High Throughput and Precision Nano-LC-MS Using an Automated Heated Dual Column Nanospray Source

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Overview

NanoLC-MS is the technique of choice for clinical proteomics because of its high sensitivity and data content. Common experimental aberrations in nanospray-MS such as loss of spray, sputtering, and emitter degradation, etc. during gradient-LC severely impact the technique's capability to obtain reliable and reproducible results from precious clinical samples. Constant monitoring of and manual intervention during a nanoLC-MS run have become standard practice. Moreover the time required to completely flush out the sample in the biological matrix and the long column equilibration time make throughput undesirably low. We present an automated nanoLC-MS source, the µAutoNano LC, that enables reliable, reproducible and completely unattended operation at double the throughput. The source accommodates two nanoLC columns: while a gradient LC-MS experiment is performed in column A, column B is equilibrating and after equilibration, loading sample off-axis from the mass spectrometer inlet to prevent the dirty matrix from contaminating the MS. Before each column begins the LC run, the spray emitter undergoes a short series of spray emitter conditioning which includes motion wiping and a nitrogen purge to ensure an optimized spray during the experiment. The results presented are on the dual-column separation of a 63-synthetic human peptide mixture with and without a human plasma matrix.

Experimental

Dual Column Method: Each column end is placed 13 mm apart on a column holder that introduces nitrogen sheath to either purge the emitter of excess eluent before analysis or as a nebulizing gas for higher flow rate applications. Motors and software control the positioning of the column before the mass spectrometer. While one column sprays before the mass spectrometer, a second column equilibrates with high aqueous mobile phase without high voltage present. The sample is then loaded onto the equilibrated column followed by a wash of the sample to remove non-retained matrix components. When the gradient analysis of the first column is complete, a N_2 purge is applied to the second column followed by a placement of the column before the mass spectrometer inlet. Movement of the column toward and back of the MS inlet removes any remaining eluent from the equilibrated column thus establishing steady spray conditions for the next analysis. This process is continuous alternating between the two columns. Each column is heated to 50ºC to increase the separation of the peptides as well as provide uniform temperatures for long, overnight analyses.

LC Method: A dual binary splitless nanoflow pump (MicroTech Scientific XtremeSimple) was run with one binary pump delivering the gradient for elution and the second binary pump isocratic for column equilibration, sample loading and wash. The gradient and isocratic systems switched between columns using an electrically-actuated six-port valve. The gradient system used 99.8 % H_2O / 0.2% formic acid in reservoir A and 99.8 % ACN / 0.2% formic acid in reservoir B. The gradient: 0-2 min. 95% A; 2-12 min. 95-80% A; 12-42 min. 80-58% A; 42-52 min. 58-30% A; 52-62 min. 30-95% A; 62-82 min. 95% A. The isocratic pump used the same reservoirs pumping a weak mobile phase of 95% A / 5% B. The injection volume of 2 µL was calculated from a partial loop injection of 7.4 min. at a flow rate of 270 nL/min. The injection was made 64.6 minutes into the isocratic equilibration. After the injection, an additional 10 minutes of isocratic flow provided the wash of the sample of non-retained matrix components.

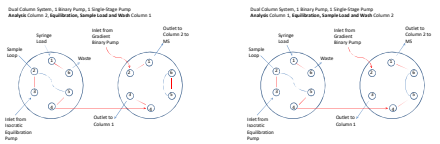

Figure 1: Two 75 µm I.D. capillaries were packed with 5 µm Thermo Hypersil Gold C18 particles, one to a depth of 27 cm, the second to a depth of 31 cm. The column ends were tapered by etching and used to spray directly to the mass spectrometer. The columns were placed 13 mm apart on an automated motorized axis that alternates the positions of the columns on-axis to the MS inlet.

Samples: A 63-synthetic peptide standard mixture at 80 fmole/µL (5% ACN / 95% H_2O) was used for all experiments. For spiked plasma experiments, 50 µL of the peptide std mix was evaporated to dryness under nitrogen and reconstituted with 50 µL plasma (40 µg plasma/ 500 µL 99.5/0.5 H_2O / ACN) to make a 80 fmole/µL Peptide Std mix in 80 ng/µL plasma. (The human plasma was initially passed through a C18 SPE cartridge to eliminate particulate and very high molecular weight proteins.)

