Supplementary results

Centrifugal microfluidic automation of the protein aggregation capture workflow for robust mass spectrometry-based proteomics

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

*Michelle Hinrichs[a,b]‡, Johanna Wallner[c,d]‡, Johanna Thiery[e,f], Carolin Kleber[b], Manuel Metzger[a,b], Stephan A. Sieber[c], Tobias Hutzenlaub[a,b], Oliver Schilling[e], Nils Paust[a,b], Hannes Hahne[d], Jan Muntel[d], Jan-Niklas Klatt[a,b]**

Supplementary results

Analysis of different bead types

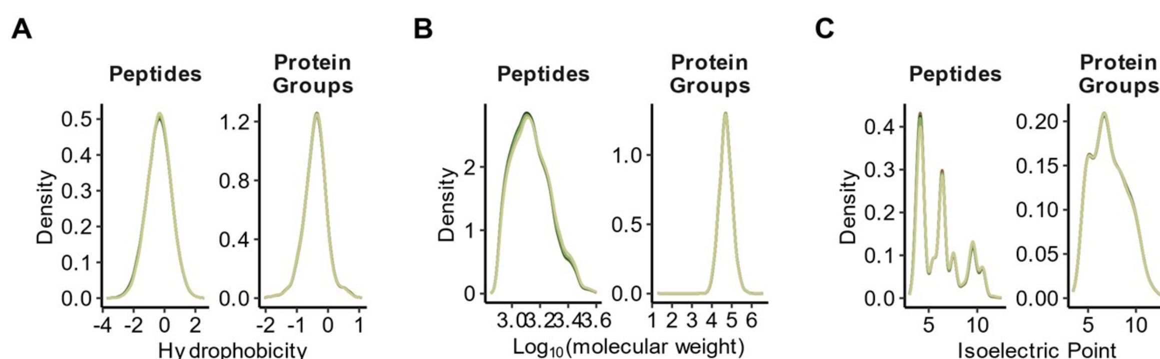


Figure 1: Comparison of physicochemical properties of different magnetic beads and bead to protein ratios for the protein aggregation capture workflow. (A) Density plot representing the Grand Average of Hydropathy score of peptides and proteins per workflow type (SpeedBeads (protein:beads 1:10, w/w) (orange) and PureCube beads (protein:bead slurry 1:0.5, 1:0.8, 1:1, 1:2 w/v) (green scales), n=3). Hydrophilic peptides receive negative values, hydrophobic peptides positive values. (B) Density plot representing the molecular weight in log10 of peptides and proteins per workflow type. (C) Density plot representing the isoelectric point of peptides and proteins per workflow type.

[a] *Hahn Schickard, Georges-Koehler-Allee 103, 79110 Freiburg, Germany*
[b] *Laboratory for MEMS Applications, IMTEK-Department of Microsystems Engineering, University of Freiburg, Georges-Koehler-Allee 103, 79110 Freiburg, Germany*
[c] *Chair of Organic Chemistry II, Center of Functional Protein Assemblies (CPA), TUM School of Natural Sciences, Technical University of Munich, Ernst-Otto-Fischer Strasse 8, 85748 Garching, Germany*
[d] *OmicScouts, a Momentum Biotechnologies Company, Lise-Meitner-Strasse 30, 85354 Freising, Germany*
[e] *Institute for Surgical Pathology, Medical Center, University of Freiburg, Breisacher Strasse 115a, 79106 Freiburg, Germany*
[f] *Faculty of Biology, University of Freiburg, Schaenzlestrasse 1, 79104 Freiburg, Germany*
‡, Contributed equally
*, Corresponding Author

Please do not adjust margins

Reducing the number of different organic solvents and pre-storage on the AutoPAC-Disk

