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APPLICATIONS

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When not advancing the forefront of HPLC Jason enjoys scuba diving with his wife and being the tickle monster for his daughter.

Investigating the Influence of Selectivity in bioZen™ Nano Column and Trap in Bottom Up Proteomics

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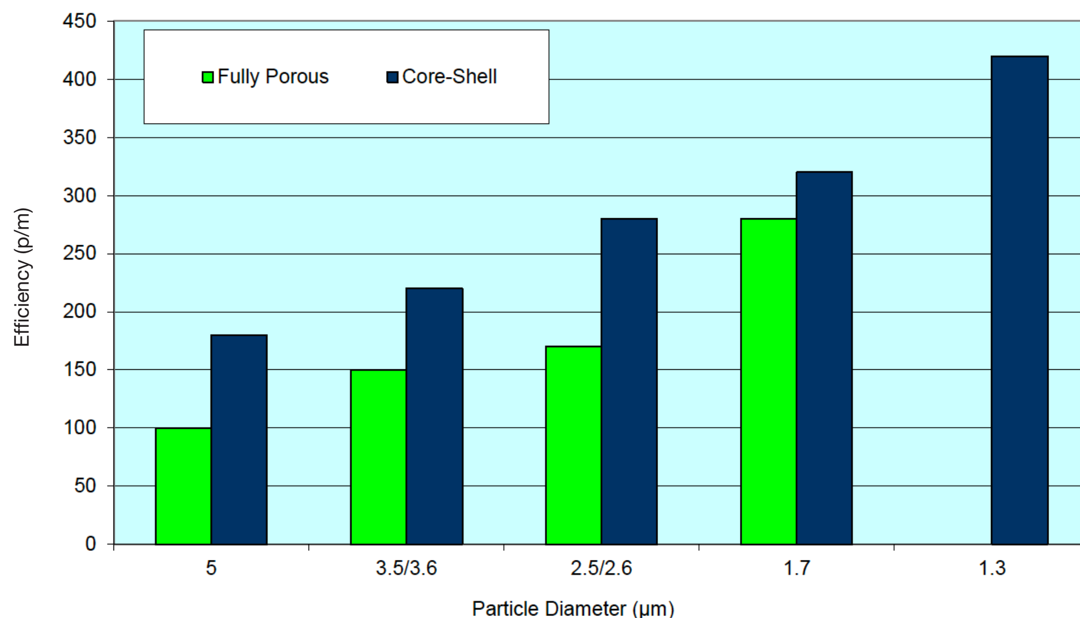
Introduction

For many years there has been a great interest in miniaturized separations, especially in LC-MS. Most often, miniaturized LC-MS is employed in situations where the amount of sample for analysis is very small.[1] Some common application areas where miniaturized LC-MS and specifically nano LC-MS are employed are Omics application including proteomics, metabolomics, lipidomics, and foodomics.[1,3,6-7] Moving to smaller I.D. (internal diameter) columns while maintaining the same mass injected onto the column, improves the ionization efficiency since there is less sample dilution due to the chromatographic process.[1-5] The increased ionization efficiency leads to improved sensitivity.

Another interesting area of research in LC optimization over the last 10 years has been the use of core-shell particles. In general, these particles consist of a solid non-porous core surrounded by a porous silica layer containing the chromatographic stationary phase. While there have been many different explanations for the chromatographic behavior of these particles, it is generally observed and reported that by going from a fully porous particle to a core-shell particle there is a substantial increase in chromatographic efficiency, leading to narrower peaks and higher peak capacities.[8-11] In **Figure 1** some typical values of fully porous and core-shell particles of various sizes are shown. While there has been some variability in the reported performance benefit between different size core-shell and fully porous particles, this has been generally shown to be a function of the performance and availability of different instrumentation.[12-13] Most of the investigations into core-shell particle performance has been limited to analytical scale columns and the ability to utilize the performance benefits of core-shell based nano columns using commercially available LC-MS equipment has received little attention.[1-5, 8-11]

In this application, we investigate the effect of column selectivity and demonstrate its importance in the quality of data that is achieved in protein and peptide identifications in bottom-up proteomics applications. We also look at the influence and importance of selectivity between the trapping column and the nano analytical column when performing forward trap and elute in nano LC-MS.

Figure 1.
Typical efficiency values for columns packed with different size particles; fully porous vs. core-shell particle structure.



Column: bioZen™ Nano 3 µm Polar C18
bioZen Nano 3 µm Peptide PS-C18
bioZen Nano 2.6 µm Peptide XB-C18
Dimensions: 10 x 0.075 mm
Part No.: 00F-4782-AW-21
00F-4771-AW-21
00F-4768-AW-21
Trap: bioZen RP1
bioZen RP2
Dimensions: 10 x 0.075 mm
Part No.: 05N-4252-AW
05N-4754-AW
Pressure (bar): 300 bar
Mobile Phase: A: 98 % water, 2 % Acetonitrile, 0.2 % Formic Acid
B: 80 % Acetonitrile, 20 % water, 0.2 % Formic Acid

Gradient:

Time (min)	%B
0	3
30	40
35	70
40	80
41	3
45	3

Flow Rate: 350 nL/min
Temperature: 25 °C
LC System: EASY-nLC™ 1200 (HeLa Digest)
NanoLC™ 425 (SCIEX®) (PepCalMix)
Detection: nanoESI
Detector: Orbitrap™ HF (HeLa Digest)
6500 QTRAP® (SCIEX) (PepCalMix)
Injection Volume: 0.5 µL

