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A Robust Method for Packing High Resolution C18 RP-nano-HPLC Columns

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Abstract

The high complexity prevalent in biological samples requires chromatographic separations with high sensitivity and resolution to be effectively analyzed. Here we introduce a robust, reproducible and inexpensive protocol for preparation of a nano-flow reversed phase high performance liquid chromatography (RP-HPLC) columns for on-line separation of analytical peptides before introduction into and detection by a mass-spectrometer in traditional bottom-up proteomics workflows. Depending on the goal of the experiment and the chemical properties of the analytes being separated, optimal column parameters may differ in their internal or outer diameters, length, particle size, pore size, chemistry of stationary phase particles, and the presence or absence of an integrated electrospray emitter at the tip. An in-house column packing system not only enables the rapid fabrication of columns with the desired properties but also dramatically reduces the cost of the process. The optimized protocol for packing a C18 AQ (aqueous) fused silica column discussed here is compatible with a wide range of liquid chromatographic instruments for achieving effective separation of analytes.

Introduction

HPLC columns have contributed immensely to productivity in the fields of pharmaceutical, medical and environmental research[1,2,3,4]. Having access to high-quality chromatography columns is a pivotal step in the fractionation of complex analytes. In shotgun proteomics, high analytical sensitivity is routinely accomplished by coupling electrospray ionization (ESI) mass spectrometry (MS) to nanoflow chromatography[5,6,7,8]. The efficient separation of thousands of peptides is paramount in this application as it allows the mass spectrometer to identify and quantify analytes with high sensitivity and resolution.

The field of column packing for mass-spectrometric applications has witnessed tremendous growth in recent years with advances in the understanding of fundamental column packing principles related to stationary phase morphology, solvent-particle interactions and hardware design, making possible the detailed characterization of a wide range of biomolecules in complex biological

settings[9,10,11,12,13,14]. Efforts highlighting practical considerations in packing analytical columns for LC-MS purposes have paved the way for proteomic laboratories to develop in-house packing systems to meet their specific interests with the promise of maximum performance[15,16,17,18].

Nanospray columns with internal diameters in the range of 50-150 µm and tapered ends are well-suited for the purpose of electrospray ionization. In the field of shotgun proteomics, separations are typically carried out using a solvent gradient flowing through a packed non-polar stationary phase, most commonly hydrophobic carbon chain bonded silica (C8-C30) with particle sizes varying between 1.7 to 3.5 µm[19,20,21,22]. The eluting analytes are emitted through an ESI emitter integrated within the column, which ensures soft ionization of solution phase analytes to gaseous ions. Coupling LC columns with ESI-MS has significantly advanced the application of tandem mass spectrometry to proteomic strategies in biomedical sciences.

LC columns with narrow inner diameters result in narrower chromatographic peaks and higher sensitivity relative to higher bore, microflow columns and hence are particularly advantageous with proteomic workflows. Although commercially available pre-packed LC columns are attractive options due to their convenience and ease-of-use, they can be prohibitively expensive and less flexible than in-house options. The goal of this work is to describe a technically simple and low-cost slurry packing approach to prepare narrow inner diameter reversed phase HPLC columns using fused-silica capillaries and an in-house built pressure bomb system for proteomic applications.

Protocol

1. Preparation of the capillary tip

1. Using a ceramic cleaving stone, cut about 60-70 cm of a polyimide coated fused silica capillary with an internal diameter (ID) of 75 µm and an outer diameter (OD) of 360 µm.
2. Hold the capillary with your hands at approximately the middle of its length, leaving a 4-5 cm gap between fingers and heat the area in the gap while rotating it over the flame of an alcohol lamp. Polish the burnt area clean using a methanol-soaked low lint tissue until the glass is visible. (**Figure 1**).
   NOTE: 4-5 cm of the polyimide coated fused-silica capillary needs to be exposed and polished before it can be loaded into the laser puller.
3. In order to pull a cone-shaped emitter for ESI, use a laser tip puller with special program settings of a heat value of 300 (equivalent of 2 Watts of laser power), a velocity of 10 (equivalent of 0.25 mm/s) and a delay of 180 milliseconds in order to obtain an ~1-5 µm diameter tip (**Figure 2** and **Figure 3A**). Load the polished part of the capillary into the laser puller and press pull, resulting in two empty capillary columns ready to be packed.
   NOTE: Frequent inspection of the tip at any step is done using a microscope. Settings will likely differ between laser pullers and will need to be determined empirically.

2. Polymerization/etching of the tip

1. In order to retain the stationary phase particles in the capillary tube, prepare a porous frit from a mixture of two potassium silicate solutions and formamide. Make the

solution immediately before use in a 2 mL tube and mix using a vortex.

