Key UHPLC Characteristics Required for High-throughput LC-MS

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With high sensitivity and selectivity, LC-MS is a workhorse technique for quantitative assays. Mass spectrometry (MS) detection allows simultaneous identification and quantitative analysis of multiple compounds, even if the chromatographic separation is imperfect. This ability dramatically increases the throughput for multiple component assays. In an ultra-fast UHPLC-MS method, the analytical cycle time is the most critical consideration. New generation UHPLC columns packed with sub-2 ¬µm or superficially porous particles for high efficiency, allow for shorter column length and faster analysis. However, ultra-high throughput is not achieved simply by using the shortest columns. First and foremost, a rapid injection speed and high sample capacity are required. Secondly, the system must reduce other time-consuming factors, such as the rinsing time. If the target compounds have a strong tendency to adsorb to a system's inner surface, the LC instrument must be rinsed carefully after every injection to avoid carryover that causes quantitative errors. This rinsing phase can considerably increase the analytical cycle time. And in addition, 'non-data-acquisition time', for column rinsing and system equilibration, has to be considered.

This article introduces the key front-end UHPLC competencies required for high-throughput LC-MS assays.

1. Injection Speed and Sample Capacity

Injection speed plays a pivotal role in achieving high sample throughput, as the duration of an injection sequence (= time required for the autosampler to inject a sample - not including post-injection rinsing) adds to the analytical cycle time. If it takes 30 seconds to inject a sample in a 30 second gradient run, half of the analytical cycle time is 'non-data-acquisition time'. This is not conducive to a high-throughput objective. New generation UHPLC equipment, such as the Nexera series Sil-40 autosampler offer high-speed injections, with an injection cycle time of only 6.7 seconds to not impede a rapid analysis.

An example application that highlights the effectiveness of sample analysis using an ultra-fast injection sequence, is the high-speed analysis of drugs in blood plasma by LC-MS/MS [1]. The plasma samples, spiked with verapamil and its isotopically labelled analogue, were analysed in an 18 second isocratic method using a 5 x 2.1 mm UHPLC column. *Figure 1* shows a representative chromatogram of the assay.

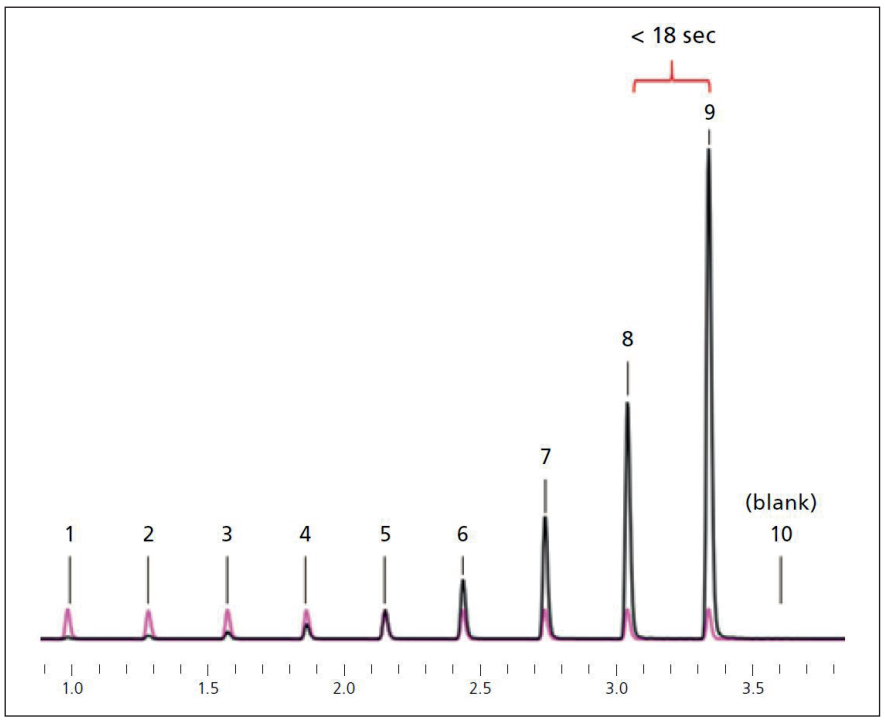

Figure 1. Linearity over the bioanalytically relevant concentration range (0.4 – 100 g/L). Black: Verapamil chromatogram. Pink: Verapamil-D6 chromatogram.

