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Application Note

Future-proofing the Biopharmaceutical QC Laboratory: Using the ACQUITY UPLC H-Class Bio System for HPLC Peptide Mapping

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Abstract

The application of the ACQUITY UPLC H-Class Bio System for HPLC-based peptide mapping is described in this application note. By making a simple adjustment to the legacy HPLC method in the form of a gradient start offset, nearly identical chromatograms were obtained on the ACQUITY UPLC H-Class Bio System compared to the HPLC chromatogram.

Benefits

- Transfer peptide map applications from HPLC to UPLC
- Future-proof laboratory for UPLC methods

Introduction

This application note represents the first in a series focusing on transitioning HPLC-based biopharmaceutical separations to UPLC-based methods. The procedure for transferring a peptide mapping method from an HPLC quaternary system to the ACQUITY UPLC H-Class Bio System is described here. The goal of this first step is to perform the identical HPLC assay on the UPLC system operating as an HPLC. This approach enables the QC laboratory to employ UPLC technology for legacy HPLC methods while transitioning to UPLC methods at a later time. Improvements in assay reproducibility and, ultimately, incorporating UPLC based technology will be discussed later in the series.

Peptide mapping represents a typical assay for QC labs dealing with routine analyses of large molecule therapeutics. Peptide separation is a considerable challenge in a QC environment due to the numerous parameters that can influence retention time and selectivity. Consequently, adoption of new separation technology such as UPLC can be a challenge in the QC environment due to the qualification requirements for new instruments together with the need to establish and validate new methods for UPLC instrumentation. Regulatory agencies and pharmacopoeias are becoming increasingly aware of the advantages UPLC offers for product characterization. Correspondingly, regulatory guidelines and pharmacopoeial monographs are beginning to outline new analytical requirements for future drug applications. Companies that manufacture such drugs are, therefore, under pressure to begin the adoption process of new analytical technology.

As a first step in transitioning from HPLC to UPLC technology for routine biopharmaceutical analysis in the

QC laboratory, a simplified process for running legacy HPLC peptide mapping methods on the ACQUITY UPLC H-Class Bio System is demonstrated. This allows the laboratory to implement UPLC technology without the burden of developing, validating, and qualifying new UPLC separation methods. HPLC-based peptide mapping performed on the ACQUITY UPLC H-Class Bio System generates comparable chromatograms compared to equivalent peptide maps acquired on HPLC instrumentation. With UPLC technology established in the lab, efforts can be directed to transferring legacy HPLC methods to UPLC without disruption to the HPLC-based analytical workflows. Users can then deploy the ACQUITY UPLC H Class Bio System for UPLC-based separations at a suitable time in the future, having previously qualified the instrumentation.

Experimental

UPLC conditions

System:	ACQUITY UPLC H-Class Bio
Detector:	ACQUITY UPLC Tunable UV (TUV)
Extension loop:	100 µL
Bio mixer volume:	250 µL
Column:	XBridge BEH C_{18} 130 Å 4.6 x 100 mm, 3.5 µm
Column temp.:	40 °C
Flow rate:	0.500 mL/min
Injection volume:	95 µL
Mobile phase A:	H_2O with 0.1% (v/v) TFA
Mobile phase B:	Acetonitrile with 0.1% (v/v) TFA

Detection wavelength: 214 nm

Gradient:

Time(min)	FlowRate(mL/min)	%A	%B	%C	%D	Curve
–	0.500	95	5	0	0	–
5.00	0.500	95	5	0	0	6
45.00	0.500	50	50	0	0	6
47.50	0.500	5	95	0	0	6
52.50	0.500	5	95	0	0	6
52.60	0.500	95	5	0	0	6
60.00	0.500	95	5	0	0	6

Sample description

Three peptide preparations were used in this study: Waters MassPREP peptide mixture standard, ribonuclease B (Sigma Aldrich, USA), and infliximab with the latter two undigested protein samples prepared as follows. Five hundred µg of ribonuclease B or infliximab was reduced with dithiothreitol, alkylated with iodoacetamide, and isolated using NAP-5 columns (GE Healthcare, USA). Sequence-grade trypsin (Promega, USA) was added to each protein to a final composition 1:20 enzyme/substrate, and samples were digested at 37 °C overnight. Following digestion, trypsin was deactivated by incubation at 70 °C for 15 min whereupon 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.

