

Advantages of Multiple Time-Slice Injections in Sensitive, High-throughput LC-MS Analyses

David W. Neyer and Don W. Arnold

Eksigent Technologies, 5875 Arnold Road, Dublin, CA 94568



Introduction

Microflow LC-MS with column diameters ≤ 1 mm is proving to be a valuable approach for high-throughput LC-MS in bioanalytical development, including ADME-Tox measurements. We recently described a chromatography system designed for this application that allows very fast separations (1 to 1.5 minute cycle times). The system can deliver a wide range of injection volumes by using a time-slice injection approach. We will present data showing how multiple, rapid separations can be conducted on a single small sample volume. Such an approach can provide expanded dynamic range and improved quantitation. In addition, since such cycle times are not limited by the overhead of the autosampler, one can gain this additional data in a shorter time.

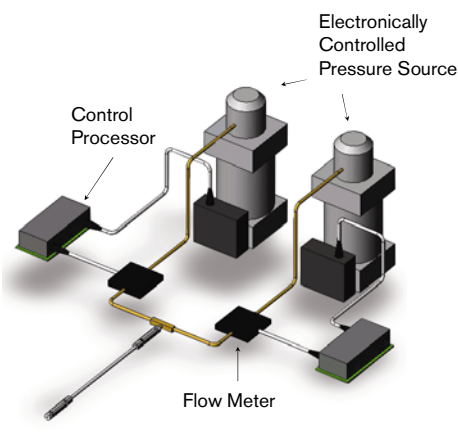
Methods

A microflow UHPLC system designed for high-throughput LC-MS analysis (ExpressHT-Ultra, Eksigent Technologies) is coupled with a triple quadrupole MS (TSQ Quantum Access, Thermo Scientific). An industry standard autosampler is used to overfill an injection loop with a few microliters of sample. By combining accurate flow metering with high speed valve actuation, precise sample volumes are metered onto a column for separation and MS analysis. A range of sample injection volumes from 500 to 9000 nL are employed depending on the experiment. Repeated injections of available drug-like compounds and peptides (Sigma-Aldrich) from the same sample volume are conducted in succession.

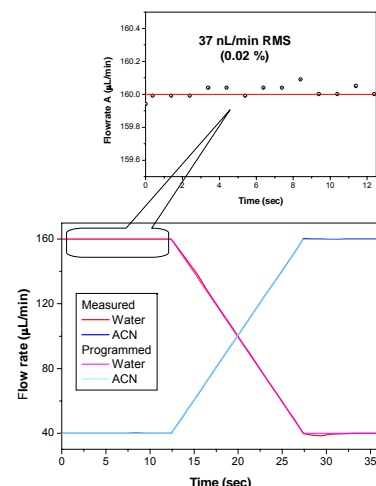
Microfluidic Flow Control for micro UHPLC/MS

The ExpressHT-Ultra solvent delivery system is based on a binary gradient pumping system designed for HPLC columns with diameters of 1 mm or smaller. A benefit of small diameter columns is a reduction of the required mixing volume. Less volume means shorter gradient delays and faster separations. Column and system re-equilibration also take much less time. The result is a system with fast cycle times for high analytical throughput. The entire system is designed to operate at pressures up to 10,000 psi. This allows the use of columns with small diameter stationary phase particles (1.5 to 3 μ m) delivering fast, high resolution separations

Eksigent's Microfluidic Flow Control ensures precise and stable flow rates. With MFC technology, direct pumping is used to deliver accurate, repeatable gradients. Each pressure source delivers fluid with the actual flow rate monitored using the flow modules in each channel. A feedback loop makes real-time adjustments to the flow rate. By continuously monitoring the flow from each of the binary system's pumps, the flow rate can be adjusted many times per second. The result is retention times with an RSD typically below 0.3%.



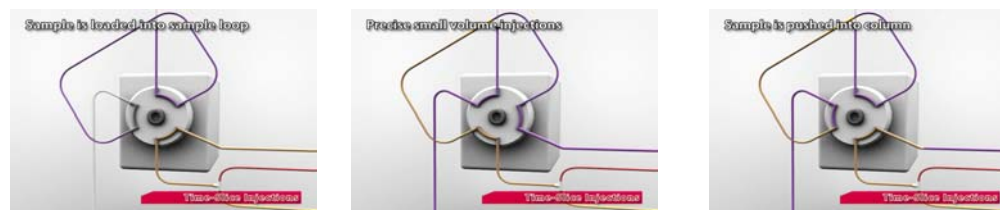
Schematic of the Microfluidic Flow Control (MFC) setup. In-line flow meters provide high speed measurements of the delivered flow rate to a built-in control processor. The processor performs real-time feedback control of the electronically controlled pressure sources to provide accurate flow for microflow LC.



