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Title

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

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Keywords:
Chromatography column, RP-HPLC, stationary phase, nanoflow chromatography, column frit, C18, bottom-up proteomics, Mass Spectrometry, Quantification

Summary:
Here, we present and evaluate a protocol for making low cost reversed phase nano-flow liquid chromatography columns for peptide characterization using LC-MS/MS proteomic workflows.

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[1–4]. 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[5–8]. 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 tostationary phase morphology, solvent-particle interactions and hardware design, making possible the detailed characterization of a wide range of biomolecules in complex biological settings[9–14]. 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[15–18].

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[19-22]. 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**

1.1. 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.

1.2. 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.

1.3. 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

2.1. 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.

2.1.1. 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.

2.2. 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.

2.3. 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**).

2.4. 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".

2.4.1. 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

3.1. 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.

3.2. 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**).

3.3. 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

4.1. 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.

4.2. 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**).

4.2.1. 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**).

NOTE: For a column with an ID of 75 µm, it typically takes ~30-60 minutes to pack a length of about 30 cm.

4.2.2. 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.

4.3. 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.

4.4. 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.

4.4.1. 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

5.1. 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.

5.2. 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.

5.3. 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.

5.3.1. 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 FWHM at 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 (**Table of Materials**) 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.

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.

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.

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.

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.

Figure 6. Column packing station. (**A**) An overall view of the column set up on the packing bomb (**B**) A close-up view of the column tip during packing on bomb. Note the solvent droplet formed at the tip of the column.

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.

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.

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.

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.

Acknowledgments

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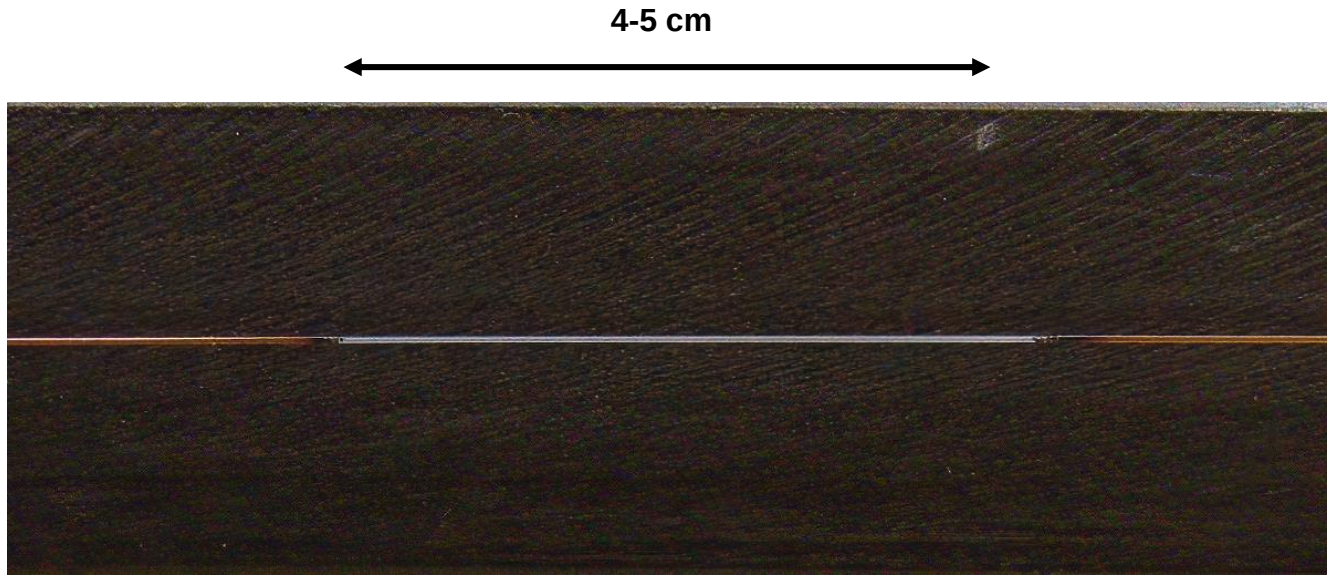

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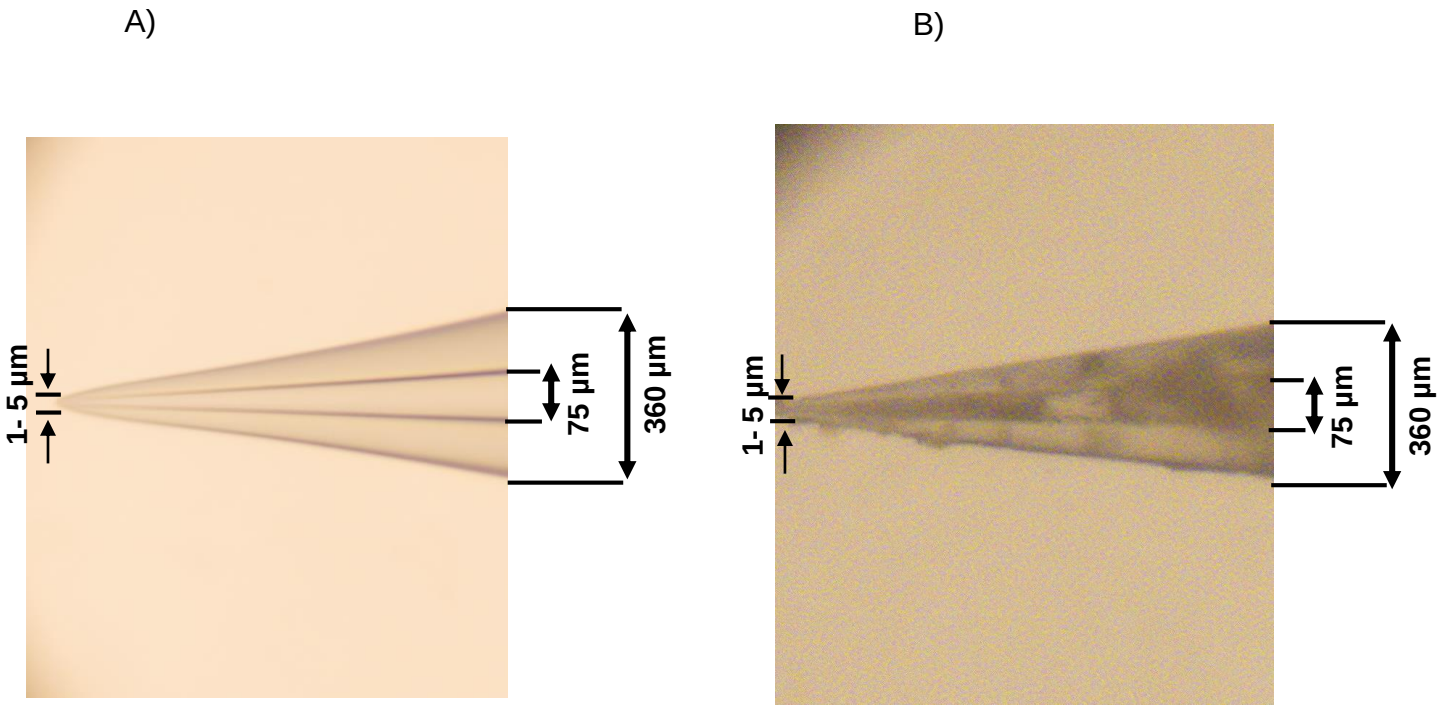

