



application note # n-03

**microfluidic flow control allows for splitless
nanoLC with unprecedented retention time
repeatability**

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The nanoLC product line from Eksigent uses direct (splitless) pumping in combination with microfluidic flow control to deliver precise gradients even at nano flow rates (20 nL/min–1000 nL/min). For protein digests, the retention time repeatability is shown to be better than 0.5% for all resolved peaks with either UV or MS detection.

introduction

Limited amounts of sample available for proteomics research have created the need for nanoscale (column ID 50–150 μm) chromatography featuring flow rates between 20 nL/min and 1000 nL/min. This is far below the 0.1–5 mL/min flow rate range of a typical HPLC instrument used for conventional chromatography (column ID 1.0–4.6 mm).

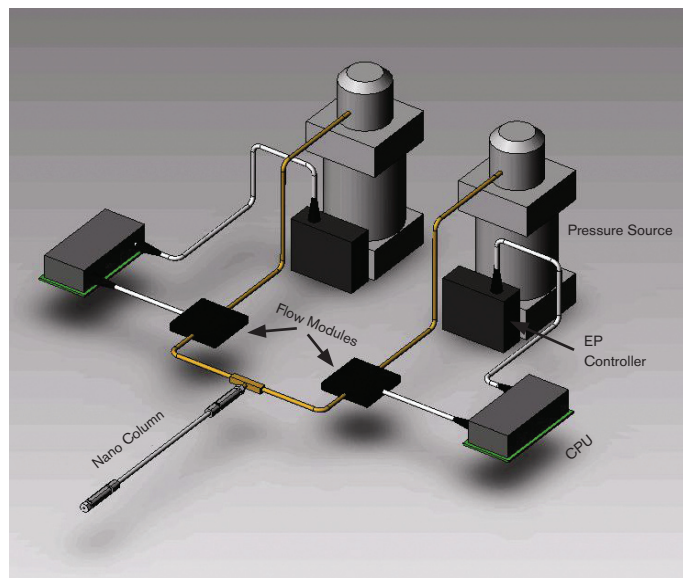
Various approaches exist to deliver flow rates in this range. One approach uses conventional HPLC in combination with a splitter. For example, delivery of 200 nL flow rates is accomplished by taking a conventional pump, operating at 200 $\mu\text{L}/\text{min}$, and splitting it through a tee where 0.1% of its total flow would be routed to the nanoLC column while the remaining 99.9% goes to waste. This approach typically suffers from the difficulty of maintaining a constant split ratio. As the back pressure on the nanoLC column changes, the split ratio of the setup changes as well. In such a case the conventional pump, well upstream of the column, will still accurately be delivering its 200 $\mu\text{L}/\text{min}$ flow, but the flow through the nanoLC column itself will not be accurate. This causes the retention time of the separated analytes to drift.

A second approach uses displacement pumps, specifically designed for low flow rates. In this case, mobile phase from a servo driven pump is displaced and routed to the nanoLC column at pressure. Careful initial calibration of the displacement will give reasonable results with equipment of this sort. However, without feedback, the user cannot be sure that the delivered flow rates remain accurate as column pressures and mobile phases change.

Figure 1. Microfluidic Flow Control

Direct pumping is used to deliver accurate, repeatable gradients. Each pressure source delivers fluid with an actual flow rate monitored using the flow modules in each channel. A CPU closes the loop by making any necessary adjustments to the flow rate using electro-pneumatic controllers (EP Controller).

figure 1.



The Eksigent nanoLC product line uses a third technique, microfluidic flow control, which ensures high quality, repeatable chromatography even when conditions downstream of the pumps are changed. With microfluidic flow control, nano flow rates are delivered using direct pumping. As shown in Figure 1, a dynamically adjusted pressure source delivers fluid through a flow module which monitors the actual flow rate. Any discrepancy between the actual and programmed flow rates are corrected for by a control loop.

The benefits of using microfluidic flow control can be seen in the quality of the chromatography. By using real-time closed loop control, retention time variation can be kept to a minimum. Splitless flow also saves up to 1000x the amount of solvent that would be required with a split system.

The repeatability of the chromatography from the Eksigent NanoLC-2D is investigated using both nanoLC-UV and nanoLC-MS. Data measured over a wide range of retention times have deviations less than 0.5% in all cases.

experimental

The retention time repeatability of the Eksigent NanoLC-2D system equipped with an Eksigent AS1 autosampler is evaluated using two samples by two different techniques. For nanoLC/UV a cytochrome c (in house prepared) digest is used, while a BSA digest (Michrom Bioresources, Inc., Auburn, CA) is used for the nanoLC/MS measurements. In the case of nanoLC/UV column temperature is controlled at 35°, while for nanoLC/MS the column temperature is uncontrolled at ambient.