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Results and Discussion

In this investigation nano LC columns with three different stationary phase bonding chemistries were investigated. Two of the chemistries were bonded on a new generation of traditional fully porous 3 µm particles whereas the third was bonded upon a core-shell based particle platform. **Table 1** summarizes the three different stationary phases. When investigating the number of peptide and protein identifications obtained on the different columns studied in this investigation, a quality control standard of digested HeLa S3 cells was used. In **Figure 2** the total ion chromatograms from these three columns are shown. Overall, there is good peptide distribution observed for all three columns. The absence of large peaks at the end of the run during the high organic section of the gradient is a good indication that there is no contamination or bleed coming from the columns or column bodies. In **Figure 3** the number of proteins and the number of peptides that were successfully identified from the HeLa sample are shown. The highest number of identifications for both proteins and peptides was realized on the core-shell based bioZen™ Nano XB-C18 column. Core-shell based columns provide narrower peak widths for a given particles size and it is this reduction in peak width that is directly contributing to the bioZen Nano XB-C18 column producing more protein and peptide identifications in comparison to the bioZen Nano Polar C18 and PS-C18 columns based on fully porous particles. Having narrower peak widths leads to better resolution and higher peak capacities, which in turn decreases sample identification redundancy by allowing the mass spectrometer to perform scans on a larger analyte pool.[14]

In **Figure 4** the separation of 20 isotopically labeled peptides is shown on nano columns having three different stationary phase selectivities. As would be expected the core-shell based bioZen Nano XB-C18 column had narrower peaks overall. The bioZen Nano Polar C18 column however had greater overall retention of the peptides. Interestingly the bioZen Nano PS-C18 column showed very short retention for the most hydrophilic IGN peptides. While the overall retention of the peptide was short on the bioZen Nano PS-C18 column, the peak is extremely narrow. Because of the positive charge at the surface, the ionic interaction between the positively charged analyte and the negative charge of any unbonded silanols is eliminated on the bioZen Nano PS-C18 column, leading to the peak shape improvement. Overall, in the chromatograms there are several elution order differences because of the differences in the stationary phase selectivities, which affords the researcher choices for their method development.

We investigated two different trap stationary phase selectivities that were packed into 10 x 0.075 mm traps. The first stationary phase is a 5 µm particle with a C18 chemistry (bioZen RP1) and the second was also a 5 µm particle bonded with two different ligands. The two different ligands on the second trap consisted of a C18 and a polar moiety that provides for better retention of polar compounds as well as providing better stationary phase stability under 100 % aqueous conditions. In **Figures 5-7** are plots of the number of proteins and peptides that we identified in direct inject, and trap and elute with both the bioZen RP1 and RP2 traps for the bioZen Nano XB-C18 column, bioZen Nano Polar C18, and bioZen Nano PS-C18 columns, respectively. In the case of all three columns, moving from direct inject to the trap and elute workflow resulted in a small loss of identified peptides and proteins in the range of 10 % for the best column trap pairs and as low as 30 % for the worst performing pair. It is interesting to note that the core-shell based column was the most impacted in terms of the number of protein and peptide identifications when moving to the trap and elute in the forward elute direction.

In **Figure 8**, the peptide ion chromatograms of the bioZen Nano XB-C18, Polar C18, and PS-C18 are shown in direct inject (pink), with the bioZen RP1 trap (green) and bioZen RP2 trap (blue). In all cases when the traps were used, there is a significant delay in elution between the direct inject and the trap and elute chromatograms. This would make sense because in order to perform trap and elute there is an addition of an extra valve and significant lengths of connection tubing, all of which adds extra volume which therefore delays the gradient getting to the analytical nano LC column. This directly leads to a reduction in overall peak capacity (**Table 2**).

An area of interest in proteomics is phosphorylation. We looked specifically at phosphorylated protein identifications that were obtained on the 3 different columns and two different trap combinations. As would be expected in direct inject mode, the core-shell based bioZen Nano XB-C18 column showed a larger number of phosphorated protein identifications in comparison to the bioZen Nano Polar C18 and PS-C18 columns. However, when we moved to trap and elute mode, the bioZen Polar RP column showed a greater number of identifications with the best trap and elute combination being the bioZen Polar C18 column with the RP2 traps (**Figure 9**). We also looked at the identification of N-terminal acetylation proteins with the three different columns and two different trap combinations (**Figure 10**). Unlike the case of the phosphorylated proteins, the acetylation results were much more in line with what was seen when looking at the HeLa sample as a whole. In direct inject mode, the core-shell based column showed the highest number of identifications, whereas when moving to trap and elute mode, the core-shell bioZen Nano XB-C18 column performed similarly to that of the bioZen Nano Polar C18 column. In this experiment the RP1 traps performed slightly better with both the bioZen Nano XB-C18 and Polar C18 columns than that of the RP2 traps.

Table 1.
Outline and description of the stationary phases investigated in nano LC-MS.

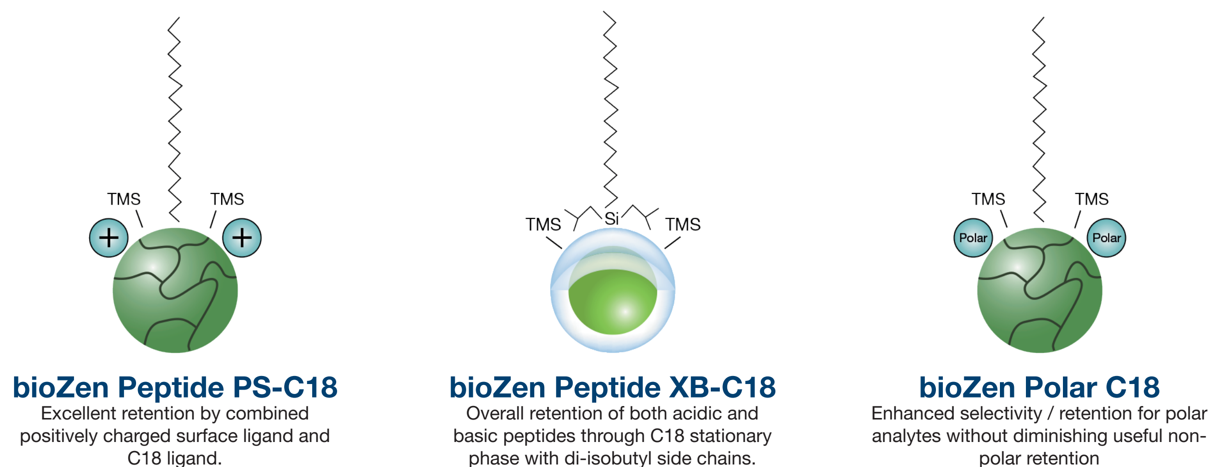


Figure 2.
Total ion chromatograms obtained by injecting 200 ng of a digested HeLa on A) bioZen 2.6 µm XB-C18 150 x 0.075 mm B) bioZen 3 µm Polar C18 150 x 0.075 mm C) bioZen 3 µm PS-C18 150 x 0.075 mm.

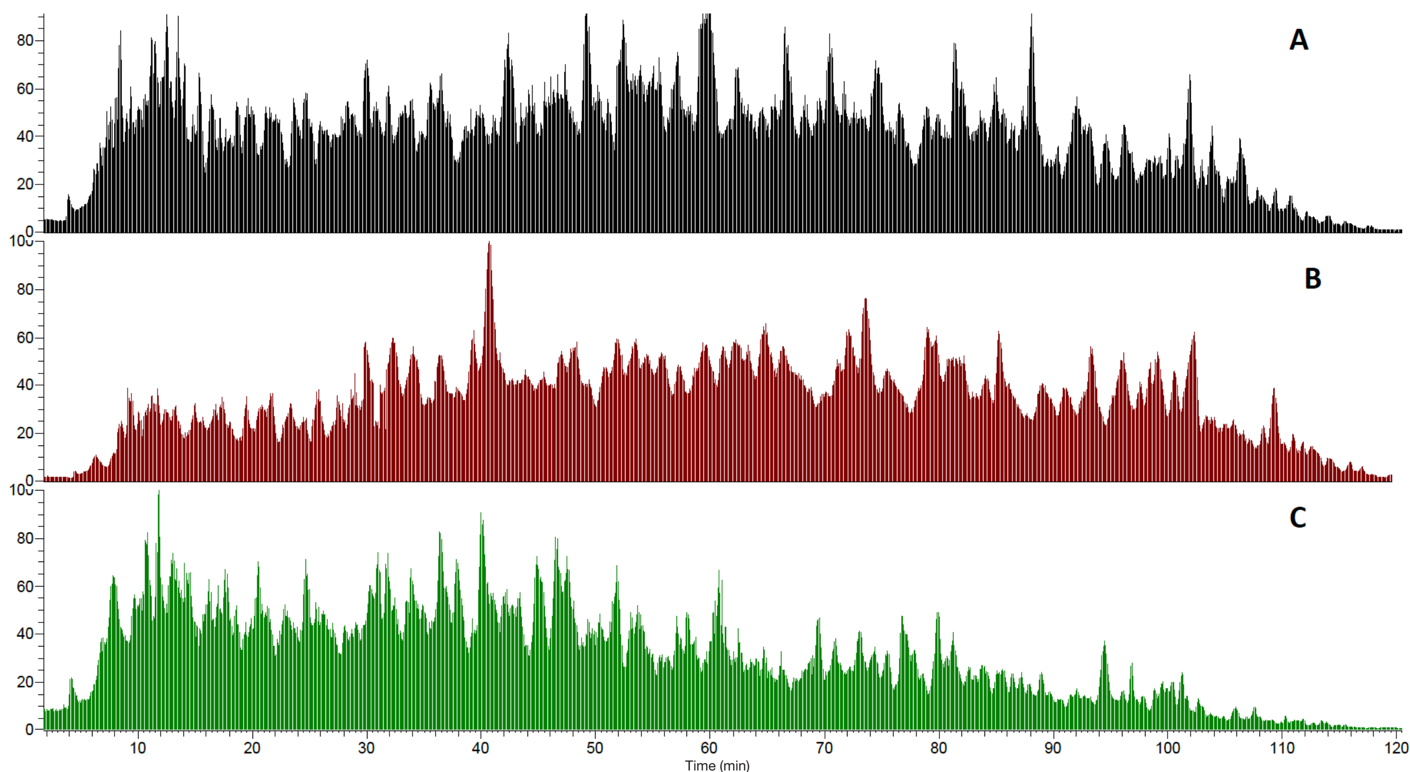
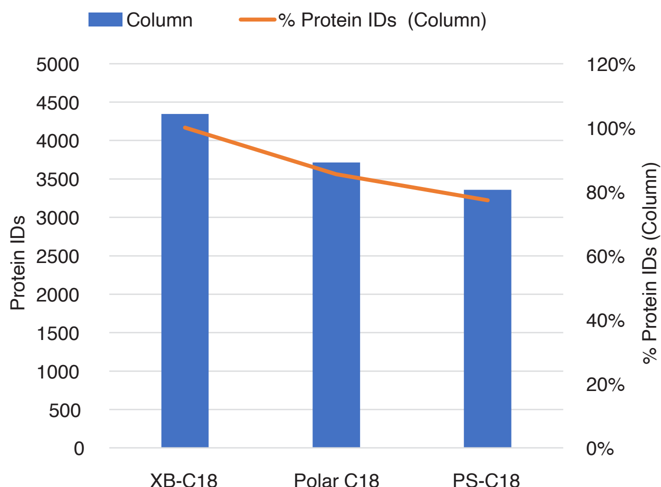


Figure 3.
The total number of proteins and peptides that were identified using nano LC-MS analysis while using bioZen™ 2.6 µm XB-C18, bioZen 3 µm Polar C18, and bioZen 3 µm PS-C18 columns (150 x 0.075 mm).



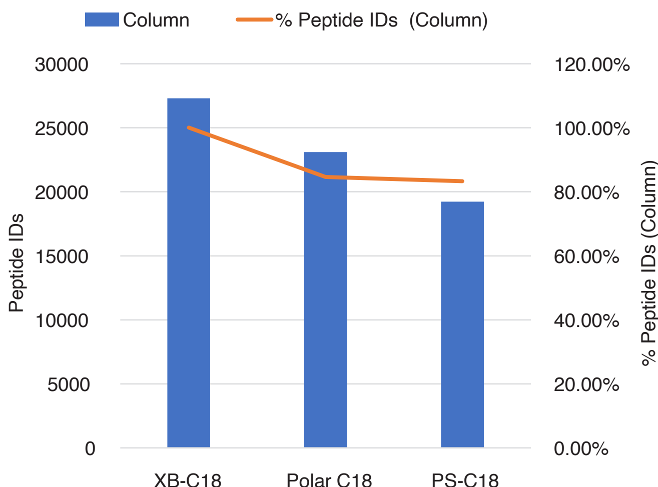


Figure 4.
Nano LC-MS chromatograms obtained on a mixture of 20 isotopically labeled peptides on 150 x 0.075 mm columns packed with A) bioZen 2.6 µm XB-C18 B) bioZen 3 µm Polar C18 and C) bioZen 3 µm PS-C18.

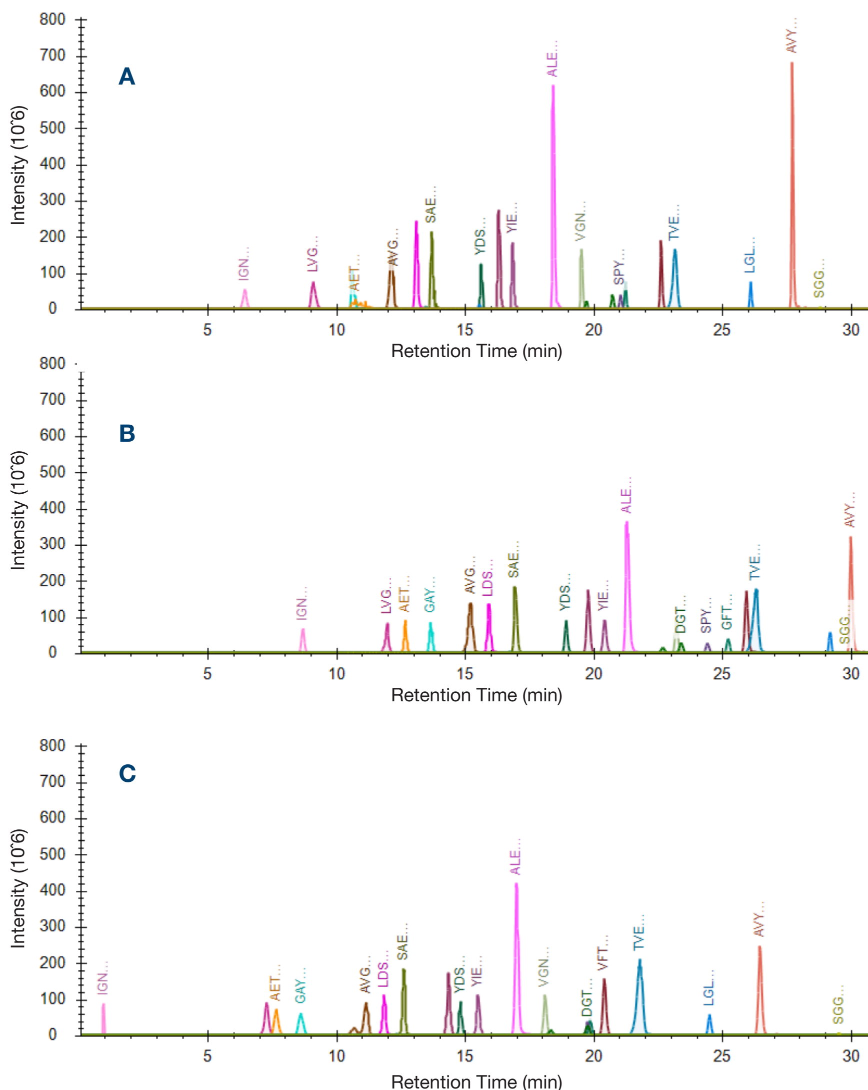
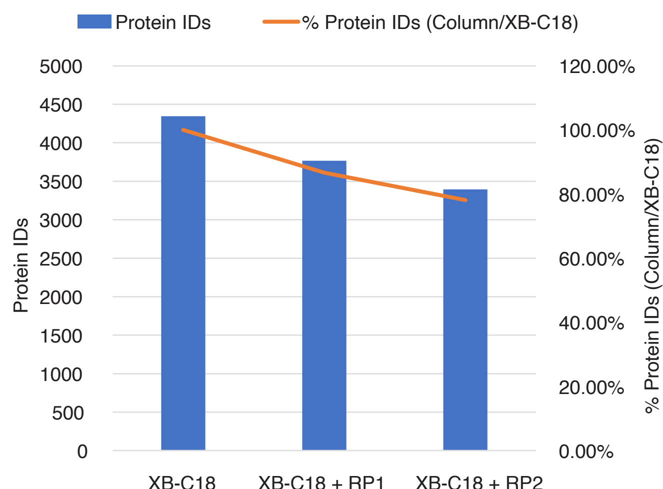


Figure 5.
Number of proteins and peptides that were identified on a nano LC-MS analysis of a digested HeLa sample using a bioZen™ 2.6 µm XB-C18 column (150 x 0.075 mm) formatted in direct inject, trap and elute using a 10 x 0.075 mm RP1 trap and a 10 x 0.075 mm RP2 trap respectively.



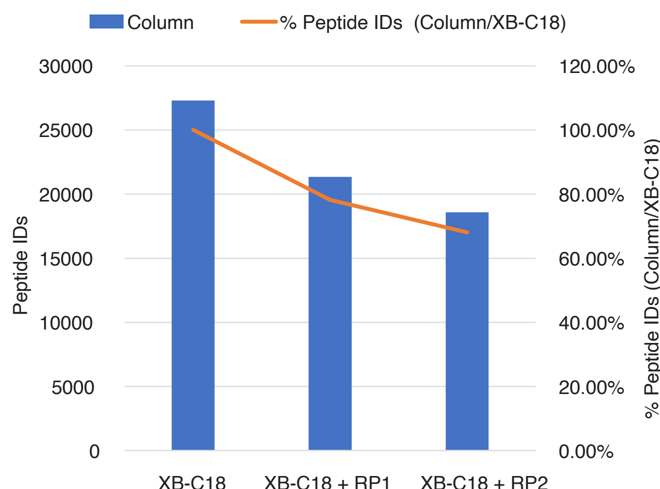
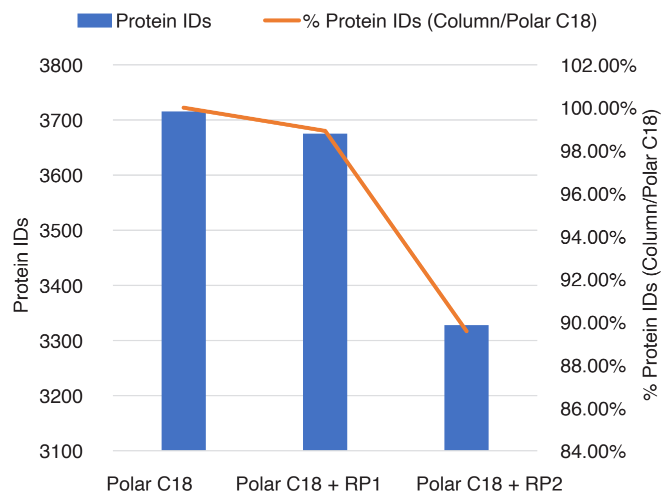


Figure 6.
Number of proteins and peptides that were identified on a nano LC-MS analysis of a digested HeLa sample using a bioZen 3 µm Polar C18 column (150 x 0.075 mm) formatted in direct inject, trap and elute using a 10 x 0.075 mm RP1 trap and a 10 x 0.075 mm RP2 trap respectively.



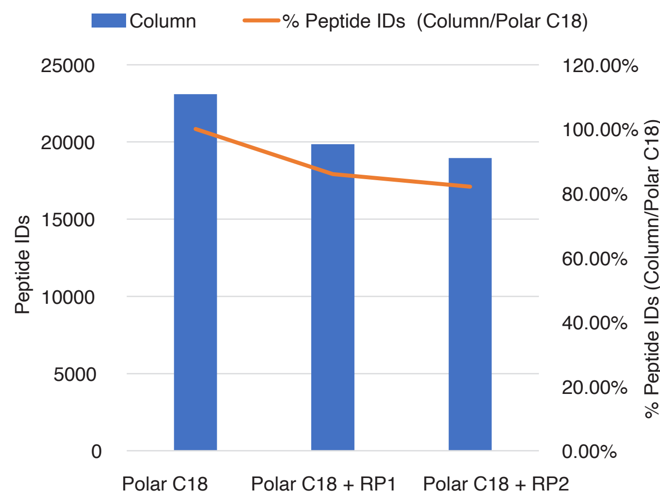
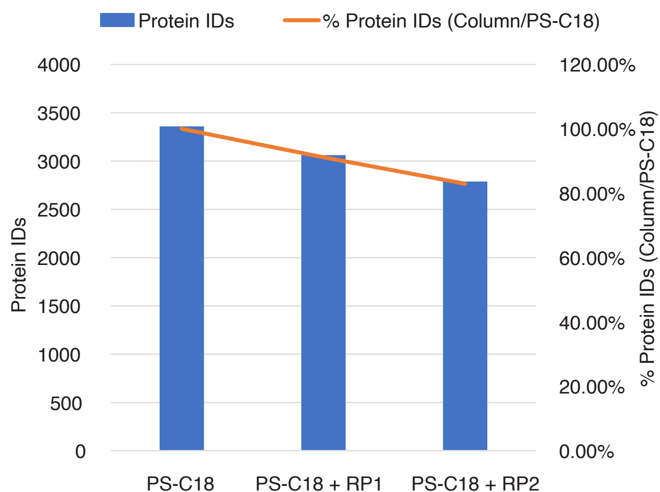


Figure 7.
Number of proteins and peptides that were identified on a nano LC-MS analysis of a digested HeLa sample using a bioZen™ 3 µm PS-C18 column (150 x 0.075 mm) formatted in direct inject, trap and elute using a 10 x 0.075 mm RP1 trap and a 10 x 0.075 mm RP2 trap respectively.

PS-C18 + Trap Selectivity Protein Identifications



PS-C18 + Trap Selectivity Peptide Identifications

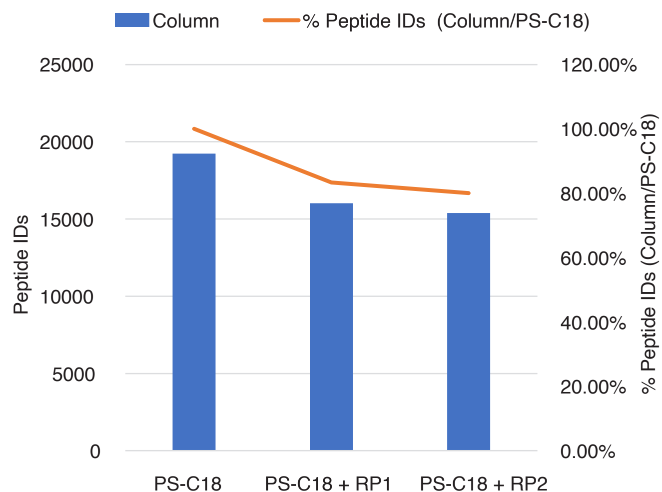


Figure 8.
Peptide ion chromatograms obtained during a nano LC-MS analysis of a digested HeLa sample using bioZen 3 µm Polar C18 column, bioZen 3 µm PS C18 column, and bioZen 2.6 µm XB-C18 columns (150 x 0.075 mm) formatted in direct inject (pink), trap and elute using a 10 x 0.075 mm RP1 trap (green) and a 10 x 0.075 mm RP2 trap (light blue).

Peptide Ion Chromatogram

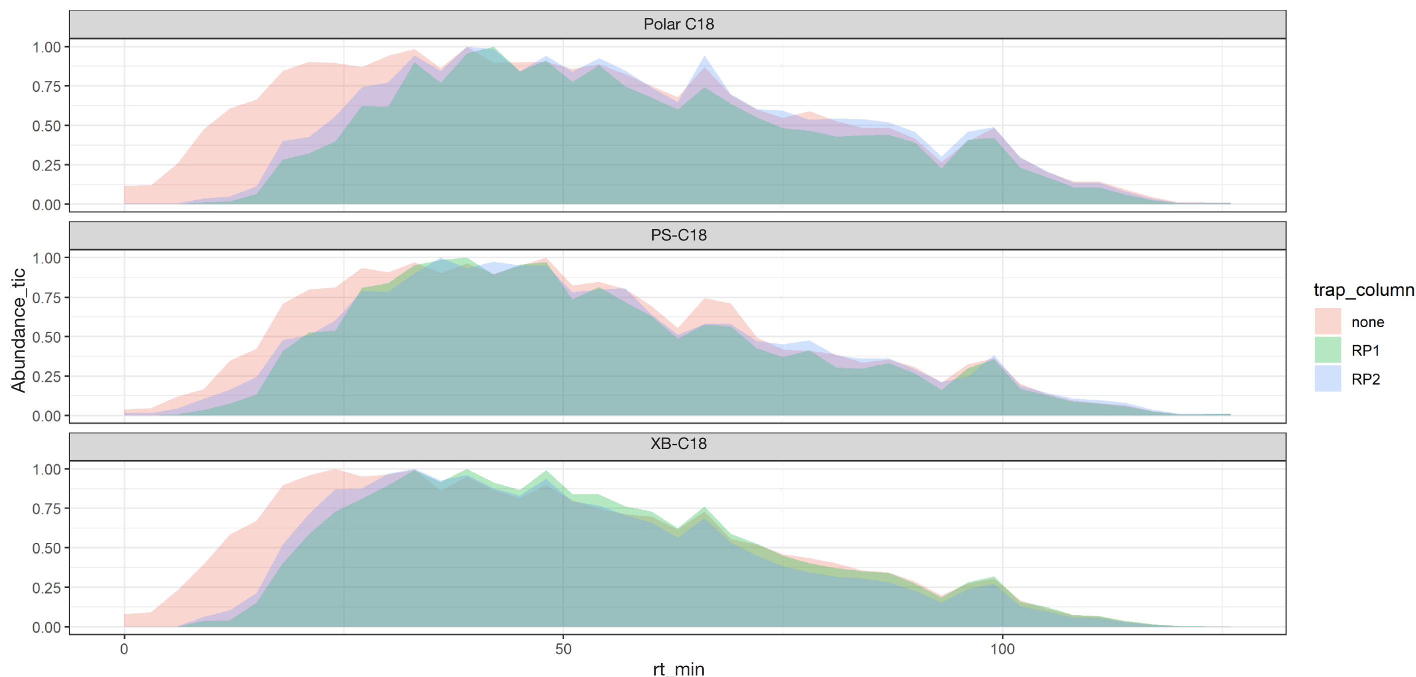


Table 2.
Average peak width, and peak capacity for nano column and trap combination with the theoretical loss of peak capacity due to peak broadening and gradient window reduction as well as the observed reduction in protein identifications.

Column	Trap	Average Peak Width (s)	Peak Capacity	Theoretical % Identification	Observed % Identifications
bioZen™ 2.6 µm XB-C18 150 x 0.075 mm	None	10	720	—	—
bioZen 2.6 µm XB-C18 150 x 0.075 mm	RP1 10 x 0.075 mm	11	545	76	78
bioZen 2.6 µm XB-C18 150 x 0.075 mm	RP2 10 x 0.075 mm	11	545	76	68
bioZen 3 µm Polar C18 150 x 0.075 mm	None	13	554	—	—
bioZen 3 µm Polar C18 150 x 0.075 mm	RP1 10 x 0.075 mm	12	500	90	86
bioZen 3 µm Polar C18 150 x 0.075 mm	RP2 10 x 0.075 mm	12	500	90	82
bioZen 3 µm PS-C18 150 x 0.075 mm	None	13	554	—	—
bioZen 3 µm PS-C18 150 x 0.075 mm	RP1 10 x 0.075 mm	12	500	90	83
bioZen 3 µm PS-C18 150 x 0.075 mm	RP2 10 x 0.075 mm	12	500	90	80

Figure 9.
Number of phosphorylated proteins that were identified on a nano LC-MS analysis of a digested HeLa sample using a bioZen 2.6 µm XB-C18 column, bioZen 3 µm Polar C18 column, bioZen 3 µm PS-C18 column, and in a 150 x 0.075 mm formatted in direct inject, trap and elute using a 10 x 0.075 mm RP1 trap and a 10 x 0.075 mm RP2 trap.

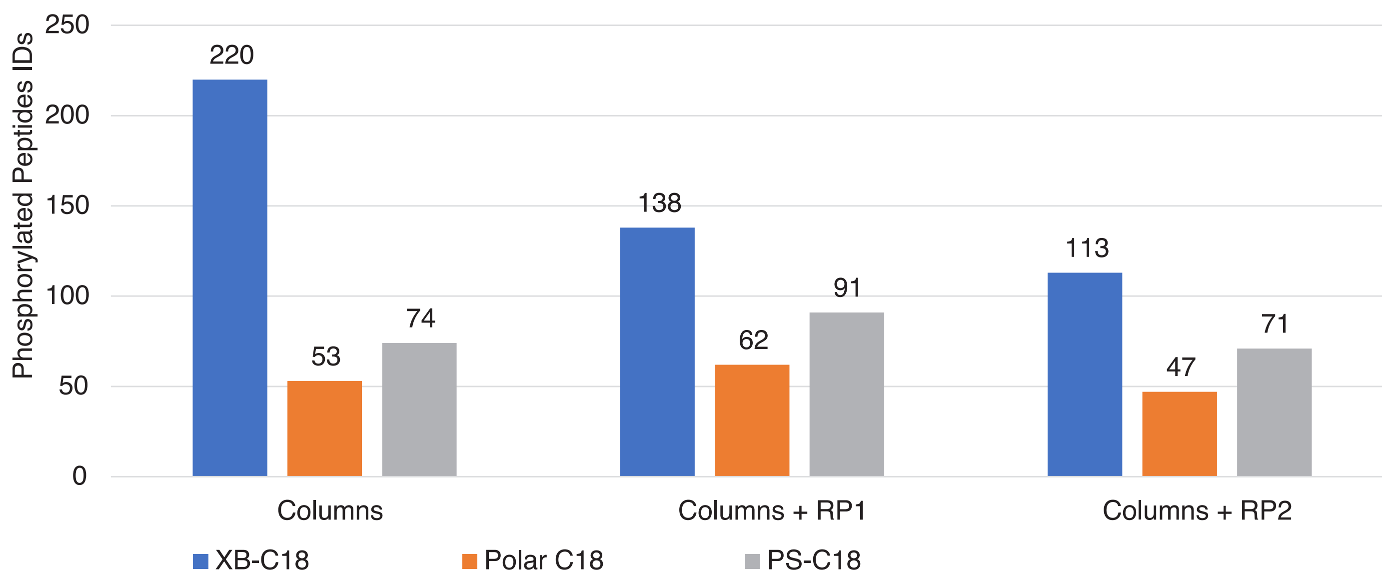
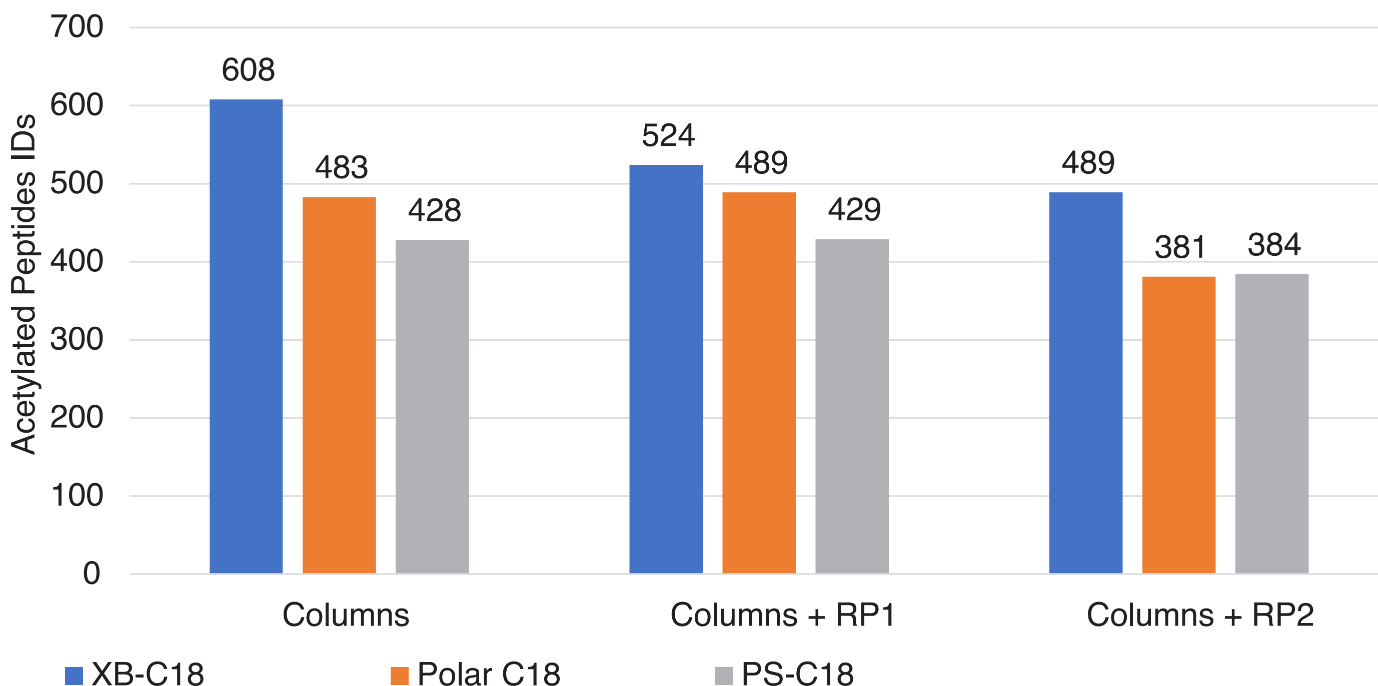


