This article focuses on liquid chromatography column miniaturization and explores the many benefits and challenges this analytical tool can bring.

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While mass spectrometry coupled to liquid chromatography has proven to be a powerful tool in the area of discovery Omics (prote-, lipid-, metabol-, food-, and glycomics). Omics research is often challenged by the complex nature of its samples. The identification and quantification of molecules of interest can prove particularly difficult when dealing with small amounts of samples, small volumes, and complex sample matrices (biofluids and single-cell analysis). Liquid chromatography column miniaturization can overcome these challenges. Reducing column inner-diameter (ID) results in a reduction in chromatographic dilution and increased sensitivity allowing for more efficient MS/MS sampling and thus a higher number of molecule identifications.

Though there is no doubt that LC-MS is a powerful tool in discovery Omics, it is also often challenged by the complex nature of its samples. Metabolomic biomarkers tend to be present in extremely low amounts in biological matrices that contain high concentrations of other compounds and contaminants, making these matrices extremely complex[5]. Consequently, the search for metabolomic biomarkers can be hampered when using analytical flow LC-MS (**Table 1**).Analytical LC-MS tends to favor the detection of metabolites already present at a high concentration, this is in part due to the difficult ionization that requires the use of high mass spectrometer source temperatures, voltages,

and nebulization gases. Similarly, lipidomic analysis often use non-volatile salts which exhibit poor desolvation and ionization efficiency.[6,7] Ionization is not the only challenge; sample amount can be a dramatic limiting factor such as in the case of biomedical and discovery proteomics that deal with low amounts of sample such as single cells, biopsies, and tissues that do not provide the required millions of cells or hundreds of micrograms of protein that are required in common proteomics workflows.[1]

Liquid chromatography column miniaturization can overcome these challenges. Reducing column inner-diameter results in a reduction in chromatographic dilution. This dilution happens when a sample amount is injected into the column and it gets diluted by the surrounding solvents. In this case, less solvent means less chromatographic dilution which is the result of using a smaller inner column diameter. Reduction in chromatographic dilution results in an increase in ion sensitivity allowing for more efficient MS/MS sampling and thus a higher number of molecule identifications.[2]

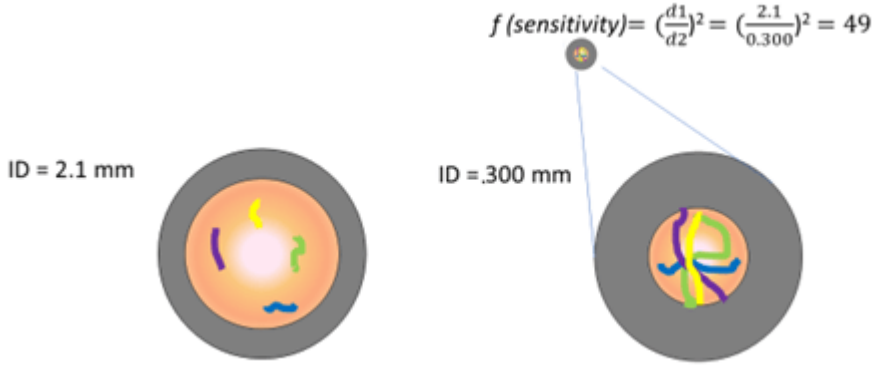

Figure 1. Gain in sensitivity (*f*) resulting from the use of a miniaturized LC column with a smaller internal diameter.

Theoretically, scaling down the column ID from 2.1 mm to 0.300 mm results in a sample concentration of 49 fold which should translate into a 49-fold increase in sensitivity.[8,9] Realistically, this is not always the case, sensitivity is measured by ion intensity which depends on other factors besides sample concentration, such as the ionization nature of the analyte, and the LC-MS equipment (emitter type) and source parameters. Nonetheless, as shown in **Figure 2**, scaling down the column ID from 2.1 to 0.300 mm can result in an increase in sensitivity of almost 10-fold for 50 ng of Oxycodone.