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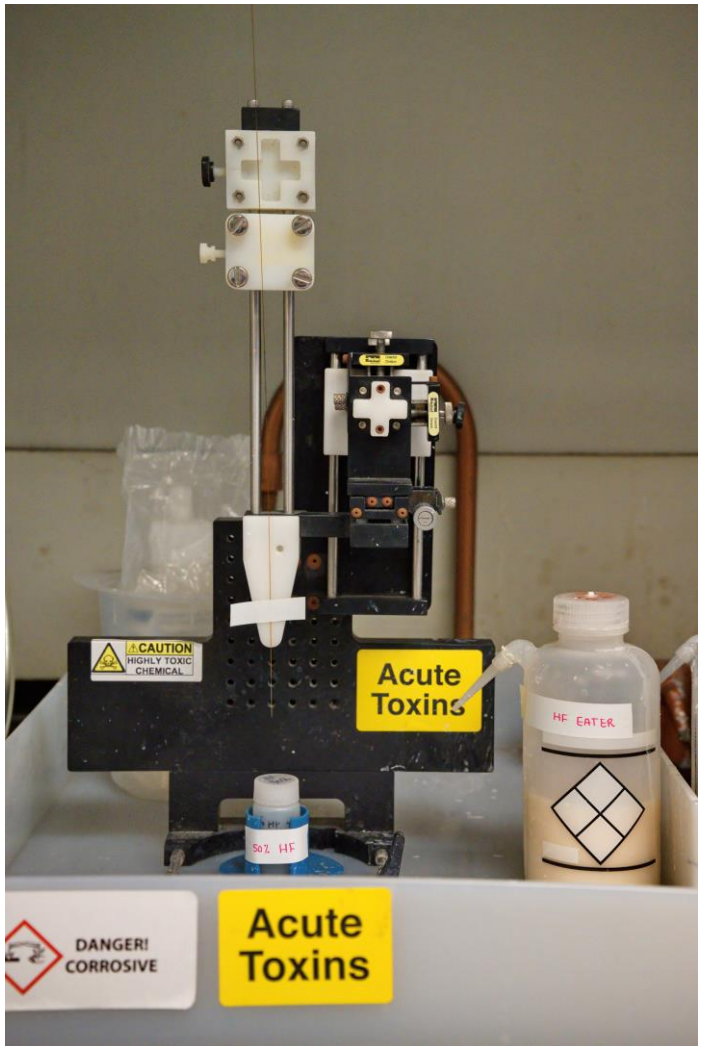

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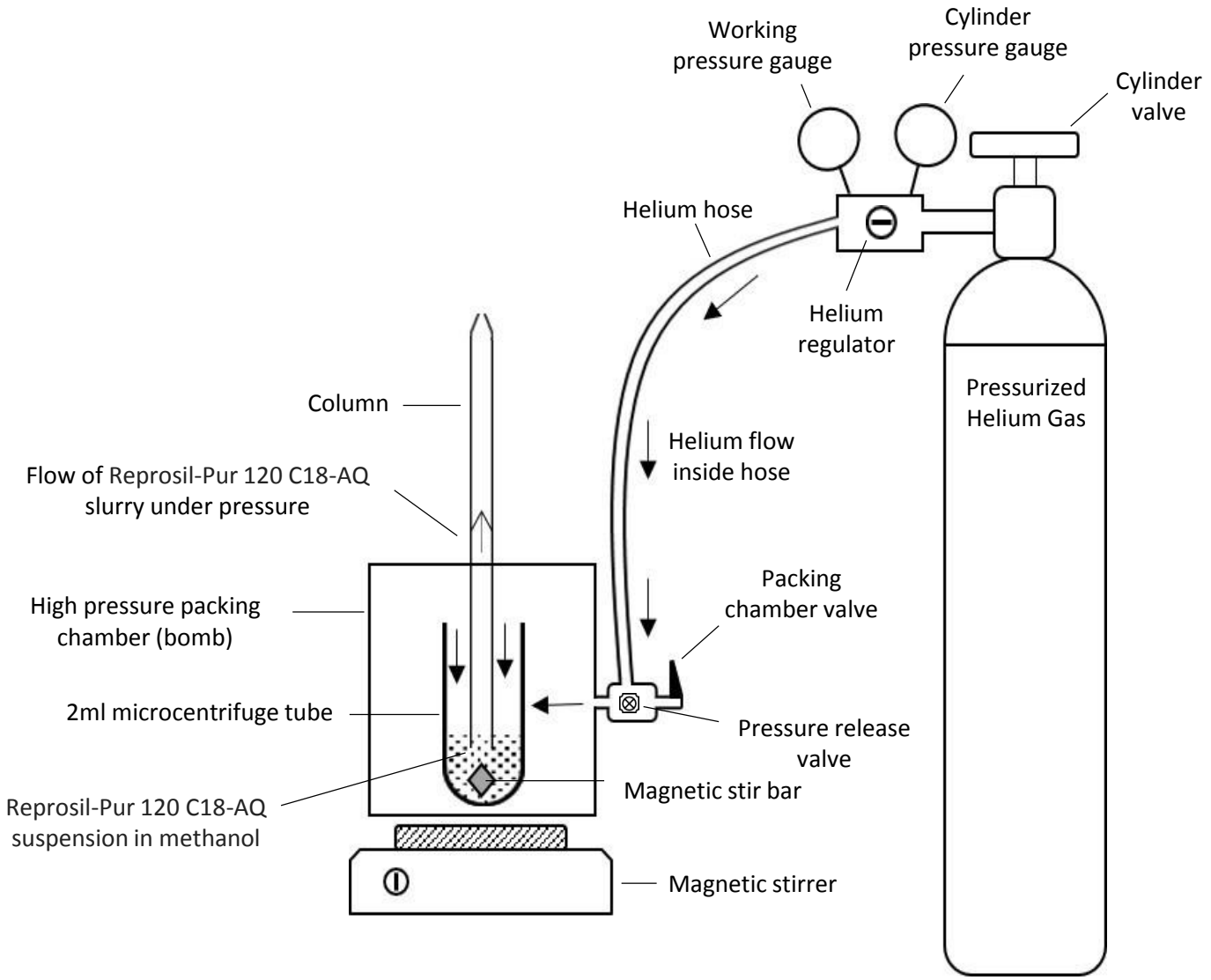

