C190-E282

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Technical Report

[White Paper] Prominent Features of Shimadzu UHPLC for an LC/MS Assay

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Abstract:

Although mass spectrometry (MS) offers high sensitivity and selectivity, the quality of the HPLC/UHPLC system is critical to maximizing a mass spectrometer's ability. Since a quantification assay is validated using accuracy and precision as key parameters, some essential performance attributes of an LC system should be investigated carefully. An ultra-fast LC/MS assay relying on high-throughput LC performance requires additional considerations. In this white paper, we report on various principles to consider when utilizing a front-end LC system and highlight the superior features of Shimadzu's UHPLC system as an LC/MS front-end platform.

Keywords: HPLC, UHPLC, LC/MS, solvent delivery pump, autosampler, carryover, throughput, multiplex analysis

Which LC Characteristics Make for a Great LC/MS Front-end System?

To get great data from your LC-MS system, you must first have a great HPLC or UHPLC.

MS detection allows the simultaneous determination and quantitative assay of multiple compounds, even if the column separation is imperfect. And old-school mass spectroscopists will tell you that the HPLC is nothing more than a way to get material to the MS inlet. "The mass spec can do it all", they say. However, in today's laboratories we do not have the time to dedicate to a lengthy analysis of the raw MS data nor do many of the analysts have that ability. A proper chromatographic separation of the components adds another identifying factor (retention time) and allows for easier data reduction. To serve the purpose well, the HPLC system needs to be a reliable and stable platform. This white paper introduces the key front-end UHPLC capabilities to consider when purchasing an HPLC for your LC/MS system.

1. Variation of Solvent Delivery

Gradient elution is a popular separation technique for an LC/MS assay because it balances chromatographic resolution and analytical cycle time to simultaneously analyze multiple components. It is also effective for reliable quantification by separating the target compound(s) from the matrices in the sample solution. The Shimadzu Nexera LC-40 solvent delivery unit (pump) series is the latest iteration of our micro plunger design, dual-piston solvent delivery unit. These pumps provide stable and accurate flows with very low pulsation, ideal for gradient formation.

There are two popular configurations for gradient formation: high-pressure gradient and low-pressure gradient (Fig. 1). The high-pressure gradient configuration consists of multiple solvent delivery units. Each unit pumps only one solvent. By altering the flow rate of each unit over time, a gradient is formed. This configuration provides an accurate, stable, and repeatable mobile phase composition. Another advantage is its smaller gradient delay volume due to the solvents being mixed at the mixer after the pump, immediately prior to the autosampler. The internal volume of the pump or any earlier component in the flow path contributes nothing to the delay volume. This small delay volume aspect is desirable for fast analysis as less delay volume shortens the method run time. In contrast, the low-pressure gradient configuration consists of a single solvent delivery unit and a quaternary solenoid valve. While this configuration has a lower initial cost, it also comes with several drawbacks, one of which is a much larger gradient delay volume compared to the high-pressure gradient configuration. This larger delay volume is a result of the gradient delay volume beginning when the mobile phase components mix. In this configuration, the components mix at the solenoid valve. As a result, one must add the solenoid valve internal volume, the pump internal volume, and any connecting tubing to the mixer volume to get the total gradient delay volume. This can add significant delay to a method.

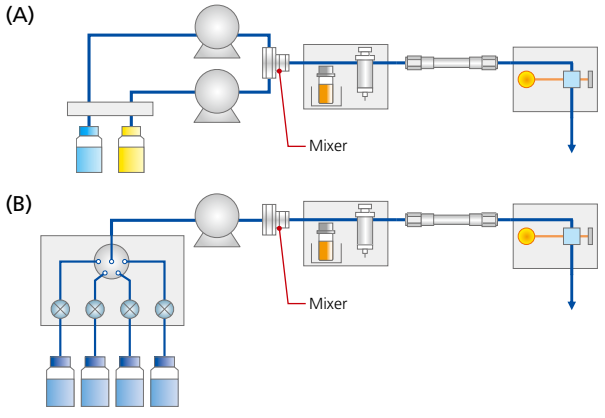

Fig. 1 Gradient configuration (A) high-pressure gradient system, (B) low-pressure gradient system

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The chromatographic separations obtained with high-pressure and low-pressure gradient configurations often don't match due to their different gradient delay volumes. One must also be aware that a low-pressure gradient configuration will typically require a larger mixer volume. This is due to the fact that the gradient-forming solenoid valve delivers "packets" of each pure mobile phase component. These packets are harder to mix properly than the individual streams of a high-pressure configuration impinging at high pressure directly in the mixer. When looking to match published results, it's important to check which gradient configuration was used for the assay in question. Additionally, managing the gradient delay volume of the system is preferable to obtain a "similar" retention time to that of the reference data.

