Improving Retention Time Precision and Chromatography of Early Eluting Peptides with Acetonitrile/Water Blends as Solvent B

Stephan Meding[1], Aran Paulus[2], and Remco Swart[1]
[1]Thermo Fisher Scientific, Germering, Germany
[2]Thermo Fisher Scientific, San Jose, CA, USA

Application Note 637

Key Words
EASY-nLC 1200, solvent B, retention time precision, carry-over, system robustness, system stability

Goal
Show that a reduced acetonitrile concentration results in:

- improved chromatographic performance, especially for early eluting, hydrophilic peptides
- no additional sample carry-over due to insufficient column washing
- no reduced long term retention time stability due to acetonitrile evaporation from solvent B

Introduction
Most proteomics workflows rely on bottom-up identification. They consist of an enzymatic protein digest, chromatographic separation of the resulting peptides, and mass spectrometric detection. The acquired data is compared to a database of theoretical protein digests to identify the proteins present in the analyzed sample. The analytical depth of the bottom-up approach has continuously improved over the last decade through advances made in the mass resolution and sensitivity of the mass spectrometer and the sensitivity and resolution of the chromatographic separation. This was done mainly by using lower flow rates, reducing the particle size of the stationary phase, reducing the column diameter, and increasing the column length. As a result, the number of proteins identifiable in a single analysis was increased to more than 5000 with the latest generation instruments. However, changing these chromatographic parameters results in higher system backpressure. Therefore, modern liquid chromatography systems must very reproducibly deliver gradients at very high pressures and at flow rates well below 1 µL/min. Currently, the Thermo Scientific™ EASY-nLC™ 1200 (Figure 1) can deliver up to 1200 bar pressure during gradient delivery while delivering flow rates as low as 100 nL/min.

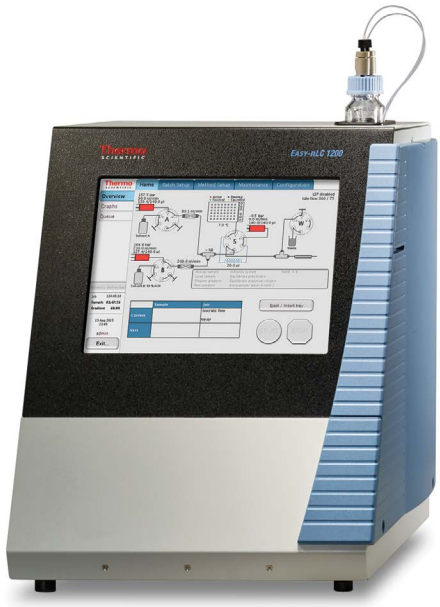

Figure 1. The EASY-nLC 1200.

Apart from sensitivity, data reproducibility is a core requirement in proteomic research. Thus, instruments have to operate in a reliable and robust way. The EASY-nLC 1200 was designed to meet this expectation while delivering simultaneously excellent chromatographic performance. In order to further improve chromatographic performance, the EASY-nLC 1200 should be operated with 80% acetonitrile in water blends as solvent B. For ease of use, pre-mixed 80% acetonitrile as solvent B can be purchased from Fisher Scientific.

This application note demonstrates that 80% acetonitrile as solvent B results in equal or better chromatographic performance for early eluting, hydrophilic peptides. No negative effects, such as increased carry-over or reduced long term stability, were observed.

Thermo SCIENTIFIC

Experimental

Reagents

All solvents were Optima™ LC-MS grade from Fisher Scientific (Table 1).

Solvent A and B were degassed using an ultrasonic bath before use.

For LC-UV experiments, cytochrome c digest (P/N 161089) was used at a concentration of 500 fmol/µL (in water with 0.1% formic acid). For LC-MS experiments, BSA digest (LC722) was used at a concentration of 50 fmol/µL (in water with 0.1% formic acid).

Table 1. LC-MS solvents

Name	Bottle Size	Product Number
Water with 0.1% formic acid	500 mL	LS118-500
	2.5 L	LS118-212
Acetonitrile with 0.1% formic acid	500 mL	LS120-500
	2.5 L	LS120-212
80% acetonitrile, 20% water with 0.1% formic acid	500 mL	LS122-500

Columns

Separation columns used for each experiment are listed in Table 2.

Table 2. Separation columns used throughout experiments.

