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Future-proofing the Biopharmaceutical QC Laboratory: Integrating Auto•Blend Technology to Improve Routine Peptide Mapping

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APPLICATION BENEFITS

- Auto•Blend™ Technology for acidic modifier control
- Transfer peptide map applications from HPLC to UPLC®
- Future-proof laboratory for UPLC methods

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

XBridge™ C_{18} 130 Å 3.5 µm, 4.6 x 100 mm Column

Empower® 3 Chromatography Data Software

KEY WORDS

Auto•Blend Technology, peptide mapping, automated mobile phase management

INTRODUCTION

As an initial step towards transferring peptide mapping methods from HPLC to UPLC, we previously presented an approach using the ACQUITY UPLC H-Class Bio System for legacy HPLC-based peptide mapping.[1] Our method transfer discussion continues here, focusing on improving the consistency of peptide mapping separations during routine analyses.

Peptide mapping methods generally include an acidic modifier to improve peak shape. However, accurate and reproducible management of the modifier content within mobile phase solvents can be variable, consequently affecting the peptide map quality and reproducibility.

This application note demonstrates the ability of Auto•Blend Technology to control the trifluoroacetic acid (TFA) component of the mobile phase during routine peptide mapping analyses, thereby producing chromatograms of equivalent performance to HPLC-acquired chromatograms with conventionally modified mobile phases.

TFA is commonly used as a modifier in peptide mapping methods with optical detection because it provides peak shape and chromatographic resolution benefits. Concentrations of TFA are typically low in most applications, accounting for 0.02% to 0.20% of the final mobile phase. Subtle changes in the modifier concentration can have profound effects on peptide retention time, resolution, and elution order, causing concern over chromatographic reproducibility and the occurrence of out-of-specification results. Such issues ultimately affect productivity due to time-consuming resolution of QC issues as opposed to moving product to the marketplace.

The reason for this classical approach using TFA in peptide mapping chromatography has been to modify the two mobile phases, normally water and acetonitrile, with a pre-determined amount of TFA. Here, we demonstrate the benefits of allocating TFA to an independent solvent line using Auto•Blend to manage its contribution to the solvent composition throughout gradient delivery. The result is not only consistency in chromatographic performance but a significant benefit in terms of chromatographic reproducibility with minimal solvent preparatory requirements. Auto•Blend Technology in peptide mapping enables QC labs to spend less time in the prep labs, instead focusing on driving productivity.

EXPERIMENTAL

Sample Preparation

Two peptide preparations were used in this study: Ribonuclease B (Sigma Aldrich, USA), and infliximab, both prepared as follows. Five hundred µg of protein was reduced with dithiothreitol, alkylated with iodoacetamide, and isolated using NAP-5 columns (GE Healthcare, PA, USA). Sequence-grade trypsin (Promega, CA, USA) was added to each protein to a final composition 1:20 enzyme/substrate with samples subsequently digested overnight at 37 °C. Following digestion, trypsin was deactivated by incubation at 70 °C for 15 minutes, and 60 µL of digested protein material was reconstituted in 40 µL of 5% MeCN/0.1% TFA, generating a final peptide concentration of 0.6 µg/µL.

UPLC conditions

System:	ACQUITY UPLC H-Class Bio with Tunable UV Detector with 10-mm titanium flow cell
Extension loop:	100 µL (p/n 430002625)
Mixer:	250 µL (p/n 205000737)
Column:	XBridge BEH C_{18} 130 Å 3.5 µm, 4.6 x 100 mm
Column temp.:	40 °C
Injection volume:	95 µL
Mobile phase A:	Water
Mobile phase B:	Acetonitrile
Mobile phase C:	1% (v/v) TFA in water
Detection wavelength:	214 nm

Time (min)	Flow rate (mL/min)	%A	%B	%C	%D	Curve
–	0.500	85	5	10	0	6
5.00	0.500	85	5	10	0	6
45.00	0.500	40	50	10	0	6
47.50	0.500	0	90	10	0	6
52.50	0.500	0	90	10	0	6
52.60	0.500	85	5	10	0	6
60.00	0.500	85	5	10	0	6

RESULTS AND DISCUSSION

Evaluation of TFA effect on peptide retention time

To illustrate the impact of changing TFA concentration on peptide retention time, a series of trypsinized Ribonuclease B peptide separations were performed with a TFA concentration ranging from 0.08% to 0.10% in 0.01% increments. The resulting chromatograms (Figure 1) show significant differences in peak retention times with changes in TFA concentration of 0.01%. Changing TFA concentrations is observed to affect not only the peak retention time but also selectivity, where various peaks are observed to change elution order based on TFA concentration (Figure 1). Based on such sensitivity to TFA concentration, accurate and reproducible preparation of solvents containing TFA must be considered critical for improving consistency in peptide mapping.