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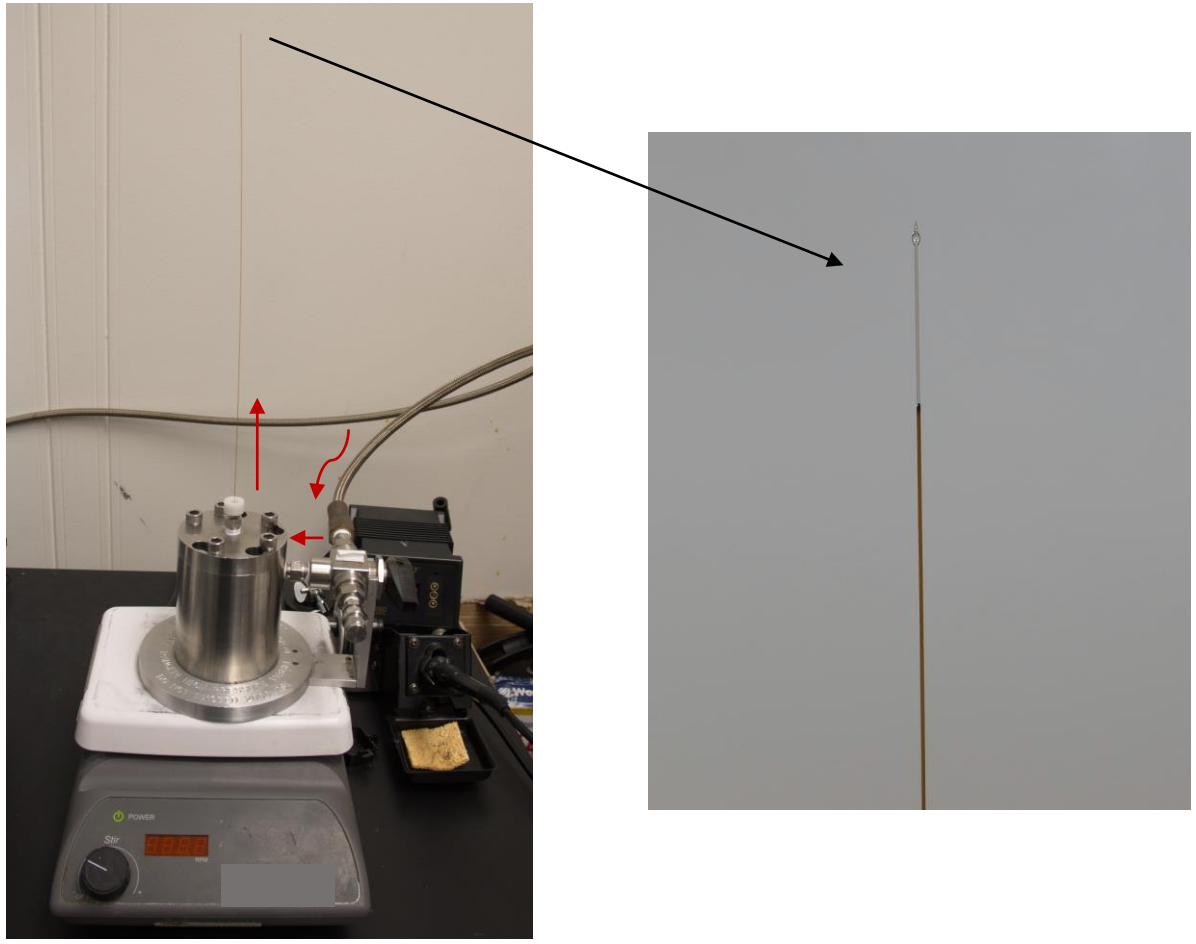
A)

B)



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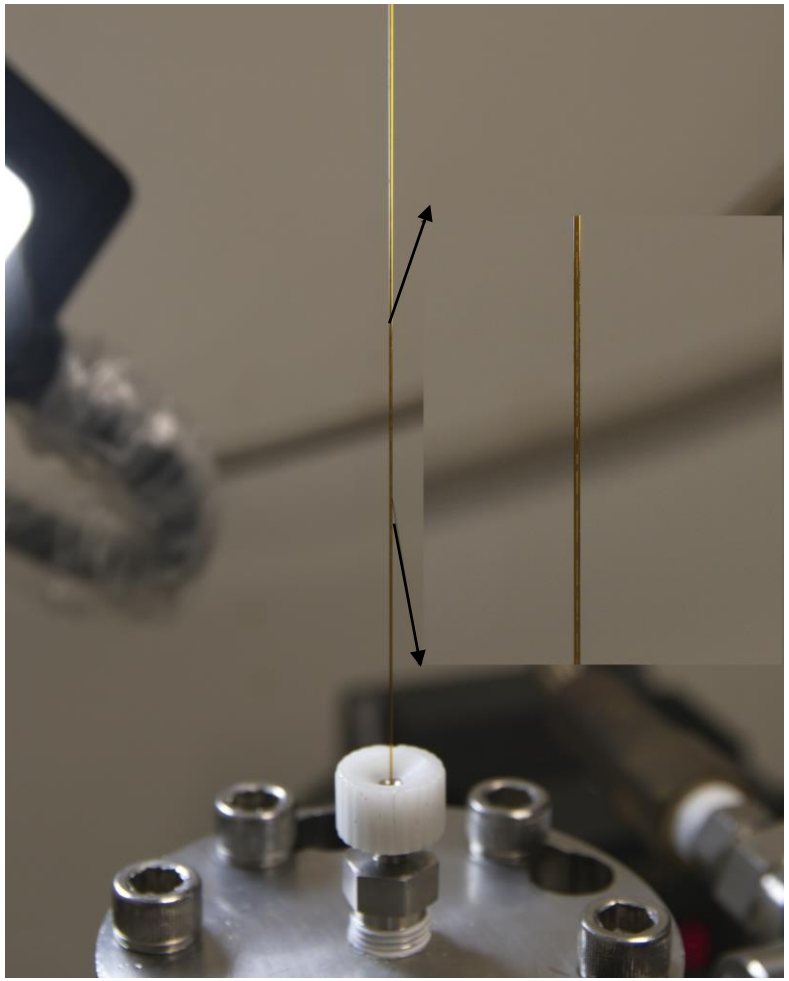