Experiment	Separation Column	Product Number
Chromatographic robustness at reduced acetonitrile concentration in solvent B	Thermo Scientific™ Acclaim™ PepMap™ C18 100 Å, 2 µm particle size, 75 µm ID × 150 mm bed length	164534
Sample carry-over at reduced acetonitrile concentration	Acclaim PepMap C18 100 Å, 2 µm particle size, 75 µm ID × 150 mm bed length	164534
Long-term retention time stability	Acclaim PepMap C18 100 Å, 2 µm particle size, 75 µm ID × 750 mm bed length	164939

Instrumental Set-up

The EASY-nLC 1200 was set up in one-column (direct injection) mode. In order to place the separation column in a column compartment (TCC-3000SD) it was connected to the venting tee using a 350 mm long 20 µm ID Thermo Scientific™ Dionex™ nanoViper™ capillary (P/N 6041.5240) and a nanoViper union (P/N 6040.2304).

A sample pick-up volume of 2 µL and a sample loading volume of 6 µL were used. Sample loading and equilibration were done at 1180 bar, except for the comparison of different solvent B compositions (900 bar). The flow rate during the gradient was kept constant at 300 nL/min.

For UV detection, a multi-wavelength detector was used (VWD-3400RS) and data was acquired at 214 nm. For MS detection, a Thermo Scientific™ Velos Pro™ with a Thermo Scientific™ Nanospray Flex™ ion source and steel emitters (P/N ES542) was used. Full scan MS was performed in positive mode. The measurement range was 400–1200 *m/z*.

Results and Discussion

Chromatographic Robustness at Reduced Acetonitrile Concentration in Solvent B

Usually, nano LC-MS gradients for bottom-up proteomics start with 1–5% acetonitrile and increase to 35–40%. Afterwards, the column is washed with high acetonitrile concentrations to remove remaining analytes and contaminants. Using a 15 cm column, we looked at the reproducibility of retention times when the B solvent has different acetonitrile concentrations. We chose 100%, 95%, and 80% acetonitrile as B solvent. Reducing the acetonitrile concentration in the B solvent will reduce the actual acetonitrile concentration delivered during the gradient.

$$\%\,ACN_{delivered} = \%\,B_{gradient} \times \%\,ACN_{solvent\ B}$$

To compensate for the reduced acetonitrile concentration in the 95% and 80% acetonitrile solvent B experiments, the gradient conditions were adjusted. As a result the absolute concentrations of acetonitrile were nearly consistent in all three experiments.

Figure 2 depicts the gradient with both the nominal percentage B solvent delivery on the top and the actual acetonitrile concentration on the bottom for all three solvent B compositions. At the onset of the gradient, the delivered percentage of acetonitrile varied slightly. This is caused by the fact that EASY-nLC systems have a default start value of 2% solvent B. After the end of the separation gradient at 30 minutes, all methods had an actual acetonitrile concentration of 32%, rising subsequently to 72% for column washing.

Figure 3 shows the overlay of six UV traces recorded at 214 nm for the separation of 1 pmol cytochrome c digest run under the above described conditions. The nine major peaks of this digests elute in a window from 6 to 22 minutes. Because the acetonitrile concentration differs at the beginning of the gradient between the 100%, 95%, and 80% acetonitrile conditions, the gradients start off with an actual organic concentration of 2%, 1.9%, and 1.6%, resulting in a shift of retention time of the first eluting peptide from 7.15 min to 8.46 min for the least hydrophobic 80% acetonitrile solvent B. In the course of the gradient, the actual acetonitrile concentrations align more closely. The robustness of a chromatographic method can be measured in the variability of the retention times for multiple injections under the same conditions.