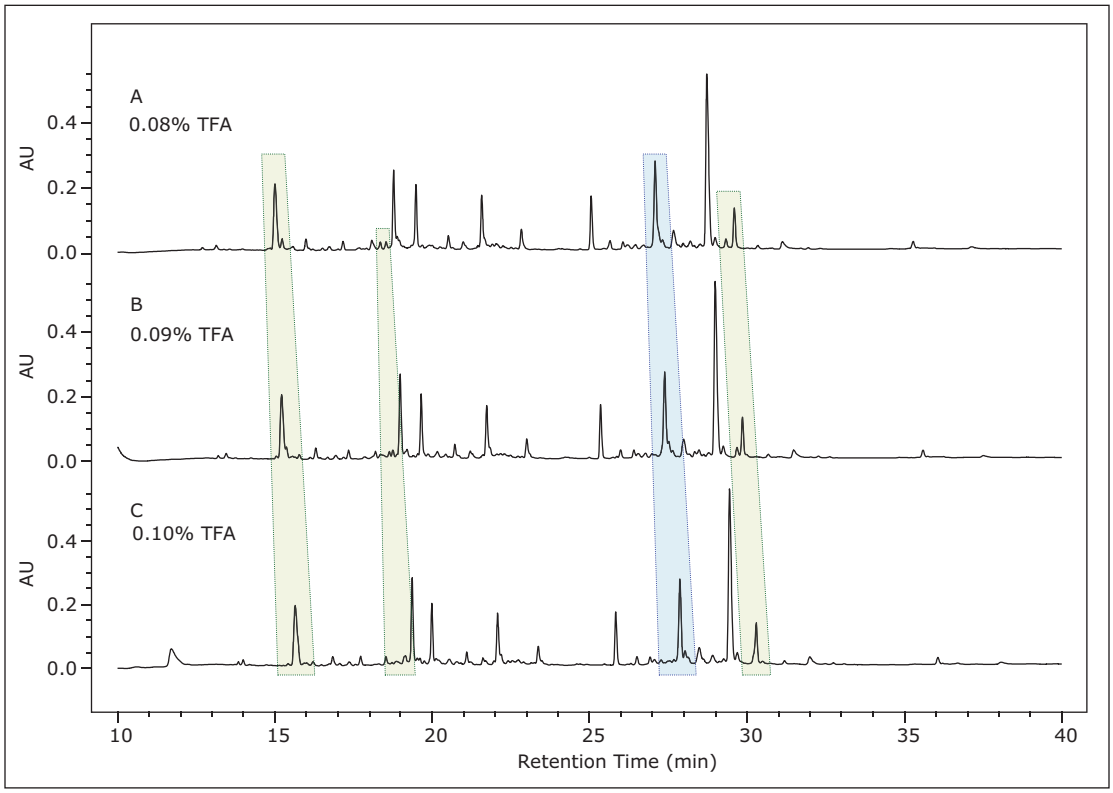

Figure 1. Effect of TFA concentration on peptide separation. Trypsinized Ribonuclease B peptides were separated with varying amounts of TFA ranging from 0.08% to 0.10%. Changes of as little as 0.01% TFA resulted in changes in peak retention times. Peaks within green enclosures indicate a loss of resolution while peaks contained within blue enclosures illustrate an increase in resolution. (A) 0.08% TFA. (B) 0.09% TFA. (C) 0.10% TFA. Adjustments to TFA concentration were obtained using Auto•Blend Technology.