For nanoLC/UV 1 µL of cytochrome c digest at 2 pmol/µL concentration, is directly injected onto a 0.075x150 mm, 3 µm, 100 Å, C18 column. Absorbance data is collected at 214 nm using the analog input of the NanoLC-2D while the LC method (Table 1) is run at a 200 nL/min flow rate.

For MS detected runs the bovine serum albumin (BSA) digest is diluted in mobile phase A to 0.5 pmol/µL and again 1 µL is injected directly onto the analytical column, which is a 0.075x100 mm, 5 µm, 300 Å, Integrafrit column packed with ProteoPep II™ (New Objective Inc., Woburn, MA).

The MS Data itself is acquired on a LTO linear ion trap mass spectrometer, (Thermo Electron, San Jose, CA) in full scan mode (300-2000 amu), using a Picoview nanospray source (New Objective, Inc.) to interface the Eksigent NanoLC to the mass spectrometer. High voltage (2.4 kV) is applied to a distal-coated SilicaTip (50 µm ID tapering to 15 µm ID) using the coated tip module of the source.

The gradient profile used for the runs is shown in Table 2 and is performed at 300 nL/min.

The LTO mass spectrometer is controlled using version 2.0 of the XCalibur™ (Thermo Electron, San Jose, CA) software package. This software also allows full control of the Eksigent NanoLC as well as the Eksigent AS1 autosampler. Methods for the mass spec, autosampler, and LC can be seamlessly programmed from a common interface.

Table 1. LC/UV Method

table 1.

Time, min	0.05% TFA/water	0.04%TFA in 20/80 water/acetonitrile
0.0	98%	2%
30.0	50%	50%
30.1	20%	80%
35.0	20%	80%
35.1	98%	2%
60.0	98%	2%

Table 2. LC/MS Method

table 2.

Time, min	0.1% FA/water	0.1% FA/acetonitrile
0	95%	5%
10	95%	5%
80	60%	40%

results and discussion

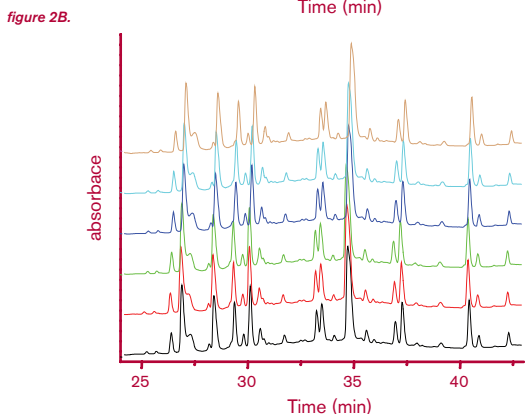
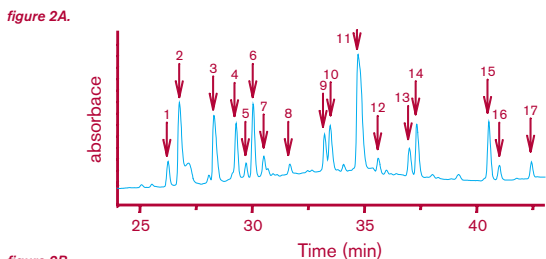
Repeated injections of cytochrome c digest are performed using the Eksigent NanoLC-2D and are detected at 1 Hz using the UV absorbance at 214 nm. The 17 major peaks (16 peptides with ≥ 2 residues are expected) eluting between 25–45 minutes are automatically integrated using the user customizable integration routines available with the PeakViewer (v 2.08) application. Integration is performed on the unsmoothed data shown in the figure on peaks ranging in height between 55 mAU (peak 11) and 3 mAU (peak 8).

Figure 2A shows these 17 peaks which are used for the study, while 2B shows the runs which are analyzed for repeatability. The mean and standard deviation of the retention times, together with the relative standard deviation (RSD) are reported in Table 3. Standard deviations range from 0.06–0.11 minutes (3.6–6.6 sec) corresponding to relative standard deviations of 0.16–0.37%. As can be seen, the earliest eluting peptides show retention time relative standard deviations well below 0.5%. As the retention times increase this value decreases further with times below 0.2% for the later eluting peptides. Low retention time variations like those shown here demonstrate the high repeatability afforded by microfluidic flow control.

In addition to highly repeatable retention times, the peak shape remains constant throughout the chromatograms. As an example of this, consider the shoulder on peak 2 (Figure 2B). This shoulder remains pronounced throughout the series of data shown here, a demonstration of precision flow control, as even small variations in the fluid composition are enough to affect this feature.

Figure 2. NanoLC/UV Data

A close up of a single cytochrome c digest chromatogram is shown in A, together with arrows indicating which peaks are used for evaluating the relative standard deviation of the retention times. The six runs used for the total evaluation are shown in B.