The Shimadzu Nexera series can be configured as both high- and low-pressure gradient systems (Fig. 2). The binary pump, which includes two pump units in a single device, results in a space-saving design in the case of high-pressure gradient configurations (XR and X3 models). Additionally, a wide variety of mixer volumes is available to support modifying the gradient delay volume as necessary.

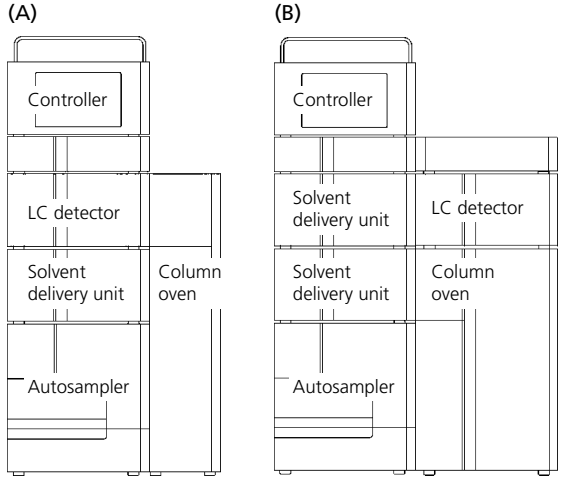

Fig. 2 Examples of Nexera system configurations: (A) high-pressure gradient with binary pump or low-pressure gradient, (B) high-pressure gradient with two pump units

A combination of solvent-blending and a high-pressure gradient configuration can dramatically reduce the analyst's burden in some applications. The method development, modification, optimization, or validation phases will benefit greatly with the use of such a system (Fig. 3). With this configuration, there is no need to prepare numerous mobile phases in advance or exchange them one by one. The HPLC system can make these mobile phase changes for you. The addition of column switching valves improves working efficiency even more by enabling the screening of a wide variety of separating conditions automatically. [1]

To improve sensitivity, miniaturization of the mobile phase flow is one of the most effective solutions. Nexera Mikros delivers the mobile phase in the high-pressure gradient mode from 1 µL/min to 500 µL/min, resulting in enhancing the MS signal dramatically. [2]

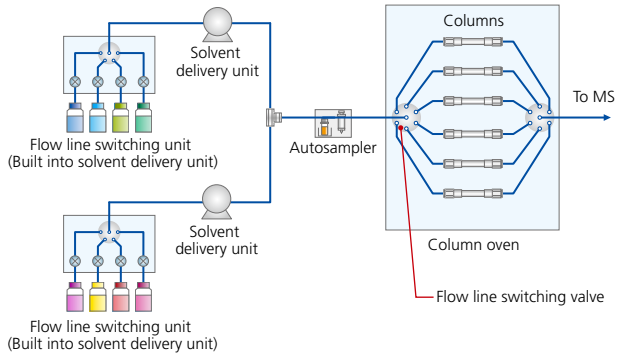

Fig. 3 Flow diagram of a Method Scouting system with solvent blending, high-pressure gradient, and column switching configuration

2. The Best Autosampler for LC/MS Assay

An autosampler injects the standard or sample solution into the LC system and delivers it to the column. It generally consists of a sample container, sampling needle, sample loop, measuring pump, injection port, rinsing unit, switching valves, etc. From a hardware design point of view [2], Nexera's autosamplers (SIL-40 series) are categorized as total-volume-injection or needle-in-the-flow-path design (Fig. 4). We introduce Nexera autosampler's features considering the requirements for an LC/MS assay.