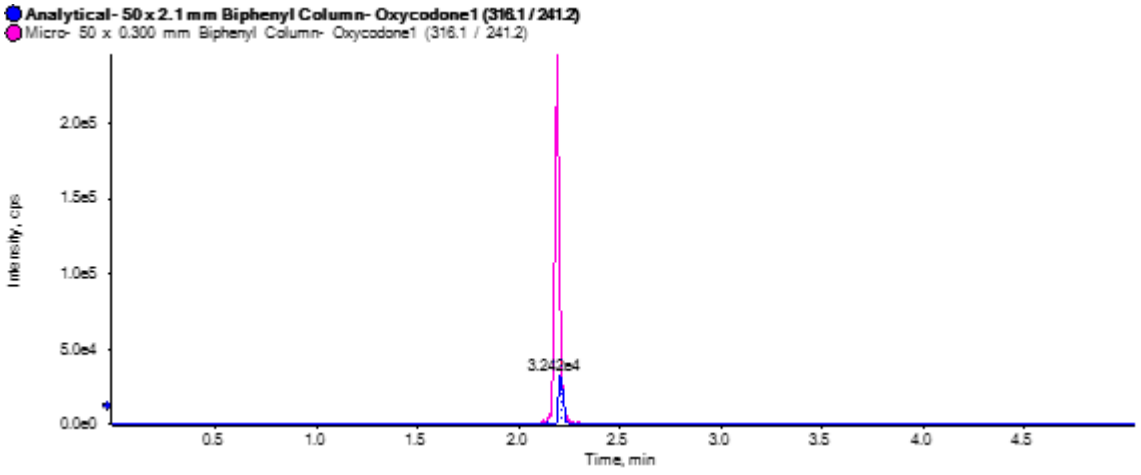

Figure 2. Extracted ion chromatogram of Oxycodone using Analytical flow (blue) and Micro flow (pink).

Furthermore, calculated by the following equation [1] ***f2= f1*** $(^{d2}/_{d1})^2$ *where* ***f= flow rate*** *and* ***d= column ID*** *and* ***f2>f1*** *and* ***d2>d1***, a decrease in column inner diameter also requires a decrease in flow rate to maintain the same (or similar) linear velocity; with a reduction in flow rate comes an increase in ionization efficiency. In mass spectrometry, Electrospray ionization (ESI) is used when analytical (**Table 1**) LC flow rates are used. Analytical flow produces a large volume of liquid at the MS emitter, the large volume of liquid makes ionization efficiency troublesome. Consequently, ESI uses high voltages, high temperatures, and high amounts of nebulization gases to produce gas phase ions from these large charged liquid droplets.

Table 1. Typical flow rates and sample loads used in liquid chromatography according to column type (ID).

Column Type	Column Internal Diameter (ID)	Typical Flow Rates	Typical Sample Load
Nano LC	50-75 µm	200-500 nL/min	100-300 ng
Micro LC	0.15-0.5 mm	1.0-50 µL/min	1-10 µg
Low-Flow Analytical	1.0-2.1 mm	0.02-0.1 mL/min	10-50 µg
Analytical	2.1-8 mm	0.1-3.0 mL/min	0.1-1.5 mg
Semi-prep	9-15 mm	5-10.0 mL/min	1-10 mg
Preparative	16-100 mm	20-250 mL/min	20-250 mg

Flow reduction decreases droplet size at the MS emitter and increases the concentration of charged analytes (**Figure 3**). Typically, a voltage of 1.5-3 kV is applied allowing for those analytes to transition from liquid to gas phase without the need of nebulizing gases, high

temperatures, and high voltages that tend to hamper ionization of analytes present in low amounts. In comparison to ESI used in analytical flow, nano-ESI (NSI) produces a stable spray that can provide better sensitivity of analytes containing undesirable but perhaps necessary amounts of salts such is in the case in discovery lipidomics,[6] but more importantly it provides better sensitivity overall.