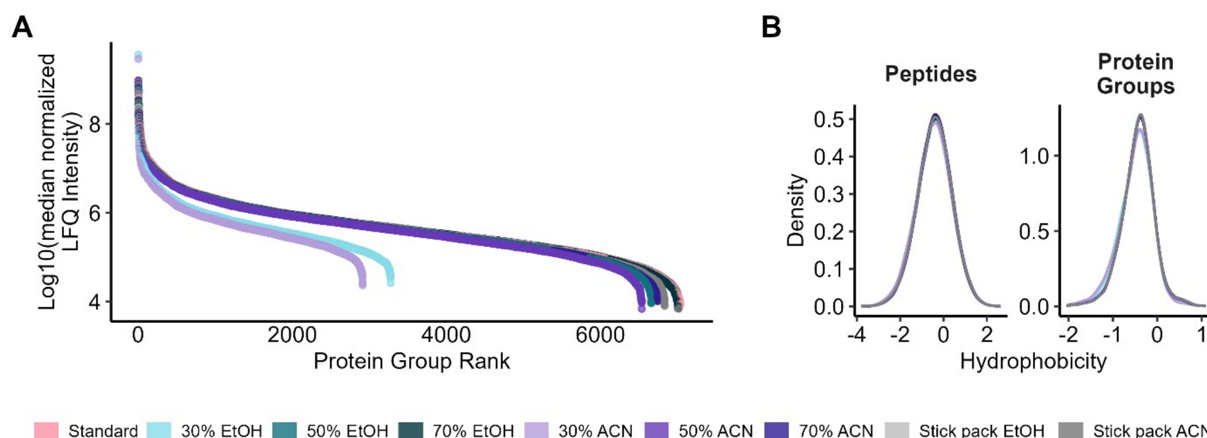


Figure 2: Comparison of different organic solvents for the protein aggregation capture (PAC) workflow. (A) Rank plot depicts the median normalized log10-transformed LFQ intensity of protein groups against their rank in the dynamic range for the standard workflow (protein precipitation on PureCube beads with 70 % ethanol, 1st and 2nd wash with 80 % ethanol, 3rd wash with 100 % acetonitrile), 30 % / 50 % / 70 % ethanol (protein precipitation on beads with 30 % / 50 % / 70 % ethanol, 1st, 2nd and 3rd wash with 80 % ethanol) and 30 % / 50 % / 70 % acetonitrile (protein precipitation on beads with 30 % / 50 % / 70 % acetonitrile, 1st, 2nd and 3rd wash with 100 % acetonitrile). Stick pack samples were precipitated with 70 % of the respective organic solvent. All conditions were tested using the KingFisher Apex. (pink: standard control; bright grey: stick pack with ethanol; dark grey: stick pack with acetonitrile; blue range: 30 %, 50 %, 70 % ethanol precipitation; purple range: 30 %, 50 %, 70 % acetonitrile precipitation, n=3). (B) Density plot representing the Grand Average of Hydropathy score of peptides and proteins per workflow type (for color coding see (A)). Hydrophilic peptides receive negative values, hydrophobic peptides positive values. EtOH = ethanol, ACN = acetonitrile.

