Alliance iS HPLC System provides tools to benefit challenging TFA gradients, allowing for improved precision and sensitivity.

References

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