Investigating the Performance Attributes of Stationary Phase Selectivity and Particle Morphology in Nano LC-MS

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Introduction

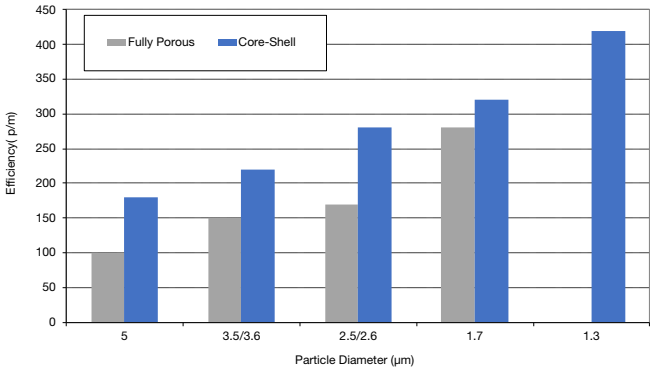
For many years there has been a great interest in miniaturized separations, especially in LC-MS. There are a good number of review papers available that detail the approaches and achievements in LC-MS miniaturization including common approaches and application areas.[1-5] Most often miniaturized LC-MS 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 applications including proteomics, metabolomics, lipidomics, and foodomics.[1,3,6-7] In many of these applications the amount of sample available can be 1 ng or even less, highlighting the need for increased MS sensitivity. While there can be some inconstancies in terminologies in miniaturized LC, it is generally accepted that "Nano LC" relates to LC performance in columns having an inner diameter (ID) of 100 µm or less and operated at flow rates in the 100-800 nL/min range.[1] Moving to smaller ID 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.

A very common workflow in nano LC-MS is the use of trap and elute injection. In this workflow, the sample is moved from the injection loop onto a short column or cartridge containing a stationary phase material that traps the analytes of interest. During this trapping step the sample can be washed with an appropriate solvent to remove unwanted and insoluble matter before injection onto the analytical nano LC column. This transfer from the trap to the analytical nano LC column can be done either in the same flow direction as the loading step (forward elute) or in the opposite flow direction to the loading step (back elute). The two primary drivers for performing a trap and elute injection are injection time and column protection. While it is very common in LC to have small samples in terms of mass, it is also common for those samples to be dissolved in a disproportionately large volume of diluent. For example, to inject a 20 µL sample at a typical nano LC flow rate (350 nL/min) would take 57 minutes. By using a trap and elute workflow and loading the trap at 4 µL/min, the loading step only takes 5 minutes. The advantage of the forward elute workflow in trap and elute is that it provides the greatest column protection as potential contaminants must pass all the way through the trap to reach the analytical nano LC column. The drawback to forward elute mode is that the sample also must pass all the way through the trap to reach the column. This means there is less chance for peak recompression on the head of the analytical column and any selectivity differences between the trap and the column could influence the ultimate chromatographic separation efficiency obtained in the analytical nano LC column. The back elute workflow, while not providing the same level of column protection, does allow for greater flexibility in both trap size and stationary phase chemistry because it is possible to take advantage of sample refocusing on the analytical nano LC column.[5,8]

Another interesting area of research in LC optimization over the last 10 years has been the use of core-shell particles. These particles have been described using several different names, including but not limited to core-shell, Fused-Core®, superficially porous, and solid core. In general, these particles consist of a solid non-porous core surrounded by a porous silica layer containing the chromatographic stationary phase. There have been numerous publications and investigations on the performance and behavior of these particles. At one count there were over 100 publications on the subject.[9-12] 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.[9-12] 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.[13-14] 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, 9-12]

In this paper, we investigate several parameters that affect the performance of nano columns and traps in nano LC-MS separations of proteins and peptides. We investigate the effect of column selectivity and demonstrate the importance of different selectivities 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. We also evaluate the influence of trap dimensions and their impact on peak capacity and band broadening. Finally, we look at the impact of sub-2 µm fully porous particles and core-shell particles in nano LC-MS with commercially available instrumentation and the effect they have on separation performance as well as protein and peptide identifications in proteomics.

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



Data Analysis

Proteome Discoverer (Thermo Fisher Scientific) version 2.5 was used to obtain the number of protein and peptide identifications. Skyline (University of Washington) version 19.1.0.193 was used for qualitative purposes.

Experimental Methods

Since multiple methods were used throughout this paper, experimental methods can be found near the figures where they were employed.

Sample:

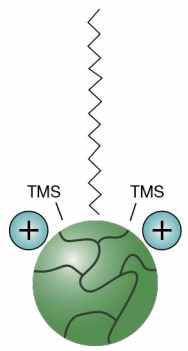
PepCalMix Standard Peptides (SCIEX®) 10 fmol/µL mixture was used with SCIEX instruments for Multiple Reaction Monitoring (MRM) purposes. HeLa tryptic digest (Pierce) was used with Thermo Fisher instrumentation for protein and peptide identification at a 100 ng/µL concentration.

Results and Discussion:

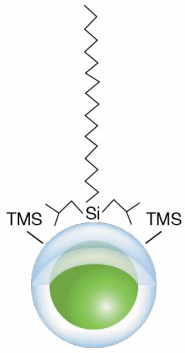
In this investigation Nano LC columns with three different stationary phase bonding chemistries were investigated. Two of the chemistries were bonded on a new generation of traditional fully porous 3 µm particles whereas the third was bonded upon a core-shell based particle platform. **Table 1** summarizes the three different stationary phases. When investigating the number or peptide and protein identifications obtained on the different columns studied in this investigation a quality control standard of digested HeLA S3 cells was used. This sample was chosen as it is a readily available QC standard with well documented characteristics.[15] 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. It is interesting to note the relatively higher abundance of peptides eluting earlier in the chromatogram for the Peptide PS-C18 column than that of the Polar C18 or Peptide XB-C18 columns. This will be discussed in further detail in the next section.

One of the most important goals of proteomic experiments, especially in discovery, is the ability to identify peptides and subsequently proteins. 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 Peptide XB-C18 column. As was stated in the introduction, core-shell based columns provide narrower peak widths for a given particles size. This will be discussed in more detail in a subsequent section of this whitepaper. It is this reduction in peak width that is directly contributing to the bioZen™ Nano Peptide XB-C18 column producing more protein and peptide identifications in comparison to the Polar C18 and Peptide PS-C18 columns. 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.[16]

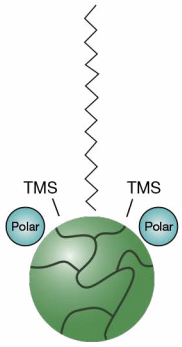
Table 1.
Outline and description of the stationary phases investigated in nano LC-MS.



bioZen Peptide PS-C18
Excellent retention by combined positively charged surface ligand and C18 ligand.



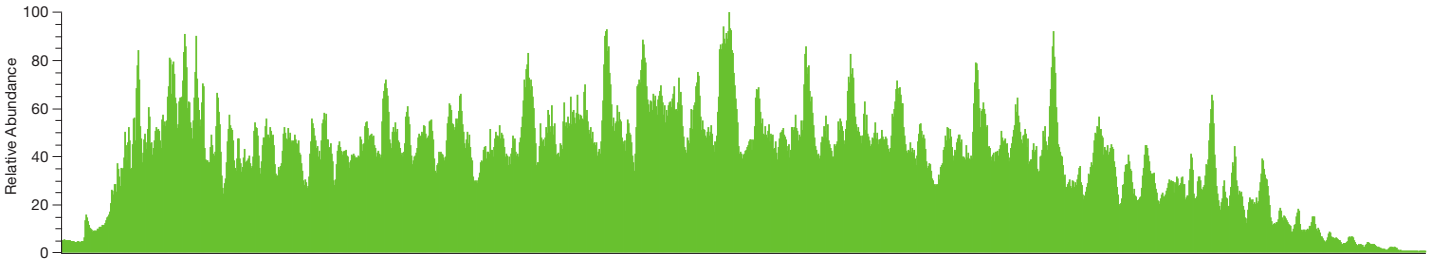
bioZen Peptide XB-C18
Overall retention of both acidic and basic peptides through C18 stationary phase with di-isobutyl side chains.



