Single Source Solution for Low Flow Chromatography

OptiFlow® Turbo V™ Source – Always Working in the Sweet Spot of Sensitivity and Robustness

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When performing electrospray ionization mass spectrometry, it is well understood that reducing chromatographic flow rates can improve sampling efficiency and therefore increase sensitivity.[1] When operating in the nanoflow regime (50-500 nL/min) very high sampling efficiencies have been measured for favorable solvent conditions.[2] This is particularly beneficial when sample amounts are limited, such as in proteomics applications, where nanoflow LC-MS is frequently used. One drawback to this approach is that typical run times are quite long; often 1-2 hour gradients are used to achieve optimal separations.

In other application areas, throughput and robustness are more important, such as with pharma bioanalysis studies. For the analysis of 100s of samples daily, analytical flow rates of 300-1000 µL/min are typically combined with gradients of less than 5-10 minutes.

In recent years, there has been a resurgence in the interest in using microflow LC coupled to mass spectrometry as evolving research demands increasingly require more sensitivity while still maintaining good throughput and robustness.

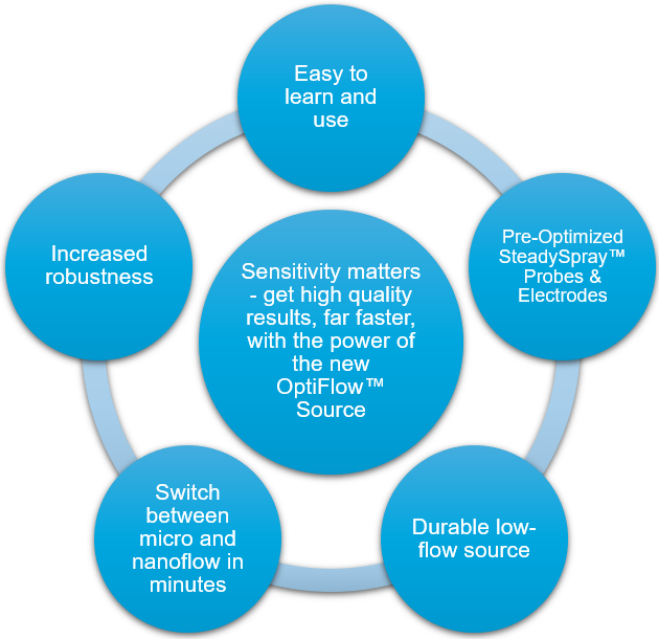

Figure 1. Benefits of the OptiFlow Turbo V Source for Low Flow Chromatography. This single source can be easily switched between nanoflow rates and microflow rates, allowing the researcher to choose the best chromatography for every application.

This led SCIEX to develop the OptiFlow Source, a single source that would cover the full spectrum of low flow rates for high sensitivity LC-MS analysis. Significant research was done to develop a low flow source that was sensitive, while maintaining high robustness and ease of use of higher flow sources. The ability to easily switch between flow regimes was also critical in the source design. Removing the barrier of switching helps researchers choose the right flow rate for every project.

Key Features of the OptiFlow Turbo V Source for Low Flow Chromatography

- Available on the QTRAP® 6500+, 6500 and 5500 systems as well as the TripleTOF® 6600+ System
- SteadySpray™ Probes and Electrodes are pre-optimized for consistent performance
  - Easy to use with no positional tuning required
  - Electrodes designed for robustness
- Switch between flow regimes in minutes, enabling the researcher to use the optimal flow regime for every project (Table 1)
  - Supports microflow rates of 1 – 200 µL/min
  - Supports nanoflow rates of 100 – 1000 nL/min

Methods

Sample Preparation: SWATH® Acquisition Performance Kit[3] was used for the nanoflow comparison experiments. Signature peptide from Kadcyla (FTISADTSK) was used for the microflow robustness experiments. A small molecule mixture was used for the microflow vs analytical flow sensitivity testing as well as the polarity switching experiments.

Chromatography: The nanoLC testing was performed using the NanoLC™ 425 system with either the nano flow module or the low micro flow module in trap-elute mode. The microflow testing was performed using the M5 MicroLC system.

Mass Spectrometry: Extensive source performance testing was performed on both the QTRAP 6500+ system and the TripleTOF 6600+ system equipped with the OptiFlow™ Interface.

Data Processing: Targeted quantitation data was processed using MultiQuant™ Software. SWATH Acquisition data was processed using SWATH acquisition 2.0 MicroApp in PeakView® Software. Data dependent acquisition data was processed using ProteinPilot™ Software.