Performance of MFC system for very rapid gradients at high flow rates. Shown in the bottom panel are the programmed and measured flow rates for a 15 second gradient at 200 μ L/min total flow rate. The top panel shows the RMS deviation of the flow rate during the brief isocratic hold before the gradient. Flow precision of better than 0.02% is demonstrated. Data was collected with approximately 8000 psi of back pressure.

Metered injections using time-slicing with accurate flow rates

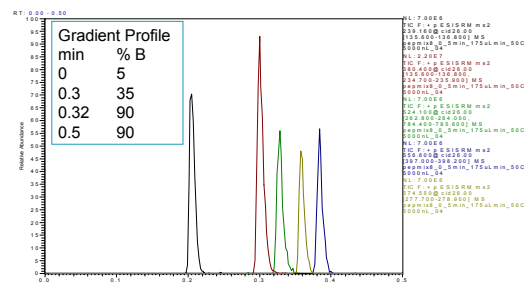
Precise and accurate pump flow control, in combination with a high speed injection valve, allow for programmable metered injections with volumes as low as 100 nL and up to 10's of μ L (depending on loop size). The filled injection loop is switched to the inject position for a pre-determined length of time to displace the programmed amount of sample. By combining a high speed injection valve with accurate flow control, metered injections can produce accurate, reproducible injections over a wide range of volumes while maintaining fast autosampler cycle times. The figures below show the steps of the metered injection process.



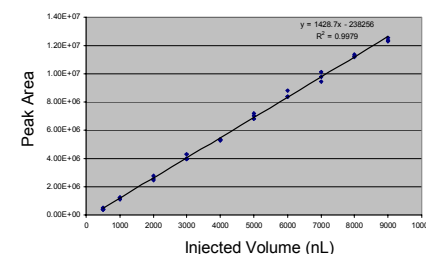
Step 1: Sample is used to overfill the sample injection loop while it is in the load position.

Step 2: The loop is switched into the inject position long enough for a specific volume of sample to be injected onto the column.

Step 3: The loop is switched back to the load position while the separation is conducted. The sample plumbing (including the sample loop) can be washed during the LC separation. Sample can also be retained in the loop for subsequent injections.



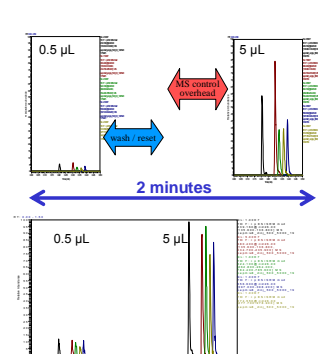
Baseline separation of five peptides in less than 0.5 minutes demonstrating high chromatographic speed. The separation was conducted on a 1 mm x 50 mm Halo 2.7 μ m C18 column at 175 μ L/min. Mobile phases are (A) water and (B) acetonitrile, each with 0.1% formic acid. Gradient is shown in the figure. SRM transitions: Gly-Tyr: 239 > 136; Val-Tyr-Val: 380 > 136 / 235; Methionine Enkephalin: 574.5 > 277; Leucine Enkephalin: 556 > 393; Angiotensin II: 524 > 263 / 785.



Peak areas as a function of injection volume. The injection volume was changed using the metered injection approach described above. Data is shown for Angiotensin II monitored by SRM transition (524 > 263/785). 20 μ L of sample was used to load a 10 μ L sample loop on the injector. Injections from 0.5 to 9 μ L are shown. The peak areas are linear with injection volume over this range with an R^2 value of >0.99.

Increasing speed and reducing sample waste using multiple injections from a single loop fill

Two or more consecutive separations can be conducted from a single filling of the sample loop. This can include repeated analyses using the same injection volume or analysis of multiple injection volumes using the same, small volume sample. Such approaches can provide improved statistics on sample analyses, increased range for quantitation, or even kinetic measurements allowing reduced total sample consumption and improved throughput.