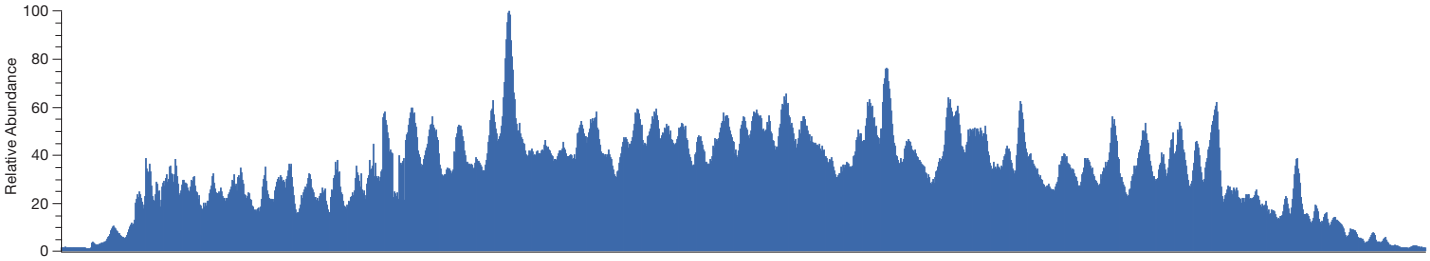
bioZen Polar C18
Enhanced selectivity / retention for polar analytes without diminishing useful non-polar retention

Figure 2.
Total ion chromatograms obtained by injecting 200 ng of a digested HeLa sample.

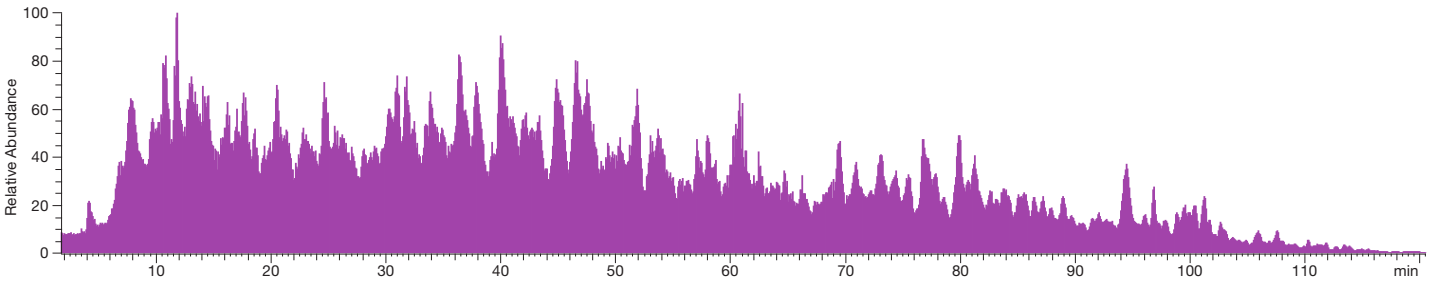
bioZen™ 2.6 µm Peptide XB-C18



bioZen 3 µm Polar C18



bioZen 3 µm Peptide PS-C18



The separation of 20 isotopically labeled peptides is shown on nano columns having three different stationary phase selectivities in **Figure 4**. As would be expected, the core-shell based Peptide XB-C18 column had narrower peaks overall. The Polar C18 column, however, had greater overall retention of the peptides. Interestingly the Peptide PS-C18 column showed very short retention for the most hydrophilic IGN peptide. Even though overall retention of the peptide was short on the Peptide PS-C18 column, the peak is extremely narrow. Due to 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 Peptide PS-C18 column, leading to the peak shape improvement. Overall, there are several elution order differences because of the differences in the stationary phase selectivity, which affords the researcher choices for their method development.

All of the trap and elute experiments presented in this work were performed in the forward elute configuration. This was done because this workflow provides the best column protection but is also the most chromatographically sensitive and therefore requires the most attention and optimization.

LC Conditions (fig 1-10. Table 1-2)

Column: bioZen™ Nano 3 µm Polar C18
bioZen Nano 3 µm Peptide PS-C18
bioZen Nano 2.6 µm Peptide XB-C18
Dimensions: 150 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	2
7.5	6
90	25
120	40
121	98
136	98

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

ID	Q1 M/Z	Q3 M/Z	CE
PEPCALMIX.AETSELHTSLK.+3Y5.HEAVY	408.55	593.35	24.3
PEPCALMIX.AGLIVAEGVTK.+2Y7.HEAVY	533.32	711.41	26.5
PEPCALMIX.ALENDIGVPSDATVK.+2Y7.HEAVY	768.9	725.39	35.6
PEPCALMIX.AVGANPEQLTR.+2Y6.HEAVY	583.31	753.41	31.5
PEPCALMIX.AVYFYAPQIPLYANK.+2Y6.HEAVY	883.47	713.41	47.9
PEPCALMIX.DGTFAVDGPGVIAK.+2Y7.HEAVY	677.86	649.41	33.6
PEPCALMIX.GAYVEVTAK.+2Y5.HEAVY	473.26	555.32	23.9
PEPCALMIX.GFTAYYIPR.+2Y5.HEAVY	549.29	721.39	26.9
PEPCALMIX.IGNEQGVSR.+2Y8.HEAVY	485.25	856.41	26.7
PEPCALMIX.LDSTSIPVAK.+2Y4.HEAVY	519.8	422.29	34
PEPCALMIX.LGLDFDSFR.+2Y6.HEAVY	540.27	796.35	26.7
PEPCALMIX.LVGTPAEER.+2Y7.HEAVY	491.27	769.37	23.4
PEPCALMIX.SAEGLDASASLR.+2Y7.HEAVY	593.8	729.38	30
PEPCALMIX.SGGLLWQLVR.+2Y5.HEAVY	569.83	711.42	29.6
PEPCALMIX.SPYVITGPGVVEYK.+2Y9.HEAVY	758.91	957.51	37.8
PEPCALMIX.TVESLFPEEAETPGSAVR.+2Y6.HEAVY	964.98	596.34	42.4
PEPCALMIX.VFTPLEVDVAK.+2Y8.HEAVY	613.35	878.51	26.3
PEPCALMIX.VGNEIQYVALR.+2Y6.HEAVY	636.35	759.44	33.5
PEPCALMIX.YDSINNTEVSGIR.+2Y9.HEAVY	739.36	999.51	40.3
PEPCALMIX.YIELAPGVDNSK.+2Y7.HEAVY	657.34	724.37	29

Multiple Reaction Monitoring

Source Parameters	
GS1:	35
GS2:	0
CAD:	Low
CUR:	30
IS:	2500
IHT:	150
DP:	80
EP:	10
CXP:	15