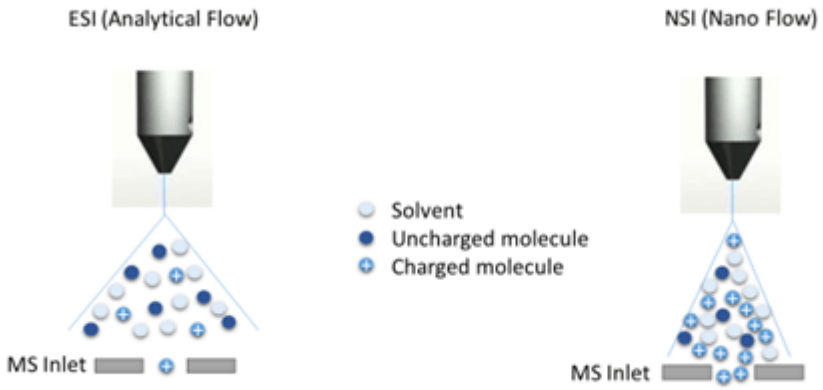

Figure 3. Depiction of analytical vs. low flow ionization. NSI allows for the concentration of charged droplets that can easily transition to the gas phase and enter the mass spectrometer inlet more efficiently in comparison to the more diluted ESI charged particles.

Column miniaturization reduces chromatographic dilution and greatly improves ionization efficiency, this combination makes nano flow a highly desirable technique in the world of Omics when only low amounts of sample are available and/or better ionization is needed due to the addition of non-volatile solvents or salts. Besides improved sensitivity, higher S/N (Signal-to- Noise) and lower LLOQ (Lower Limits of Quantitation), it also reduces solvent

consumption, which reduces costs and benefits the environment. At the same time, with a reduction in flow rate sometimes there is the need to increase gradient length. An increase in gradient length reduces sample throughput. Nonetheless, when sample throughput, sensitivity, and robustness are desired, micro flow is a great intermediate option (**Figures 4 & 5**). **Figure 5** illustrates the increase in sensitivity achieved by column miniaturization when scaling down the column ID from 0.300 mm (micro flow) to 0.075 mm (nano flow).

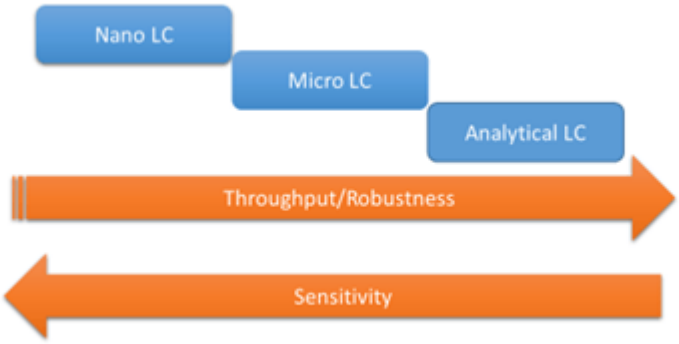

Figure 4. Relationship between Nano, Micro, and Analytical flow.