Table 1. SteadySpray Probes and Electrodes for OptiFlow Source.

Flow Rate	Electrode ID	Probe
100 – 1000 nL/min	*20 µm*	*SteadySpray Nano*
1 – 10 µL/min	*25 µm*	*SteadySpray Micro*
10 – 50 µL/min	*50 µm*	*SteadySpray Micro*
50 – 200 µL/min	*50 µm*	*SteadySpray Analytical*

Optimization of Source Design

The OptiFlow Source is the next evolution in the Turbo V Source design. The heater design was improved the Ion Drive™ Turbo V source, producing a larger “sweet spot” for ionization. For compatibility with the nanoflow interface, the orientation of the heaters in the OptiFlow source were further optimized.

Design of Experiments (DOE) work was performed to explore the range of tuning options to find the optimal settings across various flow regimes including the nanoflow regime and low and high microflow regimes. Key parameters explored during the DOE investigations were sprayer positioning, sprayer tip protrusion, nebulizer gas flows and electrospray potential. An example of this type of investigation for the nanoflow sprayer is shown in Figure 2. In this example, a wide range of sprayer positions relative to the center of the orifice aperture were tested at a range of different ionization potentials, measuring analyte sensitivity. From the shape of the surface, the optimal setting can be determined as well as the size of the sweet spot for optimization.

This detailed characterization data showed that many of the traditionally user tunable source settings could be predetermined and fixed.

This led to the development of the SteadySpray Electrodes and Probes. The user can select the correct set for the flow regime of interest (Table 1). The insertion of the electrode into the probe will produce the correct protrusion of the spray tip for optimal nebulization and signal (Figure 3 and 6). Then the probe is inserted into the source port and once tightened, the electrode position is secured in the optimal location. The standard settings provided allow the user to set the source parameters and begin, making the source ready to spray in minutes.

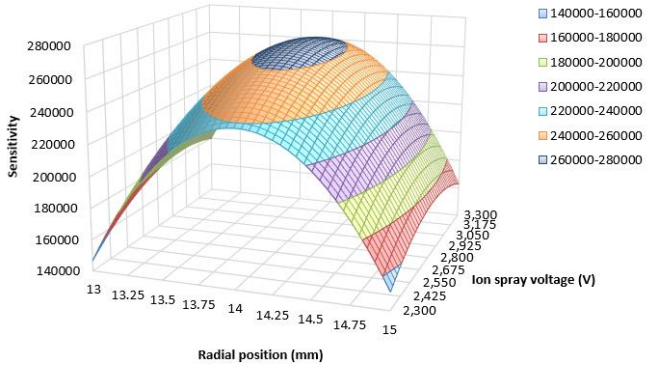

Figure 2. Design of Experiments For Optimization of Source Design. During DOE, a wide range of experimental parameters are explored and evaluated efficiently. In this nanoflow example, both radial position and ionization voltage were varied over a functional range. The shape of the 3D surface shows that the radial positioning has a large effect on sensitivity and therefore must be positioned with tight tolerances. The ionization voltage shows a broad optimum and therefore it is not necessary to undergo extensive optimization. These data on all key parameters allowed for design of the plug and play source.

Microflow LC-MS Performance

Microflow chromatography is increasingly being used in multiple application areas because of the favorable balance between robustness and sensitivity that it provides. Whether it is moving high flow rate applications down to microflow for enhanced sensitivity, or moving nanoflow applications up in flow rate for increased robustness and throughput, microflow chromatography is compelling for many applications.

However, because of the reduced flow rates relative to high flow rate ESI, it is important to minimize dead volume across the full LC flow path including the source and ionization sprayer. The OptiFlow source has been optimized for flow rates down to 1 µL/min with the short probe and electrode (half the length of current electrodes, Figure 3). Also, mounting the column directly onto the electrode ensures minimal post column dead volume. The metallized tapered tip has also been designed and optimized for flow stability and performance at low flow rates (Figure 3C).

To demonstrate utility of the OptiFlow source for higher throughput applications, a series of robustness experiments were performed. Using a simple peptide mixture, long term spray robustness was investigated using a targeted MRM experiment on the QTRAP 6500+ system (Figure 4). Over 1150 injections, very good spray stability and therefore low peak area ratio variance was observed (3.6% CV).