R1 – Microfluidic AutoPAC-Disk protocol

Loading: The AutoPAC-Disk is loaded with 50 µL of sample into the sample inlet (chamber 3), the digestion mix into chamber 6 and the PureCube bead suspension in the bead inlet (chamber 2) (Figure 3a, b). Immediately after loading, the disk is loaded into the Rhonda Player and the processing protocol is started. All subsequent actions are performed in an automated manner.
First, the rotational frequency is increased to 50 Hz. The PureCube beads are centrifuged into the bead column chamber (chamber 1) and are retained by a geometrical restriction at the radially outward end, thus forming a bead bed. Afterwards, the frequency is decreased to 30 Hz to transfer the supernatant of the bead suspension to the waste chamber via the downstream fluidic switch, which enables waste and eluate routing (Figure 3c).
Binding: To initiate the binding step, the rotational frequency is increased to 60 Hz and zone 1 of the turntable is heated up to 110 °C for 32 s. The combination of centrifugal force and the vapor pressure generated within the stick pack leads to the opening of the stick pack, and the binding buffer (BB) is released. For transfer of the buffer to the bead column chamber, the rotational frequency is reduced to 15 Hz. At this rotational frequency the generated vapor pressure within the stick pack chamber is high enough to propel the liquid from chamber 4 into the bead column chamber (Figure 3d). After the transfer of the 180 µL binding buffer, heating zone 1 is deactivated. The binding buffer stick pack was filled with 180 µL to ensure the minimum sample-to-buffer ratio of 70 % until a sample volume of 70 µL. To achieve the ratio of 70 % with the used sample volume of 50 µL, the excess binding buffer is transferred at 30 Hz to the waste chamber via the downstream fluidic switch. After 20 s, the sample transfer begins by rotating the disk at 13 Hz while zone 2 is heated up to 105 °C for 30 s. The enclosed air in the pneumatic chamber placed above heating zone 2 expands and generates overpressure and displaces the 50 µL sample from the inlet to the bead column chamber (Figure 3e). The sample and binding buffer are then mixed in the bead column chamber via shake mode with a maximum rotational frequency of ±13 Hz, and a rotational acceleration of 25 Hz s^{-1} for 10 cycles. After the mixing step, zone 2 was heated to 105 °C for 30 s at 13 Hz to perform an additional transfer step, ensuring complete delivery of the sample–binding buffer mixture into the bead column chamber. This step compensates for minor fluid redistribution that can occur during oscillatory mixing across the zero-g condition and prewetted channel interfaces. Next, the mixture is transferred to the waste chamber at a frequency of 20 Hz via the volume siphon in within 10 minutes (Figure 3f).
Washing: After the binding step, the column is washed with 80 % ethanol. To initiate the washing process, the rotational speed is increased to 60 Hz, and zone 5 is heated up to 110 °C for 40 s to open the stick pack, as already described for the BB stick pack. The washing step is performed with 250 µL washing buffer (WB) at a rotational frequency of 30 Hz (Figure 3g).
Digestion: The digestion mix in chamber 6 is transferred to the bead column chamber using thermopneumatic valving. Heating zone 3 of the turntable is heated up to 70 °C for 60 s while the system operates at a constant rotational frequency of 50 Hz. The elevated temperature within the pneumatic chamber leads to an increased pressure as the air cannot displace the digestion mix from chamber 6 at this high rotational frequency. Once the rotational frequency is decreased to 10 Hz, the centrifugal force no longer suppresses the rise of the digestion mix within the siphon. As the meniscus climbs, the air volume in the adjacent pump