Results and Discussion

Adding a gradient start offset to an instrument method

As a first step in transferring the method from HPLC to UPLC, the ability of the ACQUITY UPLC H-Class Bio System to generate a comparable chromatogram using identical conditions outlined in a legacy HPLC peptide mapping method was evaluated. This study included the use of a MassPREP peptide mixture standard containing peptides of varying hydrophobicity eluting regularly across a delivered gradient. The MassPREP peptide mixture was analyzed using both the ACQUITY UPLC H-Class Bio System and an HPLC instrument, each fitted with an XBridge C_{18} 130 Å, 3.5 µm column. As shown in Figure 1A, separation on the ACQUITY UPLC H Class Bio System resulted in early elution positions for all peaks in the peptide mixture standard. This result indicated that the reduced dwell volume of UPLC compared to the HPLC was causing a change in peak retention times.

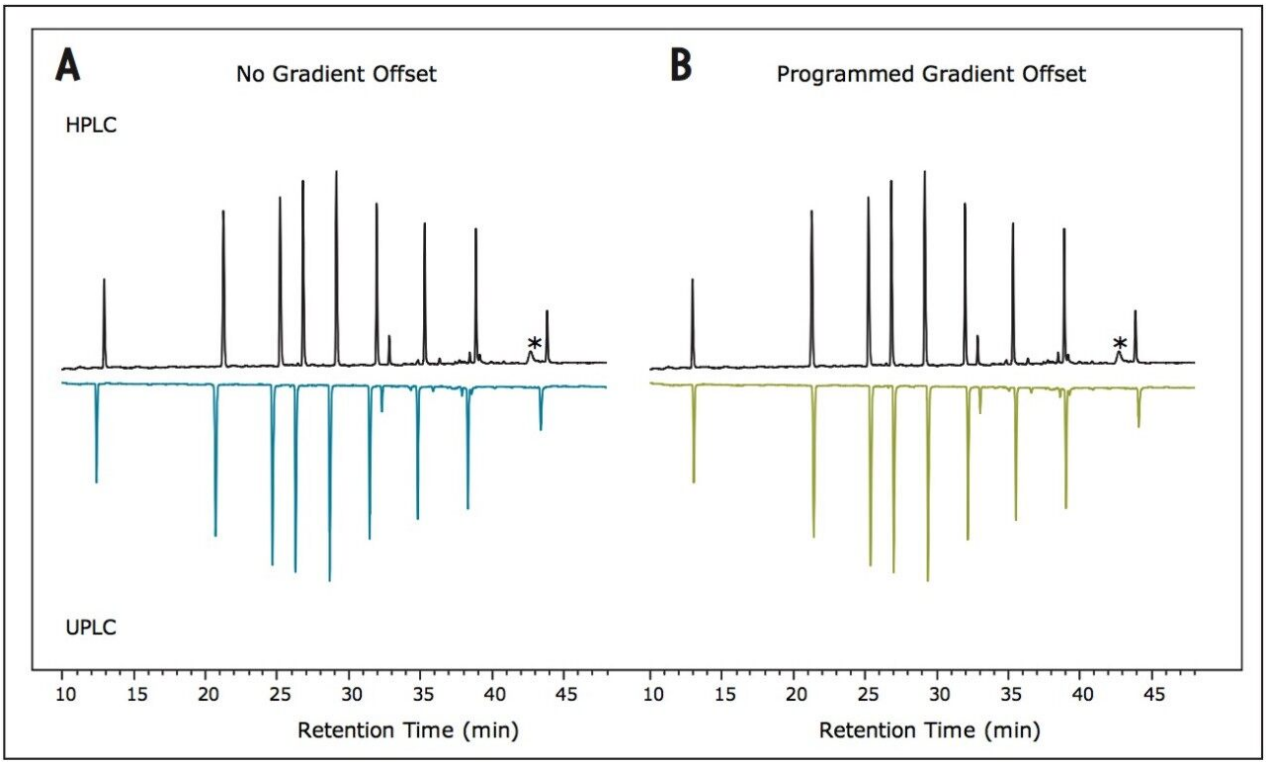
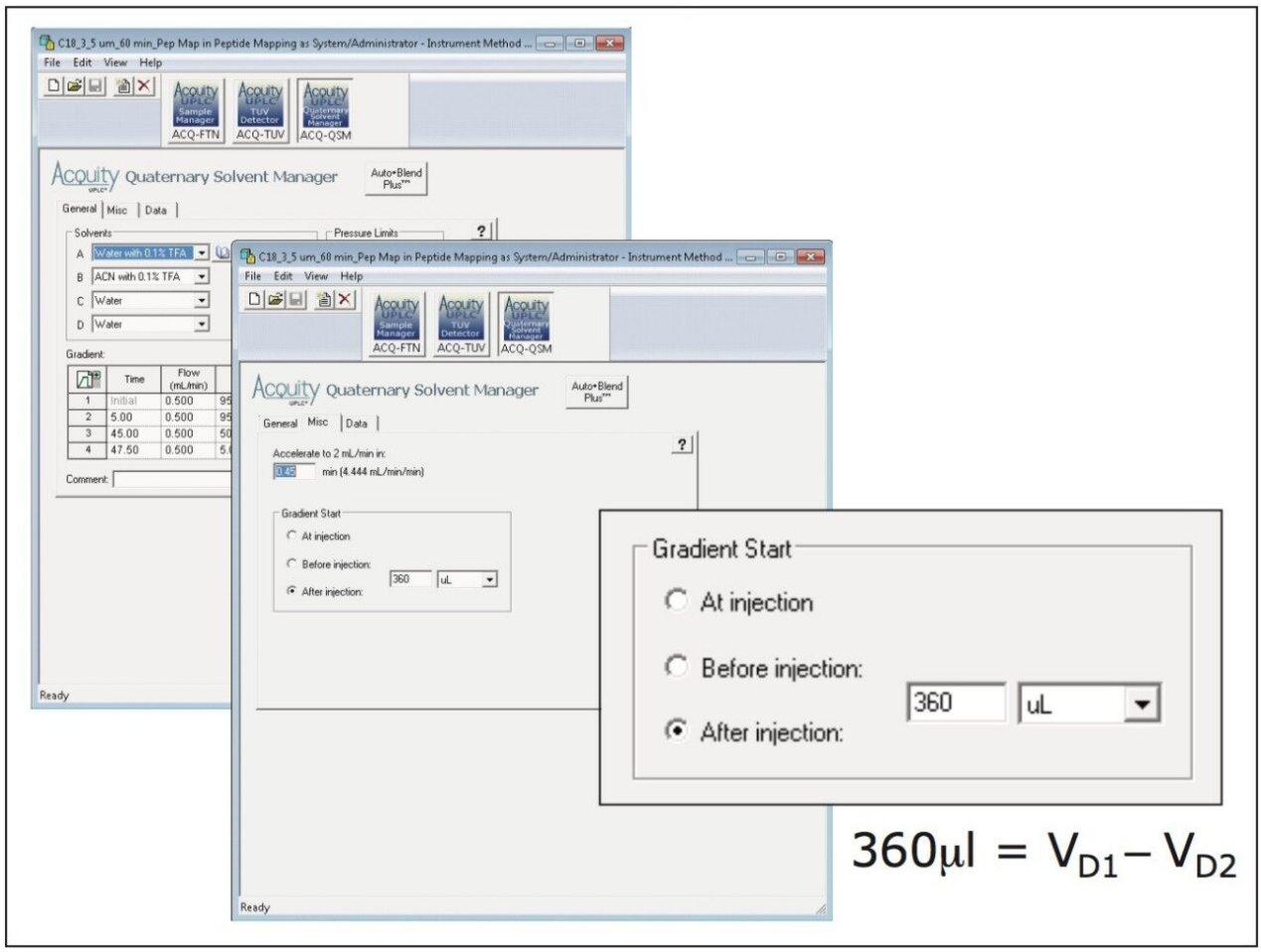