Using Auto•Blend Technology to automate accurate delivery of TFA

One approach for eliminating TFA concentration variability is to remove the additive as a component of each mobile phase, instead providing a stock concentration of TFA as its own solvent line. This results in three solvents contributing to a peptide map gradient, all of which can be accurately managed using Auto•Blend. To demonstrate the use of Auto•Blend in peptide mapping using this solvent arrangement, 1% TFA in water was prepared and configured on solvent line C beside MilliQ dH_2O and acetonitrile as solvents A and B, respectively. Evaluation of Auto•Blend Technology for controlling TFA in peptide mapping was performed using trypsinized infliximab as a model protein therapeutic.

Previous peptide mapping of trypsinized infliximab monitored a total of 56 peaks.[1] For comparative purposes between the HPLC and ACQUITY UPLC H-Class Bio instruments, and instrument methods (with or without Auto•Blend), the same 56 peaks were monitored. Trypsinized infliximab was separated using either a standard configuration of solvents modified with 0.1% TFA or in an Auto•Blend configuration with three solvents (pure acetonitrile, pure water, and 1% formic acid in water) used for gradient formation. Each of these configurations was compared to the legacy method generated on an HPLC instrument (Figure 2a).

In the conventional mobile phase delivery using the ACQUITY UPLC H-Class Bio System, comparable chromatography can be observed to that obtained on the HPLC instrument (Figure 2b). Using the Auto•Blend configuration with 1% TFA as a separate solvent line, no difference in selectivity and nearly identical retention times are observed (Figure 2c). Importantly, no difference was observed between the chromatogram obtained using Auto•Blend Technology and the chromatogram obtained on the HPLC instrument; a result that supports the use of ACQUITY UPLC H-Class Bio and Auto•Blend for running legacy HPLC peptide mapping methods.