Nine peptides were tracked for reproducibility and separation. Only one of the nine was not listed on the table provided by the vendor. This mass, $_{m/z}$ 1095, was included as it was one of the most intense ions in the sample analysis.

Mass Spectrometer: Thermo-Scientific LCQ Advantage. Full scan 395 – 1500 amu. N_2 was supplied to the source from the MS at 15 psi.

Sequence	Mass	[MH+2]2+
LSITGTYDLK[HeavyK]	1118	559.8
SKEQLTPLIK[HeavyK]	1164	582.9
LKEFGNTLEDK[HeavyK]	1301	651.3
GAYPLSIEPIGVR[HeavyR]	1381	691.4
GFGGLTGQIVAALSTAK[HeavyK]	1599	800.0
QLSLPETGELDSATLK[HeavyK]	1710	855.5
IAPQLSTEELVSLGEK[HeavyK]	1722	861.5
IFFYDSENPPASEVLR[HeavyR]	1894	947.5

Table 1: Peptides monitored in experiments

Improved Resolution using Nano-Column Heaters

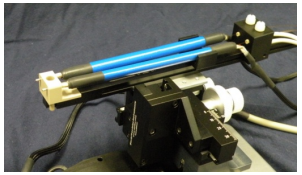

Figure 2: µAutoNano LC Source with PST Column Heaters

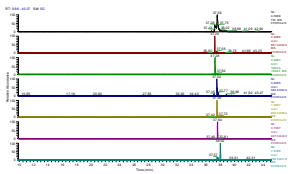

Figure 3: Column A at Room Temperature

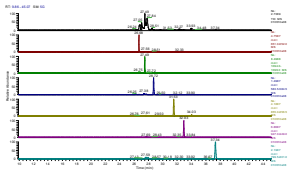

Figure 4: Column A at 50ºC

Nano-Column Heating for Improved Separation: 25 cm PST nano-column heaters were used for all experiments presented in this poster. The heaters are dynamically controlled by thermocouple feedback. The experiments in this paper were run at 50ºC using the same LC gradient. Figure 3 on the left shows the analysis of the 63-peptide mix with no column heat applied to Column A. This analysis shows little to no separation of the selected peptides. Figure 4, bottom left, shows the same analysis but at 50ºC. All the selected peptides, which closely eluted at room temperature, are well separated at 50ºC. Column heating also guarantees stable column temperatures for lengthy, overnight analyses.

Robustness: Unattended Dual Column Performance

Multiple, sequential overnight experiments were performed to demonstrate the robustness of the dual column approach. In each set of experiments, no spray failures occurred both in initiating spray after off-axis equilibration , sample loading and wash as well as during the gradient analysis.

Peptide Standard Runs: **9.6 hours**

Peptide Standard in Plasma Runs: **15 hours**

Peptide Standard 155 min analysis: **18 hours**

Peptide Standard 155 min analysis, RT: **15.5 hours**

Peptide Standard Comparison Room Temperature vs. 50ºC: **18.4 hours**

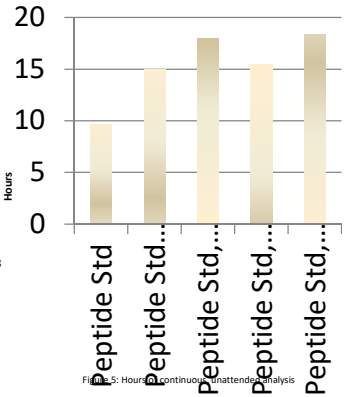

Figure 5: Hours of continuous unattended analysis

Precision: Retention Time Reproducibility

Obtaining acceptable retention time precision by nanoLC-MS can be a challenge due, in part, to the long equilibration times required to ensure consistent performance. Although 5 to 20 column volumes of equilibration can be readily achieved, adequate flushing of the pump tubing, mixer, injection valve, and transfer tubing require total equilibration times that can sometimes exceed the actual analysis time. By implementing the dual column approach, equilibration of a column occurs off-axis as a second column undergoes a gradient analysis. This approach allows adequate time for column equilibration while improving sample throughput.

