# Capillary Flow LC-MS Unites Sensitivity and Throughput

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There is a continuous demand for improved analytical depth and higher throughput capabilities for liquid chromatography mass spectrometry (LC-MS) based analyses in both research and applied markets. The need for greater sensitivity, selectivity and faster cycle times has led to impressive improvements in instrumentation and column technology.

Liquid chromatography has seen the adoption of high performance systems (HPLC) and then a transition to ultra-high performance systems (UHPLC, >400 bar) [1]. Nowadays, pressure capabilities beyond 1000 bar are widely available for analytical flow LC-MS systems. Together with ultra-high performance columns, packed with small particle size stationary phases (2 µm and below), fast separations with high resolution are now possible. Additionally, instrument development has led to commercially available LC systems capable of nano flow rates and UHPLC performance levels [2].

The combination of LC systems with mass spectrometers, for analyte identification and quantification, has further improved the analytical capabilities. The development of mass spectrometers has led to detection limits in the attomole range, with the latest generation high-resolution accurate-mass (HRAM) mass spectrometers being able to achieve sub-part-per-million (ppm) mass accuracy and mass resolution approaching 250,000 [3]. At the same time commercial LC-MS manufacturers have worked hard on increasing the system robustness and usability.

Unfortunately, obtaining the best sensitivity and the highest throughput is not always possible, as these two demands often counteract each other. Reducing flow rates increases the efficiency of the electrospray ionisation (ESI) process but at the same time increases the run time, and will also have an effect on the chromatographic performance. This results in the use of different instrumentation and the development of different methods which provide either ultimate sensitivity with limited throughput

*Table 1. Flow rate regimes in HPLC*

| Flow range | |
|---|---|
| Analytical flow | >100 µL/min |
| Micro Flow | 10-100 µL/min |
| Capillary Flow | 1-10 µL/min |
| Nano Flow | <1 µL/min |

or highest throughput with limited sensitivity. As a result, chromatographers need to consider carefully which requirement is more important to them.

Capillary LC-MS can provide the solution to the problem of delivering high sensitivity and throughput at the same time [4,5]. It is usually defined by the flow rate (1-10 µL/min) used, which is a consequence of using column diameters in the range 100-500 µm i.d. (see Table 1) [6]. As a consequence, the ESI source, which interfaces the LC with the MS system, has to be modified from the sources used for analytical flow rates (>100 µL/min). The inner diameter of the flow emitter needles used in analytical flow systems is too wide resulting in sample dispersion and reduced sensitivity when used in capillary systems. Efficient electrospray ionisation at analytical flow rates requires a supporting gas flow around the emitter, to aid the desolvation process. At lower flow rates, such as nano or capillary flow rates, this is no longer needed. Therefore, existing LC-MS sources designed for nano flow rates can often be adapted to capillary flow rates. This is done by using emitters with an appropriate inner diameter for capillary flow rates (20-50 µm). To facilitate operation these emitters are usually

incorporated into easy-to-install transfer lines or chip based devices.

As mentioned previously, reducing flow rate results in increased sensitivity due to better desolvation of the analytes, a more efficient electrospray ionisation process and a higher sampling of analytes into the mass spectrometer [7]. Wilm and Mann observed an improvement in sensitivity of approximately two orders of magnitude for nano compared to analytical flow LC-MS systems [7]. The effect of the sample matrix on ionisation efficiency can also contribute considerably to sensitivity improvements, when using reduced flows. Unfortunately, the combined effects are hard to predict and would require experimental data for each analyte / sample combination. As a rule of thumb, however, capillary LC-MS has approximately a 10-20 times higher sensitivity than analytical flow LC-MS (with 2.1 mm columns), when optimised LC conditions and appropriate emitter needles are used (Figure 1). We have verified these findings with our own set-up and observed significant impact of the flow rate and column diameter on the sensitivity. At a given internal diameter sensitivity increases with lower flow rates.
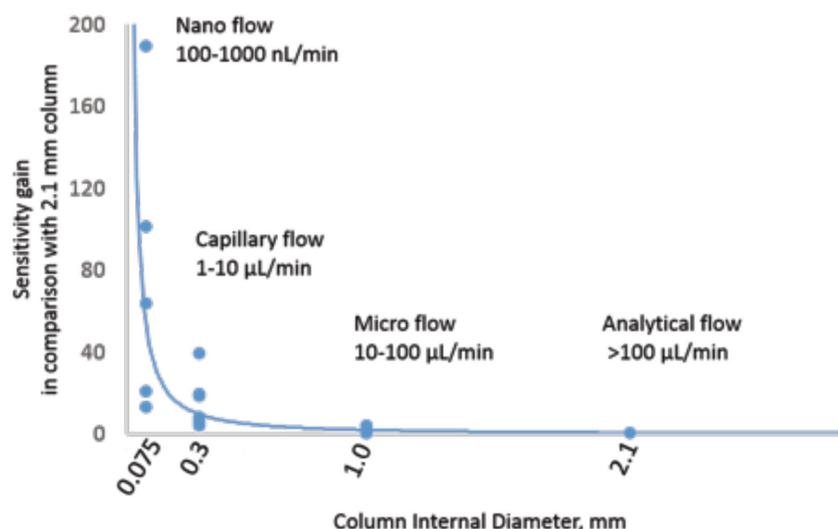
*Figure 1: Relative sensitivity gain for different column inner diameters and flow rates from 100 nL/min to 450 µL/min. Data is shown for EDLIAYLK peptide of Cytochrome C tryptic digest. Each dot represents results for a particular column inner diameter and flow rate. 75 µm x 15 cm , 0.3 mm x 15 cm, 1.0 mm x 15 cm Thermo Scientific Acclaim PepMap columns with 2 µm particle size, and a 2.1 mm x 10 cm Thermo Scientific Accucore AQ column with 2.6 µm particle size were used.*

Often it is believed that the sensitivity increase for nano and capillary flow compared to analytical flow LC-MS is the result of downscaling column dimensions. It is true that reducing column inner diameter can result in an increase in sensitivity [6] However, in LC-MS analysis this effect is only a minor contributor to the overall sensitivity gain. Hence, the theoretical gain in sensitivity can often not be achieved by only downsizing the column.