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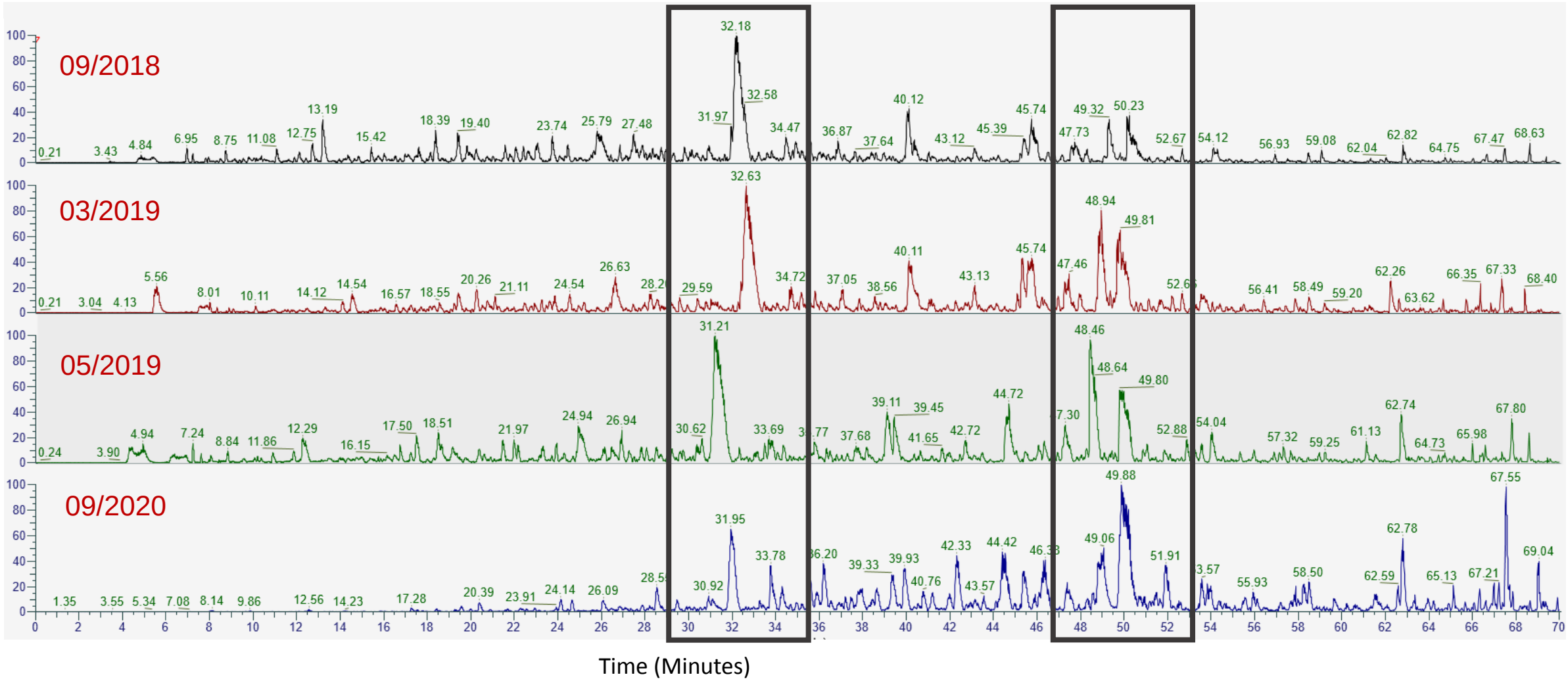

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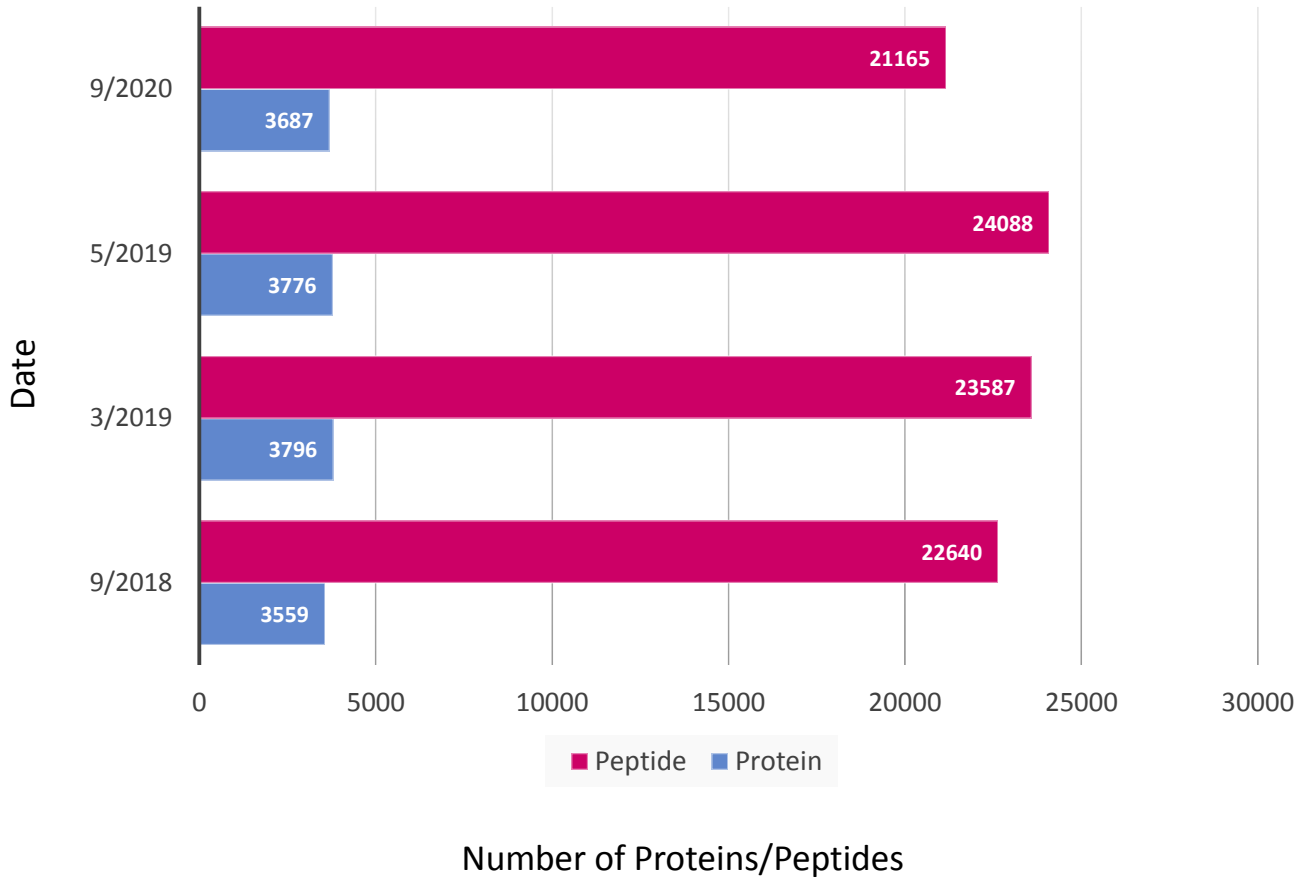