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

Please do not adjust margins

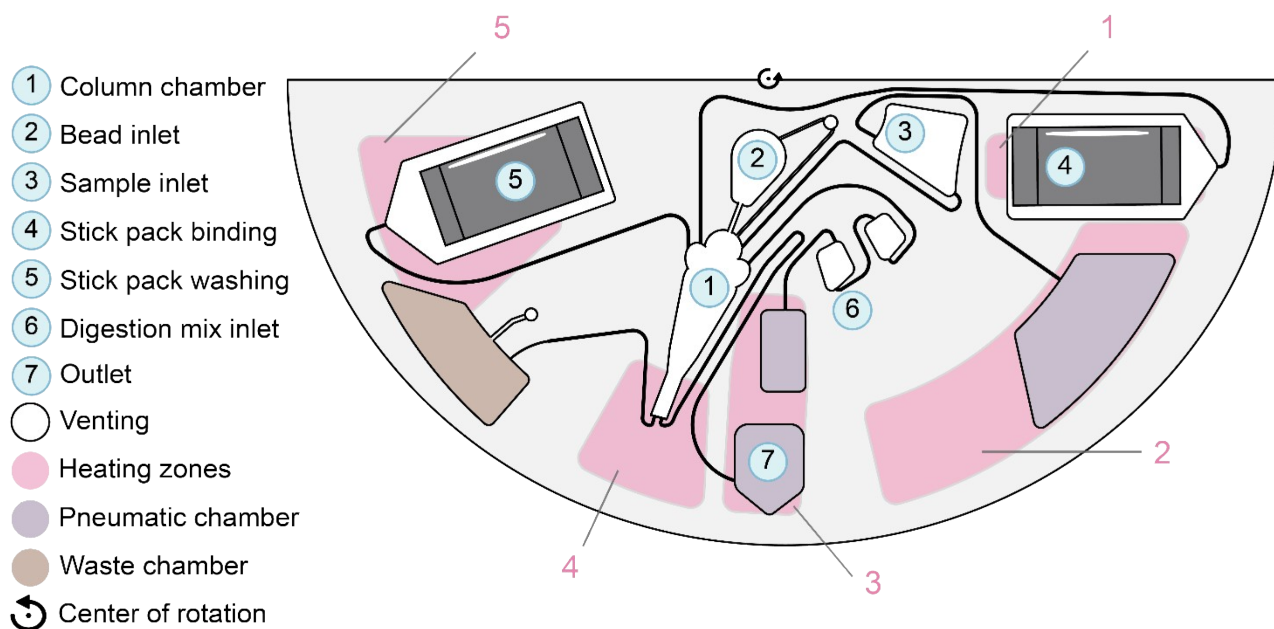
chamber positioned on the heating zone expands, thereby driving the complete transfer of the digestion mix into the bead column chamber. (Figure 3h). The 14 µL volume of the digestion mix completely covers the bead bed (Figure 3i), enabling on-bead digestion. As this volume is lower than 30 µL, the volume siphon leading to the waste chamber is not primed, thereby ensuring that the digestion mix is retained in the bead column chamber. For the subsequent digestion step, zone 4 is heated to 37 °C, and digestion is carried out at a constant rotational frequency of 25 Hz for 4 h. While zone 4 is heated to initiate digestion, zone 3 is gradually cooled from 70 °C to 30 °C, and subsequently switched off during the digestion step.

Sample collection: After the 4 h digestion step, the sample is transferred to the outlet chamber via thermopneumatic pumping. Zone 3 of the turntable, which contains the outlet chamber (chamber 7), is heated to 105 °C. The pneumatic pressure in the outlet chamber is increased while the air remains encapsulated, as the centrifugal force at 40 Hz maintains the meniscus in the adjacent siphon channel connecting the bead column chamber and the outlet chamber (Figure 3j). To enable the transfer of the peptide solution into the outlet chamber, the rotational frequency must be decreased to 10 Hz, thereby reducing the centrifugal force acting on the meniscus. This reduction triggers an overpressure release via the formation of air bubbles passing from the outlet chamber through the liquid (peptide solution) in the bead column chamber. Subsequent cooling of zone 3 to 20 °C is used to contract the confined air, thereby generating a pressure lower than ambient pressure in the outlet chamber, by which the peptide solution is drawn into the outlet chamber (Figure 3k). This heating and cooling sequence are repeated to ensure full transfer of the peptide solution. The peptide solution is then transferred to a clean reaction tube.

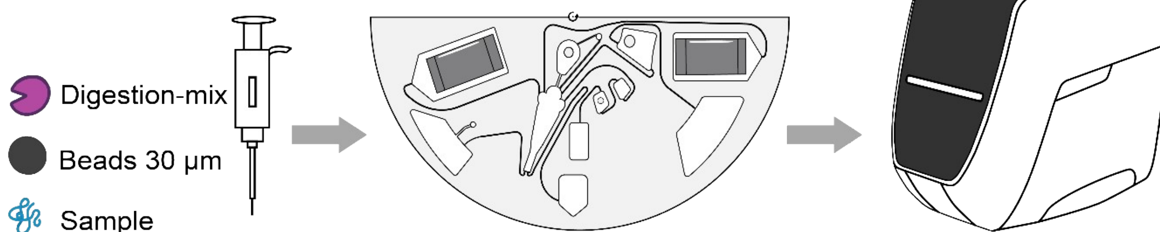
This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