Figure 1. A gradient start offset aligns chromatograms generated on instruments with different dwell volumes. A MassPREP peptide mixture standard was separated using either HPLC or UPLC. (A) Comparison of the peptide mapping method transferred directly across without any modifications to the ACQUITY UPLC H-Class Bio instrument method. (B) The resulting chromatogram after the addition of a 360 µL gradient start offset volume on the ACQUITY UPLC H-Class Bio instrument method. The asterisked peak (*) refers to a system peak observed on the HPLC instrument.

A simple adjustment was made to the ACQUITY UPLC H-Class Bio System method to account for this difference, which worked to simulate a larger dwell volume. To implement this adjustment, the dwell volume was calculated for each instrument where a difference of 360 µL was determined to exist between the instruments. This value was then included as the sole change on the ACQUITY UPLC H-Class Bio instrument method as a gradient start offset volume, as shown in Figure 2.



The addition of a gradient start offset following sample injection acts to increase the length of the isocratic hold step, providing a means for simulating an instrument with a larger dwell volume. This offset does not affect void markers that may elute within the isocratic hold as the injection step occurs in a typical manner, ensuring all unretained molecules will appear at a similar position in the chromatogram. This allows for continuity in relative retention times should the reference peak(s) of interest be calculated against a void marker. As shown in Figure 1B, separation of the MassPREP peptide mixture standard following implementation of the volume correction yields nearly identical chromatographic performance to that of the original HPLC system.

Impact of a gradient start offset on trypsinized ribonuclease B

While the gradient start offset feature was able to align chromatograms generated on two separate instruments, the MassPREP peptide mixture standard contained a limited number of peaks. To challenge the applicability of this approach, a more complex sample in the form of trypsinized ribonuclease B was selected for analysis. With the gradient start offset enabled, the ribonuclease B peptide map produced a highly homologous chromatogram on the ACQUITY UPLC H-Class Bio System compared to the legacy HPLC, as shown in Figure 3. Slight differences were observed in retention times of similar peaks; however, these differences were limited to no greater than a two-second window, as shown in Table 1. Relative retention times were also calculated for all of the 33 monitored peaks on each instrument to determine the extent of comparability between the HPLC and UPLC instruments, as shown in Table 1. Peptide maps from each instrument generated highly similar relative retention time values.

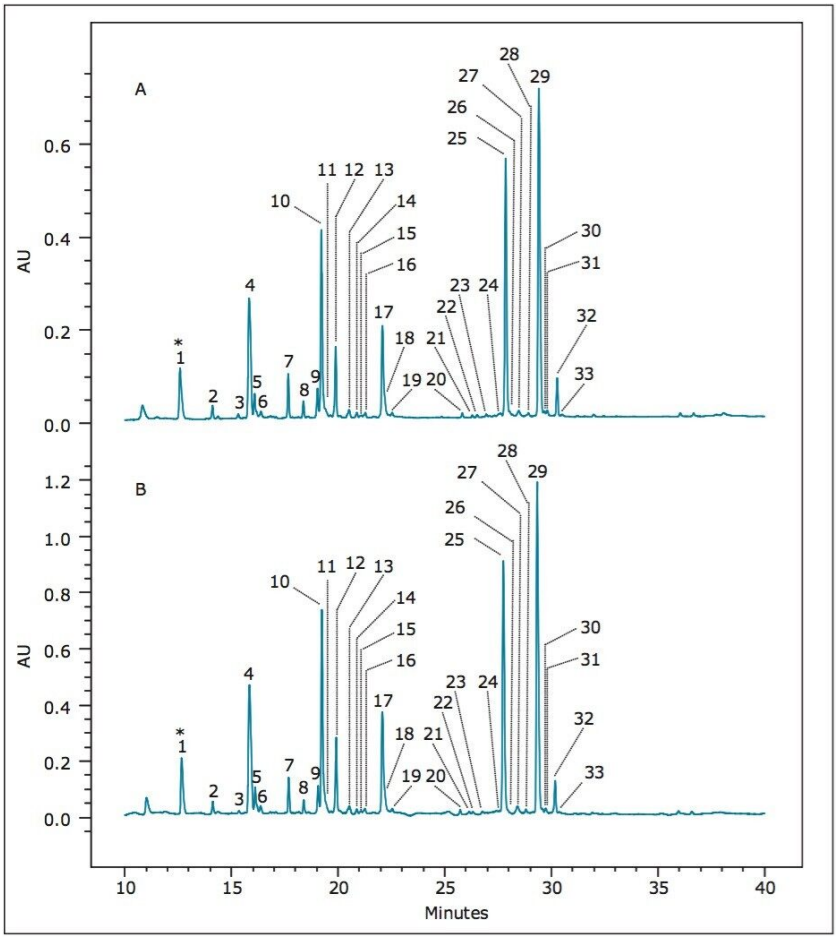


Figure 3. Trypsinized ribonuclease B peptide maps acquired on an HPLC and the ACQUITY UPLC H-Class Bio System are aligned using the gradient start offset feature. A peptide mixture generated from trypsinization of ribonuclease B was used to evaluate the robustness of the gradient start offset feature. Using the same 360 µL offset, the peptide map produced on the HPLC (A) can be generated on the ACQUITY UPLC H-Class Bio System (B) with nearly an identical separation. Peaks denoted by an asterisk (*) refer to the peak used for calculating relative retention times.