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Name	Company	Catalog Number	Comments
Alcohol lamp	Any brand		for providing heat
Brechbuehler helium pressure cell	BioSurplus		for packing column
Ceramic column cutter	Thermo Fisher		for cutting silica capillary
Dimethyl sulfoxide (DMSO) ≥ 99%	Sigma-Aldrich		Stored in a flammable cabinet
Formamide ≥ 99.5%	Sigma-Aldrich		for making frit
Hydrofluoric acid (HF) (50%)	Fisher Scientific		for opening the emitter after polymerization
KASIL (Potassium Silicate Solution)	PQ Corporation PO Box Valley Forge PA 19482		for making frit
Orbitrap Fusion Lumos	Thermo Fisher Scientific		for MS data acquisition
P2000 Laser Puller	Sutter		for pulling capillary
PTFE 1/16" Ferrule 0.4 mm ID (long) for Tube Fitting	Chromre	214104	For bomb setting
Reprosil-Pur 120 C18-AQ, 1.9 um, 1g	Dr. Masch GmbH	r119.aq.0001	Batch 5910
Soldering	Any brand		For initiating polimerization
Stainless Steel Pipe Fitting, Hex Coupling, 1/4 in. Female NPT	Swagelok	SS-4-HCG	for bomb setting
TSP075375 fused silica, 75 µm ID x 360 µOD	MOLEX/Polymicro	1068150019	For column tubing
Ultimate 3000 UHPLC	Dionex		HPLC type

Table of material.

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_ofMaterials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
99.99% Formamide acid	Sigma-Aldrich		for making frit
alcohol lamp	Any brand		For providing heat
Brechbuehler helium pressure cell	BioSurplus		for packing column
Ceramic column cutter	Any brand		for cutting silica capillary
Dimethyl sulfoxide (DMSO) ≥ 99%	Sigma-Aldrich		Stored in a flammable cabinet
Formamide ≥99.5%	Sigma-Aldrich		for making frit
Hydrofluoric acid (HF) (50%)	Fisher Scientific		for opening the emitter after polymerization
KASIL (Potassium Silicate Solution)	PQ Corporation		for making frit
Orbitrap Fusion Lumos	Thermo Fisher Scientific		for MS data acquisition
P2000 Laser Puller	Sutter		for pulling capillary
PTFE 1/16" Ferrule 0.4 mm ID (long) for Tube	Chromre	214104	For bomb setting
Reprosil-Pur 120 C18-AQ, 1.9 um, 1g	Dr. Masch GmbH	r119.aq.0001	Batch 5910
Soldering	Any brand		For initiating polimerization
Stainless Steel Pipe Fitting, Hex Coupling, 1/4	Swagelok	SS-4-HCG	for bomb setting
TSP075375 fused silica, 75 µm ID x 360 µOD	MOLEX/Polymicro	1068150019	For column tubing
Ultimate 3000 UHPLC	Dionex		HPLC type

Detailed responses and changes to
"A robust method for packing high resolution C18 RP-nano-HPLC columns"

By
Yasaman Jami-Alahmadi, Vijaya Pandey, Adarsh K Mayank, James A Wohlschlegel

First of all, we thank the reviewers for their very thorough review of our paper. We strongly believe that by taking into account their comments the paper is much stronger.

Editorial comments:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have addressed this issue in the revised manuscript.

2. Please provide email addresses of all authors.

We have addressed this issue in the revised manuscript.

3. For in-text citations, use "… research1-4." Instead of "…research.1-4 " (line 35, 38, 45, etc.)

We have addressed this issue in the revised manuscript.

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

We have addressed this issue in the revised manuscript.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note.". E.g. Line 92-94: Use "Place a hot soldering iron along the tip to initiate the polymerization of the frit solution…" instead.

We have addressed this issue in the revised manuscript.

6. Use appropriate symbols and abbreviations of SI units. E.g. use "µL / µm" instead of "uL/um" (line 80,84, 112-113, etc.), "nL" instead of "nl" (line 172). Add a single space between quantity and its unit e.g. "1.7-3.5 mm" instead of "1.7-3.5mm" (line 45), "1300 psi" instead of "1300psi" (line 128), "275 oC" instead of "275oC" (line 174). Check the same for the Table of Materials as well.

We fixed this.

7. Line 68: Please add appropriate units for the settings of heat value, velocity and delay.

We fixed this.

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. Ploymicro technology, Kimwipe, Sutter, Kasil, Eppendorf, Orbitrap Fusion Lumos etc. Also mask the manufacturer/model name on instruments used (e.g. Figure 5a).

We agree with the editor's comments about removing commercial language from the manuscript. However, one of the reviewers has suggested sharing in the protocol, the commercial source of empty fused silica capillary with integrated ESI emitter. These capillaries have not been used in our protocol, so we are not adding them to the table of materials. In this situation, where do you recommend, we mention the commercial source?

9. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We highlighted the essential steps of the protocol for the video.

10. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

We fixed this.

11. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend. E.g. Figure 3

We inserted dimension of the column to provide context to the magnification in Figure 3.

12. Figure 7: Please adjust the Y-axis length. Include appropriate axis labels and shift the legend (it is covering the bar).

We fixed this.

13. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included

We fixed this.

14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate the journal name. Do not use

“& / and” in the author list in the references. Please include volume and issue numbers for all references.

We have addressed this issue in the revised manuscript.

15. Please sort the Materials Table alphabetically by the name of the material.

We have addressed this issue in the revised manuscript.

Reviewers' comments:
Reviewer #1:
Major Concerns:

One of the major concerns in this article is that the authors have entirely disregarded the recent fundamental studies done in the field of column packing science and yet claim their method as "state of the art" in the abstract. It may not be fair to call the method outlined here "state of the art" because authors are perhaps unaware of recent contributions in this field of column packing science.

We agree with the reviewer’s comment that the “state of the art” claims made in the protocol are overstated (not to mention not really our intention) and have revised the text accordingly. Importantly, the revised protocol attempts to better emphasize the true goal of the protocol which is to provide an efficient approach for preparing reverse-phase LC columns that offer a strong blend of performance and durability in proteomic applications at a fraction of the cost. This protocol has been routinely and reproducibly employed in our laboratory to publish >100 proteomics-based publications and we believe that this protocol can be easily replicated in laboratories equipped with basic supplies for proteomic applications [1-10].

This reviewer recognizes the importance of well-packed capillaries, but have you considered what others have investigated and what were their findings? Those findings are certainly relevant to this work because the whole paper revolves around developing a protocol for packing a good capillary.

We apologize for largely ignoring the fundamental studies in the field of column packing science. We agree that the literature suggested by the reviewer is extremely relevant for putting into perspective the column packing approach described in the manuscript. We have revised the introduction to reflect the importance of previously published work and have incorporated the corresponding references.

I suggest the authors go through the following references and notice the importance of optical microscopy of suspensions before packing their capillaries. How did they choose the solvent? Is that solvent choice universal? More importantly, does that capillary represent the highest efficiency?

We agree with reviewer's comments about the significance of optical microscopy in selecting the most appropriate solvent for slurry packing. We also appreciate that when dealing with slurry particles of sub- 2mm size range, as is often the case in modern proteomics, the adhesion forces between the particles are strong enough to necessitate a stable suspension before packing can commence. Hence it is critical to experimentally determine solvent or mixture of solvents for efficient suspension of the slurry. Although we did not perform optical microscopy and sedimentation velocity measurements to study the behavior of the slurry particles in solvent, our choice of methanol as the slurry packing solvent was informed by previously published literature[11-15] and also subsequently empirically determined in our laboratory. Methanol is a commonly used and frequently reported solvent for sample preparation and chromatography for its ready availability and low costs. As suggested by the reviewer we have noted the importance of solvent-particle interaction in the introduction and cited references where these interactions have been discussed extensively.

2) In any packing protocol, the design of the packing hardware is also critical. Please show the inside of the packing chamber and schematically show how helium pressurizes the slurry in this particular chamber design. Also, show the nature of connections from the helium tank to the packing chamber.

We agree with the reviewer and have added the schematic as represented in Figure 5.

3) The authors must include a quality control test that shows the packed capillary chromatographic efficiency. For that, the authors must include a quality control peak efficiency test using a compound of their choice after packing a capillary. How would the user know that the efficiency of their packed capillary is at the optimum? Again, please see the flow chart in the above references.

We agree that it is important to assess the quality of packed columns for proteomic applications. Based on our experience, the number of peptide identifications resulting from a standardized LC-MS/MS analysis (e.g. 750 ng of HEK293 cell tryptic digest in a 70-minute water-acetonitrile gradient) is a very sensitive measure of column quality and one that we use in conjunction with classic properties such as retention time reproducibility and peak width. Importantly, we track these metrics longitudinally to ensure reproducibility over time. Fig. 8 shows the consistency of our approach for columns packed at different points in time. It is also worth noting that using complex peptide digests to benchmark LC-MS/MS setups is common in the field and the columns described here provide results that are comparable to previously

published reports using either commercial or in-house packed columns based on similar column length, slurry, solvent and MS parameters similar to the current study[12,16,17].

4) The word nano repeatedly appears like a buzzword in the introduction. Please reduce its usage and define the dimensions of the columns instead of using nanoLC. These are just commercial names, and hence should be avoided.

We have revised the text as suggested by the reviewer.

6) In the protocol, point no. 3. "heat value of 300, velocity of 10 and delay of 180" write the units (these numbers are meaningless without their units).

We have added the proper units to the text.

7) In Step 3, under the heading of "Preparation of Stationary Phase,": Please do not call C18 silica as a resin until and unless polymers are being used. C18 silica is a fully porous silica particle, not a resin. What is the particle size?

We agree with the reviewer and have revised the text accordingly.

8) A good practice in writing is to write the figure caption as a stand-alone statement and detail everything so that the readers do not have to search the main text. There might be some repetition, but it is better to repeat in a caption. For example, in Figure 4, explain what HF-eater is and what is being done there.

We have revised the figure captions based on the reviewer's suggestions.

Minor Concerns:
1) Add a cautionary note in "column tip is immersed in a 50% hydrofluoric acid (HF)".

We have added the cautionary note per the reviewer's suggestion.

2) Correct reference 10.

We have corrected the reference.

Reviewer #2:
Manuscript Summary:
The abstract reads well. However, I would advise to replace the statement: "the size and nature of stationary phase particles" to a more specific and clearer statement: "the particle size, pore size and chemistry of stationary phase particles"

We have corrected this error.

Major Concerns:

Protocol
1. Preparation of capillary tip
Protocol Point 2 When talking about the bare-fused silica capillary from (Polymicro Technology) it should be clarified that the capillary is "coated with Polyimide buffer" as documented in the manufacturer's website. It is important to state this as this is the justification for the procedure in point 2 of the protocol: removing the polyimide coating from the capillary along 10 cm in the middle. The authors should also explain the purpose of wanting to have these 10 cm of bare-fused silica capillary with clear glass windows and where they intend to locate this 10 cm segment.

We have revised the text based on the reviewer's suggestion.

Protocol Point 3 The units should be included to the value in this list: heat value of 300, velocity of 10 and delay of 180,

We have revised the manuscript to indicate the units.

Question: are the authors talking about 1-5 mm or 1-5 µm diameter tip? Take care with the units.

We have revised the manuscript to make sure the units are correct. µm is the correct unit for the diameter tip as shown in figure 3.

Related figure 3: A size bar should be shown in Fig. 3 as to get a clear idea about the difference between the tip of the capillary and the main ID of the capillary.

We agree with the reviewer and have revised Fig. 3A accordingly

2. Polymerization/etching of the tip
Protocol Point 1: These instructions are misleading: "the emitter is blocked with a porous frit comprised". It reads as the composition of this solid/gel frit is composed by these chemicals. Instead, the porous frit is prepared from these chemicals as starting materials a suspension or gel which later need to be allowed to polymerise.

We apologize for the confusion and have clarified this point in the revised manuscript.

This Note can be misleading: For example, one can use 100uL of Kasil 1, 300 uL of Kasil 1624 and 100uL of formamide acid to get the polymerized frit solution.
Do the authors mean here:" …and 100uL of formamide acid to ensure the frit solution will be able to polymerize and form the frit with the required porosity"?

We have clarified the description of this step of the protocol in the revised manuscript.

Protocol Point 2: This sentence is unclear: "Immerse the laser-pulled capillary tip in the clear solution of the frit (not the precipitate)". Do the authors mean: "in the clear mother liquor of the frit suspension (not the precipitate)

We have revised the text based on the reviewer's suggestion.

Protocol Point 4: "To account for the possibility" may be more accurately described as "to prevent"

We have revised the text accordingly.

The authors should add 3 extra pictures of the nano-column tip: 1) before etching with HF; 2) after etching with HF and 3) after washing the HF from the capillary tip with water.

Figure 3B shows the column tip before etching. However, taking an image of the column after HF-etching and before washing it, does not align with the safety protocols followed in the lab. Since HF is a highly corrosive chemical, we strictly avoid contact with etched tip unless it has been thoroughly washed first with HF-neutralizer and then with copious amounts of water. In our experience, the emitter tip before and after etching looks very similar such that an image of post-etching tip provide little useful information. We have revised the text to make this point.

Under notes, No 3: the authors could suggest from which suppliers could "the empty fused silica capillary columns with an integrated electrospray emitter" be purchased.

We have included the requested information.

3. Preparation of stationary phase
Protocol Point 1: C18 reversed phase is not a resin as it is covalently bonded to silica. It is a C18 stationary phase. The term resin can only be used when the C18 is bonded to an organic polymer. This is not the case here.

We thank the reviewer for his/her insight and have revised the text accordingly.

C18 stationary phase material that the authors are using to pack the nanocolumn is not a straightforward C18; they are using the "Reprosil-Pur 120 C18-AQ". The authors should learn from the manufacturers more about the chemistry of this stationary phase. I am disappointed with the lack of sensible information on the chemistry and applications of this column in dr-maisch.com website. AQ typically stands with most column manufacturers for aqueous, and these C18 AQ phases have been designed with a polar group (amide, carbamate, ether, other) embedded in the C18 strand so as to prevent the stationary phase from collapsing when using 100% aqueous mobile phase. Stationary phase collapse means that the alkyl strands in the presence of highly aqueous phase collapse away from the highly aqueous mobile phase just as oil droplets will collapse in water. The effect of phase collapsing will result over time in shorter retention times for the analytes.

The phrase: Resuspend ~ 0.05 g of is wrongly used. Reprosil-Pur 120 C18-AQ is sold as a solid powder, not as a suspension. So, it would be less confusing to the reader if the instruction is written as ": Suspend ~ 0.05 g. The authors should not use this rough quantity (0.05g) but should instead use 50 mg. It is important for the column to column reproducibility how the concentration of the suspension/slurry of the stationary phase is prepared. The authors should give more careful advice.

We agree with and value the points being made by the reviewer. We have now corrected the text wherever possible to reflect the correct nomenclature of the particles being used as the stationary phase. As suggested by the reviewer, we have also accurately stated the weight of Reprosil-Pur 120 C18-AQ particles used for preparing the stationary phase suspension.

The authors use MeOH to prepare the slurry of the C18 Aq. stationary phase. From my own experience, the solvent for packing is carefully selected to give an ideal slurry preventing the particles from sedimenting or agglomerating. Different solvents are more suitably for different stationary phases.

We agree with reviewer's comments about the significance of meticulous selection of appropriate solvent for slurry packing. We also appreciate that when dealing with slurry particles of sub- 2mm size range, as is often the case in modern proteomics, the adhesion forces between the particles are strong enough to necessitate a stable suspension before packing can commence. Hence it is critical to experimentally determine solvent or mixture of solvents for efficient suspension of the slurry. Although we did not perform optical microscopy and sedimentation velocity measurements to study the agglomeration behavior of the slurry particles in solvent, our choice of methanol as the slurry packing solvent was informed by previously published literature[11-15] and also subsequently empirically determined in our laboratory. In the same note, we have also stated the importance of solvent-particle interaction in the introduction and have cited references where these interactions have been discussed extensively.

The authors should report with accuracy the details of their stationary phase: "C18 reversed phase resin with 1.9 um diameter and 100 Å pore". Two points:
1) 1.9 µm is the particle size which is most likely spherical but it should not be referred as 1.9 µm diameter; dr-maisch.com website claims particle size= 1.9 µm;

We have revised the text accordingly.

2) the details of the pore size are incorporated into the name Reprosil-Pur 120 C18-AQ; looking at dr-maisch.com, this stationary phase is reported as having 120 Å pore size.

We thank the reviewer for noting this issue and have corrected it in the revised manuscript.

Protocol Point 2: When describing the HPLC packing bomb, the authors should explain to the reader the marked difference between the operation of HPLC pumps and HPLC packing bombs.

HPLC pumps gives very accurate constant flowrates in order to reproduce the retention time of analytes. On the other hand, HPLC packing bombs have to be operated at constant pressure to avoid disrupting the HPLC slurry during packing.

We have revised the text based on the reviewer's suggestions.

I am surprised that the packing Bomb is operated with helium as this gas is expensive and not very sustainable. Have they authors checked if the packing pump could be also operated with other gases like nitrogen? This should be discussed.