Sample capacity also plays a critical role in enabling high-throughput analysis, as it allows an extended time of unattended operation. The ease of adding samples to the running system is another important characteristic for high-throughput devices. An open-access design allows to add samples to an analytical sequence without interrupting an ongoing analysis.

2. Carryover and Rinsing Capability

With their ultra-high sensitivity, LC-MS/MS systems can detect trace levels of analyte that remain in the system and elute in a subsequent run. This so-called 'carryover' negatively impacts an analysis and can result in quantitative errors. It is also a serious issue with regards to throughput, as it requires additional rinsing phases within an analytical sequence, leading to longer cycle times. Autosampler and separation column are the most likely sources of carryover. Column-based carryover is best dealt with during method development - making sure that the mobile phase/gradient conditions are sufficient to remove any trace amounts of the analytes. As for samples remaining in the autosampler, there are two ways to minimise the risk of system carry-over - the sampler design, and effective countermeasures.

In a 'needle-in-flow-path' design, the sample is aspirated into the needle and the total volume is swept directly onto the system. The sample loop is therefore part of the flow path, hence continually washed with the method gradient, aiding in reducing chemical adsorption. This design eliminates the intermediate step of loading a separate sample loop and the required valve openings and closings, which can trap the sample and lead to carryover. The total injection method is fast and clean - a pre-requisite for high-throughput LC-MS [2].

However, not all molecules allow to skip post-sample injection cleanup, they require additional methods to remove any remaining compound from the system. Typical areas of concern are the injection port and the autosampler needle. Rinsing the needle is one of the most effective solutions to flush out any remaining analyte on its surface. Most of the newer autosamplers have rinsing functions that allow dipping the needle into a washing solution. But it's important to note that not only the outer but also the internal surface of the needle is exposed to a risk of adsorption. Additionally, the inside wall of the injection port can also be contaminated if the needle is not perfectly clean. Compounds may accumulate around these parts and become a source of carryover.

Overcoming this issue requires a well-designed washing program. Various rinsing methods and multiple rinsing solvents to wash the inside and outside of the needle, the sample loop, and the inside and surface of the injection port are helpful to address a wide range of chemical properties of the target compounds. One can design a wash routine to eliminate any carryover - strong organic wash, acidic or basic wash, ionic wash - whichever is needed for the class of compound in question. *Figure 2* highlights the reduction of carryover with an additional internal rinse of the needle and injection port, compared to only an external rinse.

Of course, any wash/rinse routine will add time to the total autosampler sequence. And this may have an impact on the analytical sequence time.