1. Mix two potassium silicate solutions with SiO_2/K_2O ratio of 2.50 (w/w), and 1.65 (w/w) and formamide in a ratio of 1:3:1 (v/v/v), respectively. For example, mix 100 µL of potassium silicate with SiO_2/K_2O ratio of 2.50 (w/w), 300 µL potassium silicate with SiO_2/K_2O ratio of 1.65 (w/w)), and 100 µL of formamide followed by vortexing. This solution will polymerize when heated and produce a porous frit.

2. Immerse the laser-pulled capillary tip in the clear mother liquor of the frit suspension (not the precipitate) for about 10-20 seconds, allowing it to penetrate about 5 mm into the tip by capillary action. Depending on the width of the tip, keeping the tip immersed longer in the frit solution may be required. Generally, the dipping time is shorter if the tip is wider since the solution can enter the tip faster.

3. Place a hot soldering iron (set at 350 °F) along the tip to initiate the polymerization of the frit solution while inspecting the capillary under the microscope. Please refer to **Figure 3** to see the pulled tip before (**Figure 3A**) and after polymerization (**Figure 3B**).

4. To prevent blockage of the emitter after frit polymerization, immerse the column tip in a 50% hydrofluoric acid (HF) solution for 5 minutes (setup is illustrated in **Figure 4**). HF etching also imparts a flat cone-shape geometry to the column increasing its longevity. After HF etching, ensure the column's tip is thoroughly washed, first with HF neutralizer and then generously with water to safeguard against contact with the acid.

   CAUTION: HF is a highly dangerous and corrosive chemical. Extreme caution is advised while handling to prevent exposure. Ensure that HF is handled at all times with appropriate protection inside a fume hood that is identified with a sign stating "Danger! Acute Toxins".

   1. For safety, use both regular and anti-flammable lab coats, in addition to double layers of nitrile and neoprene gloves, while handling HF.

      NOTE: It is optional to frit capillary tips; however, it renders the column significantly more resistant to clogging and increases its longevity. Empty fused silica capillary columns with an integrated electrospray emitter are commercially available and can be used to replace steps 1 and 2 if needed.

3. Preparation of stationary phase

1. Suspend 25-50 mg of fully porous ReproSil-Pur 120 C18-AQ silica particles with 1.9 µm particle size and 120 Å pore size in 300 µL of methanol. Pipet up and down for about 20 times to ensure homogenous mixture of the slurry particles in methanol.

2. Place the tube containing slurry suspension inside the pressure cell chamber of an in-house built column-packing bomb system, which in turn is set up atop a magnetic stirrer allowing the slurry particles to remain in suspension. Connect the bomb to helium tank (<1500 psi) which operates at constant pressure to avoid disrupting the HPLC slurry during packing. (Schematic represented in **Figure 5**).

3. Secure the lid of the pressure cell by tightening it in place with the bolts as shown in **Figure 6A**.

   NOTE: It is important to appreciate the difference in operation of an HPLC pump and HPLC packing bomb. While the former is designed to operate at constant flow rate regardless of the back pressure exerted by the stationary phase in the column, HPLC packing

pressure systems operate at a constant pressure to ensure unbroken and dense packing of the stationary phase particles throughout the length of the capillary column.

4. Packing the column with stationary phase

1. Thread the column bottom first (un-fritted open end) through the finger-tight fitting on the top of the pressure bomb such that the column's tip points upwards. Push the column through until it touches the base of vial containing slurry and retract it 1-2 mm above the base. Tighten the fingertight fitting to secure the column in position.
2. Connect the packing bomb to a helium gas tank (recommended pressure < 1500 psi) and turn it on to a pressure of ~ 1300 psi. The helium gas enters the pressure cell housing the vial containing slurry through a three-way valve. Open the valve by slowly turning it 180° clockwise.
   NOTE: As soon as the helium gas begins to flow inside the chamber, it pushes the slurry from the tube into the capillary. As the slurry passes through the column, the particles are retained in the capillary while the solvent forms a liquid droplet at the tip of the column (**Figure 6B**).
   1. If the formation of liquid droplet on the column tip is delayed, quickly flame the tip to ensure it is open. At this stage, a light source placed behind the column can help observe the progress of the packing process (**Figure 7**). For a column with an ID of 75 µm, it typically takes ~30-60 minutes to pack a length of about 30 cm.
   2. If the flow of the slurry through the column stops or slows down, hold the capillary down tightly above the fingertight fitting of the packing bomb and slightly loosen it by turning about quarter of a turn (hissing sound of depressurization may be heard).
      NOTE: This allows for the column to be repositioned without the need to completely depressurize the bomb.
   3. Now gently reposition the column by moving it up and down and then retighten the fingertight fitting making sure that the column is not touching the base of the vial. This ensures a uniform flow of the slurry through the capillary at all times.
   4. If the aforementioned measure fails to resume packing, close the valve, vent the packing bomb, unscrew the lid and inspect the slurry to make sure there are no precipitates. It is also possible that the column end is clogged with solid stationary phase particles in which case, cutting a small length from the back of the capillary may help resume flow.
3. Pack a few centimeters longer than the desired length at the end of the column to ensure complete packing of the stationary particles and minimizing the possibility of getting helium bubbles into the packed column.