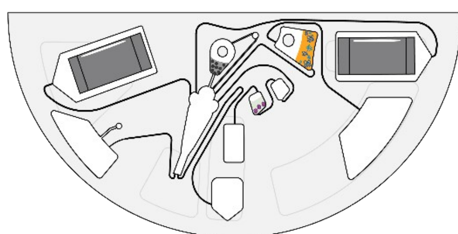
Please do not adjust margins



a) Sample loading and disk insertion

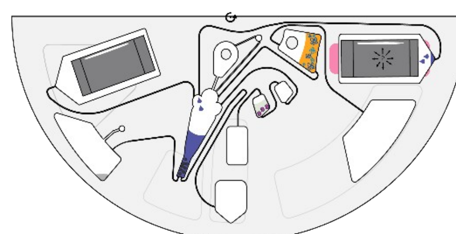


b) Initial condition at rest

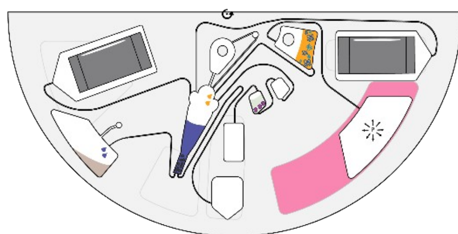


c) Bead column

d) BB to bead column chamber

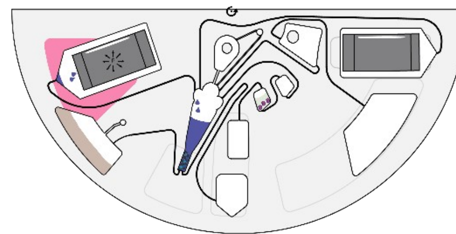


e) Sample to bead column chamber



f) Mixing

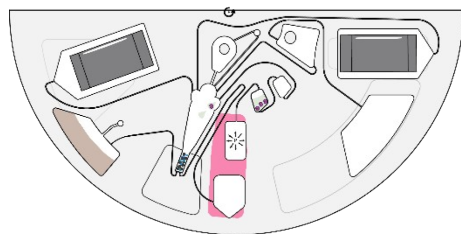
g) WB to bead column chamber



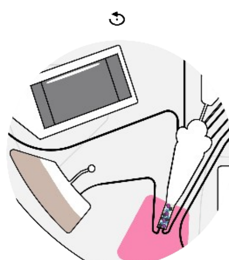
This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

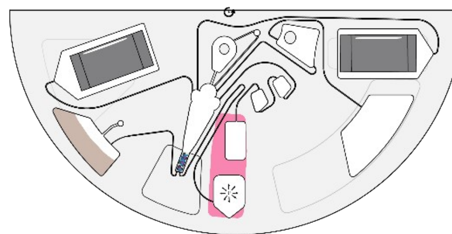
h) Digestion mix to bead column chamber



i) Digestion



j) Elution 1/2



k) Elution 2/2

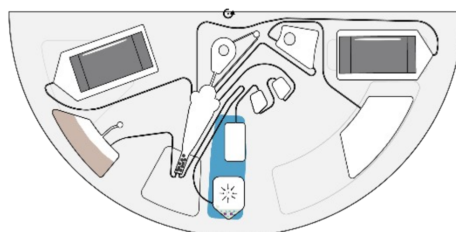


Figure 3: Schematic illustration of the liquid handling steps on the AutoPAC-Disk. The upper panel depicts the disk structure, highlighting fluidic chambers and all heating zones of the turntable in relation to the chambers on the disk. The lower panel illustrates the microfluidic protocol sequence: (a) Loading, (b) Disk at rest in the player, (c) bead column, (d) transfer of the binding buffer (BB) into the bead column chamber, (e) transfer of the sample into the bead column chamber, (f) Shake mode mixing, (g) transfer of washing buffer (WB) into bead column chamber, (h) transfer of digestion mix into the bead column chamber, (i) digestion step at 25 Hz, (j/k) elution is shown in the lower part.