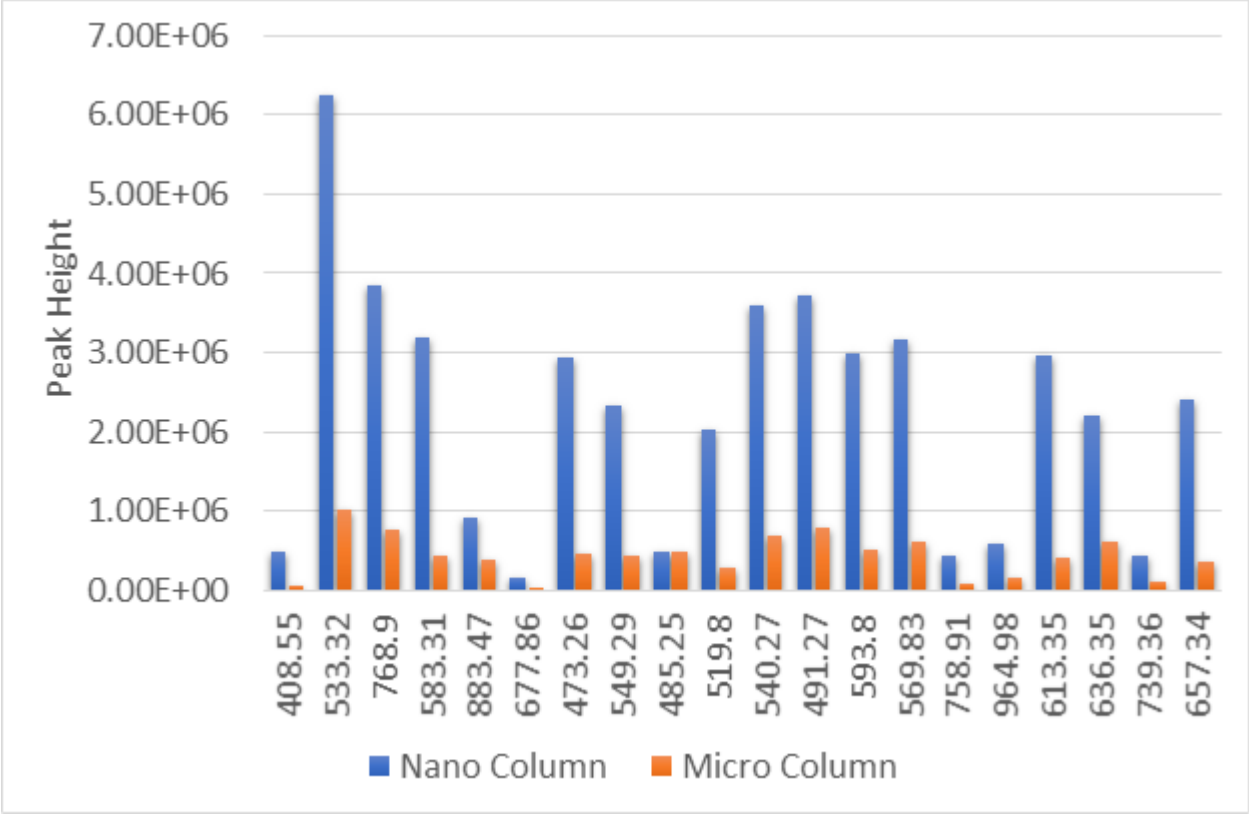

Figure 5. Ion intensity increases when scaling down from Micro (orange) to Nano (blue) column for each of the 20 synthetic peptides (10 fmol of each in column), x axis = peptide m/z.

Despite the many benefits of low flow chromatography, there are also a few considerations that must be taken into account. When moving towards nano and micro flow, one must consider and be careful about dwell volume. This is the volume it takes from when the gradient is formed, to when it reaches the head of the column and is responsible for gradient delays. In the case of analytical flow, a dwell volume of 5 µL does not cause a noticeable gradient delay, but the same dwell volume when operating at 250 nL/min would

cause a gradient delay of about 20 min. To minimize this volume, low flow instrumentation must be plumbed with 10-75 µm ID capillary tubing.

Extra column volume refers to the volume found between the injector and the detector and is responsible for mixing effects that lower observed efficiency since it adds extra column broadening. It can be found in any of the components of the LC system (needle seat, frits, tubing, connections) and can contribute to peak broadening. To minimize extra column volume effects, low flow chromatography uses miniaturized LC systems in which each of its components transports only a small volume in comparison to conventional LC systems. Furthermore, to minimize or avoid extra column volume effects, every single connection must be routinely inspected for leaks, which can be very time consuming. A leak in the low flow world is almost always undetectable by the naked eye and it requires a highly experienced operator to detect such leaks.

Additionally, column clogging and overloading also need to be considered as microscopic solids and in the case of proteomics, undigested protein, can clog the small ID column rendering it unusable. Thus, the user must be careful to properly clean the sample prior to injection and/or to use a trap (pre-column) column to eliminate undesired contaminants from reaching the miniaturized column. When using a trap column, the trap is in line with the column, the sample is loaded into it while undesirable particles go to waste. Once the sample is concentrated into the trap and free of contaminants, it is directed towards the column for analysis. Using a trap column can aid to avoid nano/micro column contamination and to extend the column’s lifetime.

Column miniaturization can bring many challenges, but there is no denying that the benefits outweigh these challenges. The increase in ion intensity brought by low flow chromatography has made it possible to identify biomarkers for clinical research[10]; to identify thousands of proteins using less than 2 ng of protein and hundreds in single cells[11] as well as metabolites.[5] The biomedical advancements and gains in fundamental scientific knowledge brought by micro and nano flow separations make this a powerful analytical technique.

Explore the latest solutions for nano LC columns here.

About the Author

Roxana Eggleston-Rangel is an Application Scientist at Phenomenex. She received her bachelors and masters degree in Chemistry and Biochemistry from California State University, Los Angeles, USA. Roxana is an LC-MS/MS enthusiast currently working on developing LC-MS/MS methods with a focus on nano and micro flow separations.

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