5. Finishing the column and making the back-frit

1. Once the desired packing length is achieved, close the main valve of the helium gas tank, and wait for a minimum of 15 minutes to ensure uniform packing of the column and to allow the system to self-depressurize.
   NOTE: In order to avoid introduction of He bubbles as a result of empty fused silica at the end of the column, a longer packing length is recommended. It is critical that packing process be continued even after the portion of the column visible to eye has been packed to account for the length of the capillary inside the pressure chamber. Additionally, a longer packing length is recommended

in cases when repeated repositioning of the column is required as this results in less air bubbles and higher packing efficiency.

2. Gently depressurize the chamber by rotating the valve 180 degrees anti-clockwise to its original position. Hold the column tightly, unscrew the fingertight fitting and remove the column gradually. This step needs to be done very gently to avoid introducing air bubbles.

3. Cut the end of the column to a length of 25.5 cm. While inspecting under the microscope, use a hot soldering iron at 350 °F to remove a length of 0.5 cm of the slurry from the back end of the column.

    1. Dip the back end of the column into the leftover frit solution from step 2.1 for about 10 seconds and polymerize it by placing a hot soldering iron along the back of the column while inspecting it under the microscope. The back frit ensures that the ReproSil-Pur 120 C18-AQ particles remain in the column and prevents backflow during chromatographic washes.
    NOTE: The frit in the back of the column is also optional but makes the column more robust as was also noted for the front frit in step 2.

Representative Results

To evaluate the performance of the columns, 750 ng of tryptic peptide digests prepared from whole cell lysates of HEK293 cells were fractionated online using a 25 cm long, 75 µm ID fused-silica capillary packed in-house with bulk ReproSil-Pur 120 C18-AQ particles as described in the protocol. Prior to sample loading, the column was washed using 6 µL of a mixture of acetonitrile, isopropanol and H_2O in a ratio of 6:2:2 and pre-equilibrated with buffer A (Buffer A: water with 3% DMSO). The tryptic peptide digest was analyzed using a 70-minute reverse phase gradient. The solvent gradient began with buffer B (Buffer B: acetonitrile with 3% DMSO and 0.1% formic acid) increasing from 0 to 6% over 4 minutes at a flow rate of 400 nL/min. The flow rate was then reduced to 200 nL/min and a linear gradient starting at 6-25% buffer B was applied to the column over the course of next 58 minutes. Buffer B was further increased to 25-32% for a period of 8 minutes, followed by a rapid ramp-up to 85% for washing the column. The gradient composition was dropped to 1% buffer B for the remaining 4 minutes of chromatographic separation.

Peptides were ionized using a distal 2.2 kV spray voltage with an ion transfer capillary temperature of 275 °C and analyzed by tandem mass spectrometry (MS/MS) on an orbitrap mass spectrometer.

Data were acquired by a Data-Dependent Acquisition (DDA) method comprised of a full MS1 scan resolution of 120,000 FWHMat m/z 200 followed by sequential MS2 scans (Resolution = 15,000 FWHM) obtained using higher-energy collisional dissociation (HCD) to induce peptide fragmentation.

In this study, we used the Integrated Proteomics pipeline 2 to generate peptide and protein identifications. MS2 spectra were searched using the ProLuCID algorithm against the EMBL Human reference proteome (UP000005640 9606) followed by filtering by DTASelect using a decoy database-estimated false discovery rate of < 1%.

We evaluated column performance using a series of columns made at different points in time. The extracted chromatograms of 750 ng of HEK293 cell tryptic digest in 70-minute gradient runs are depicted in **Figure 8**. Retention time alignment, peak width, and peak intensity are reproducible across columns regardless of when the column was prepared suggesting the reproducibility of the protocol. As illustrated

in **Figure 9**, the columns produced using our approach also demonstrate consistent performance in LC-MS/MS runs in terms of the number of peptide and protein identifications.

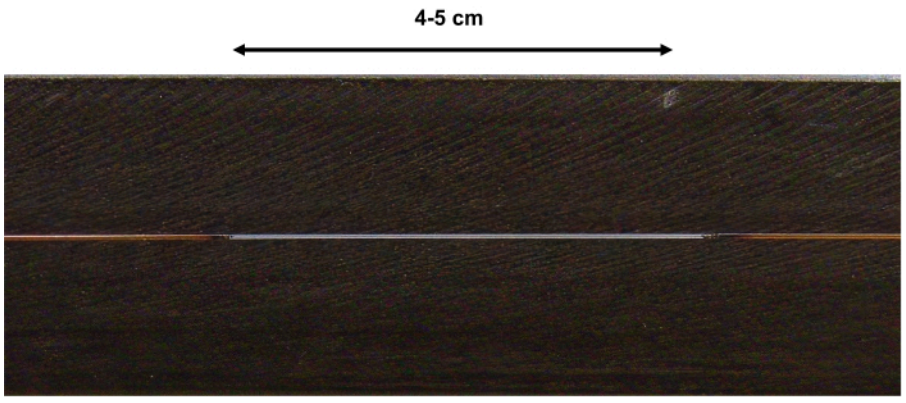

Figure 1. Representative image of fused-silica capillary portion with removed polyimide coating. Please click here to view a larger version of this figure.