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

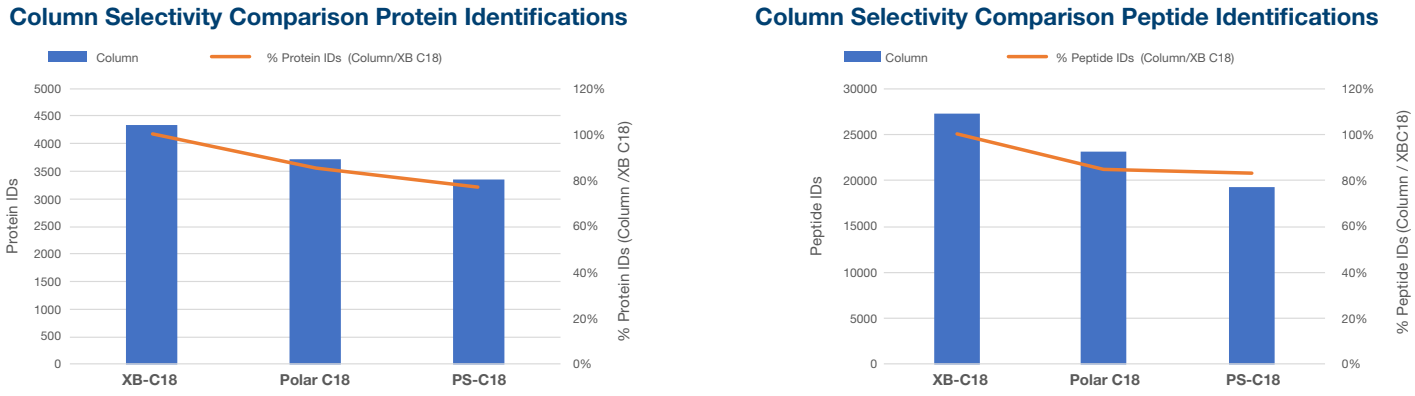
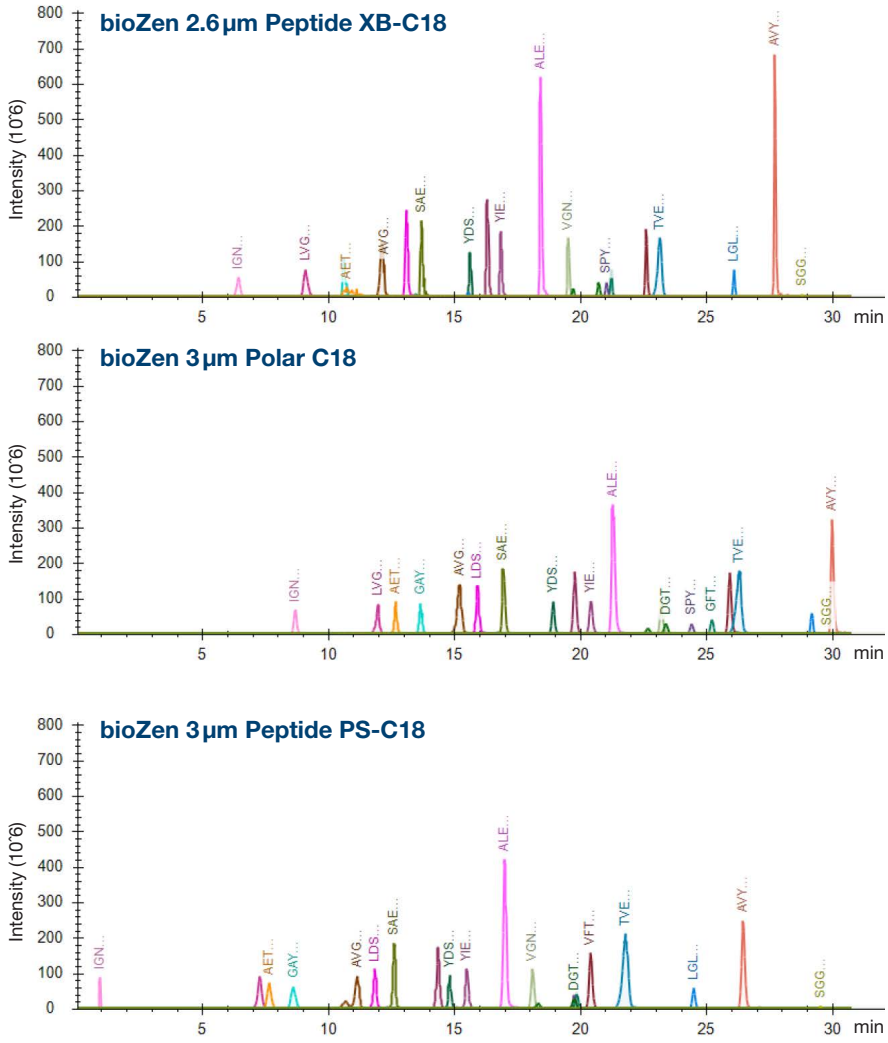
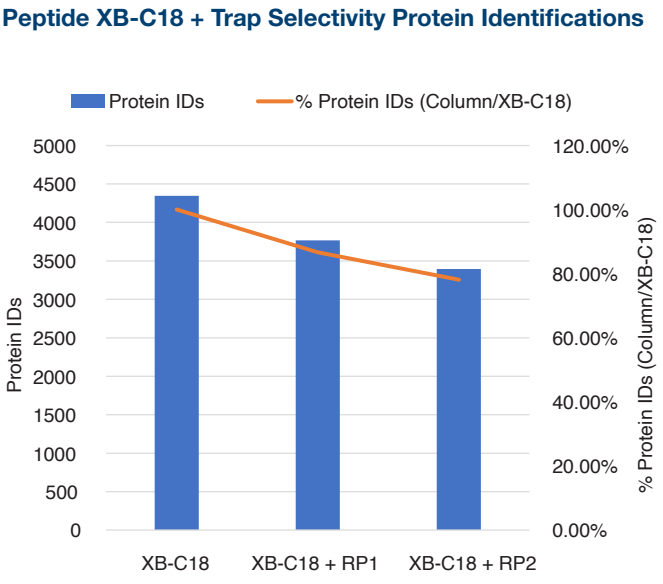


Figure 4.
Nano LC-MS chromatograms obtained from a mixture of 20 isotopically labeled peptides.



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 (RP1) and the second was also a 5 µm particle bonded with two different ligands (RP2). 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 SP stability under 100 % aqueous conditions. **Figures 5-7** show plots of the number of proteins and peptides that we identified in direct inject, and trap and elute with both the RP1 and RP2 traps for the bioZen™ Peptide XB-C18, bioZen Polar C18, and bioZen Peptide PS-C18 columns respectively. In the case of moving from direct inject to the trap and elute workflow, there was 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. Loss of peptide, and subsequently protein identifications, is common when moving to trap and elute workflows and generally impacts the most hydrophilic peptides the most, as they are the most difficult to retain in reversed phase workflows.[17] 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. As we saw in the previous section, the core-shell based columns have the narrowest peak widths that affords them the very high number of identifications. The core-shell columns therefore stand the most to lose with any disruptions to chromatographic performance. Even in the most optimized trap and elute system, the additional connections and volume by the addition of the trap and the trap valve will impact the chromatographic performance, resulting in wider peaks. Even with this small loss in identifications, the core-shell based columns in trap and elute provide better performance than other popular trap and elute nano LC-MS solutions that are available which will be shown in later sections of this paper. It is interesting to note that the difference in protein identifications when using the core-shell column with RP1 versus RP2 traps is greater than 10 %, whereas with the Polar C18 and Peptide PS-C18 columns the differential between them is <5 %. Again, this demonstrates that the high performance of the core-shell based columns is more sensitive to disruptions in chromatographic performance through volumetric or selectivity mismatches.

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 Peptide XB-C18 column formatted in direct inject, trap and elute using a RP1 trap and a RP2 trap respectively.



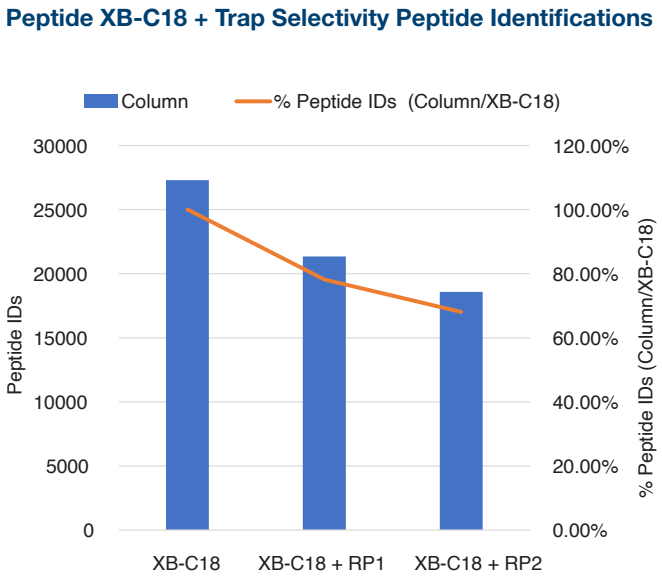
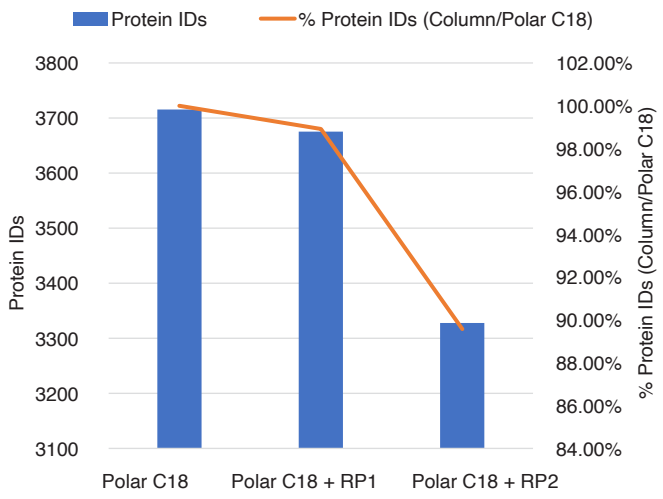


Figure 6.
Number of proteins and peptides that were identified on a nano LC-MS analysis of a digested HeLa sample using a bioZen™ 3 µm Polar C18 column formatted in direct inject, trap and elute using a RP1 trap and a RP2 trap respectively.