The ion traces below are of the same peptide on four different analyses of the same column. The retention time RSD of the four runs is 0.30%. The experiment was a dual column experiment, thus analyses were performed alternating between the two columns.

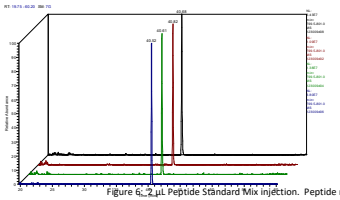

Figure 6: 2 µL Peptide Standard Mix injection. Peptide m/z 800 ion trace

The retention time results of a dual column experiment are reported in Table 2. These results are from six injections, three on each column. Nine peptides of the standard mixture are reported.

	Column A								
	m/z 651	m/z 1094	m/z 583	m/z 560	m/z 855	m/z 861	m/z 947	m/z 691	m/z 800
	31.49	31.75	32.73	32.7	33.39	34.17	35.03	34.7	40.14
	31.25	31.56	32.76	32.73	33.27	34.06	34.75	34.67	41.56
	31.12	31.4	32.7	32.68	33.38	34.28	35.24	34.91	41.93
AVE	31.29	31.57	32.73	32.70	33.35	34.17	35.01	34.76	41.21
SD	0.15	0.14	0.02	0.02	0.05	0.09	0.20	0.11	0.77
RSD	0.49	0.45	0.07	0.06	0.16	0.26	0.57	0.31	1.87
	Column B								
	m/z 651	m/z 1094	m/z 583	m/z 560	m/z 855	m/z 861	m/z 947	m/z 691	m/z 800
	18.68	20.66	23.19	23.47	30.34	32.31	33.13	32.4	40.82
	19.19	20.25	22.95	22.98	29.63	32.34	33.72	32.34	40.63
	18.35	19.9	22.68	22.77	29.39	31.54	32.73	31.65	40.52
AVE	18.74	20.27	22.94	23.07	29.79	32.06	33.19	32.13	40.66
SD	0.35	0.31	0.21	0.29	0.40	0.37	0.41	0.34	0.12
RSD	1.84	1.53	0.91	1.27	1.35	1.15	1.23	1.06	0.30

Table 2: Retention Time Data for Peptide Standard Mix, Columns A and B

Column A shows ~0.5% or better RSDs for all but one peptide. For Column B, the RSDs were ~1.5% or better for all but one peptide. Differences in retention times between the two columns can be attributed to the different column lengths. Column A had a bed length of 31 cm while Column B's bed length was 27 cm.

Table 3 reports retention times for 8 injections, 4 on each column, of the peptide standard mix in human plasma at a 1:1 fmole/ng ratio. The off-axis sample loading and wash is designed to remove non-retained matrix components from the column before analysis to the MS. Slight changes in retention time from Table 2 (peptide standard mix neat) are observed with greater, but acceptable RSDs. Theses RSDs are exceptional as the presence of plasma on the column alters the elution times of the peptides.