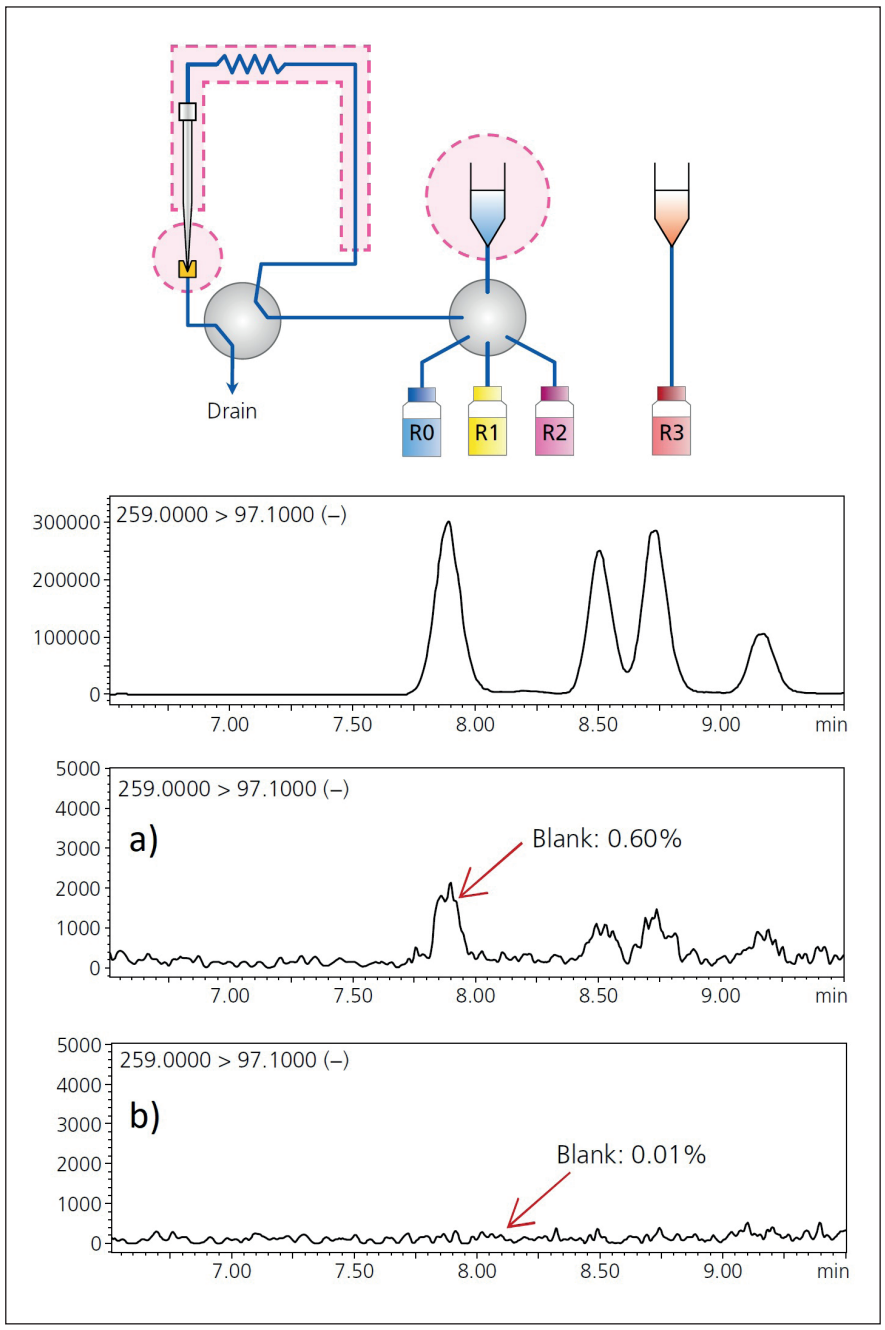

Figure 2. Example of carryover of a standard solution of Glucose 6-phosphate.
a) Outside needle rinse by dipping into R0 (50 % methanol aq.)
b) Outside needle rinse by dipping into R0 (water), internal needle surface and injection port rinse by R1 (mixture of formic acid, methanol, acetonitrile and isopropyl alcohol) [2].