Please do not adjust margins
Please do not adjust margins

Please do not adjust margins

Additional information for the geometry of bead column chamber

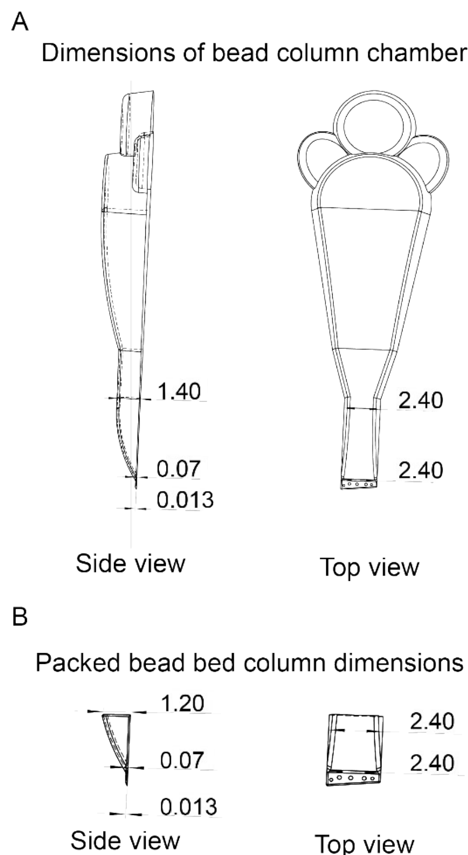


Figure 4: Illustration of bead column chamber. A: Depicts the full bead column chamber with annotated dimensions. B: Highlights the bead bed region within this chamber. The bead bed volume is 4.3 mm^3, corresponding to 2927 particles of 30 µm beads based on the manufacturer's information.

Additional results for comparative performance of PAC workflows

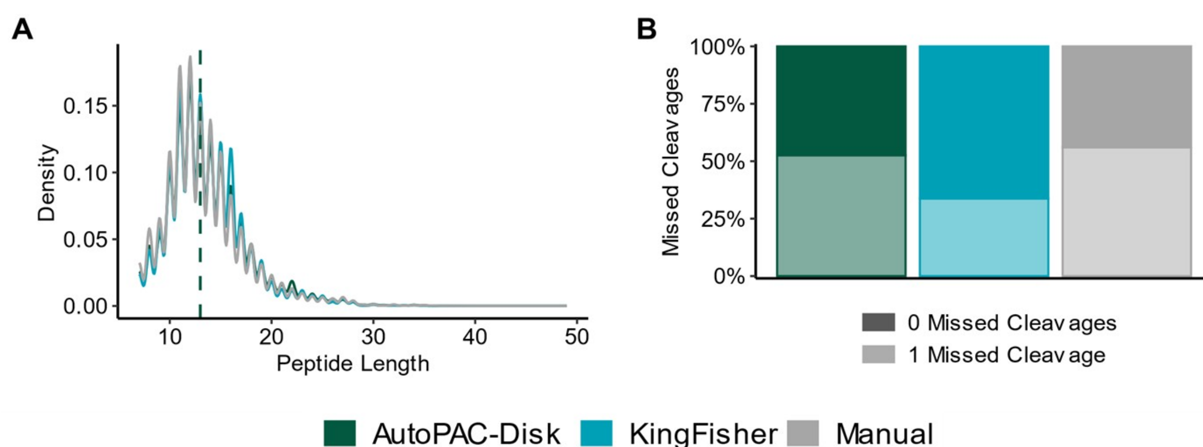


Figure 5:Comparison of peptide length and digestion efficiency of the three different workflows. AutoPAC-Disk with 4 hours digestion (green), KingFisher with 16 hours digestion (blue), Manual 4 hours digestion (grey), n=4.