Column A									
	m/z 651	m/z 1094	m/z 583	m/z 560	m/z 855	m/z 861	m/z 947	m/z 691	m/z 800
	30.43	30.77	32.98	33.02	35.12	37.74	39.52	38.32	41.56
	29.77	30.28	32.31	32.06	34.87	38.13	39.15	38.32	41.93
	29.5	30.23	32.06	31.87	33.95	37.54	38.09	37.68	42.15
	29.25	29.61	32.6	32.69	35.34	37.09	37.62	37.2	43.2
AVE	29.74	30.22	32.49	32.41	34.82	37.63	38.60	37.88	42.21
SD	0.44	0.41	0.34	0.46	0.53	0.37	0.77	0.47	0.61
RSD	1.48	1.36	1.05	1.43	1.52	1.00	1.99	1.24	1.44
Column B									
	m/z 651	m/z 1094	m/z 583	m/z 560	m/z 855	m/z 861	m/z 947	m/z 691	m/z 800
	17.26	18.84	21.8	22.23	28.52	32.25	33.51	32.17	41.29
	16.4	18.78	21.41	21.59	27.55	30.77	32.32	30.73	40.53
	16.45	18.78	21.35	21.69	27.52	30.94	32.25	30.85	40.23
	17.06	20.57	22.73	22.86	29.5	31.38	32.06	31.4	39.89
AVE	16.79	19.24	21.82	22.09	28.27	31.34	32.54	31.29	40.49
SD	0.37	0.77	0.55	0.51	0.81	0.57	0.57	0.57	0.52
RSD	2.23	3.99	2.53	2.29	2.88	1.83	1.75	1.82	1.28

Table 3: Retention Time Data for Peptide Standard Mix in Plasma, Columns A and B

Off-Axis Plasma Sample Load and Wash

The dual column configuration is uniquely designed to maximize throughput while providing options for sample loading and wash. In the experiments presented in this paper, sample was loaded onto the column undergoing mobile phase equilibration ~18 minutes before analysis. This approach provided a 10 minute wash of the column with starting mobile phase after a 2µL injection, all performed off-axis (away from the MS inlet) without high voltage. When the µAutoNano LC received a signal from the MS to switch columns, a N_2 purge of the off-axis column was applied. This was followed by positioning the column emitter in front of the MS inlet, applying high voltage using TipGuard™ technology, and moving the emitter along the Z-axis (forward and back to the inlet). This motion increases the field strength and assists in initialing spray.

The standard mix in plasma chromatographic results are shown in Figure 7. The neat standard mix chromatogram is displayed in Figure 8. Both chromatograms show similar results for the selected ion traces with slight shifts in retention times, common for analyses of samples in plasma. The total ion chromatograms (TIC) are also similar with more peaks present in the plasma sample chromatogram, plasma components retained by the column and eluted during the gradient analysis.

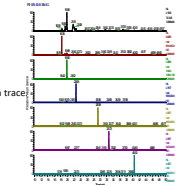

Figure 7: 2 µL Peptide Standard Mix Neat, 160 fmole inj.

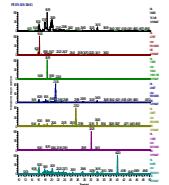

Figure 7: 2 µL Peptide Standard Mix in Plasma, 160 fmole /:160ng

Throughput Increase with Dual Columns

The dual column approach is designed to maximize throughput for nanospray applications. In the standard mix in plasma experiment, the experiment would have taken over 26 hours using a single column method. The experiment using the dual column approach took 15 hours, an ~75% increase in throughput. A 100% throughput increase is possible using a dual binary gradient LC system rather than the single binary, single isocratic LC systems used for these experiments. The additional increase of throughput is derived from the fact that the additional 20 minutes of equilibration for the gradient pump to reach starting mobile phase conditions before switching columns would not be needed.

Summary and Discussion

A dual column nanospray source has been shown to provide high throughput, precision, and robustness in overnight and unattended analyses. By utilizing gradient and isocratic LC systems, sample throughput was demonstrated to increase ~75% over a single column method. In addition, 100% increase in throughput is possible with two gradient LC systems. The off-axis sample loading and wash not only increased the throughput, but also allowed the washing of non-retained matrix components away from the MS inlet. This resulted in consistent MS performance for 15 hours of spiked plasma injections. Retention time precision of ~0.5% for one column of the neat peptide standard mix demonstrates the power of a dual column system allowing the necessary, lengthy equilibration times while actually increasing throughput. The use of nano-column heaters at 50ºC provided consistent temperatures for overnight analyses. More importantly, they provided separation of peptides where there was little to no separation at room temperature. With the implementation of a N_2 purge, TipGuard HV, and Z-axis motion, spray was successfully initialized and maintained over 76 hours of analysis.

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