Peak	Legacy HPLC		ACQUITY UPLC H-Class Bio		D
	RT (min)	RRT	RT (min)	RRT	
1	12.680	1.000	12.604	1.000	0.000
2	14.140	1.115	14.127	1.121	0.006
3	15.372	1.212	15.332	1.216	0.004
4	15.856	1.250	15.845	1.257	0.007
5	16.128	1.272	16.099	1.277	0.005
6	16.381	1.292	16.396	1.301	0.009
7	17.699	1.396	17.678	1.403	0.007
8	18.403	1.451	18.383	1.459	0.007
9	19.072	1.504	19.042	1.511	0.007
10	19.250	1.518	19.228	1.526	0.007
11	19.680	1.552	19.647	1.559	0.007
12	19.923	1.571	19.900	1.579	0.008
13	20.540	1.620	20.529	1.629	0.009
14	20.886	1.647	20.889	1.657	0.010
15	21.091	1.663	21.109	1.675	0.011
16	21.272	1.678	21.294	1.689	0.012
17	22.083	1.742	22.086	1.752	0.011
18	22.402	1.767	22.408	1.778	0.011
19	22.546	1.778	22.543	1.789	0.010
20	25.822	2.036	25.832	2.050	0.013
21	26.150	2.062	26.301	2.087	0.024
22	26.332	2.077	26.526	2.105	0.028
23	26.780	2.112	26.956	2.139	0.027
24	27.580	2.175	27.590	2.189	0.014
25	27.755	2.189	27.863	2.211	0.022
26	28.030	2.211	28.171	2.235	0.025
27	28.413	2.241	28.474	2.259	0.018
28	28.810	2.272	28.928	2.295	0.023
29	29.338	2.314	29.414	2.334	0.020
30	29.578	2.333	29.674	2.354	0.022
31	29.734	2.345	29.811	2.365	0.020
32	30.181	2.380	30.276	2.402	0.022
33	30.365	2.395	30.512	2.421	0.026

Table 1. Trypsinized ribonuclease B separated on HPLC and the ACQUITY UPLC H-Class Bio System generates nearly identical chromatographic data when the gradient start offset feature is employed. Retention times (RT) and relative retention times (RRT) were recorded for the 33 monitored peaks observed in the ribonuclease peptide map. The difference in relative retention time (D) between the HPLC and the UPLC separations was calculated and recorded.

Gradient start offset approach aligns a trypsinized monoclonal antibody peptide map

To further challenge the applicability of the gradient start offset approach, infliximab, a therapeutic monoclonal antibody, was selected for analysis as it represents a typical protein used in routine peptide mapping analyses in QC labs. A total of 56 infliximab peptide peaks were selected for monitoring between the UPLC and HPLC systems, despite approximately 90 peaks identified within each chromatographic space. Peak selection was based on a number of factors including signal intensity, elution position, and relative resolution. This approach was chosen to simplify the comparative analysis. When separated using the ACQUITY UPLC H-Class Bio System, the infliximab peptide map illustrated significant chromatographic alignment compared to its equivalent acquired on the HPLC instrument, shown in Figure 4. Assessing the relative retention times, virtually no differences were observed with values of less than 0.005 reported, as shown in Table 2. These data provide convincing evidence that employment of a gradient start offset is a suitable strategy for transferring legacy HPLC peptide mapping methods to the ACQUITY UPLC H-Class Bio System.