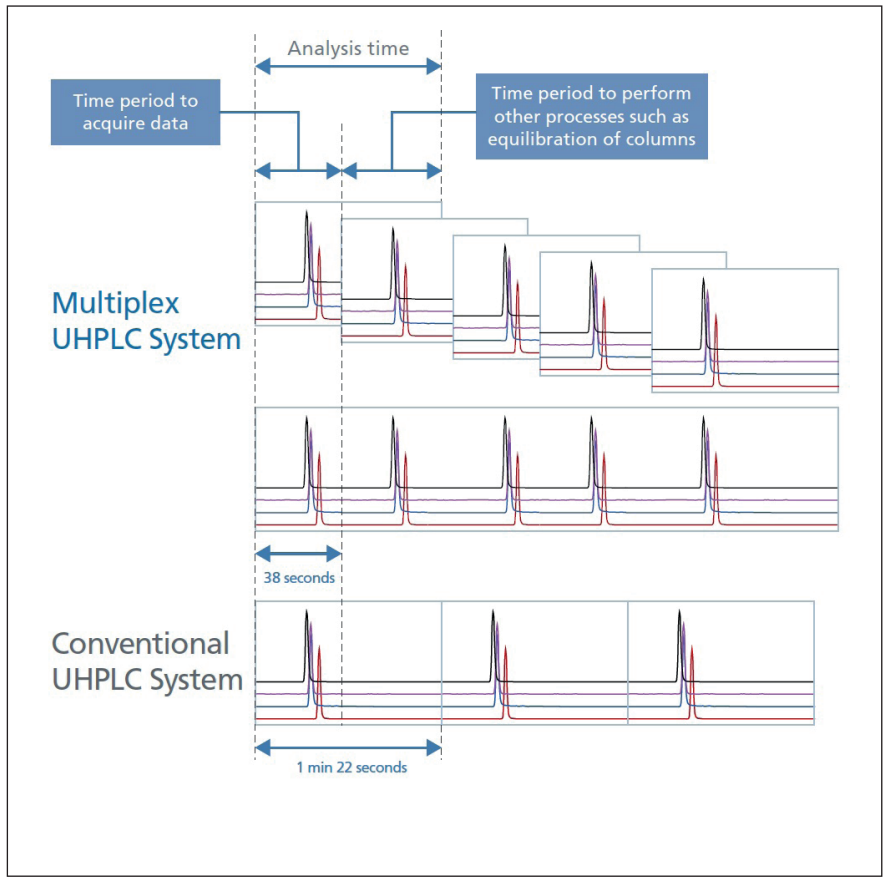

Figure 3. Example of multiplex analysis for Cytochrome P450 assay.

3. Maximise Throughput with a Multiplex Configuration

RPLC gradient analysis requires performing various processes, such as the needle rinse, column washing and re-equilibration as preparation for the next sample injection. While these are essential processes to generate meaningful data, they result in additional 'non-data-acquisition time' that impacts throughput. Reducing the time where the MS sits idle, waiting for the LC to wash and re-set, could dramatically increase throughput and lead to higher laboratory productivity. Hence, a front-end configuration that offers to overlap the data acquisition time and washing/equilibration phase by switching between two streams into one LC-MS is an ideal solution to close to double sample throughput in one system. *Figure 3* [3] shows an example where this Dual Stream Technology was used for analysis of four biomarkers for the four major molecular species in the Cytochrome P450 family. Analysis could be completed in only 38 seconds compared to 1 minute 22 seconds using the conventional single stream approach, saving 44 seconds per sample.

Conclusion

LC-MS/MS is a powerful technique for quantitative assays, as it offers high selectivity and sensitive detection. For a laboratory to achieve and maintain high sample throughput and gain as much information as possible from each sample, it is essential to carefully consider the characteristics of the front-end UHPLC system. Certainly, sample injection speed and capacity, the autosampler's ability to reduce carry-over, while maintaining a high-speed injection cycle, and the possibility to perform multiplex analysis should be considered in an advanced UHPLC front-end configuration, to achieve high efficiency and a maximum in sample throughput.

References

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2. K. Watanabe, C. Campbell et. al., Prominent Features of Shimadzu UHPLC for an LC/MS Assay, Shimadzu Technical Report (C190-E282)
3. Nexera MX brochure (C190-E190), Shimadzu Corporation, 2020