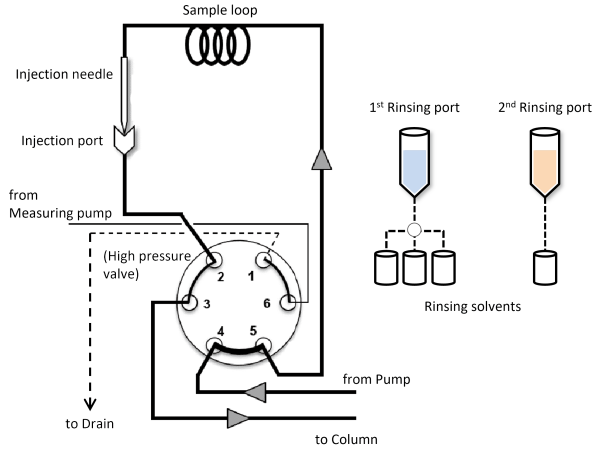

Fig. 4 Flow diagram and injection process on Nexera autosamplers

Carryover

Compared to other major LC detectors, an LC/MS offers the distinct advantage of providing better limits of quantitation (LOQ). With its high selectivity and sensitivity, an LC/MS enables trace-level compound determination, especially on a triple-quadrupole platform. However, this sensitivity comes at a cost. Injection carryover or residual sample from injection to injection must now be considered.

Carryover is a phenomenon wherein the target compound (or other interference) is detected when a blank sample is injected after a sample or standard (Fig. 5). It really becomes a problem when the quantitative value of the carryover is a non-negligible amount. Carryover sources that are known to exist in an HPLC/UHPLC system include the column and sample flow path. The autosampler can also be a major source of carryover.

For this reason, Shimadzu has continually focused its attention on minimizing carryover issues and, as a result, has established a reputation for having the world's lowest carryover models. Here, we introduce some key features on the latest Nexera series autosamplers, and how we incorporate two philosophies to combat carryover in our autosamplers: *1. effective countermeasures, and 2. innovative design*.

Effective Countermeasures

We sometimes require other methods to remove any remaining compounds. Typical areas of concern are the autosampler needle and the injection port. Rinsing the needle is one of the most effective solutions to flush out the compounds on the surface of the needle. Most of the latest autosamplers have rinsing functions that allow dipping of 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 (Fig. 6).

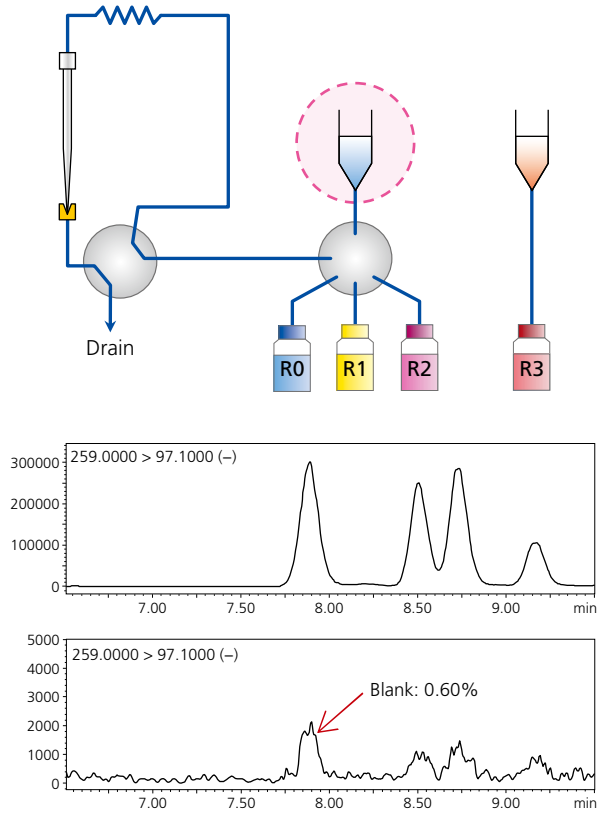

Fig. 5 Example of carryover; Standard solution; Glucose 6-phosphate (1 µmol/L), Blank; water, injection volume; 3 µL, reversed-phase mode, ESI (negative). Rinsing needle's outside by dipping into R0 (50% methanol aq.). The flow path is simplified to illustrate.