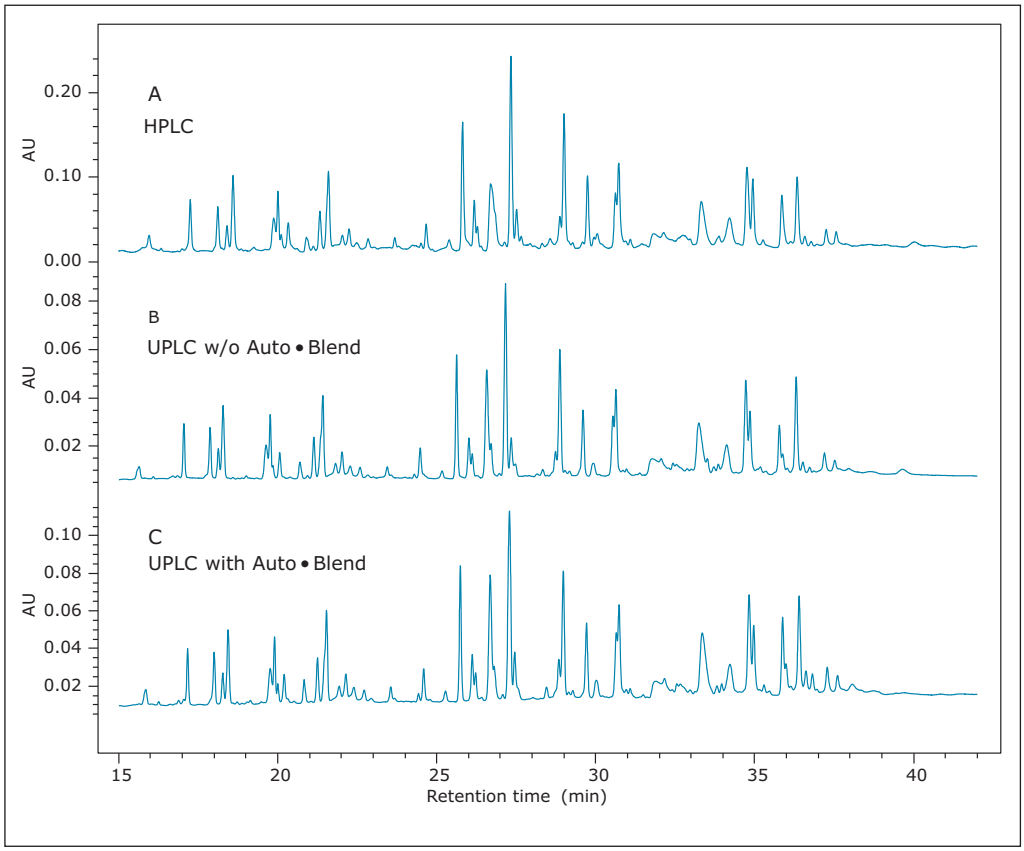

Figure 2. Incorporation of Auto•Blend Technology for peptide mapping produces chromatograms comparable to conventional solvent. The challenge for Auto•Blend control of multiple solvent lines is to replicate chromatographic performance of a legacy HPLC peptide mapping method. A trypsinized infliximab peptide map was produced on an HPLC using a legacy HPLC method with the acidic modifier as a component of two solvents (A), establishing the benchmark for separation on the ACQUITY UPLC H-Class Bio System. The same approach for solvent use was set up on the ACQUITY UPLC H-Class Bio System to establish comparability to the HPLC chromatogram (B). TFA was then removed as a component and provided its own solvent line, where the resulting chromatography was found to be significantly comparable to both the HPLC method and the HPLC method run on the ACQUITY UPLC H-Class Bio System.

Adoption of Auto•Blend Technology for peptide mapping methods allows more consistent mobile phase composition and delivery, which ultimately benefits chromatogram reproducibility over wider time spans and reduces analyst bench time preparing stock solvents adjusted with acidic modifiers.

Auto•Blend control of TFA results in reproducible peptide mapping

For QC labs performing peptide mapping on a routine basis, reproducibility and reliability are key factors that ultimately drive productivity. To determine the reproducibility, and hence consistency, of the ACQUITY UPLC H-Class Bio System with Auto•Blend for peptide mapping applications, five injections of trypsinized infliximab were run on the ACQUITY UPLC H-Class Bio System. Results of the evaluation demonstrated comparable chromatograms with standard deviations no greater than 0.011 (Figure 3a and Table 1). This finding was confirmed by measuring the relative peak area across each chromatogram, where low standard deviation was found with the relative peak area of each monitored peak (Figure 3b).

Measurement of reproducibility within a peptide map is best reported in terms of percent relative standard deviation (%RSD) of peak retention times. The performance of the ACQUITY UPLC H-Class Bio System in terms of retention time reproducibility was also evaluated through determination of relative standard deviation (RSD) with calculated retention times indicating a maximum %RSD value of 0.089% (Table 1). This represents a value significantly lower than that required by existing regulatory guidelines. Despite the inclusion of an additional solvent line, Auto•Blend Technology demonstrated a capacity to generate highly consistent and reproducible chromatograms in both retention time (Figure 3a) and peak area values (Figure 3b).