Compared to capillary LC-MS, nano LC-MS exhibits even greater sensitivity. Thus the question is raised, why is nano LC-MS not more popular given the obvious benefit. The reason for this is the limited throughput and the high level of expertise required to set up the fluidics. The latter problem can be easily resolved by using ready-to-use capillary connections and integrated plug-and-spray consumables but increasing throughput with a nano LC-MS set-up is very difficult. For example, the gradient delay and sample loading volumes contribute to the much longer analysis times in nano flow compared to analytical flow applications. In absolute terms the gradient delay volumes and loading volumes can often be reduced to a 1 µL or less. However, these volumes have a large impact on analysis times in relative terms. A delay volume of 1 µL will already result in 3.3 minutes delay time at 300 nL/min flow rate. As a result, nano LC-MS run times of one hour or longer are the average rather than the exception. Capillary flow solutions result in sample analysis times of 10-15 minutes per sample, which is comparable to the cycle times usually experienced in analytical flow analyses.

On the MS side, reducing the flow rate is actually beneficial for the system stability. Less system cleaning is required because there is less contamination accumulating on the ion source and in the ion optics of the mass spectrometer. On the LC side nano flow rates can impair robustness. Fluidic leaks which have no impact on data quality in capillary and analytical flow analyses will cause huge effects in nano flow applications. For example, a leak rate of 30 nL/min is 0.006% flow loss at 500 µL/min but 10% flow loss at 300 nL/min. Developments in capillary connections have resulted in the ability to have leak tight connections within seconds without using any tools. Leaks in other components such as pumps and valves are harder to minimise, but modern LC systems can compensate for leakages by active flow control.

Increasing the flow rate to capillary flow further reduces the risk of small leaks impacting on results. Hence, capillary flow LC-MS results in high sensitivity, high sample throughput and good system robustness. Chromatographers using analytical flow rates who require higher sensitivity can move to capillary flow rates without sacrificing sample throughput and instrument robustness. Chromatographers using nano flow rates and who require higher throughput can move to capillary flow rates because it still provides good sensitivity in combination with modern MS detectors.

Capillary flow LC-MS has attracted most attention for bioanalysis applications in pharma and especially biopharma laboratories. Dedicated solutions have been put forth by LC-MS vendors in order to provide robust, easy-to-use applications. The targeted detection of small molecules, such as drugs and their metabolites, and large molecules, such as peptide hormones and drug antibodies, in biological fluids can be a big challenge with analytical flow LC-MS.

Ligand binding assays, such as ELISA, were the method of choice for protein quantification. However, antibody specificity has been questioned recently, with several studies finding that antibodies may target a lot more than only the intended target [8,9]. Additionally, polyclonal antibodies cannot be produced with the same specifications repeatedly requiring revalidation of tests after receiving a new batch of antibodies. This consideration has resulted in the search for alternative methods and hence, the adoption of LC-MS analyses and for better sensitivity capillary flow LC-MS analyses. The latter provides sensitivity high enough to detect analytes at pharmacokinetically relevant levels; its selectivity is unambiguous if the right ion targets are selected, and LC and MS instrumentation has matured enough to provide robust methods.

Another great advantage of LC-MS based assays is the fast, straight-forward method development and multiplexing capabilities. For multiple analytes from the same sample type near identical method conditions can be applied differing only in the MS detection settings. Often, multiple analytes can be detected in a single analysis. Most likely, LC-MS will gradually replace most antibody based applications.

LC-MS analysis of peptide hormones or biotherapeutics is mostly done after enzymatic digest of target proteins into smaller peptides. This results in loss of information since post-translational or chemically introduced modifications cannot be evaluated in the context of the respective intact protein species. The first attempts are being made to detect whole proteins in biofluids but major development work is still needed to overcome the technological hurdles [10]. Once the challenges have been mastered whole protein bioanalysis by capillary flow LC-MS will become an important part of the toolkit for biopharma analysis.

Other fields are also likely to adopt capillary flow LC-MS for their applications.

Environmental and food safety standards are rising. Wherever higher sensitivity is required but sample material is limited or analyte enrichment steps are impractical, capillary flow LC-MS will become a useful alternative. In clinical proteomics research there is a push towards higher throughput while good sensitivity has to be maintained. Therefore, shotgun profiling of clinical samples will benefit greatly from this technology. It can be envisioned that research driven proteomics will generate marker signatures on large patient cohorts. In a second step the identified markers could be used for an individualised patient diagnosis.

In conclusion, capillary flow LC-MS is becoming a useful alternative for highly sensitive and selective analysis of complex samples. In bioanalysis it will continue to penetrate different application areas. It has already shown a lot of promise and more and more laboratories are adopting capillary LC-MS for routine applications. Still, further proof is required to convince more conservative LC-MS users that this method is mature enough to be broadly used. However, the first steps are made and we are confident that further evidence and proof of the true capabilities of this technique will be generated in the next years.

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