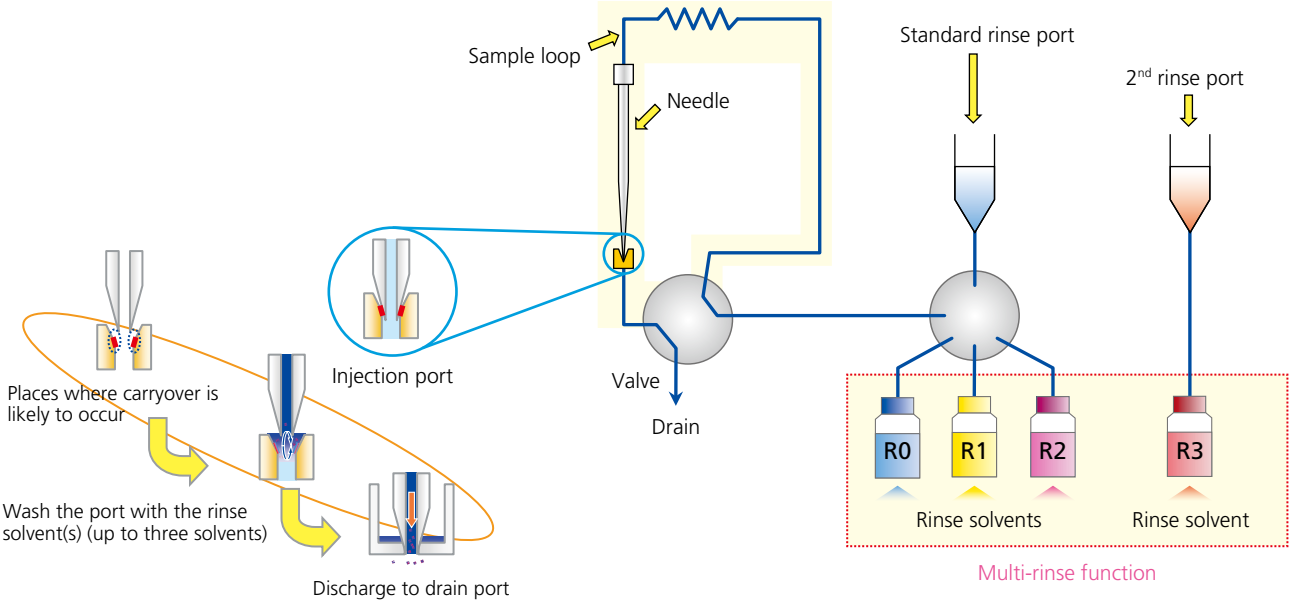

Fig. 6 Various rinsing methods and multiple rinsing solutions. The flow path is simplified to illustrate.

Overcoming this issue requires a well-designed washing program. Various rinsing methods and multiple rinsing solvents are helpful to address a wide range of chemical properties of the target compounds. Fig. 6 illustrates the parts to be taken care of with multiple rinsing solutions — inside and outside of the needle, the sample loop, and the inside and surface of the injection port. By carefully choosing rinsing solutions (R0, R1, and R2 in Fig. 6), one can design a wash routine to eliminate any carryover — strong organic wash, acidic or basic wash, ionic wash — whatever is needed for the class of compound in question.

Fig. 7 shows that an internal rinse of the needle and injection port reduced the carryover significantly compared to what's shown in Fig. 5 when only a needle dip was utilized. Within the rinsing program, you can customize when and how rinsing takes place to optimize cleaning ability and instrument performance.

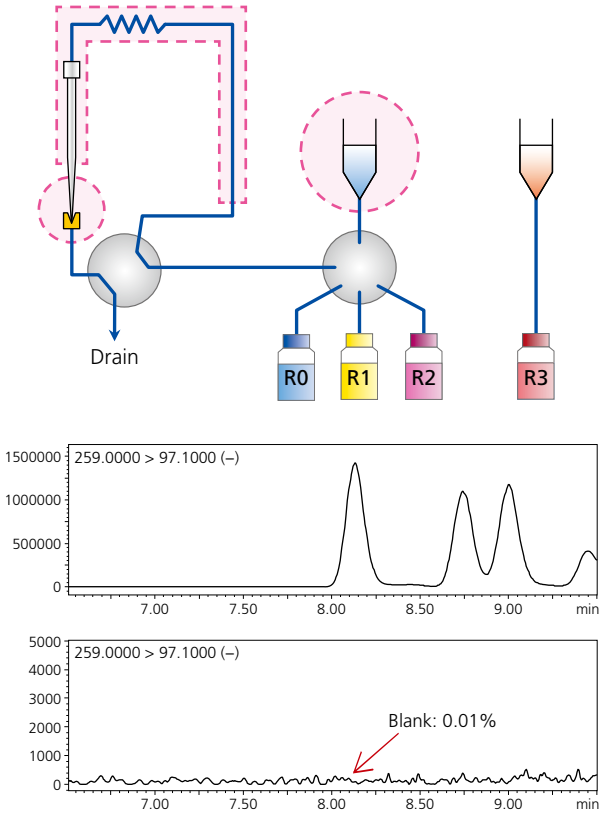

Fig. 7 Standard solution; Glucose 6-phosphate (5 µmol/L). Rinsing outside the needle by dipping into R0 (water), and an internal surface of the needle and injection port by R1 (mixture of formic acid, methanol, acetonitrile, and isopropyl alcohol). Other conditions are the same as Fig. 5. The flow path is simplified to illustrate.

Innovative Design

The SIL-40 series of autosamplers is the ninth generation of Shimadzu's total-volume-injection or needle-in-the-flow-path design. In this design, the sample is aspirated into the needle and the total volume is introduced directly onto the system (Fig. 4). The "sample loop" is actually part of the flow path and is continually washed in the gradient method cycle, 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 sample and lead to carryover. The total injection method is faster and cleaner — a necessity for high-throughput LC/MS analysis mentioned in the next section.

With each iteration of the design, Shimadzu made subtle but effective changes to obtain the world's best carryover performance without the need for rinsing. Simple changes like reducing the contact surface area between the needle and needle seal; less area means less chance for material sticking. The flow characteristics through the injection port and high-pressure valve have been optimized to reduce eddies or stagnant areas that can trap small amounts of the injected sample. The internal and external surfaces of the needle are finely polished and treated to minimize active adsorption sites. The sample loop and needle in the autosampler are connected with the "zero-dead-volume" design. These and other proprietary design characteristics allow the SIL-40 to claim the world's best carryover performance of 0.0015% without rinsing (under specific analytical conditions).

By combining an optimized hardware design and rinsing method with the appropriate rinsing solution, the carryover can be significantly reduced. Fig. 8 shows what can be obtained by utilizing the internal rinsing options on the SIL-40 autosampler. [4]

Of course, any rinsing routine will add time to the total autosampler sequence. And this may have an impact on the analytical sequence time. However, with the many options and flexibility available with the SIL-40 series, coupled with the built-in innovative design characteristics, the effects will be minimal.

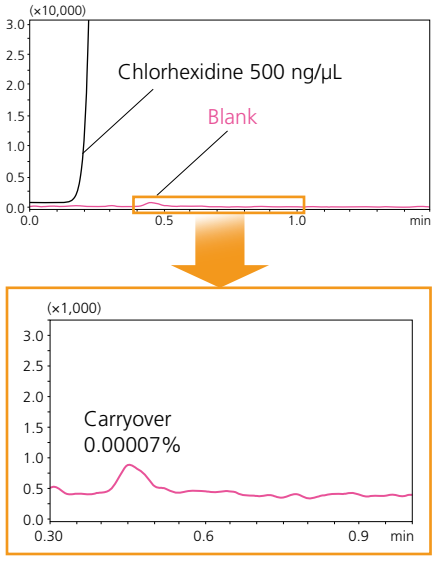

Fig. 8 Improvement in carryover property on SIL-40 series autosampler.