Data and timing for different approaches to obtaining multiple injection volume data (0.5 and 5 μ L injections) from the same sample. Top left shows the standard workflow of separate methods for each analysis controlled from within the MS software (Xcalibur). AS syringe is washed and new sample loaded in between methods. Bottom panel shows data from two consecutive injections from a single filling of the 10 μ L sample loop. Sample and chromatography conditions as described above. By reducing the autosampler overhead needed to wash and refill the sample loop as well as the MS control overhead, the combined separations can be conducted in less than the time needed for two separate analyses. The table at right shows the quantitative accuracy and relative peak areas for repeated analyses of the two injections covering a 10x range of volumes (no IS correction has been applied).

Run #	Peak Area (500 nL injection)	Peak Area (5000 nL injection)	Ratio (5000 nL / 500 nL)
1	582905	5863706	10.06
2	624847	5799001	9.28
3	599693	5983000	9.98
4	533803	6056460	11.35
5	602262	5720834	9.50
6	614081	5392250	8.78
7	634510	5981904	9.43
8	556989	5878267	10.55
9	583587	5951782	10.20
%RSD	5.4%	3.4%	7.7%

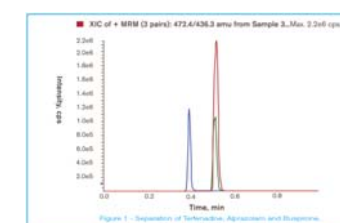
Analysis of nine repeated analyses with the dual injection approach shown at lower left. Data is shown for the Gly-Tyr peptide transition (239 > 136).

Variable injection volumes to extend quantitation in fast LC/MS/MS for bioanalysis

Fast LC/MS/MS using triple quadrupole mass spectrometers is used for many bioanalytical methods monitoring potential drug compounds. MRM monitoring of compounds in biological matrices is conducted in both discovery, preclinical, and clinical evaluation of compounds. Fast separations and cycle times allow for increased throughput to address an increasing sample load.

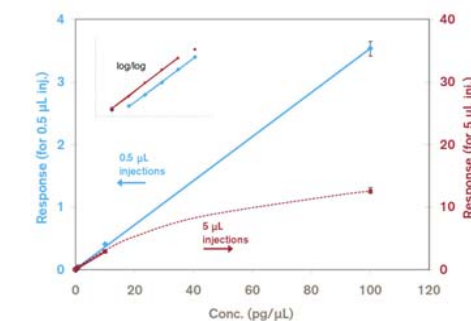
A typical measurement of three different compounds in precipitated rat plasma is shown below. Separations were conducted with a 1 mm x 50 mm column packed with 2.7 μ m diameter HALO particles. Metered injections provide an easy approach for conducting high throughput experiments with a wide range of injection volumes. This feature can be extremely useful in cases where the upper dynamic range of a linear calibration curve is insufficient to cover samples of unexpected high concentration. Instead of diluting the entire series of samples and re-analyzing them, one can simply re-inject the same samples using a smaller injection volume.

The data on the left shows the peak area as a function of injection volume using the metered injection approach. As illustrated at right, by reducing the injected volume from 5 μ L to 0.5 μ L, the upper linear dynamic range is effectively extended by an order of magnitude. There is no need for additional sample dilution steps or exchange of the injection loop.



Analysis of terfenadine, alprazolam, buspirone in rat plasma

50 x 1 mm Halo C18 column
Water/ACN 0.1% FA gradient:
5% to 90% in 0.6 min
Flow rate = 150 μ L/min; 2 μ L injection
API 5000 Triple Quadrupole Mass Spec



Calibration curves for haloperidol in 80/20 water/acetonitrile with verapamil as the internal standard. An ExpressHT-Ultra was used in conjunction with an ABI API 5000 triple quadrupole mass spectrometer. MRM transitions were m/z 376 > 165 and 455 > 165 for haloperidol and verapamil, respectively. The sample concentration range is from 1 fg/ μ L to 100 pg/ μ L, and the internal standard concentration is 3 fg/mL. Inset is a log/log plot of the data showing the linearity over the concentration range.

Conclusions

An ultra high performance micro LC system for LC/MS analysis employing Microfluidic Flow Control and a high speed injection valve has been used to conduct high throughput separations using variable volume, metered injections. This approach allows a broad range of injection volumes to be employed while maintaining the speed and cycle time of a high speed autosampler. In addition, multiple injections from the same loop fill allow even greater utilization of small sample volumes and faster injection to injection times for repeated analyses of the same sample. Metered injections with over 10x range of volumes have been demonstrated using samples extracted from biological matrix (plasma) and provide opportunities for increased quantitation range without additional sample preparation in DMPK and ADME/Tox workflows.