For the nanoLC/MS work eight replicate injections of BSA digest are performed. The base peak chromatograms are shown here. That is, the intensity of the major peak in the mass spectrum at each time point is shown for every time point in the chromatogram.

Figure 3A shows (on the following page) the 14 peaks (73 peptides with ≥ 2 residues are expected) which are well enough resolved to integrate accurately while Figure 3B shows the balance of the data taken. Full scan MS spectra are acquired every 1.3 sec, while integration is performed using XCalibur™ 2.0 with a 5 point smoothing window.

The standard deviation of the retention times ranged from 0.07–0.17 min (4.2–10.2 sec) corresponding to relative standard deviations between 0.26–0.45% for peaks across the entire range of the chromatogram.

The precision gradient control is illustrated by the repeatable separation of the 2 peaks circled in Figure 3A. This feature remains split throughout the eight runs. Given that the slope of the gradient during the run is 0.5% B/minute and the fact that the peaks elute within 0.3 minutes of each other, being able to clearly see both peaks throughout all of the runs demonstrates flow accuracy better than 0.15%B. To put this number in perspective consider that this data is measured running at 300 nL/min and that these peaks are eluting at ca. 10% B, which corresponds to 30 nL/min of flow on the B side of the gradient. Then 0.15% flow accuracy requires that the system be accurate to within 450 pL/min, which can be readily achieved.

Table 3. LC/UV Retention Time Variation

table 3.

peak	mean, min	stand. dev, min	RSD, %
1	26.30	0.10	0.37
2	26.80	0.09	0.34
3	28.34	0.09	0.33
4	29.31	0.10	0.32
5	29.75	0.10	0.33
6	30.07	0.10	0.34
7	30.55	0.11	0.35
8	31.70	0.09	0.30
9	33.24	0.09	0.28
10	33.49	0.10	0.28
11	34.73	0.08	0.24
12	35.62	0.09	0.24
13	36.99	0.08	0.21
14	37.33	0.08	0.21
15	40.54	0.07	0.16
16	41.00	0.06	0.16
17	42.44	0.07	0.16

conclusions

The Eksigent NanoLC system effectively uses microfluidic flow control to produce highly repeatable chromatograms in both nanoLC/UV and nanoLC/MS applications.

For the nanoLC/UV runs the average RSD for the 17 peaks studied is 0.27%, while the value for the 14 peaks in nanoLC/MS runs is 0.35%. While both of these values are substantially lower than the specification of 0.5% on the instrument they are somewhat different. The most likely reason for this is the fact that in the nanoLC/UV case the column is placed in a temperature controlled oven, while for nanoLC/MS the column is at ambient.

It is shown here that relative standard deviations can be kept below 0.5% throughout the runs even while doing shallow gradients that demand flow rate accuracy of better than 500 pL/min.

Figure 3. NanoLC/MS Data

A close up of a single BSA digest base peak chromatogram is shown in A, together with arrows indicating which peaks are used for evaluating the relative standard deviation of the retention times. The eight runs used for the total evaluation are shown in B. A feature referenced in the text is circled.

figure 3A.

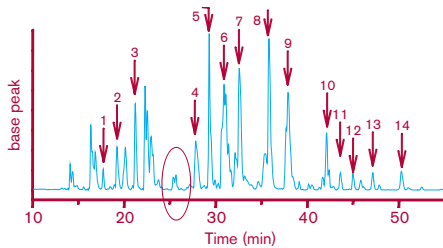


figure 3B.

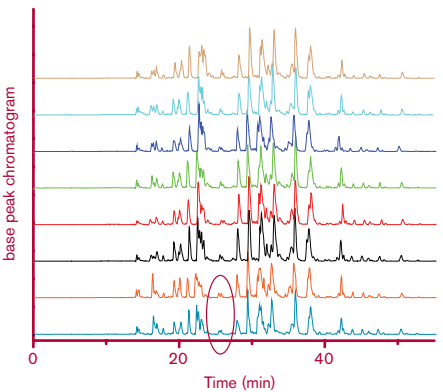


Table 4. LC/MS Retention Time Variation

table 4.

peak	mean, min	stand. dev, min	RSD, %
1	17.62	0.08	0.45
2	19.13	0.07	0.37
3	21.13	0.08	0.38
4	27.92	0.10	0.37
5	29.36	0.10	0.34
6	31.04	0.10	0.33
7	32.68	0.14	0.43
8	35.72	0.09	0.26
9	37.78	0.12	0.33
10	42.04	0.13	0.31
11	43.61	0.17	0.38
12	45.02	0.14	0.32
13	47.29	0.16	0.34
14	50.30	0.15	0.29



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