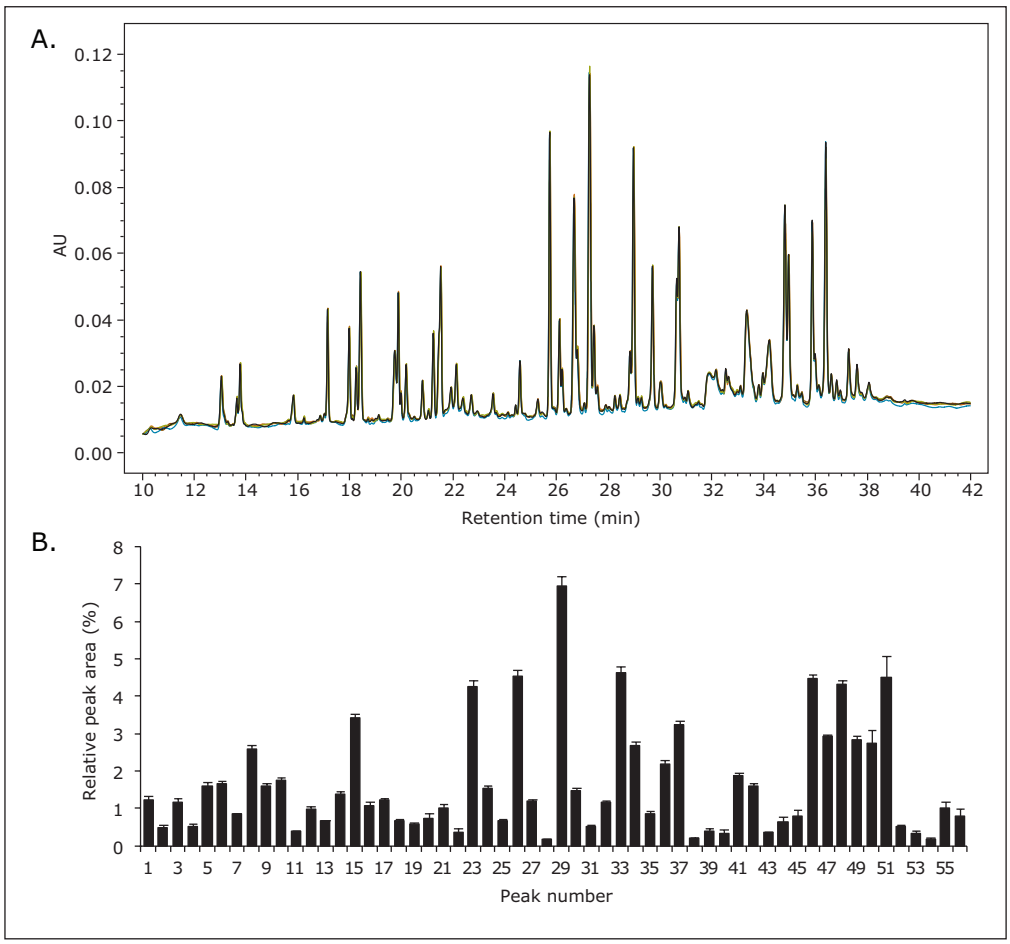

Figure 3. Auto•Blend Technology provides accurate and consistent reproducibility for peptide mapping on the ACQUITY UPLC H-Class Bio System. To ascertain the reproducibility of the ACQUITY UPLC H-Class Bio and Auto•Blend Technology, a total of five injections were performed with retention times tabulated for a total of 56 common peptide peaks identified in the infliximab peptide map. The resulting five chromatograms were overlayed to illustrate the reproducibility of a separation using Auto•Blend (A). Reproducibility of relative peak area was also assessed, with average relative peak areas and associated standard deviations provided as a column chart (B).