Small molecule applications often require use of both instrument polarities due to the diversity in molecular structure. To ensure negative mode performance, the most challenging type of experiment was used for testing: polarity switching experiments were conducted with MRM analysis on the QTRAP 6500+ system (Table 2). Using a mixture of 11 analytes, peak areas across 1050 injections were monitored with very good reproducibility.

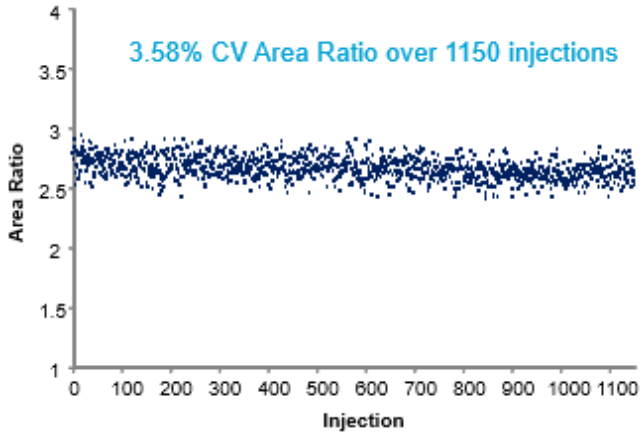

Figure 4. Source Robustness Testing in Positive Mode. Here, the signature peptide (FTISADTSK) from an IgG was monitored by MRM on a QTRAP 6500+ system using a trap elute workflow and a flow rate of 3 µL/min. Monitoring the peak area relative to a stable isotope labeled peptide, very good peak area ratios across 1150 injections were observed.

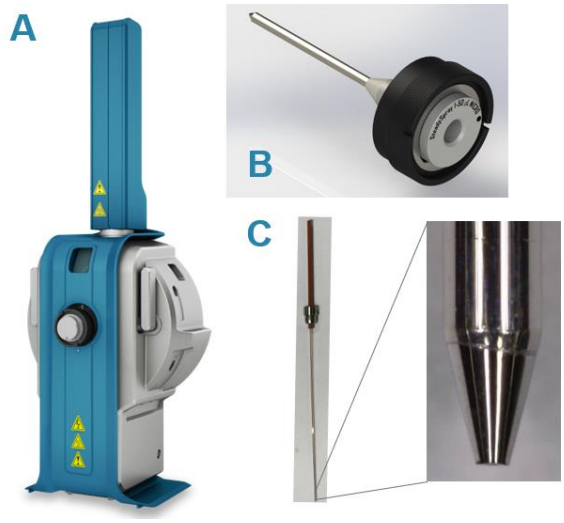

Figure 3. OptiFlow Source Configuration for Microflow LC-MS. A) Column and column heater install directly on top of the orthogonal probe and electrode for minimized post column dispersion. B) Electrode installs easily in probe and C) unique electrode design ensures robust performance.

Sensitivity comparisons were also performed between analytical and microflow ESI to demonstrate the gains in observed peak areas and S/N (Table 3 and Figure 5) resulting from decreasing the solvent flow rate.

Table 2. Long Term Robustness of OptiFlow Source and Electrodes. As source robustness in both polarities is key for small molecule research, a long-term robustness test was performed on the QTRAP 6500+ system using polarity switching. An MRM method with 11 analytes was monitored, 7 in positive mode and 4 in negative mode. 1050 injections were performed which translates to 437.5 hours (18.2 days) of acquisition using a 14min gradient (20 min total run time). Good peak area reproducibility was observed across the experiment, with the average %CV across compounds of ~6%.

Compound	Polarity	%CV over 1050 Injections
Methadone	*Positive*	*4.24*
Methadone – d9	*Positive*	*4.16*
Tapentadol	*Positive*	*3.6*
Tapentadol – d3	*Positive*	*3.74*
Haloperidol	*Positive*	*5.52*
Midazolam	*Positive*	*8.66*
Midazolam – d4	*Positive*	*8.84*
2,4-D	*Negative*	*7.26*
Bentazon	*Negative*	*4.34*
Bromoxynil	*Negative*	*6.69*
Fluorescein	*Negative*	*9.17*

Table 3. Comparing Sensitivity for Small Molecules Between Microflow and Analytical Flow Rates. Quantitation curves on the QTRAP 6500+ system were generated using both high flow (500 µL/min and microflow (3 µL/min) using 7 compounds in neat solution. Area and S/N gains were observed for all compounds across the concentration range analyzed when using microflow LC vs analytical flow LC.