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

Please do not adjust margins

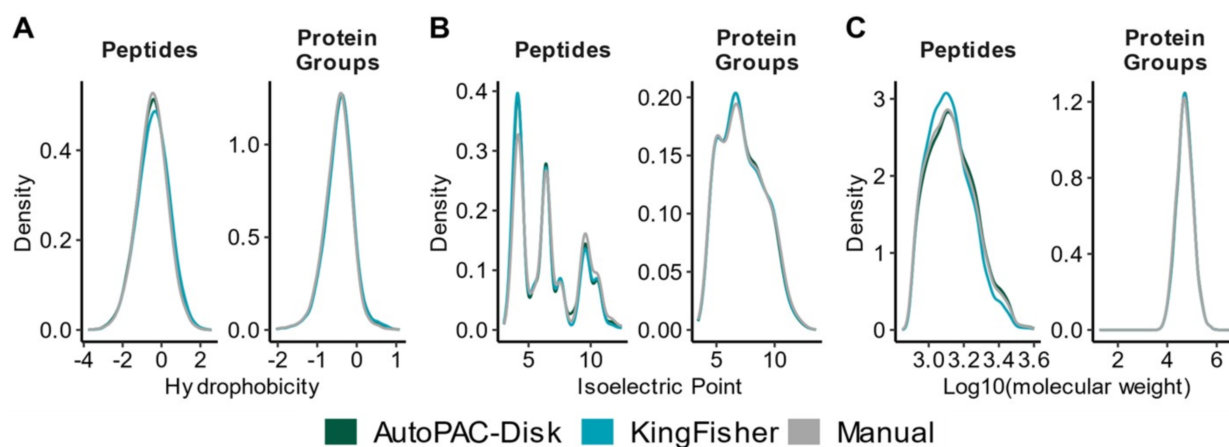


Figure 6: Distribution for all protein and peptides of (A) hydrophobicity, (B) isoelectric point, (C) molecular weight (AutoPAC-Disk: green, KingFisher: blue, Manual: grey, n=4).

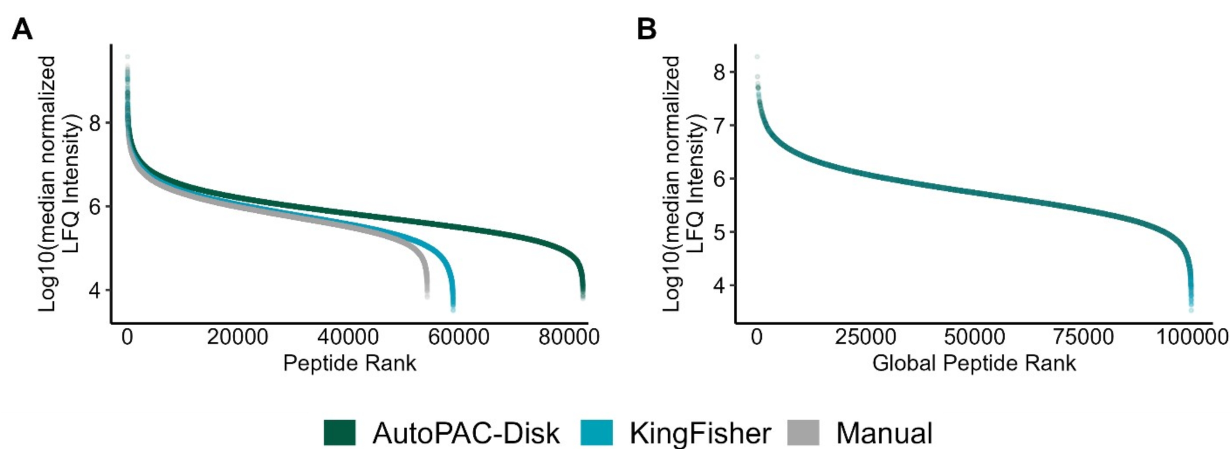


Figure 7: (A) Peptide rank plot with log10 normalized protein intensity plotted over rank for each workflow separately. Color code (AutoPAC-Disk: in green, KingFisher: in blue, Manual: in grey, n=4). (B) Global peptide rank plot with log10 normalized protein group intensity plotted for uniquely identified proteins color-coded as described in (A).

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins