ABRF 2005

Performance and Advantages of a Direct-Flow Nanoscale HPLC System Optimized for Columns Packed with Particles Less Than 2 Microns

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Introduction

Due to higher MS sensitivity, nano– and capillary-scale chromatography (column diameters requiring flow rates < 5,000 nL/min) are often the methods of choice for qualitative and quantitative analysis of complex proteomics samples. Here we present results for the Waters nanoACQUITY UPLC™ system, a new nanoscale HPLC platform designed with the following features and advantages:

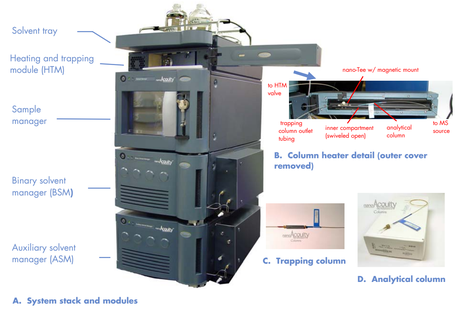
- Direct flow without splitting
- Based on ACQUITY UPLC™ platform
- Accurate gradient delivery yielding excellent retention time reproducibility
- Binary high-pressure mixing pump with wide dynamic flow range
- Operation at elevated pressures for nanocolumns packed with particles less than 2 microns
- Heating Trapping Module (HTM) with simplified "forward flush" sample trapping scheme and integrated column heater
- Consumables that deliver consistent performance and are easily replaced
- Nanocolumns packed with ACQUITY 1.7 µm bridged-ethyl hybrid (BEH) particles that provide higher separation efficiency
- Variable flow chromatography ("peak parking") capability
- Dedicated auxiliary pump for providing stable flow of "lockmass" solution to reference sprayer of Q-Tof NanoLockSpray source

Methods

- HPLC: Waters nanoACQUITY UPLC System (Figure 2A,B)
- Mass Spectrometer: QTof™ Micro or Q-Tof™ API-US (W optics mode, R=17,500) with NanoLockSpray source and custom nanoflow sprayer equipped with PicoTip (New Objective) emitter w/10 µm id tip
- Solvents A: 0.1% formic acid, B: acetonitrile w/ 0.1% formic acid
- Gradients: typically 3%-60% B in 30 min or 60 min
- Gradient delivery flow rates: 100, 250, or 300 nL/min
- Sample trapping: 3% B at 5 µL/min (from binary pump) for 3 minutes
- Columns trap: Waters nanoACQUITY UPLC™ 5 µm Symmetry® C18 180 µm x 20 mm (Figure 2C), analytical: Waters nanoACQUITY UPLC 1.7µm BEH C18 75 µm x 100 mm (Figure 2D)
- Column temperatures: trap at ambient, analytical at 35 °C
- Samples: Waters MassPREP™ Protein Digest standards or digests from protein samples (Sigma) prepared in-house at 5—200 fmol/µL
- Nanolockspray reference: 300 fmol/µL glufibrinopeptide (Sigma) in 25% acetonitrile w/ 0.1% formic acid delivered from Auxiliary Solvent Manager (ASM) at 1 µL/min, reference channel sampled every 30 sec during LC/MS acquisition

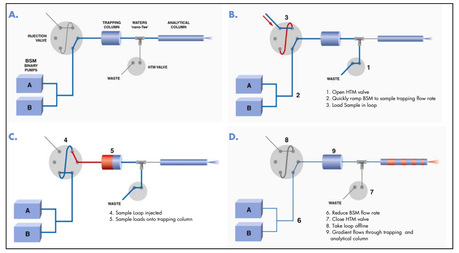
Figure 1. Nanocolumns packed with bridged-ethyl hybrid particles

Figure 2. nanoACQUITY UPLC system and consumables



Components of the nanoACQUITY UPLC system (Figure 2A) are specifically designed for operating at pressures up to 5,000 psi or higher. The column heater (Figure 2B) of the HTM is mounted on a pivot which positions the column outlet close to the MS source to minimize delay volume. All fused-silica tubing in the sample path is preassembled with PEEK protective cladding, fittings, and pre-cut polished ends to minimize dead volume. The analytical column (Figure 2D) can be replaced using a single-piece PEEK finger tight fitting. The Sample manager is completely enclosed, holds 2 microtiter plates (or 2 plates with 48 1.5 mL sample vials), and can maintain samples at 4 °C to 40 °C. The needle and sample loop can be flushed with both a strong and weak solvent wash, eliminating sample carryover, which is advantageous for quantitative proteomics applications.

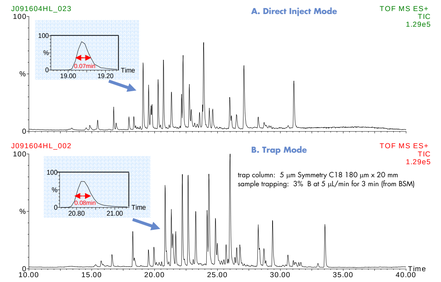
Figure 3. Simplified sample loading/trapping scheme



The wide dynamic flow range of the BSM makes it possible to configure the system for sample loading/trapping using the scheme shown in Figure 3. This greatly simplifies system fluidics, allows for robust operation in a "forward flush" mode, and eliminates the need for an extra sample loading pump. Consumable design (packing process, fritting at both ends, etc.) is critical for withstanding the rigors of pressurization/depressurization while yielding consistent performance over hundreds of injection cycles.

Results and Discussion

Figure 4. Chromatographic performance: direct inject vs. trapping



For sample loading/trapping combined with gradient elution, column dimensions and chemistries are carefully chosen so that robust operation can be achieved without compromising chromatographic fidelity. Figure 4 compares LC/MS traces for enolase digest acquired using the same analytical 1.7 µm BEH column for direct inject vs. trap modes. The data demonstrates that there is no significant loss in chromatographic resolution of the peptides with the addition of a trap column. Furthermore, the data shows that sample losses are minimized.

Figure 5. Performance at 100 nL/min, 60 min gradient

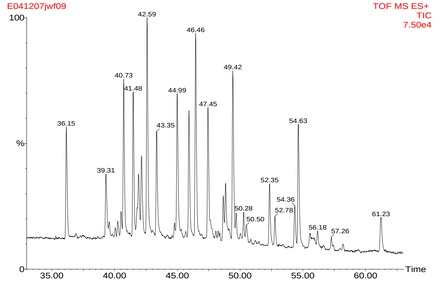
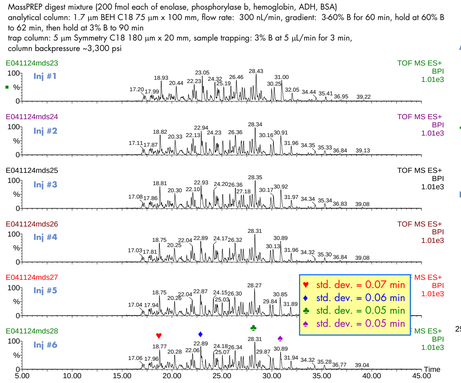


Figure 5 demonstrates that good chromatographic performance can be achieved with the nanoACQUITY system in trapping mode, even at relatively low flow rates with shallow gradients. Peak widths for this particular separation of enolase digest range from 6 to 9 sec across the gradient.

Figure 6. LC/MS Reproducibility at 300 nL/min, n = 6



Separation efficiency, reproducible gradient delivery, and consistent MS response are necessary for quantitative peptide analysis when comparing proteins expressed in animal or patient samples. Figure 6 shows traces from six consecutive injections of a 5 protein digest, along with retention time std. dev. of four peptides. The data set demonstrates that the nanoACQUITY/Q-Tof yields good retention time reproducibility (std. dev. < 0.1 min) and reproducible MS response over an extended period of time. The 1.7 µm BEH column provides greater separation efficiency for peptides compared to columns packed with conventional particles (3 to 5 µm).

Figure 7. Flow chart for automated variable flow chromatography with Data Directed Analysis (DDA)

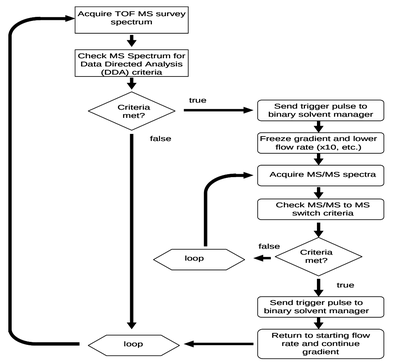


Figure 8. LC/MS/MS with variable flow chromatography

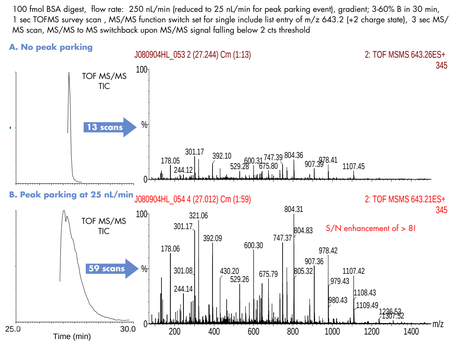


Figure 7 shows a flow chart representing the system decision making process and series of events which occur during a DDA run coupled with variable flow chromatography. Event out pulses from the Q-Tof communicate to the BSM when to trigger a peak parking event, and when to return to normal operation. To demonstrate advantages of peak parking, 100 fmol of BSA digest was analyzed both with and without peak parking enabled for a single peptide mass entered into the DDA include list (Figure 8). Without peak parking, MS/MS measurement time of the peptide is limited (~18 sec of useful MS/MS scans; S/N ≥ 3). However, the ability to rapidly reduce flow and "spread out" the chromatographic peak during the peak parking event results in a considerable increases in MS/MS measurement time (~126 sec of useful MS/MS scans; S/N ≥ 3) and significant improvement in S/N for the peptide fragment spectrum. Furthermore, peak parking does not adversely affect chromatographic peak shape of peptides eluting after the system resumes normal gradient elution.

Conclusions

Features and advantages of the nanoACQUITY UPLC coupled with a Q-Tof instrument demonstrated here include:

- Direct nanoflow delivery without splitting
- Highly reproducible retention times and MS response over long measurement times
- Binary high pressure pump with wide dynamic flow range
  - -can support analytical column flow rates from 100 to 5000 nL/min
  - -simplified sample loading/trapping configuration
  - -variable flow chromatography for improving MS/MS sensitivity
- High pressure capabilities enabling the use of nanocolumns packed with particles < 2 µm
- Columns packed with 1.7 µm bridged-ethyl hybrid particles
  - -higher efficiency
  - -higher resolution
  - -greater selectivity

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