Compound	Average Area Gain with Microflow vs Analytical Flow	Average Signal to Noise Gain
Buprenorphine	*6.7*	*13.9*
Propranolol	*6.1*	*13.4*
Alprazolam	*5.3*	*33.5*
Dextromethorphan	*5.2*	*10.7*
Busprione	*5.0*	*8.4*
Haoloperidol	*4.8*	*9.0*

Nanoflow LC-MS Performance

Very low flow chromatography can be challenging because of the narrow bore tubing and columns that are required, making clogging of columns and spray tips a constant worry. Extensive work was done to develop the electrospray electrodes for the OptiFlow Source (Figure 6). In the final design, the electrodes have 20 µm inner diameter and the tip is ground on the outside of the electrode to provide a taper. A benefit of this innovative design is that there is no internal constriction, reducing the chances of electrode clogging. Nanoflow LC-MS runs on many different sample types were performed over the course of 30 days and the system pressure showed minimal change across the runs showing that no clogging of the electrode occurred (Figure 7).

Figure 6. OptiFlow Source Configuration for Nanoflow LC-MS. Nanoflow probe and electrode are installed in the port on the front of the source and the microflow port is closed with the plug. The nanoflow column heater mounts on the front of the source and cartridge based nanoflow columns from Phenomenex can be inserted easily. (Right top) The nanoflow probe is pre-configured so the probe/electrode mount the same with the same protrusion every time. Nebulizing gas flows coaxial to the electrode. (Right bottom) Nano electrodes are designed for robustness, produced by grinding the outside of tip to produce a taper. This ensures no internal constriction at the end and reduced chance of plugging compared to a pulled tip design.

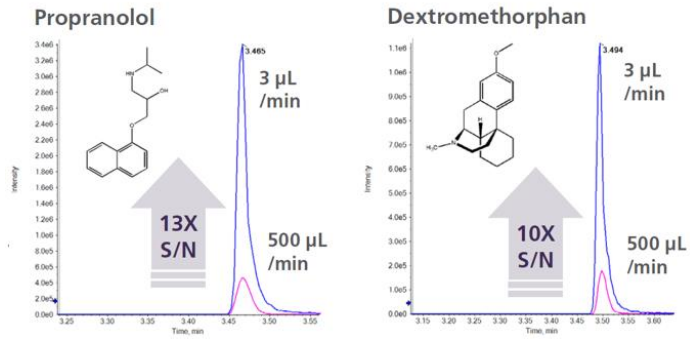

Figure 5. Small Molecule Sensitivity Testing. Example chromatograms are shown for two of the compounds in the experiment described in Table 3. Solid gains in S/N were observed for microflow (blue trace) vs analytical flow (pink trace) providing improved lower limits of quantitation.

As with microflow, significant optimization was performed during design testing with the nanoflow probe and electrode for the OptiFlow Source to determine the optimal spray tip positioning and protrusion. The user inserts the precut electrode into the liquid junction union that inserts into the SteadySpray Nano probe and screws it into place on the front of the source (Figure 6). This automatically positions the sprayer in the center of the heated inlet for optimal performance. Significant testing with different sources and probes on multiple instruments confirmed that the performance was equivalent to a highly optimized nanoflow source.

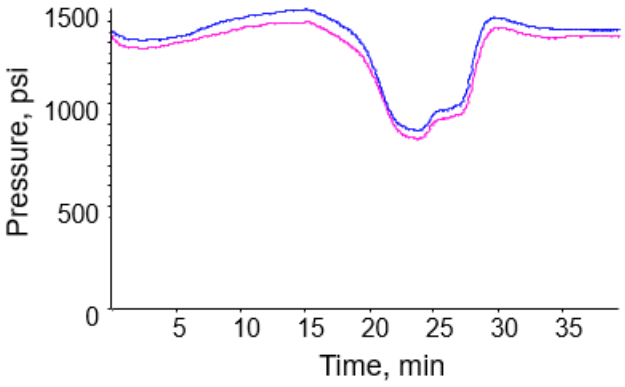

Figure 7. Long Term Nanoflow Electrode Robustness. Proteomics experiments using nanoflow LC-MS were performed using a single electrode over the course of 30 days. The system pressure was monitored for the QC runs and minimal pressure differences were observed, confirming no clogging of electrode.

The OptiFlow Source was compared to the NanoSpray® III Source to evaluate the performance in typical proteomics workflows using a TripleTOF 6600+ system. OptiFlow Interface was also used, to allow easy switching to nanoflow[2]. Manual source tuning was performed for position and settings on the NanoSpray source to obtain the best signal. On the OptiFlow source with the preset probes and electrodes, no positional tuning was required, and the standard settings were used with no additional optimization.