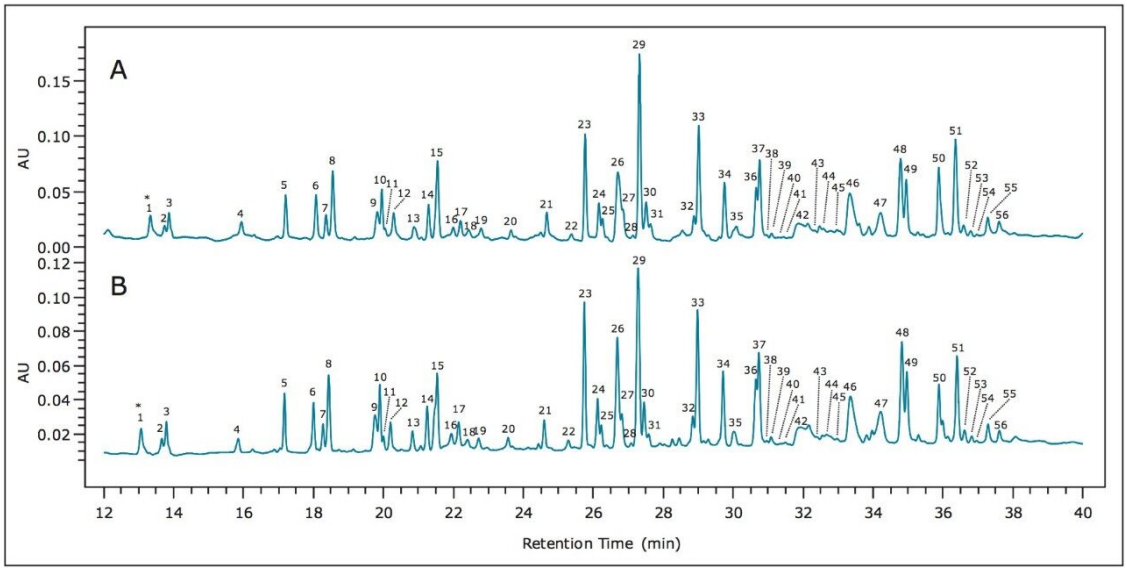


Figure 4. The gradient start offset feature aligns chromatograms for complex trypsinized infliximab. Infliximab was used to evaluate the performance of the gradient start offset option for dwell volume adjustment. The chromatogram generated on the HPLC system (A) demonstrates extensive similarity to the chromatogram generated on the UPLC system (B). Peaks denoted by an asterisk (*) in each chromatogram refer to the peak selected for calculating relative retention times.

Peak	Legacy HPLC		ACQUITY UPLC H-Class Bio		D
	RT (min)	RRT	RT (min)	RRT	
1	13.060	1.000	13.034	1.000	0.000
2	13.655	1.046	13.639	1.046	0.001
3	13.785	1.056	13.770	1.056	0.001
4	15.843	1.213	15.826	1.214	0.001
5	17.165	1.314	17.151	1.316	0.002
6	17.997	1.378	17.982	1.380	0.002
7	18.267	1.399	18.254	1.400	0.002
8	18.430	1.411	18.416	1.413	0.002
9	19.757	1.513	19.741	1.515	0.002
10	19.894	1.523	19.878	1.525	0.002
11	19.995	1.531	19.980	1.533	0.002
12	20.197	1.546	20.180	1.548	0.002
13	20.826	1.595	20.815	1.597	0.002
14	21.247	1.627	21.233	1.629	0.002
15	21.533	1.649	21.518	1.651	0.002
16	21.938	1.680	21.916	1.681	0.002
17	22.147	1.696	22.125	1.697	0.002
18	22.403	1.715	22.382	1.717	0.002
19	22.721	1.740	22.705	1.742	0.002
20	23.563	1.804	23.547	1.807	0.002
21	24.596	1.883	24.582	1.886	0.003
22	25.288	1.936	25.270	1.939	0.002
23	25.745	1.971	25.730	1.974	0.003
24	26.121	2.000	26.106	2.003	0.003
25	26.227	2.008	26.211	2.011	0.003
26	26.686	2.043	26.668	2.046	0.003
27	26.815	2.053	26.792	2.056	0.002
28	27.081	2.074	27.063	2.076	0.003
29	27.281	2.089	27.266	2.092	0.003
30	27.452	2.102	27.436	2.105	0.003
31	27.587	2.112	27.569	2.115	0.003
32	28.844	2.209	28.831	2.212	0.003
33	28.982	2.219	28.969	2.223	0.003
34	29.713	2.275	29.701	2.279	0.004
35	30.015	2.298	30.022	2.303	0.005
36	30.647	2.347	30.636	2.350	0.004
37	30.737	2.354	30.726	2.357	0.004
38	30.963	2.371	30.950	2.375	0.004
39	31.085	2.380	31.075	2.384	0.004
40	31.493	2.411	31.484	2.416	0.004
41	31.934	2.445	31.899	2.447	0.002
42	32.164	2.463	32.156	2.467	0.004
43	32.393	2.480	32.410	2.487	0.006
44	32.553	2.493	32.537	2.496	0.004
45	32.972	2.525	33.102	2.540	0.015
46	33.354	2.554	33.342	2.558	0.004
47	34.222	2.620	34.213	2.625	0.005
48	34.828	2.667	34.819	2.671	0.005
49	34.970	2.678	34.962	2.682	0.005
50	35.887	2.748	35.876	2.752	0.005
51	36.404	2.787	36.396	2.792	0.005
52	36.619	2.804	36.614	2.809	0.005
53	36.824	2.820	36.818	2.825	0.005
54	36.965	2.830	36.959	2.836	0.005
55	37.292	2.855	37.281	2.860	0.005
56	37.616	2.880	37.603	2.885	0.005

Table 2. A gradient start offset produces significantly similar chromatographic data for complex monoclonal