Although we appreciate the reviewer's concern, we and others have been routinely using this gas for the purpose of capillary column packing [4,5]. In our experience, balloon grade helium gas is readily available in our lab. Although we have not tested the suitability of nitrogen gas in our system, we expect nitrogen gas is equally suitable for this purpose. Indeed, Kovalchuk et al have used nitrogen gas in their protocol for packing columns with dimensions fairly similar to ours.[2]

Fig 5a which is relevant for this section, should indicate with arrows how the 3-way valve of the packing bomb is to be used for packing the nano-LC column.

We have added a schematic representation (Figure 5) of the column packing system where we have indicated the direction of helium gas flow inside the pressure bomb system.

4. Packing the column with stationary phase
Protocol Point 1
The specific plastic fitting shown as a white fitting on relevant Figures 5, 6 and 7 is intended to seal the end of nano- LC column to the female fitting at the outlet of the packing bomb to prevent leaking and loss of pressure during packing. This HPLC fitting is probably a PEEK One-Piece Fingertight fitting required for sealing 360µm OD tubing. This must be a very specific fitting and should be documented under the section "Name of Material and Equipment". Additionally, a picture showing the end of the nanocolumn being threatened through the One-Piece Fingertight fitting should be shown making sure that there is a clear view of One-Piece Fingertight fitting.

We have added an image as Figure 5 which shows the column being threaded through the one-piece fingertight fitting. We have also documented the information related to this fitting in the "Name of Material and Equipment" table.

Protocol Point 2 The term "slurry" is correctly used in this context and should be consistently used over the protocol.

Per the reviewer's suggestion, we have corrected the usage of the term 'slurry' in the text where relevant.

Protocol Point 3 This statement requires an explanation "If the formation of liquid droplet on the column tip is delayed, quickly flame the tip to ensure it is open."
In my mind there are at least 2 reasons why the tip could prevent MeOH from coming out. 1) either the frit pores were much too large and particles are coming through and blocking it or 2) the stationary phase has too many fine particles which are coming through a well prepare frit with the right pore size range and clogging the tip. Modern HPLC stationary phases do not tend to have fines. Even if these problems were to be resolved by flaming the tip, a higher backpressure will be obtained through a blockage of the frit or continuous blockage of the capillary end through the stationary phase fines.

Based on our experience, 5-10% of the columns packed using our approach have blocked tips that prevent the slurry from flowing through the length of the capillary column. This may happen either for the reasons cited by the reviewer or due to sub-optimal pulling of the tip by the laser puller. In this scenario, a quick flaming of the tip often releases the blockage by pushing it out enabling the packing procedure to resume. Columns rescued by this technique perform comparably to ones packed normally.

Point 4: The authors do not discuss the possibility of getting He bubbles into the packed column, if packing had to be stopped and restarted due to blockage. The authors should discuss strategies to avoid He bubbles inside the nano LC column. Any disruption of good packing, like gaps due to air bubbles, inside a typical HPLC column results in peak splitting of the eluted peaks.

We appreciate the concerns raised by the reviewer related to the possibility of He bubbles inside the column and have addressed them in the section 5. As mentioned in section 5.2, we recommend packing columns longer than the desired length. In the same note, we also advise the users to continue packing beyond the visible length of the column above the one-piece finger-tight fitting for the reason that there is 5-10 cm of column length which is inside the bomb and not visible to eyes (see the schematic). Additionally, we recommend allowing the packing apparatus to slowly self-depressurize after the helium flow has been turned off, in order to ensure that the particles remain uniformly packed throughout the length of the column, avoiding the introduction of air bubbles.

The authors talk about tightening the ferrule; this is confusing as in the picture it is clear that the authors are using a One-Piece Fingertight fitting where the ferrule is an integral part of this fitting.

We have revised the text to address the reviewer's concerns.

5. Finishing up the column and making the back-frit.
The authors jumped by mistake from Protocol Point 3 to 5, bypassing Point 4

We thank the reviewer for noticing this error which we have corrected in the revised manuscript.

Protocol Point 4: The way the instructions have been written here implies that the column end is cut with stationary phase flash with the end. If this end is dipped into the frit solution, then the polymerisation will trap stationary phase probably blocking many of the pores of this frit. This should be better explained.

We apologize for the confusion and have edited the text in section 5 to make the instructions clearer.

Representative Results
Here the authors should clearly state the starting composition of the mobile phase gradient. Is it probably implied that the gradient starts at 0% Buffer B? This is not stated. If so, it is important to state the equilibration time required to come from 80% buffer B to 0% buffer B to ensure the chromatograms are reproducible.

We have revised the manuscript to address the reviewer's concerns.

I am not familiar with this LCMS term: 120,000 "resolution". Maybe the authors could make it clearer for the readers. The supplier, quality and preferred storage conditions of DMSO should be documented.

In mass spectrometry, resolution refers to the measure of ability of instrument to distinguish two peaks or peptide ions with slight difference in their mass-to-charge ratio. We have described the resolution of the MS and MS/MS scans in results section using standard mass spectrometry nomenclature.

Per the reviewer's suggestion, we have also added the supplier and quality of DMSO to the table of materials. Storage conditions have also been added to the materials table section.

Title of Figure 8
The title inside the figure is unclear and confusing: It should say: "Example of total number of identified proteins and peptides"; It may be necessary to reduce the font of this title

We have revised the title of this figure to make it clearer.

Minor Concerns:
The titles of the selected references cited here are relevant and up to date. The chromatography examples presented in Figure 8 are as reproducible as can be expected from any HPLC analysis.
Title: the concept of nano- such as C18 RP-nano-HPLC column is important and should be incorporated into the title.

We have renamed the title of the manuscript in accordance with the reviewer's suggestion.

Abstract: The abstract reads well. However, I would advise to replace the statement: "the size and nature of stationary phase particles" to a more specific and clearer statement: "the particle size, pore size and chemistry of stationary phase particles"

We have revised the manuscript in accordance with the the reviewer's suggestion.

Keywords: If only 10 keywords are allowed, I would then recommend to replace "mobile phase" which does not add value here for "nanoflow chromatography" or "nanoLC". The term "frit" is vague. "nanoLC column frit" is more specific, as this type of frit can be only used for nano LC columns.

We have implemented the reviewer's suggestion.

Correct use of terminology over the document: Better to use nanoLC column than LC column; uL or mL are incorrect; should be µL; um or mm are incorrect; should be µm; please check all the occurrences of these units in the document.

We apologize for the oversight on our part and have corrected the spellings and units throughout the text.

Introduction: Same advice for keywords. The "gradient" should be referred more correctly as "solvent gradient".

We have made necessary changes in the introduction to address the reviewer's concerns.

References:

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