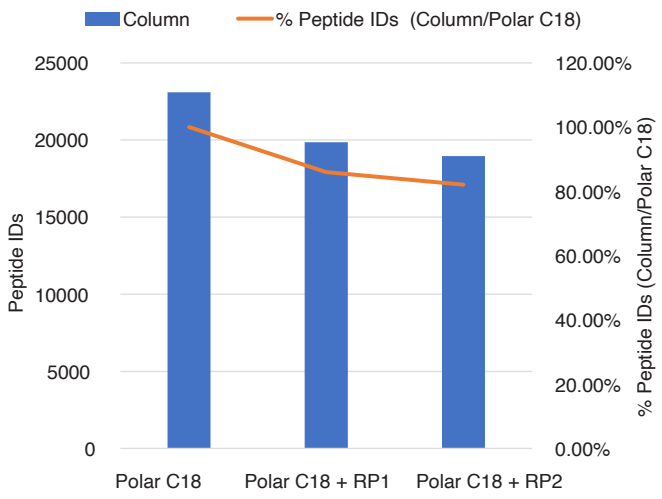
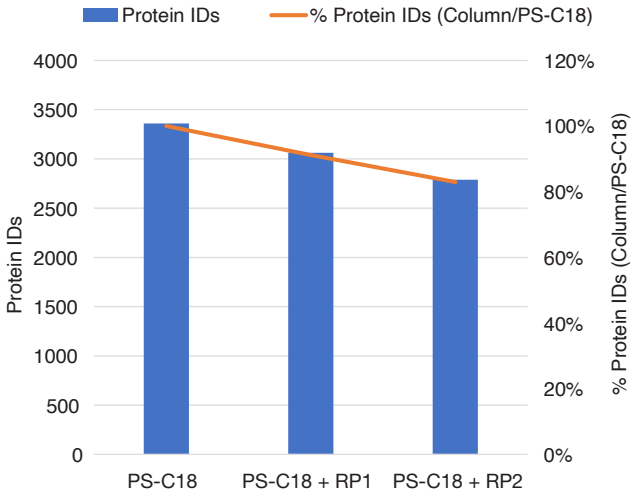
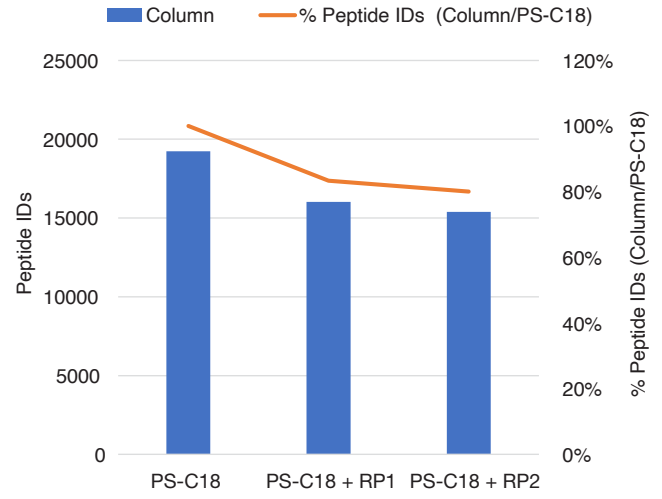


Figure 7.
Number of proteins and peptides that were identified on a nano LC-MS analysis of a digested HeLa sample using a bioZen 3 µm Peptide PS-C18 column formatted in direct inject, trap and elute using a RP1 trap and a RP2 trap respectively.





In **Figure 8**, the peptide ion chromatograms of the bioZen™ Peptide XB-C18, Polar C18, and Peptide PS-C18 are show in direct inject (pink), with the RP1 trap (green) and RP2 trap (blue). In all cases when the traps used, there is a significant delay in elution between the direct inject and the trap and elute chromatograms. This would make sense because to perform trap and elute there is an addition of an extra valve and significant lengths of connection tubing. This adds extra volume, which delays the gradient getting to the analytical nano LC column. This means that the gradient window in the trap and elute workflow was smaller than that of the direct inject. This directly leads to a reduction in overall peak capacity (**Table 2**). The reduction in peak capacity to a large extent explains the reduction in the number of peptides and proteins that were identified in trap and elute mode versus direct inject mode. In order to

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 Peptide PS C18 column, and bioZen 2.6 µm Peptide XB-C18 columns formatted in direct inject (pink), trap and elute using a RP1 trap (green) and a RP2 trap (light blue).

Peptide Ion Chromatogram

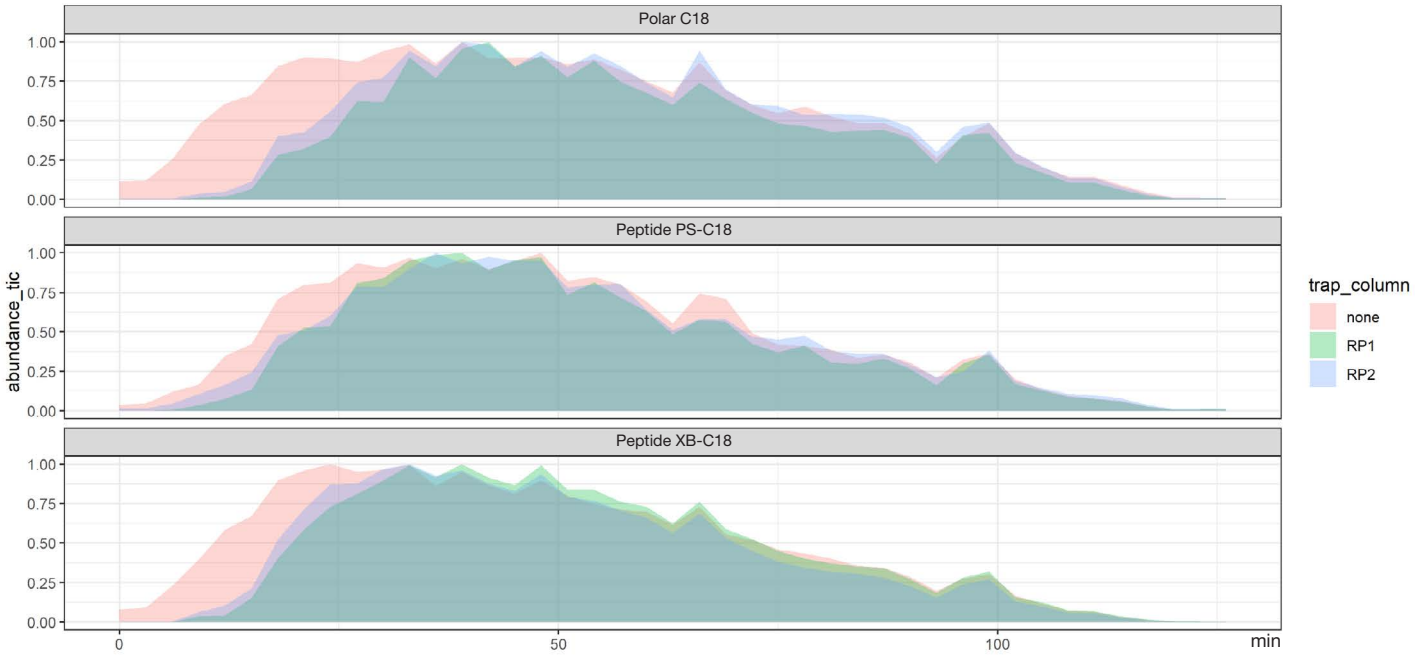


Table 2.
Average peak width, and peak capacity for nano column and trap combination with the theoretical loss of peak capacity due to peak broadening and gradient window reduction as well as the observed reduction in protein identifications.

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

normalize the comparison between direct inject and trap and elute, either an isocratic hold equal to trap and elute delay volume would need to be added to the beginning of the direct injection method, or the gradient run time on the trap and elute method should be increased so that a volume equal to the gradient delay would allow the latter half of the gradient to elute from the nano LC column. Since it is uncommon for scientists doing nano LC to switch back and forth between direct inject and trap and elute workflows, it is outside the scope of this work to show the direct scaling, but rather just highlight the impact of gradient delay volumes in nano LC and the significant impact they can have on data quality.

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 Peptide XB-C18 column showed a larger number of phosphorylated protein identifications in comparison to the Polar C18 and Peptide PS-C18 columns. However, when we moved to trap and elute mode the Polar C18 column showed a greater number of identifications, with the best trap and elute combination being the Polar C18 column with the RP2 traps. These results are shown in **Figure 9**. Even the combination of the Polar C18 column and RP2 traps still only generated 50 % of the identifications in comparison to direct inject on the core-shell based Peptide XB-C18 column. Phosphorylated peptides are generally hydrophilic, and as we have seen in the previous experiments, these hydrophilic peptides are harder to trap.

We also looked at the identification of N-terminal acetylation proteins with the three different columns and two different trap combinations. This data is shown in **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. In the direct inject mode, the core-shell based column showed the highest number of identifications. When moving to trap and elute mode, the core-shell Peptide XB-C18 column performed similarly to that of the Polar C18 column. In this experiment the RP1 traps performed slightly better with both the Peptide XB-C18 and Polar C18 columns than that of the RP2 traps.

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 Peptide XB-C18 column, bioZen 3 µm Polar C18 column, bioZen 3 µm Peptide PS-C18 column formatted in direct inject, trap and elute using a RP1 trap and a RP2 trap.

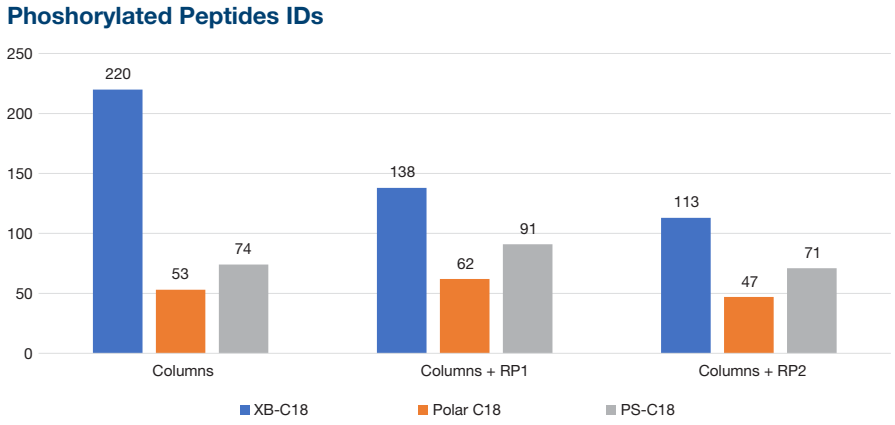
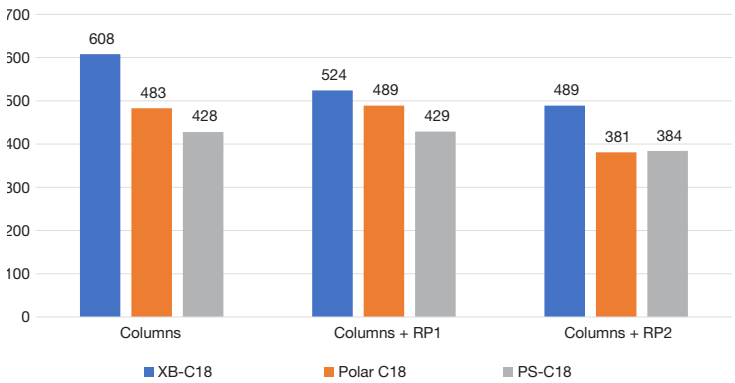


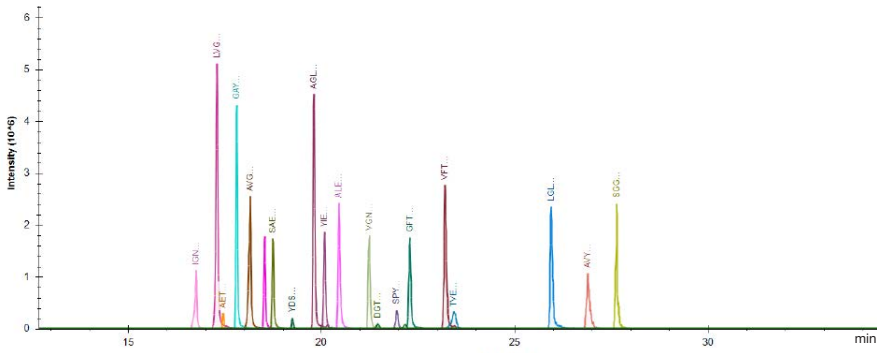
Figure 10.
Number of N-terminal acetylation proteins that were identified on a nano LC-MS analysis of a digested HeLA sample using a bioZen 2.6 µm Peptide XB-C18 column, bioZen 3 µm Polar C18 column, bioZen 3 µm Peptide PS-C18 column formatted in direct inject, trap and elute using a RP1 trap and a RP2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



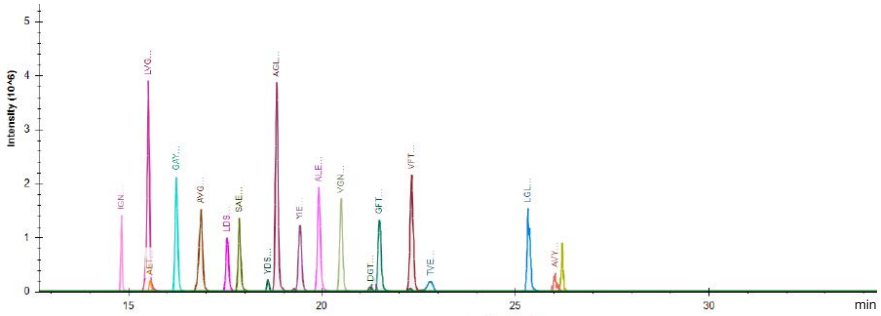
As was discussed in the introduction, the concept of core-shell particles in nano LC-MS is rather new and, unlike its analytical scale LC counterpart, has not been investigated in nearly as much depth. We investigated nano LC-MS separations done in both direct inject and trap and elute modes and compared those to not only a traditionally fully porous 3 µm column but also a fully porous sub-2 µm column. In **Figure 11**, chromatograms obtained from these three columns with a mixture of 20 isotopically labeled peptides are shown. Plots of the average peak width and tailing factor that were obtained in the separations shown in **Figure 11**, are shown in **Figure 12**. Moving from a fully porous 3 µm column to a fully porous sub-2 µm column resulted in a 53 % decrease in the average peak width, which is in close alignment to the theoretical 56 % decrease. The backpressure needed to run the separation with the sub-2 µm column was 5200 psi, as compared to 1800 psi with the 3 µm column. Backpressure increases with the square of the particle size so

Figure 11:
Nano LC-MS/MS chromatograms obtained from a mixture of 20 isotopically labeled peptides injected on columns packed with 2.6 µm core-shell Peptide XB-C18, Thermo Fisher® Acclaim™ PepMap™ 100 nanoViper™ 3 µm fully porous C18, and Waters® SpaceNanoEase™ M/Z Peptide BEH 1.7 µm fully porous C18 particles.

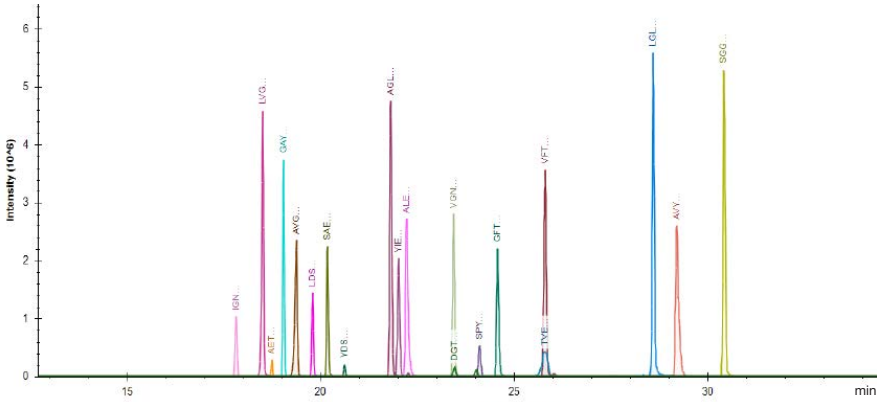
Core-shell 2.6 µm Peptide XB-C18
2000 psi at 250 nL/min



Fully porous 3 µm C18
1800 psi at 250 nL/min



Fully porous 1.7 µm C18
5200 psi at 250 nL/min



LC Conditions (Figures 11–15)

Column:	bioZen™ Nano 2.6 µm Peptide XB-C18
	Waters nanoEase 1.7 µm M/Z Symmetry C18
	Thermo Fisher Acclaim PepMap 100 3 µm nanoViper C18
Dimensions:	150 x 0.075 mm
	20 x 0.180 mm
	150 x 0.075 mm
Trap:	bioZen RP1
	nanoEase 1.7 µm M/Z Peptide BEH C18
	Acclaim PepMap 100 nanoViper
Dimensions:	10 x 0.075 mm
	150 x 0.075 mm
	20 x 0.1 mm
Pressure (bar):	250 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	2
	7.5	6
	90	25
	120	40
	121	98
	136	98

Flow Rate:	350 nL/min
Temperature:	25 °C
LC System:	EASY-nLC™ 1200
Detection:	nanoESI
Detector:	Orbitrap™ HF
Injection Volume:	2 µL

we would expect an increase of 3.1x for the sub 2 µm column. This was very close to what was experimentally observed. When we performed the same separation using a 2.6 µm core-shell column we observed peak widths identical to was obtained on the fully porous sub-2 µm column. The separation on the core-shell particle column also required a significantly lower backpressure of 2000 psi as compared to 5200 psi for the sub-2 µm column. This peak width result is aligned with what has been reported in the literature for analytical scale separations.[9-11, 13-14]

To investigate the effect that the narrow peak widths of both the core-shell and sub-2 µm column have on peptide and protein identifications, these columns were used to separate and analyze the HeLa S3 quality control standard. In **Figure 13**, the number of protein and peptide identifications that were obtained on a fully porous 3 µm, fully porous sub-2 µm and core-shell 2.6 µm column are shown. There was a substantial increase in the number of peptide (12 %) and protein (10 %) identifications obtained by moving from the fully porous 3 µm column to either the sub-2 µm fully porous or core-shell 2.6 µm column in direct inject mode. Again, the most significant difference between the core-shell column and the fully porous sub-2 µm column was the substantially higher backpressure required to use the sub-2 µm column.

We also investigated the effect of sub-2 µm fully porous and 2.6 µm core-shell particles in trap and elute mode and their impact on

Figure 12.
Peak widths and tailing factors obtained from a mixture of 20 isotopically labeled peptides injected on columns packed with 2.6 µm core-shell Peptide XB-C18, Thermo Fisher® Acclaim™ PepMap™ 100 nanoViper™ 3 µm fully porous C18, and Waters® NanoEase™ M/Z Peptide BEH 1.7 µm fully porous C18 particles, respectively

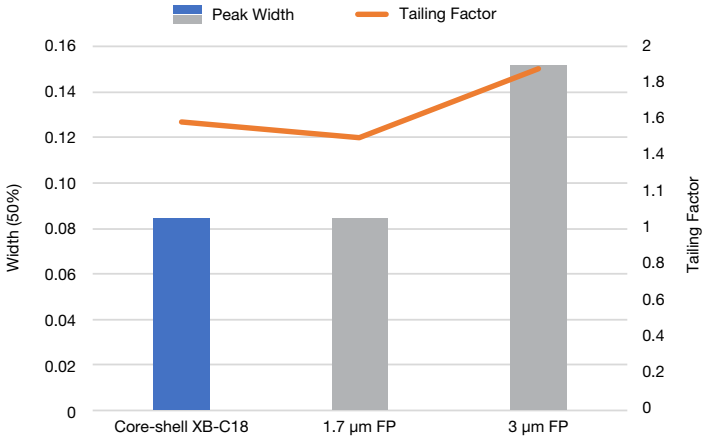
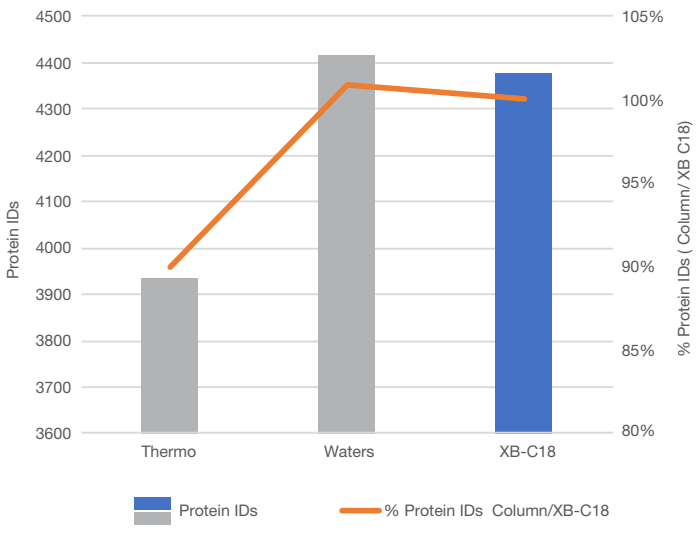
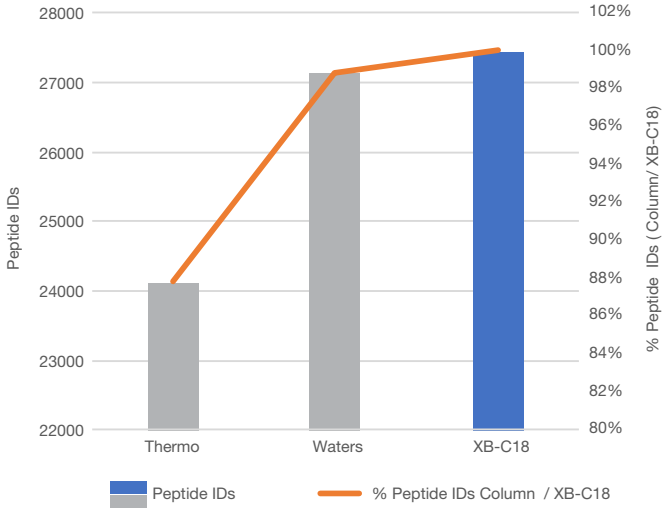


Figure 13.
Number of proteins and peptides that were identified on a nano LC-MS analysis of a digested HeLa sample using a Thermo Fisher Acclaim PepMap 100 nanoViper C18, Waters NanoEase M/Z Peptide BEH 1.7 µm C18, and bioZen 2.6 µm Peptide XB-C18, in direct inject mode.



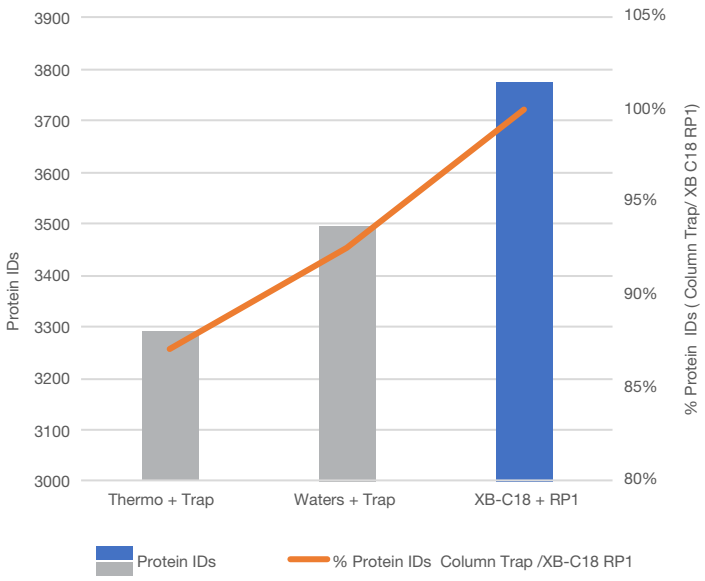


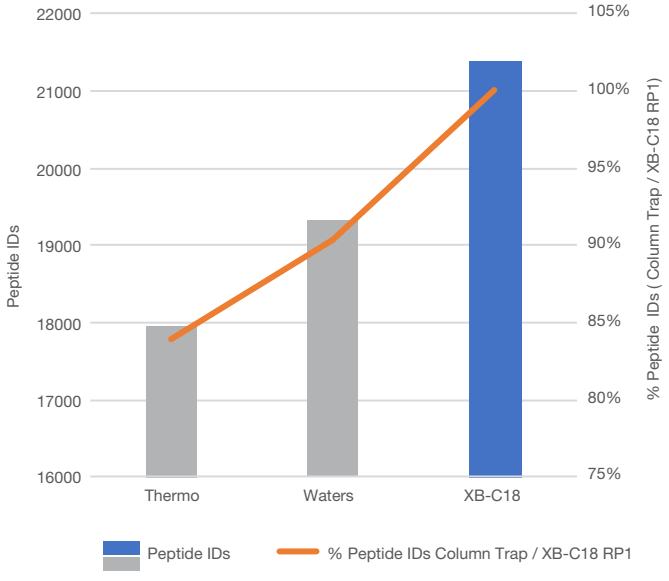
the number of protein and peptide identifications. As we saw in a previous section of this paper, the matching of trap selectivity to that of the column plays a role in the overall performance of the separation. Since the fully porous columns used for this comparison were manufactured by different vendors, the traps that we used in conjunction with those columns were the ones that were recommended by their respective vendors. In addition to having different particles and stationary phases in the traps, they also had different internal physical dimensions, and therefore volumes. It is important to note this as the experiments were performed in forward elute mode where such differences can play a role in overall performance. In **Figure 14** the number of protein and peptide identifications that were obtained on a fully porous 3 µm, sub-2 µm, and 2.6 µm core-shell column in trap and elute mode are shown. Unlike the case with the direct inject experiments, in the trap and elute experiments there was a substantial difference between the number of peptide (10 %) and protein (7 %) that were obtained when using the fully porous sub-2 µm column and trap system versus the core-shell column and trap system. This can be explained at least in part by the aforementioned differences in trap architecture. The trap for the fully porous sub-2 µm column was 20 x 0.180 mm whereas the trap for the core-shell based column was 10 x 0.075 mm. In **Table 3** the average peak widths for the fully porous 3 µm, sub-2 µm, and 2.6 µm core-shell column is shown both in direct inject and trap and elute mode. We can see that for the fully porous sub 2 µm column and trap system there is an increase in the average peak width from

Table 3.
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 % Identifications	Observed % Identifications
bioZen™ 2.6 µm Peptide XB-C18 150 x 0.075 mm	None	10	720		
	RP1 10 x 0.075 mm	11	545	76 %	78 %
Waters® nanoEase™ M/Z Peptide BEH C18 130 Å, 1.7 µm, 75 µm x 150 mm	None	9	800		
	Waters nanoEase M/Z Symmetry C18 100 Å, 5 µm, 180 µm x 20 mm	13	508	63 %	80 %
Thermo Fisher® Acclaim™ PepMap™ 100 75 µm x 15 cm, nanoViper™ C18, 3 µm, 100 Å	None	13	554		
	Thermo Acclaim PepMap 100 µm x 2 cm nanoViper	13	508	92 %	85 %

Figure 14.
Number of proteins and peptides that were identified on a nano LC-MS analysis of a digested HeLa sample using a Thermo Fisher Acclaim PepMap 100 nanoViper 3 µm C18, Waters NanoEase M/Z Peptide BEH 1.7 µm C18, and bioZen 2.6 µm Peptide XB-C18, in trap and elute mode with Thermo Fisher Acclaim PepMap nanoViper, Waters nanoEase M/Z Symmetry C18, and Nano Trap RP-1 (General RP) traps, respectively.

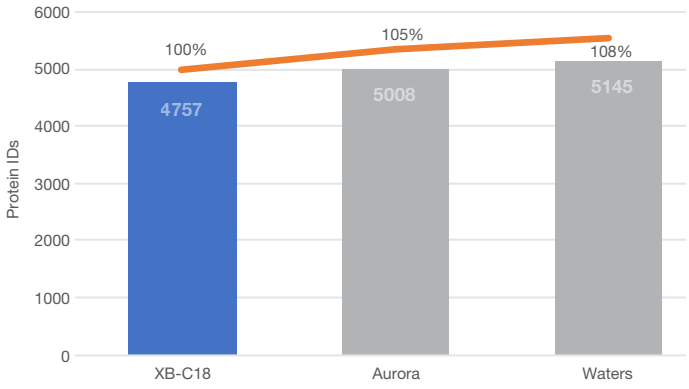


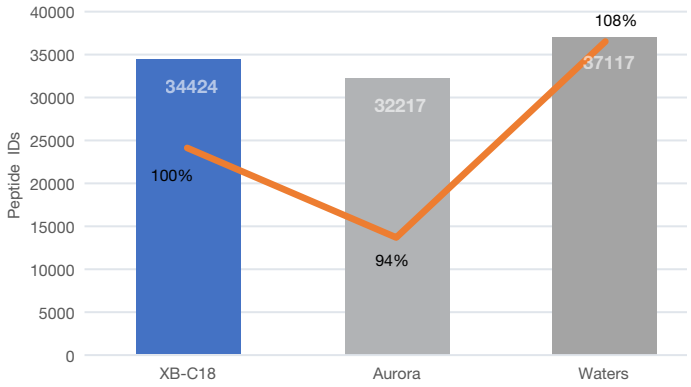


9 seconds to 12 seconds by the addition of the large volume trap, substantially lowering the peak capacity in trap and elute mode.

When using nano LC-MS, it is common practice to use longer columns in order to get better peak capacities and higher resolution. This leads to higher numbers of identifications of peptides and subsequently proteins.[16] We investigated the effect of using a longer (25 cm) 2.6 µm core-shell column in comparison to columns packed with 1.6 and 1.7 µm fully porous columns. It should be noted that the 1.6 µm column had the nano spray emitter directly coupled to the column, whereas the 2.6 µm core-shell column and the 1.7 µm fully porous column were coupled to an external spray emitter. **Figure 15** shows the number of both peptides and proteins that were identified when using these three columns. All 3 of these columns produced very similar numbers of both peptide and protein identifications. Under the conditions run here, the greatest difference was the core-shell column needed 5000 psi and the sub 2 µm columns needed 9,400 p.s.i. at 50 °C in order to operate at 350 nL/min. There are several benefits to being able to achieve the same separation quality in nano LC at lower pressures. The first is a greater flexibility in terms of flow rates and temperatures. To run at reasonable flow rates on the sub-2 µm columns, it is necessary to heat the column to 50 °C or even higher depending upon the pressure capabilities of the nano LC system, as many system have a max pressure of 10,000 psi Lower backpressures also mean that the columns are more resistant to fouling and other column degradation over time, leading to more robust columns which will be discussed in the next section. It was also interesting to note that in this example there was no major benefit to having the spray emitter built into the column in terms of the overall performance.

Figure 15.
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 Peptide XB-C18, IonOpticks Aurora 1.6 µm C18, and Waters NanoEase M/Z Peptide BEH 1.7 µm C18.





In the early days of nano LC the availability of pre-packed columns was very limited. Even to this day, the availability of packed nano LC columns lags significantly behind larger analytical dimensions, making it still a popular practice for researchers to pack their own column. This, however, can be a very time-consuming endeavor for a multitude of reasons, and not just that of the time that it takes to pack and test the columns. Besides just peak width and peak shape considerations, there are other performance attributes that are important in nano LC columns. Column-to-column and batch-to-batch reproducibility is an important performance attribute. Without reproducible columns, it could become necessary to repeat all of the injections in the batch of runs in order for retention times to be compared if a single column fails. Furthermore, having robust columns able to perform hundreds of injections without any degradation in chromatography is also of key importance. In **Figure 16**, the extracted ion chromatograms from 3 different bioZen™ 2.6 µm Peptite XB-C18 columns, 150 x 0.075 mm are shown. The retention time RSD was 1.03 % between these columns demonstrating excellent column-to-column reproducibility. **Figure 17** shows the extracted ion chromatograms for the 1st and 100th injection of a mixture of 20 isotopically labeled peptides when run on a bioZen 2.6 µm XB-C18, 150 x 0.075 mm column. There was a 1.5 % retention time RSD and a 1.2 % change in peak width over the course of the 100 injections.

LC Conditions

Multiple Reaction Monitoring (16-17)

Column: bioZen 2.6 µm Peptide XB-C18
Dimensions: 150 x 0.075 mm
Part No.: 00F-4768-AW-21
Pressure (bar): 200 bar
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile

Gradient:	Time (min)	% B
	0	3
	15	30
	18	80
	24	80
	25	3
	35	3

Flow Rate: 350 nL/min
Temperature: Ambient
LC System: NanoLC™ 425 (SCIEX®)
Detection: MS/MS
Detector: 6500 QTRAP® + (SCIEX)
Injection Volume: 1 µL

Figure 16.
Extracted Ion chromatograms from a mixture of 20 isotopically labeled peptides run on 3 different bioZen 2.6 µm Peptide XB-C18 150 x 0.075 mm columns

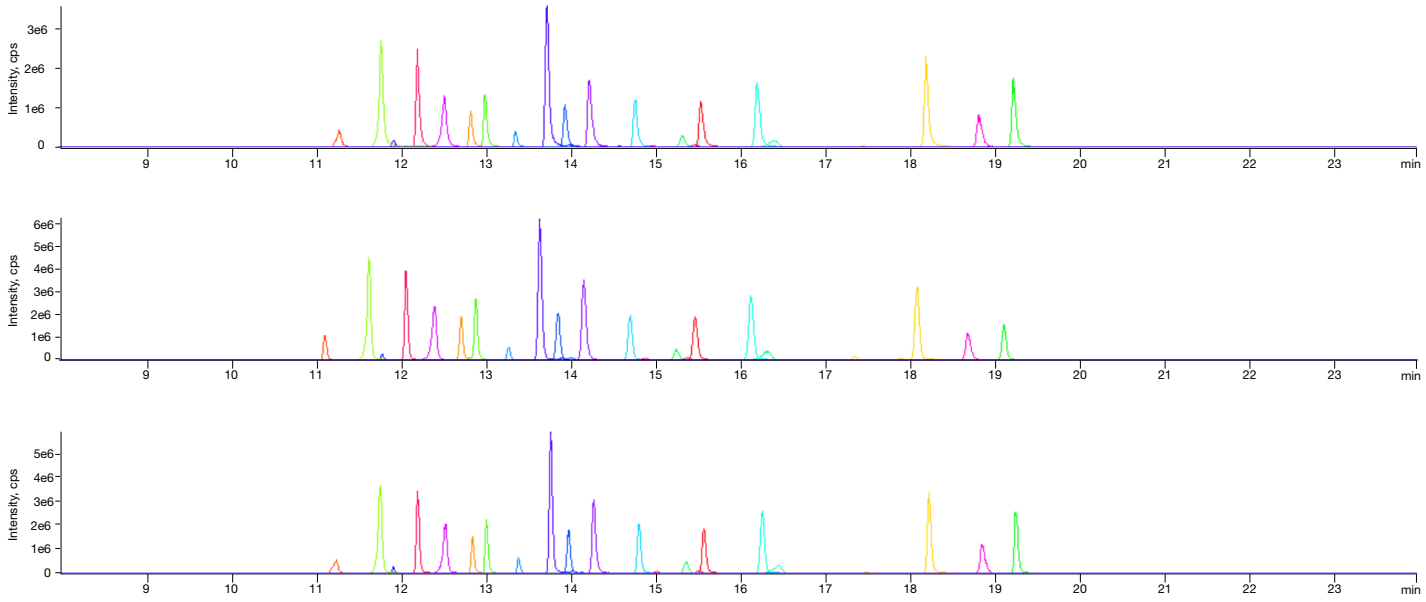
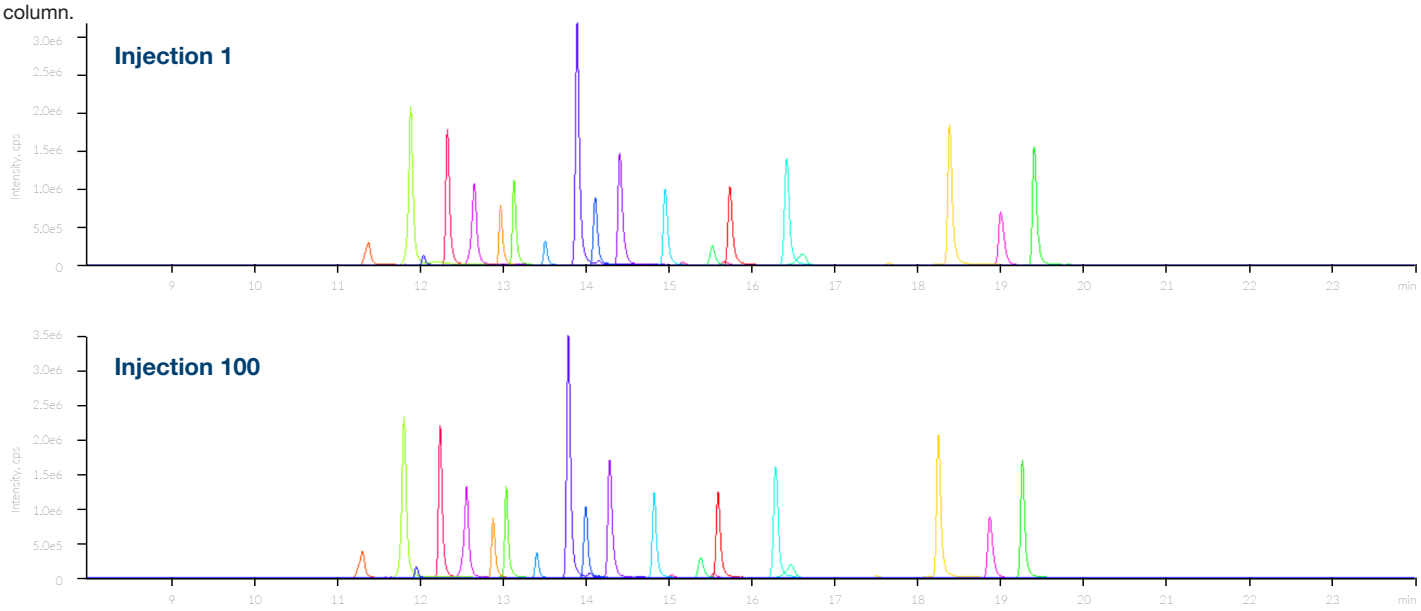


Figure 17.
Extracted Ion chromatograms of injection 1 and 100 from a mixture of 20 isotopically labeled peptides run on a bioZen™ 2.6 µm Peptide XB-C18 150 x 0.075 mm column.



Conclusion

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 can increase the benefits through narrower chromatographic peaks. 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. The core-shell particle technology also provides the added benefit of producing higher chromatographic efficiency at significantly lower pressures than sub-2 µm fully porous particles. 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. Finally, we saw that nano columns packed with core-shell materials demonstrated excellent column-to-column reproducibility and robustness over hundreds of injections.

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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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