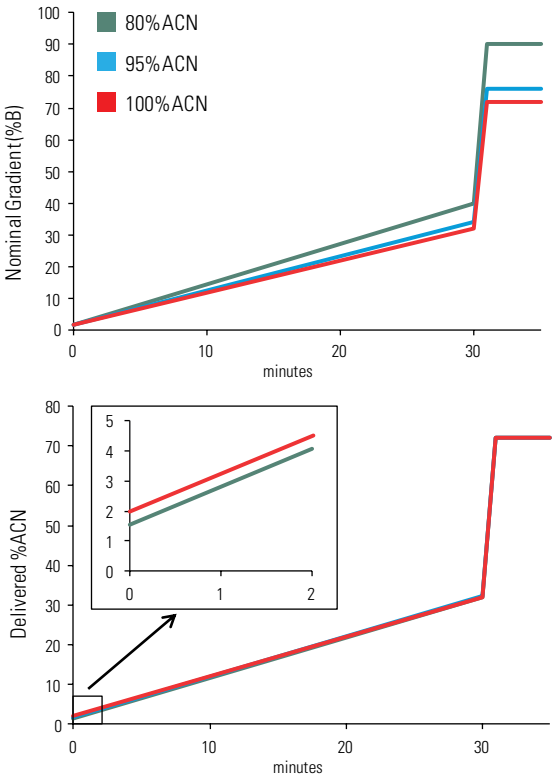

Figure 2. Nominal gradients (top) and delivered percentage acetonitrile (bottom) for 80% acetonitrile, 95% acetonitrile, and 100% acetonitrile in solvent B.

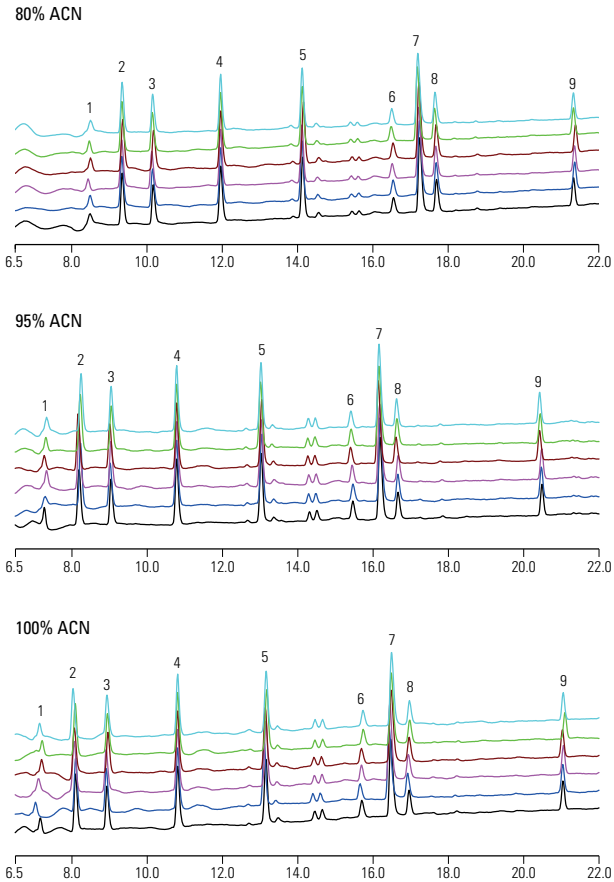

Figure 3. Chromatograms (λ = 214 nm) of six consecutive injections with 80%, 95%, and 100% acetonitrile (ACN) in solvent B. The peaks used for further analysis are indicated with numbers.

Figure 4 and Table 3 show the relative standard deviation for all nine peaks, which is typically between 0.1% and 0.3% for all peaks in all three conditions, except the first eluting peak under 100% acetonitrile conditions. The higher relative standard deviation of 0.7% for this peak is most likely due to the fact that it elutes very early and thus together with the salt front. Therefore, a reduced concentration of 80% acetonitrile in solvent B improves the chromatographic performance of early eluting, hydrophilic peptides.

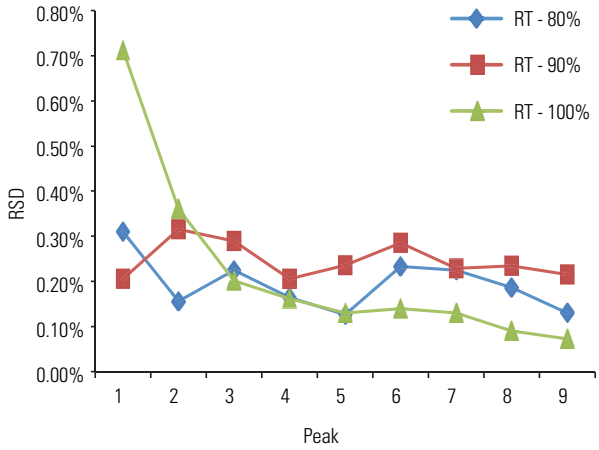

Figure 4. Retention time precision observed for nine different peaks with different solvent B compositions.