Figure 2. Representative image illustrating loading of the column on the laser puller. Note how the polished portion of the capillary is aligned inside the laser puller. Please click here to view a larger version of this figure.

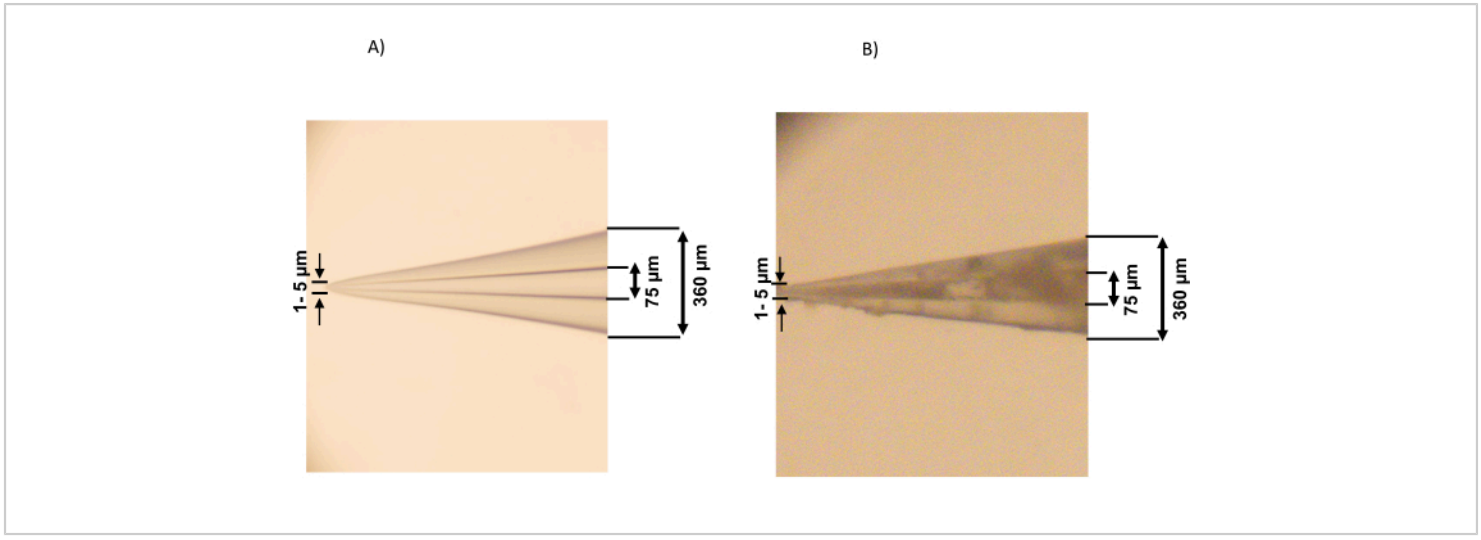

Figure 3. Comparison of pulled column tips with or without a frit. (**A**) A microscopic view of laser pulled tip before initiating frit polymerization. (**B**) A microscopic view of the laser pulled tip after frit polymerization. Please click here to view a larger version of this figure.