Figure 10.
Number of N-terminal acetylation proteins that were identified on a nano LC-MS analysis of a digested HeLA sample using a bioZen™ 2.6 µm XB-C18 column, bioZen 3 µm Polar C18 column, bioZen 3 µm PS-C18 column, and in a 150 x 0.075 mm formatted in direct inject, trap and elute using a 10 x 0.075 mm RP1 trap and a 10 x 0.075 mm RP2 trap.



Conclusions

The miniaturization of LC columns is a powerful tool to increase MS sensitivity. By combining nano LC column technology with highly efficient column packing materials such as core-shell and thermally modified particles, a laboratory can increase the benefits through narrower chromatographic peaks. The narrower peaks lead to higher sensitivity as well as increases in the number of protein and peptide identifications by as much as 10 %. As with analytical scale LC separations the choice of column chemistry can greatly affect the separation. Having multiple selectivity options in nano LC columns allows one to tailor the chromatography for the compounds of interest to the researcher. Selectivity is also an important consideration when doing trap and elute injections, especially when operating in the forward elute configuration. Having the ability to pair the appropriate trap selectivity with the column chemistry of choice greatly improves separation quality providing for better protein and peptide identifications. When moving from direct inject to trap and elute it is important to take into account the additional gradient delay volume that is added and adjust the gradient and the method appropriately as to not lose peak capacity.

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Ordering Information

bioZen Nano LC Columns with Integrated SecurityLINK™ Fingertight Fitting

Phases	150 x 0.075 mm	250 x 0.075 mm	500 x 0.075 mm
bioZen 3 µm Peptide PS-C18	00F-4771-AW-21	00G-4771-AW-21	–
bioZen 2.6 µm Peptide XB-C18	00F-4768-AW-21	00G-4768-AW-21	–
bioZen 3 µm Polar C18	00F-4782-AW-21	00G-4782-AW-21	–
bioZen 5 µm Peptide XB-C18	–	–	00J-4792-AW-21

bioZen Nano LC Columns with Open Fused-Silica Inlet Fitting

Phases	150 x 0.075 mm	250 x 0.075 mm	500 x 0.075 mm
bioZen 3 µm Peptide PS-C18	00F-4771-AW-11	00G-4771-AW-11	–
bioZen 2.6 µm Peptide XB-C18	00F-4768-AW-11	00G-4768-AW-11	–
bioZen 3 µm Polar C18	00F-4782-AW-11	00G-4782-AW-11	–
bioZen 5 µm Peptide XB-C18	–	–	00J-4792-AW-11

bioZen Trap Columns

Nano Trap Columns		
Phases	10 x 0.075 mm	Unit
RP1 (General RP)	05N-4252-AW	3/pk
RP2 (Aqueous Stable RP)	05N-4754-AW	3/pk

PEEKLok™ Trap Fittings

Trap Fittings		
Part No.	Description	Unit
AQ0-7602	PEEKLok fittings with 6-40 thread for 1/32” OD tubing (2 x fittings, 6 x ferrules and 1 x tightening tool)	ea
AQ0-7603	PEEKLok fittings with 6-32 thread for 1/32” OD tubing (2 x fittings, 6 x ferrules and 1 x tightening tool)	ea
AQ0-7600	PEEKLok fittings with 10-32 thread for 1/32”OD tubing with low profile hex head (2 x fittings, 6 x ferrules and 1 x wrench)	ea

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