Sample Carry-Over at Reduced Acetonitrile Concentration

A consequence of using acetonitrile blends in solvent B is the fact that the maximum acetonitrile concentration during the column wash phase is reduced. Although peptides mostly elute at low acetonitrile concentrations (<30%), efficient column washing determines the carry-over. Reducing the acetonitrile concentration too much may impair the wash procedure. In order to test if there is any noticeable run-to-run carry-over, an LC-MS experiment was performed. Using standard gradient conditions, 100 fmol BSA digest was injected on-column (Table 4).

Table 4. Gradient conditions for testing system carry-over. Solvent B is 80% acetonitrile. %B states the delivered solvent B, %ACN the resulting acetonitrile concentration delivered during the gradient.

Time	%B	%ACN
0–30 min	2–40%	1.6–32%
30–35 min	40–95%	32–76%
35–60 min	95%	76%

Table 3. Average retention time precision of nine peaks. Six consecutive injections with 80%, 95%, and 100% acetonitrile (ACN) in solvent B were used for calculation. (RT: average retention time; RSD: relative standard deviation)

Peaks		1	2	3	4	5	6	7	8	9
80% ACN	RT (min)	8.46	9.32	10.13	11.94	14.11	16.50	17.19	17.65	21.33
	RSD (%)	0.31%	0.16%	0.23%	0.16%	0.13%	0.23%	0.22%	0.19%	0.13%
95% ACN	RT (min)	7.27	8.17	9.00	10.75	12.99	15.40	16.13	16.61	20.42
	RSD (%)	0.21%	0.32%	0.29%	0.21%	0.24%	0.29%	0.23%	0.23%	0.22%
100% ACN	RT (min)	7.15	8.08	8.94	10.82	13.15	15.71	16.47	16.96	21.06
	RSD (%)	0.71%	0.36%	0.20%	0.16%	0.13%	0.14%	0.13%	0.09%	0.07%

To determine the carry-over, *m/z* 722.3 (YIC*DNQDTISSK) was used. Measurement resulted in a peak area of 3.4 × 10^6 for both replicates (Figure 5 and Table 5). In the following blank injections using only 0.1% formic acid in water, an *m/z* 722.3 peak was detected at a very low level. In the first experiment the detected peak area was 2.8 × 10^3, in the second experiment 3.0 × 10^3. Therefore, the carry-over was calculated to be as low as 0.08% and 0.09%, respectively. This result is comparable to data obtained using 100% acetonitrile in solvent B and EASY-nLC 1000 systems where usually carry-over of <0.1% with the respective peptide is observed. Therefore, it was concluded that the lowered acetonitrile percentage in solvent B has no negative effect on sample carry-over.

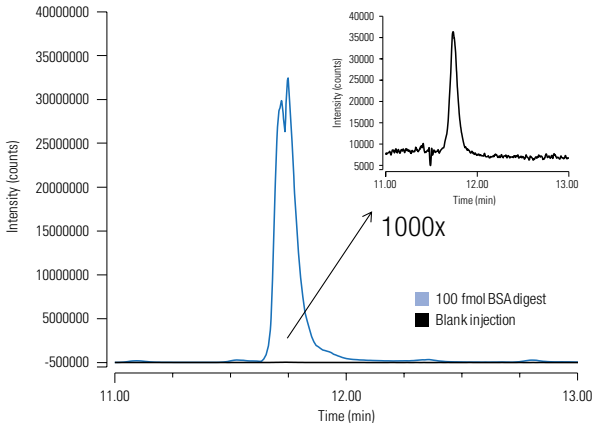

Figure 5. Extracted ion chromatograms of *m/z* 722.3 for 100 fmol BSA digest injection and subsequent blank injection.

Table 5. Peak area and carry-over determined for BSA peptide *m/z* 722.3.

	First repeat		Second repeat	
	Peak Area	Carry-over	Peak Area	Carry-over
100 fmol BSA digest	3,395,963	NA	3,370,271	NA
Blank injection	2834	0.08%	2996	0.09%

Long-term Retention Time Stability

Solvents in the EASY-nLC 1200 should be exchanged at least every two weeks. During this time period the solvent composition has to remain stable, otherwise experimental reproducibility could suffer. Since acetonitrile is more volatile than water, the composition of an 80:20 mixture of acetonitrile and water may change over time. This would result in a continuous deviation of the intended acetonitrile concentration from the delivered one during the gradient and as a consequence a drift in retention times of the separated peptides.

In order to test the solvent stability of the 80% acetonitrile solvent B, a long-term experiment using a 75 cm long column was conducted. A total of 135 cytochrome c digest injections (1 pmol each) with 2–40% solvent B in 50 min and additional 20 minutes of column washing resulted in more than eight days of continuous sample analysis. Six peaks were used for monitoring retention time stability using UV detection at 214 nm. Representative chromatograms of six consecutive injections are displayed in Figure 6. The peaks used for retention time stability analysis are marked with numbers. The retention times of these peaks throughout the whole experiment are displayed in Figure 7 and summarized in Table 6. The peaks eluted from 20 to 40 minutes and showed a relative standard deviation of the retention time of 0.4–1.3%. The early eluting peaks had the highest relative standard deviation. The values are higher than in the first experiment because they are calculated for the whole sequence of 135 injections. Selecting six consecutive injections from the middle of the sequence resulted in relative standard deviation values below 0.3% (Figure 6 and Table 6).

The results demonstrated that within the eight days operated at room temperature, the solvent compositions did not change. Otherwise a drift in retention times towards later peak elution would have been observed. For ease of use, LC-MS grade water, 0.1% formic acid and 80% acetonitrile, 20% water, 0.1% formic acid solvents can be purchased through Fisher Scientific.

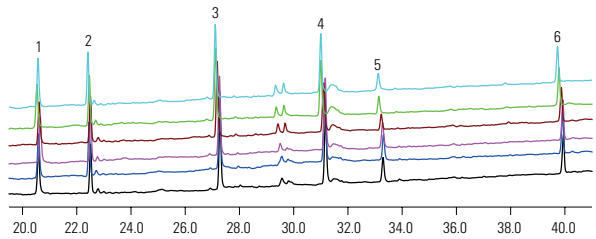

Figure 6. Chromatograms (λ = 214 nm) of six consecutive injections (# 64–69). The peptide peaks used for further analysis are indicated with numbers.

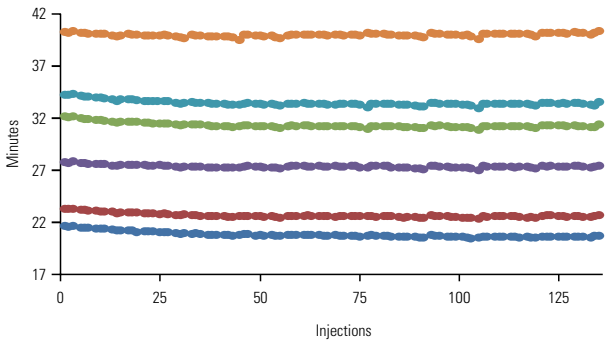

Figure 7. Retention time stability of six peptides throughout 135 injections (eight days of continuous measurement).

Table 6. Retention time stability of six peaks throughout 135 injections (eight days of continuous measurement). (RT: average retention time; RSD: relative standard deviation)

	Peaks					
	1	2	3	4	5	6
RT (min)	20.82	22.66	27.36	31.31	33.45	39.98
RSD	1.32%	0.92%	0.48%	0.79%	0.74%	0.37%
Six consecutive injections during the sequence (injections 64–69)						
RT (min)	20.59	22.48	27.21	31.10	33.23	39.86
RSD	0.20%	0.17%	0.29%	0.26%	0.25%	0.22%

Conclusion

- Using 80% acetonitrile as solvent B results in a robust separation and allows reproducible detection of early eluting hydrophilic peptides.
- No significant sample carry-over is observed with 80% acetonitrile as solvent B.
- Very good long-term retention time precision can be obtained with 80% acetonitrile as solvent B. Solvents should be exchange at least every two weeks. During this time period experiments can be successfully conducted without determinable changes in solvent B composition.

Useful Links

Further information about the system is available on:
thermoscientific.com/nanoLCMS

For more information, visit: **thermofisher.com/nanoLCMS**

Africa +43 1 333 50 34 0
Australia +61 3 9757 4300
Austria +43 810 282 206
Belgium +32 53 73 42 41
Canada +1 800 530 8447
China 800 810 5118 (free call domestic)
400 650 5118
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland +358 10 3292 200
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9494
Italy +39 02 950 591
Japan +81 45 453 9100
Korea +82 2 3420 8600
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
Norway +46 8 556 468 00
Russia/CIS +43 1 333 50 34 0
Singapore +65 6289 1190
Spain +34 914 845 965
Sweden +46 8 556 468 00
Switzerland +41 61 716 77 00
UK +44 1442 233555
USA +1 800 532 4752

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand

AN64627-EN 0716S