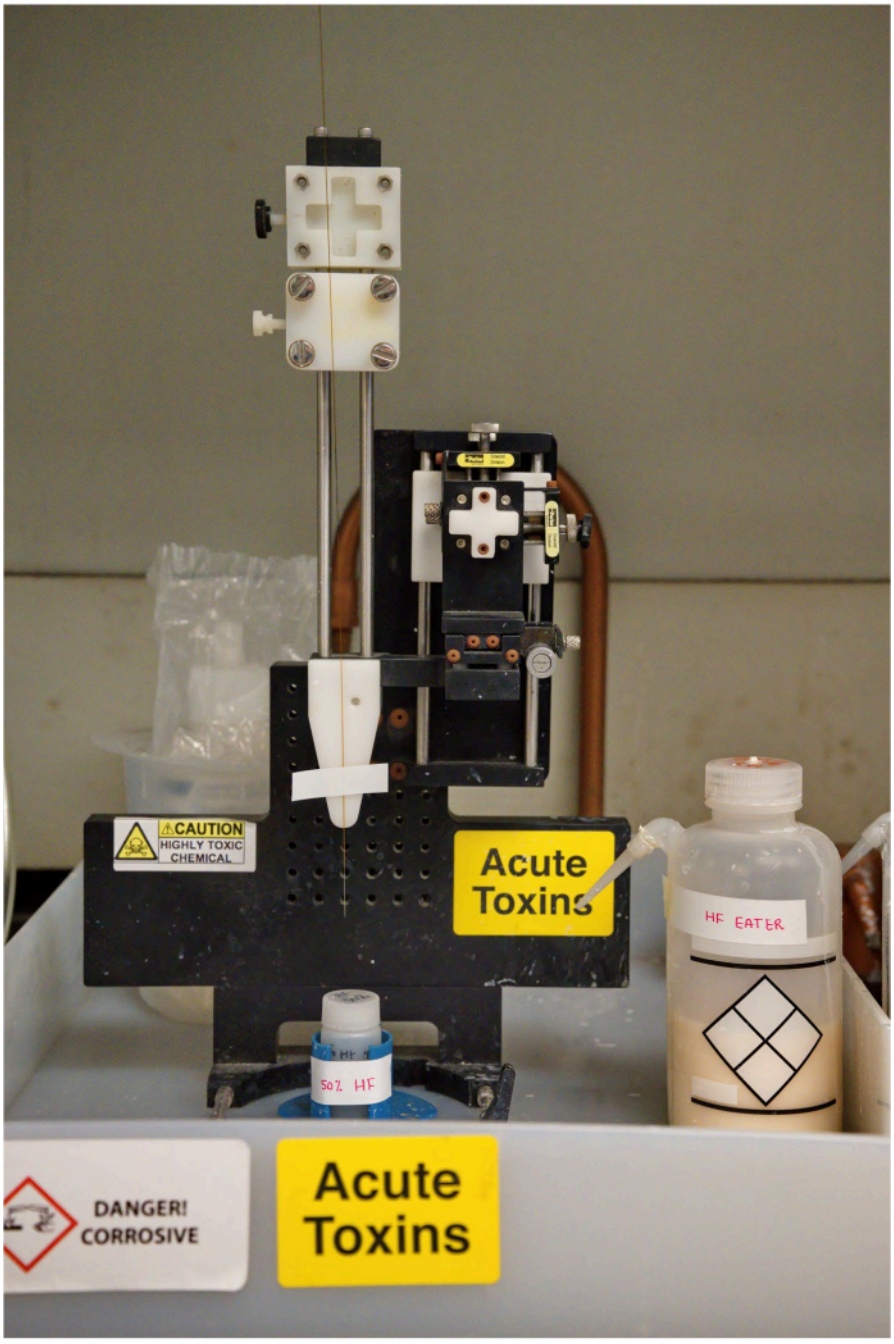

Figure 4. HF Etching station. The HF etching station for immersing the emitter after polymerization of the frit. Here, HF neutralizer solution is labeled as "HF EATER" which is used to wash the column tip after HF etching to safeguard against acid contamination. Please click here to view a larger version of this figure.