Throughput and Sample Capacity

An ultra-fast LC/MS assay utilizing columns with sub-2μm particles allows significantly higher throughput, without compromising any reliability on quantitative values. To achieve this higher throughput, however, certain parameters, including analytical cycle time, speed of injection, carryover, and sample capacity, need to be evaluated. These key competencies of an LC front-end system are described in another paper. [5]

In addition to the SIL-40 series autosampler, the SIL-30ACMP is designed for high-throughput analysis with its large sample capacity and fast injection speed. Although its sample capacity can't be expanded with a plate changer, it can mount six sample plates; meanwhile, the SIL-40 series mounts up to three (without a Plate Changer).

Injection Volume Range, Accuracy, and Reproducibility

Accuracy, reproducibility, and a wide range of injection volumes are also essential for LC/MS analyses. Nexera's SIL-40 series autosamplers fulfill all requirements, as shown in Table 1. It is easy to see that the Nexera's autosamplers meet the needs for the most demanding aspects of LC/MS analysis.

Table 1 Specification of SIL-40 series and SIL-30ACMP

	SIL-40 series (*1)	SIL-30ACMP
Range	0.1–50 μL Up to 2 mL (optional)	0.1–50 μL
Accuracy	≤ ±1% (5 μL injection, n = 20)	≤ ±1% (50 μL injection, n = 10)
Reproducibility (%RSD. Both official specifications and typical values in each representative condition are shown.)	*(Official specifications)* ≤ 0.5% (1.0 to 1.9 μL) ≤ 0.15% (more than 5.0 μL) *(Typical values)* < 0.5% (0.5 μL) < 0.25% (1.0 μL)	*(Official specifications)* ≤ 0.5% (1.0 to 1.9 μL) ≤ 0.2% (more than 5.0 μL)

(*1) SIL-40 XR/40C XR/40C XS/40C X3

3. System

The Nexera HPLC/UHPLC system for LC/MS consists of one or multiple pumps, an autosampler, a column oven, and a system controller. Other components can be added to complete the system for your desired assay (Fig. 9). [6] As mentioned, the appropriate mixer volume needs to be chosen for consistent gradient formation. Two different forced-air column compartments are available to maintain stable column temperatures — above or below ambient conditions. In addition, a wide variety of both low- and high-pressure valves for solvent and column selection applications are available. Finally, a range of complementary LC detectors, including photo-diode array (PDA), UV-Vis, fluorescence, refractive index, and evaporative light scattering detectors, complete any specialized system needed.

The Nexera HPLC/UHPLC system consists of multiple models covering a wide pressure tolerance range, from a standard HPLC to three UHPLC models. One can choose the pressure rating applicable for the laboratory's requirements: lite (440 bar), XR (700 bar), XS/XSi (1050 bar), or X3 (1300 bar). Fig. 10 shows some of the components and how they are grouped. Each model consists of a solvent delivery unit(s) and an autosampler with the same symbol, e.g., an X3 system is configured with an LC-40B X3 (or LC-40D X3) and a SIL-40C X3. SIL-30ACMP can be used as an alternative to the SIL-40C X3.

UV-VIS Detector
SPD-40/40V

Photodiode Array (PDA) Detector
SPD-M40

Baseline stability and linearity have been improved, and stability remains even under fluctuating temperatures. The PDA detector is equipped with a UV cut-off filter to improve the quantitation accuracy of photodegradable compounds. The cell and lamp are traceable via individual IDs.

Solvent Delivery Pump
LC-40 series

In addition to the four parallel double plunger models based on the maximum pressure limit, the XR and X3 models have a dual pump that reduces gradient delay volume and enables an ultra-fast high-pressure gradient. Other pumping environments (low-pressure gradient, mobile phase blending) can also be provided.

Autosampler
SIL-40 series

The autosampler boasts ultra-low carryover, less than 0.0003% (under specified conditions). Its ultra-fast injection cycle and auto pretreatment functions also contribute to more efficient analysis. The optional dual-injection system consists of two separate injection ports and flow lines, enabling different analyses to be carried out simultaneously.

Mobile Phase Monitor
MPM-40 (Optional)

The monitoring device can be placed in the reservoir tray. The volume of liquid remaining in each mobile phase bottle is measured in real time and can be checked from a PC or mobile device. Before a batch analysis is started, the amount of mobile phase required is calculated and a warning is displayed if the amount remaining is insufficient.

System Controller
SCL-40, CBM-40/40lite

The SCL-40 system controller features a touch panel and allows the user to control the instrument and carry out analysis preparation directly without the need for a PC. A graphical UI makes the controller easy to use.

Degassing Unit
DGU-403/405

3-channel and 5-channel types available. Since the degassing unit is built into the LC-40B X3 pump, a separate unit is not required.

Column Oven
CTO-40 series

The circulation oven has a slim 130 mm model (maximum temperature: 85°C) and a standard 260 mm model (maximum temperature: 100°C). Both are able to accommodate a 300 mm column and have connection ports for CMD or mixer ID recognition. Active preheater tubing is available as an option.

Plate Changer

The installation area has been greatly reduced to 170 mm. It is possible to load up to 7 racks of 1.5 mL vials or 14 microtiter plates. Up to 3 plate changers can be connected, allowing up to 44 MTPs with up to 16896 samples to be loaded at once (using 384-well MTPs).

Fig. 9 Standard system configuration of Nexera HPLC/UHPLC system

	Solvent delivery unit	Autosampler	Switching valve	Others
X3 model (1300 bar)	• LC-40D X3 • LC-40B X3 (binary pump)	• SIL-40C X3 • SIL-30ACMP	• FCV-0206H3 • FCV-0607H3 (up to 1300 bar)	• System controller (SCL-40/CBM-40/CBM-40lite) • Low-pressure gradient unit • Gradient mixer • Solvent switching valve (valve for internal installation/FCV-11AL/11ALS) • Column oven (CTO-40S/CTO-40C) • Plate Changer • Degassing unit (DGU-403/405) • LC detector (SPD-40/40V/M40/M30A, RF-20Axl etc.)
XS model (1050 bar)	• LC-40D XS	• SIL-40C XS		
XSi model (1050 bar)	• LC-40D XSi	• SIL-40C XSi	• FCV-0206H2i • FCV-0607H2i (up to 1050 bar)	
XR model (700 bar)	• LC-40D XR • LC-40B XR (binary pump)	• SIL-40C XR • SIL-40 XR (without sample cooler)	• FCV-0206H • FCV-0607H (up to 800 bar)	
lite model (440 bar)	• LC-40D	• SIL-40C • SIL-40 (without sample cooler)	• FCV-0206 • FCV-0607 (up to 440 bar)	

Fig. 10 Nexera HPLC and UHPLC unit lineup

4. Conclusions

Many factors play a role in the development of a high-throughput LC/MS assay. Prominent among these factors is the HPLC components and systems that make up the front-end configuration. Utilizing the best solvent delivery pumps, autosamplers, and other components will result in more reliable quantitative values and higher throughput. The Shimadzu Nexera HPLC/UHPLC system meets these criteria with no exceptions. Additionally, the system can be configured in multiple ways, making it suitable for a wide range of LC/MS analysis.

Coupled with LabSolutions software and a Shimadzu triple quadrupole LC/MS/MS, the Shimadzu solutions for LC/MS analysis are unmatched. Of course, we realize that purchasing a mass spectrometer requires a significant investment that cannot be changed easily. Therefore, Shimadzu works hard to benefit the entire scientific community by making our instrument control available to any and all Mass Spec vendors so that our UHPLC systems can be used to their full potential. Currently, our UHPLC can be controlled in Analyst and Sciex OS (Sciex), Xcalibur (Thermo Fisher Scientific), and Hyster (Bruker). For LC purposes only, Nexera can be controlled by Empower (Waters), Chromeleon (Thermo Fisher Scientific), and OpenLab (Agilent).

References

[1] Method Scouting System brochure (C190-E216)
[2] Nexera Mikros brochure (C146-E350)
[3] C. Paul, F. Steiner, and M. W. Dong, HPLC Autosamplers: Perspectives, Principles, and Practices, *LCGC North Am.* 37(8), 514–529, 2019.
[4] Nexera series brochure (C196-E096)
[5] K. Watanabe, C. Campbell *et. al.*, Which Key UHPLC Characteristics Are Required for a High Throughput LC/MS Assay?, Shimadzu Technical Report (C190-E283)
[6] Nexera series specification sheet (C196-E095)

First Edition: March, 2022

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3655-01206-PDFNS