First, the chromatographic quality was assessed, to ensure that the peak width and peak shape were equivalent or better on the OptiFlow Source as compared to the NanoSpray III source (Figure 8). The peaks were very similar between the two sources across all peptides, as well as the peak widths and tailing factors, indicating similar high quality chromatographic performance can be achieved on the OptiFlow source with no tuning.

Replicate DDA experiments were run on a single TripleTOF 6600+ system and 3 different sources were used for comparison, a NanoSpray Source, and two different OptiFlow sources (Table 4). Very similar protein identification results were obtained with deviations ~2% across the sources.

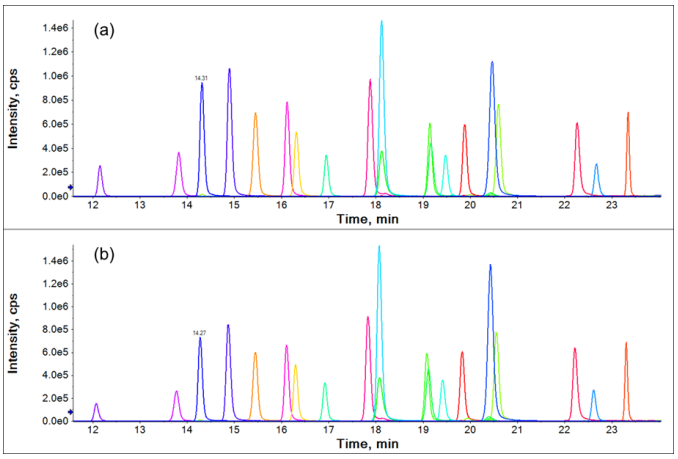

Figure 8. High Quality Chromatography. Chromatograms for the PepCalMix run at 300 nL/min using the A) OptiFlow source with the nanoflow probe and electrode with no optimization and the B) NanoSpray III Source with full optimization. Very similar LC profiles are observed.

A similar experiment was performed for SWATH acquisition, comparing the 3 sources on a single instrument (Table 4, bottom), Again, very similar results were obtained across the 3 sources, with deviations less than ±5%. These results demonstrate that the plug and play nanoflow probes and electrodes provide optimized performance.

Table 4. Proteomics Results Comparing Performance between OptiFlow Source in Nano Mode and the NanoSpray III Source. Using the same nanoLC 425 and TripleTOF 6600+ system, 3 sources were used to collect back-to-back data for comparison. Data dependent experiments were performed, and very similar protein and peptide identification numbers were obtained from ProteinPilot software searches. Also, very similar protein and peptide quantitation numbers were found between the sources for the SWATH Acquisition data, filtered at <1% FDR and <20% CV across 5 replicates.

		NanoSpray Source	OptiFlow Source in Nano Mode	OptiFlow Source in Nano Mode	% Difference from NanoSpray Source	% Difference from NanoSpray Source
			1	2	1	2
Protein Identification Results						
Avg # Peptides across 5 reps	*<1% FDR*	*31791*	*31069*	*31170*	*-2.3*	*-2.0*
	%CV	*1.4*	*1.1*	*0.8*		
Avg # Proteins across 5 reps	*<1% FDR*	*3811*	*3747*	*3755*	*-1.7*	*-1.5*
	%CV	*1.0*	*0.6*	*1.1*		
SWATH Acquisition Quantitation Results						
Avg # Peptides		*11543*	*10981*	*12107*	*-4.87*	*4.89*
Avg # Proteins		*3282*	*3173*	*3411*	*-3.32*	*3.93*

Using the SWATH Acquisition Performance Kit.[1]

Conclusions

With the innovations in the OptiFlow Turbo V Source, a new standard for ease of use has been achieved for low flow chromatography.

- The mechanical design of the OptiFlow source was optimized using DOE approaches
- SteadySpray probes and electrodes cover a broad range of flow rates by simply exchanging them for the desired flow rate
  - Switching flow regimes can be done in minutes
  - High performance across the flow rate range with very little need for tuning
- In experimental testing, the OptiFlow Source provides similar sensitivity to fully adjustable ion sources
- Selecting the right flow rate for every project means the LC-MS system is always working in the sweet spot of sensitivity, robustness and throughput

References

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3. SWATH Acquisition Performance Kit Standard Operating Protocol.
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