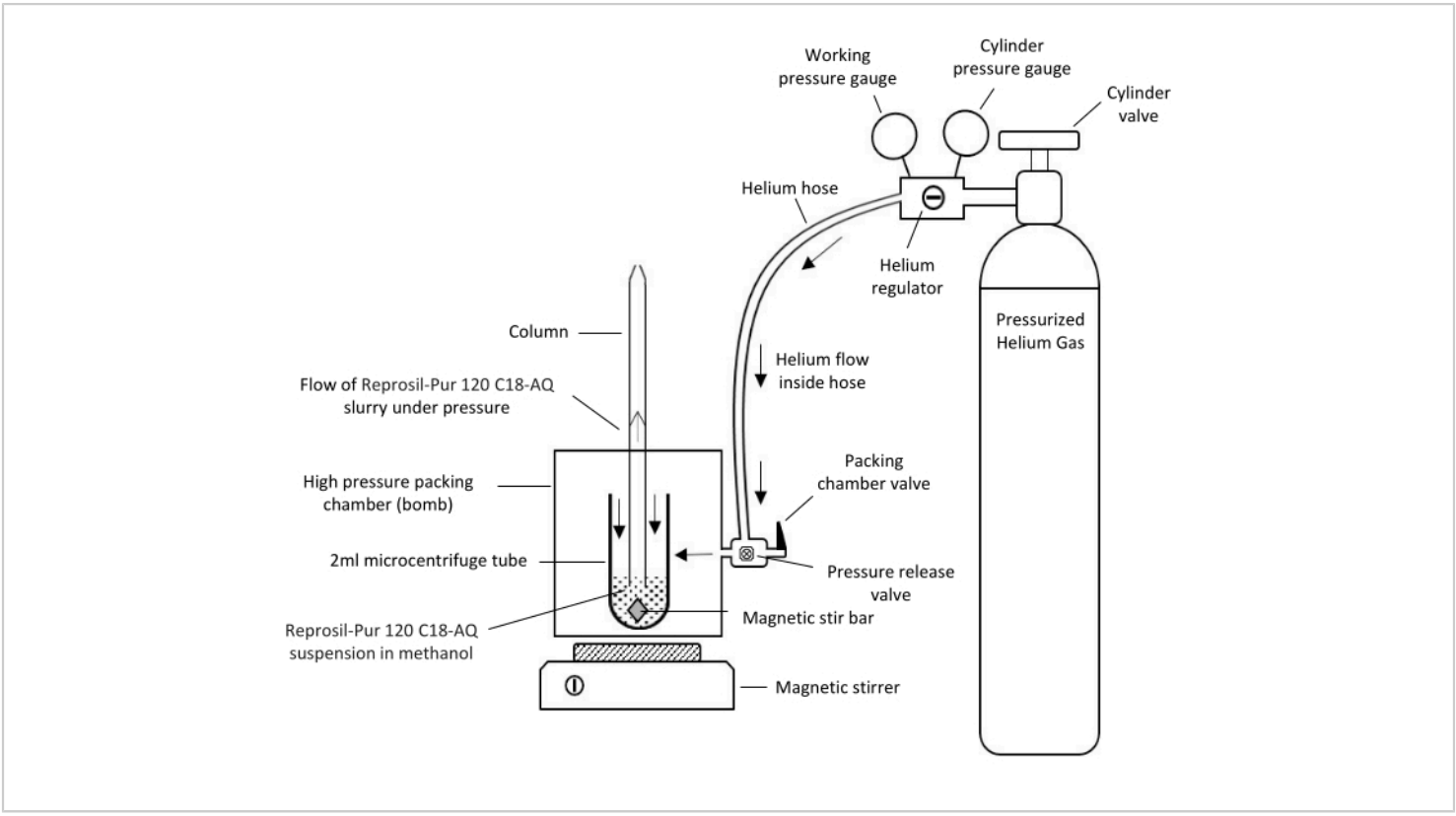

Figure 5. Schematic of column packing hardware. The hardware system basically consists of pressurized helium gas that is connected through a three-way valve to a high pressure packing chamber housing the vial containing slurry and column with its tip pointing upwards. Please click here to view a larger version of this figure.

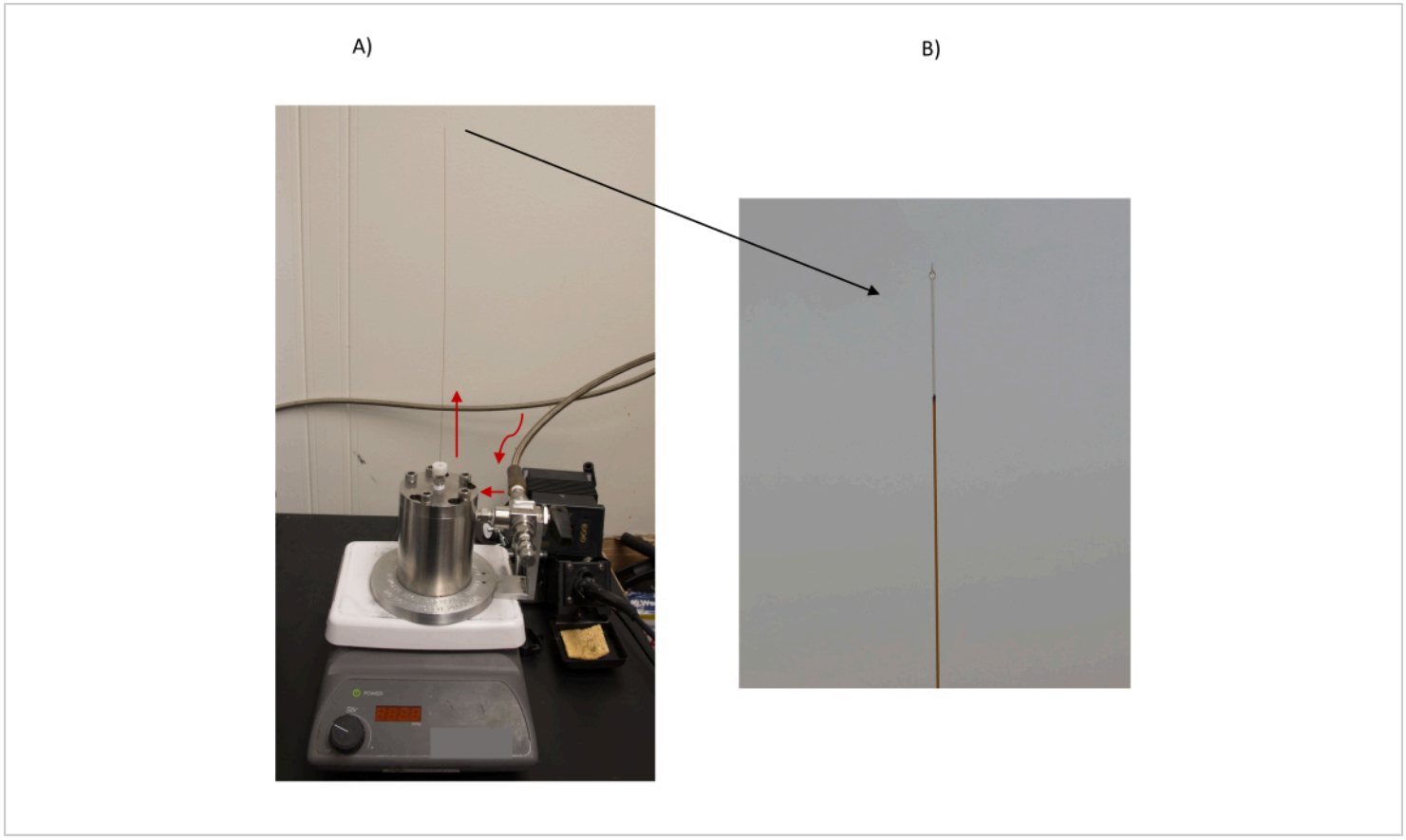

Figure 6. Column packing station. (**A**) An overall view of the column set up on the packing bomb. The red arrows indicate the direction of the flow of helium gas from the helium hose to the 3-way valve and further into the column packing bomb, finally pushing the slurry (upwards) in to the column. (**B**) A close-up view of the column tip during packing on bomb. Note the solvent droplet formed at the tip of the column. Please click here to view a larger version of this figure.

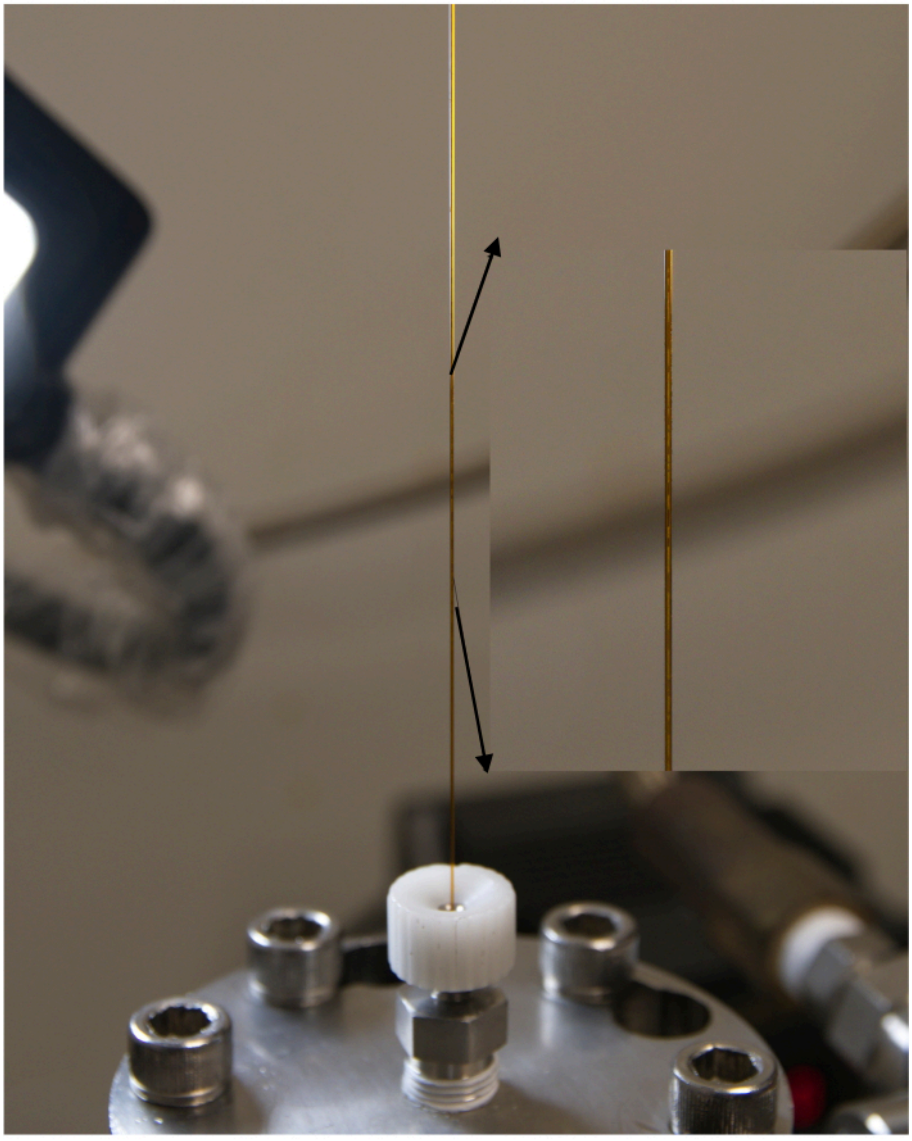

Figure 7. Visualization of slurry movement through the column while packing on bomb. Note the inset image where movement of the slurry from the bottom to the tip of the capillary is seen due to helium gas flow inside the pressurized chamber. Please click here to view a larger version of this figure.

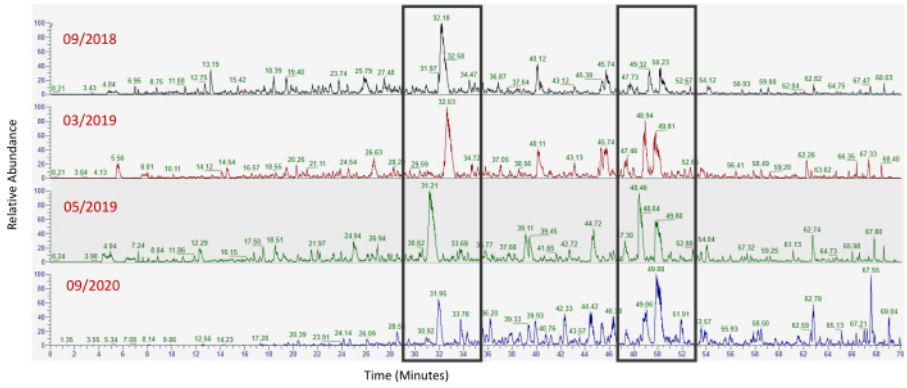

Figure 8. Evaluation of column performance using a series of columns prepared at different points in time. Comparison of the retention time alignment, peak intensity and width in a series of columns packed at different time points over a period of two years in 70 minute LC-MS/MS analysis of HEK293 cell tryptic digest. Please click here to view a larger version of this figure.

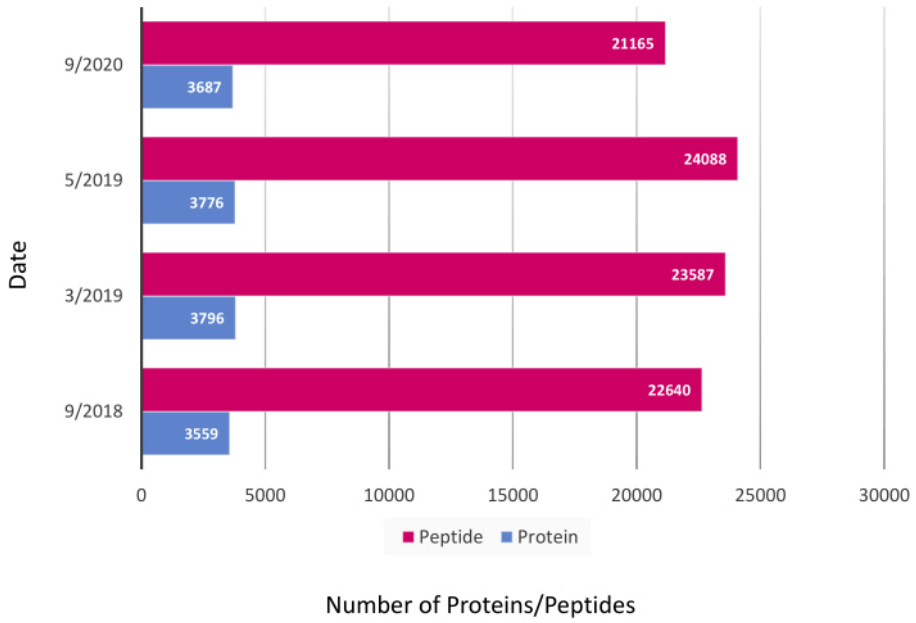

Figure 9. A bar graph representation of number of proteins and peptides identified in a 70 minute LC-MS/MS analysis of HEK293 cell tryptic digest on columns analyzed in Figure 8. Data reflect consistent performance of the columns packed using our approach. Please click here to view a larger version of this figure.

Discussion

Modern proteomic strategies are reliant upon high quality chromatographic separations to effectively analyze complex biological systems. Hence, high-performing and cost-effective nanoflow LC columns are crucial components of a successful tandem mass-spectrometry regime aimed at characterizing thousands of proteins in a single workflow.

In this study we evaluated the performance and reliability of a range of LC columns for LC-MS/MS made using the protocol described above. The performance of these columns prepared over a period of two years was tested by using them for online fractionation of a HEK293 cell tryptic digest followed by analysis using tandem mass spectrometry. As shown in **Figure 8** and **Figure 9**, a comparison of column parameters such as the alignment of chromatographic elution profiles and numbers of peptide and proteins identified is reproducible displaying less than 10% variability between different columns. These results reflect that columns made in different points in time using the protocol described above exhibit consistent performance and robust run-to-run reproducibility.

Taken together, the protocol presented here produces high-quality columns with low column-to-column variation for proteomic applications. Given the easily available raw materials and low-cost needed to adopt this in-house built column-packing approach, it can be swiftly implemented in many LC-MS laboratories for a wide range of MS-based bioanalytical applications. Further, the protocol provides flexibility for custom optimization such as column length, internal diameter, choice of particles and solvent for column packing, which are often guided by the biological questions being pursued.

Disclosures

The authors have nothing to disclose.

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