Peak	Retention Time (min)					$\bar{x}$	s	% RSD
	1	2	3	4	5			
1	13.034	13.041	13.055	13.063	13.053	13.049	0.012	0.089
2	13.639	13.639	13.647	13.660	13.649	13.647	0.009	0.064
3	13.770	13.769	13.778	13.791	13.780	13.778	0.009	0.065
4	15.826	15.828	15.838	15.847	15.843	15.836	0.009	0.058
5	17.151	17.150	17.161	17.163	17.157	17.156	0.006	0.034
6	17.982	17.982	17.994	18.000	17.990	17.990	0.008	0.043
7	18.254	18.254	18.265	18.271	18.262	18.261	0.007	0.040
8	18.416	18.417	18.428	18.434	18.425	18.424	0.008	0.041
9	19.878	19.881	19.892	19.895	19.890	19.887	0.007	0.037
10	19.980	19.983	19.994	19.995	19.991	19.989	0.007	0.034
11	20.180	20.183	20.194	20.198	20.192	20.189	0.008	0.038
12	20.815	20.813	20.825	20.828	20.822	20.821	0.006	0.031
13	21.049	21.048	21.060	21.065	21.060	21.056	0.008	0.036
14	21.233	21.232	21.244	21.249	21.244	21.240	0.008	0.035
15	21.518	21.518	21.530	21.534	21.527	21.525	0.007	0.033
16	21.916	21.915	21.928	21.937	21.925	21.924	0.009	0.041
17	22.125	22.124	22.135	22.145	22.132	22.132	0.009	0.039
18	22.382	22.377	22.392	22.404	22.386	22.388	0.010	0.046
19	22.705	22.700	22.713	22.721	22.710	22.710	0.008	0.035
20	23.547	23.541	23.555	23.561	23.555	23.552	0.008	0.033
21	24.582	24.579	24.590	24.595	24.592	24.588	0.007	0.028
22	25.270	25.272	25.284	25.285	25.284	25.279	0.007	0.029
23	25.730	25.734	25.745	25.741	25.744	25.739	0.007	0.025
24	26.106	26.108	26.121	26.117	26.122	26.115	0.007	0.028
25	26.211	26.213	26.226	26.222	26.228	26.220	0.008	0.029
26	26.668	26.673	26.689	26.684	26.690	26.681	0.010	0.037
27	26.792	26.800	26.816	26.810	26.817	26.807	0.011	0.040
28	27.063	27.070	27.082	27.076	27.082	27.075	0.008	0.030
29	27.266	27.272	27.287	27.280	27.285	27.278	0.009	0.032
30	27.436	27.440	27.454	27.449	27.455	27.447	0.008	0.031
31	27.569	27.575	27.588	27.585	27.590	27.581	0.009	0.033
32	28.831	28.833	28.849	28.843	28.848	28.841	0.008	0.029
33	28.969	28.971	28.988	28.981	28.986	28.979	0.009	0.030
34	29.701	29.704	29.719	29.711	29.717	29.710	0.008	0.026
35	30.022	30.018	30.029	30.030	30.030	30.026	0.005	0.018
36	30.636	30.640	30.653	30.645	30.653	30.645	0.008	0.025
37	30.726	30.729	30.743	30.735	30.742	30.735	0.008	0.025
38	30.950	30.953	30.967	30.961	30.966	30.959	0.008	0.025
39	31.075	31.078	31.092	31.083	31.090	31.084	0.007	0.024
40	31.484	31.488	31.503	31.493	31.502	31.494	0.008	0.027
41	31.899	31.892	31.914	31.903	31.911	31.904	0.009	0.028
42	32.156	32.152	32.172	32.161	32.169	32.162	0.008	0.026
43	32.410	32.407	32.422	32.404	32.410	32.411	0.007	0.021
44	32.537	32.534	32.550	32.537	32.549	32.541	0.008	0.023
45	33.102	33.102	33.117	33.105	33.115	33.108	0.007	0.022
46	33.342	33.344	33.357	33.350	33.356	33.350	0.007	0.020
47	34.213	34.216	34.231	34.221	34.226	34.221	0.007	0.021
48	34.819	34.821	34.834	34.826	34.829	34.826	0.006	0.017
49	34.962	34.965	34.977	34.969	34.972	34.969	0.006	0.017
50	35.876	35.881	35.893	35.884	35.888	35.884	0.007	0.018
51	36.396	36.401	36.410	36.405	36.406	36.404	0.005	0.015
52	36.614	36.618	36.626	36.622	36.624	36.621	0.005	0.013
53	36.818	36.824	36.830	36.828	36.830	36.826	0.005	0.014
54	36.959	36.966	36.974	36.967	36.971	36.967	0.006	0.015
55	37.281	37.289	37.297	37.292	37.295	37.291	0.006	0.017
56	37.603	37.609	37.619	37.612	37.616	37.612	0.006	0.017

Table 1. Auto•Blend control of TFA delivery generates highly reproducible retention times. Five individual injections of trypsinized infliximab were separated on the ACQUITY UPLC H-Class Bio System using Auto•Blend Technology, where retention times of 56 peaks were monitored. Relative standard deviation (%RSD) was calculated across all chromatograms with a maximum value of 0.089 determined.

CONCLUSIONS

Reliability, robustness, and reproducibility are cornerstones of QC laboratories. For routine analysis of complex peptide maps, LC instrumentation needs to generate consistent chromatography over extended periods of time to adhere to specifications outlined in SOP documents. Modification of mobile phases with acidic modifiers introduces the potential for loss of reproducibility due to sensitivity of peptide maps to subtle changes in modifier concentration. Auto•Blend Technology using the ACQUITY UPLC H-Class Bio System circumvents this issue by simplifying solvent preparation and automating the formulation of the peptide map mobile phase throughout gradient delivery.

As a result, Auto•Blend improves the reproducibility of complex peptide separations, thereby reducing time dedicated to reviewing instrument-related separation issues.

Reference

1. Cosgrave EFJ, McCarthy SM. Future-proofing the Biopharmaceutical QC Laboratory: Using the ACQUITY UPLC H-Class Bio System for HPLC Peptide Mapping. Waters Application Note 720004614EN. 2013 June.

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