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Considerations for Selecting the Optimal Stationary Phases for Proteomic Trap-and-Elute Nanochromatography

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This white paper reviews various experimental parameters that influence the chromatographic performance of a trap-and-elute nanoLC method. The most important step in the method development process is to select an appropriate column and trap pair that maximizes chromatographic resolution so that the method is capable of thoroughly identifying proteolytic peptides.

INTRODUCTION

Miniaturizing liquid chromatography interfaced with mass spectrometry (LC-MS) offers significant sensitivity benefits. The benefits mostly come from the reduced column diameter. The low flow rates used with small columns enhance ionization efficiency and the mass spectrometer's sampling efficiency,[1,2] while the reduced ion suppression decreases matrix effects.[3] All these effects improve MS sensitivity. The chromatographic separation also benefits from miniaturization. Because chromatographic processes are scalable, small diameter columns provide the same separation power as larger columns while requiring smaller amounts of sample. These benefits make miniaturized LC-MS a powerful tool for many challenging assays, especially when only limited quantities of sample are available. A proteomic shotgun study[4,5] is one such example. In proteomic work, nanoscale LC-MS (nanoLC-MS) is often used along with columns smaller than 100 µm in internal diameter (I.D.) and flow rates less than 500 nL/min. It is also common to use a shallow gradient on a long column to maximize chromatographic resolution, because high resolution is crucial to the thorough identification of the proteolytic peptides that are in turn used to determine the presence, post-translational modifications, and potentially the abundance of various proteins.

The resolution of nanoLC assays can be compromised by poor experimental methods. The reduced column volume makes nanoLC more vulnerable to small incompatibilities in analysis conditions that might have little or no impact on larger columns. A 75-µm-I.D. nanocolumn, for example, has about 780 times smaller column volume than a 2.1-mm-I.D. column of the same length. Injecting 5 µL of sample directly onto a 75-µm nanocolumn is thus equivalent to injecting 3.9 mL of sample onto a 2.1-mm column. Injecting such a large volume of sample can foul a nanocolumn with particulates or problematic species (e.g., proteins or lipids), although their concentrations in the sample matrix might be very low. Meanwhile, a moderately high solvent strength in the sample matrix, which is often the result of an upstream sample preparation step, can disrupt the retention of analytes on the stationary phase, and thereby ruin the chromatographic separation. A so-called trap-and-elute nanoLC method is a perfect solution to these problems. In this method, the sample is first injected onto a trap column. A trap is packed with larger particles (typically 5 µm in diameter) in larger bore hardware so that trapping can be performed at high flow rates in a short cycle time. The trap selectively captures sample components through its analyte retention mechanism, typically by isocratic reversed-phase retention. The captured species are then eluted from the trap and subsequently introduced into

the downstream analytical nanocolumn. Because the trapping process decouples the analytes of interest from the original injection sample matrix, a trap-and-elute method can accept diverse sample matrices without the risk of deteriorating the chromatographic separation. It allows the injection of a large volume of sample that has a relatively high solvent strength. The trap column can also function as a mechanical sieve to filter out particulates in the sample to prevent column plugging and thus extend the lifetime of the downstream nanocolumn. In the end, an optimized trapping condition improves the method's robustness by preventing interfering species in samples, such as salts and components that cannot be washed off easily from the column, from entering the nanocolumn.

While a well-optimized trapping method achieves all the benefits described above, a poorly designed trapping method can significantly damage the assay capability through reduced sensitivity and, even more seriously, analyte loss. A trapping method can be optimized by varying fluidic parameters, such as the trapping flow rate, the trapping time, and the composition of the trapping mobile phase. An equally important but often overlooked factor is the retention characteristics of the packing materials in the trap column and the analytical nanocolumn. This white paper presents the importance of selecting the right traps and nanocolumns for a successful trap-and-elute nanoLC-MS method. We start with the basic concepts of trap-and-elute chromatography to illustrate various factors that influence the method capability. This discussion is verified with experimental data taken from several commercially-available traps and nanocolumns that are widely used for proteomic applications. The data includes retentivity of each sorbent, trapping efficiency, and chromatographic performance for a typical nanoscale proteomic LC-MS separation. A couple of practical considerations in selecting traps and columns will be presented in summary.

TRAPPING OVERVIEW

A trap column is like a waiting area for the sample components before they are further separated with high resolution in the downstream analytical nanocolumn. Effective trapping is thus to capture all the desired components in the sample while discarding unnecessary or interfering species. In a typical reversed-phase trap-and-elute setup, the ability to capture a component depends on its reversed-phase retention, which in turn is determined by the retentivity of the stationary phase, the strength of the mobile phase, and the hydrophobicity of the component. An isocratic trapping method, with the first two factors held constant during trapping, may not effectively capture some analytes that are weakly retained by the stationary phase. These analytes may continue to move through the trap column as traveling bands along with the trapping flow, and experience significant broadening due to the large particles and the large-bore tube of the trap column. Any bands that travel further than the length of the trap will be eventually lost.

The analytes that are successfully captured in the trap are later eluted from the trap and directed into the analytical nanocolumn for high resolution separation. The ease of analyte elution, which is the reverse phenomenon of the analyte retention, also depends on the above-mentioned factors. The increasing elution strength of the solvent gradient sequentially elutes the captured analytes from the trap in the order of increasing analyte retention. The eluted analyte is again retained at the head of the nanocolumn where it may form a narrow band. This process is called refocusing. It helps to mitigate band broadening occurring in the trap and the transfer tubing. Refocusing is most effective when the nanocolumn retains the analyte more strongly than the trap. Because the analyte elution from the trap and the refocusing at the nanocolumn occur with the same mobile phase, it is therefore recommended to select a trap column that has a lower retentivity than the nanocolumn to maximize refocusing.

Waters® Nanocolumns and Traps support two trap-and-elute configurations for ACQUITY UPLC® M-Class Systems. In the forward-elute configuration (Figure 1a), the flow direction in the trap remains the same throughout the method. Because both trapping and elution flow can be provided by a single pump, the system setup is relatively simple. The reverse-elute setup requires two independently controlled pumps (Figure 1b): one pump drives the trapping flow and the other pump provides the elution flow. The flow direction in the trap is reversed between trapping and elution. While more complex, the dual-pump reverse-elute configuration has a chromatographic advantage. The captured analytes seldom travel through the whole length of the trap column because of the change in the flow direction (Figure 1c), and thus are subject to less band broadening. An earlier publication demonstrated the increased peak capacity from the reverse-elute configuration.[6]

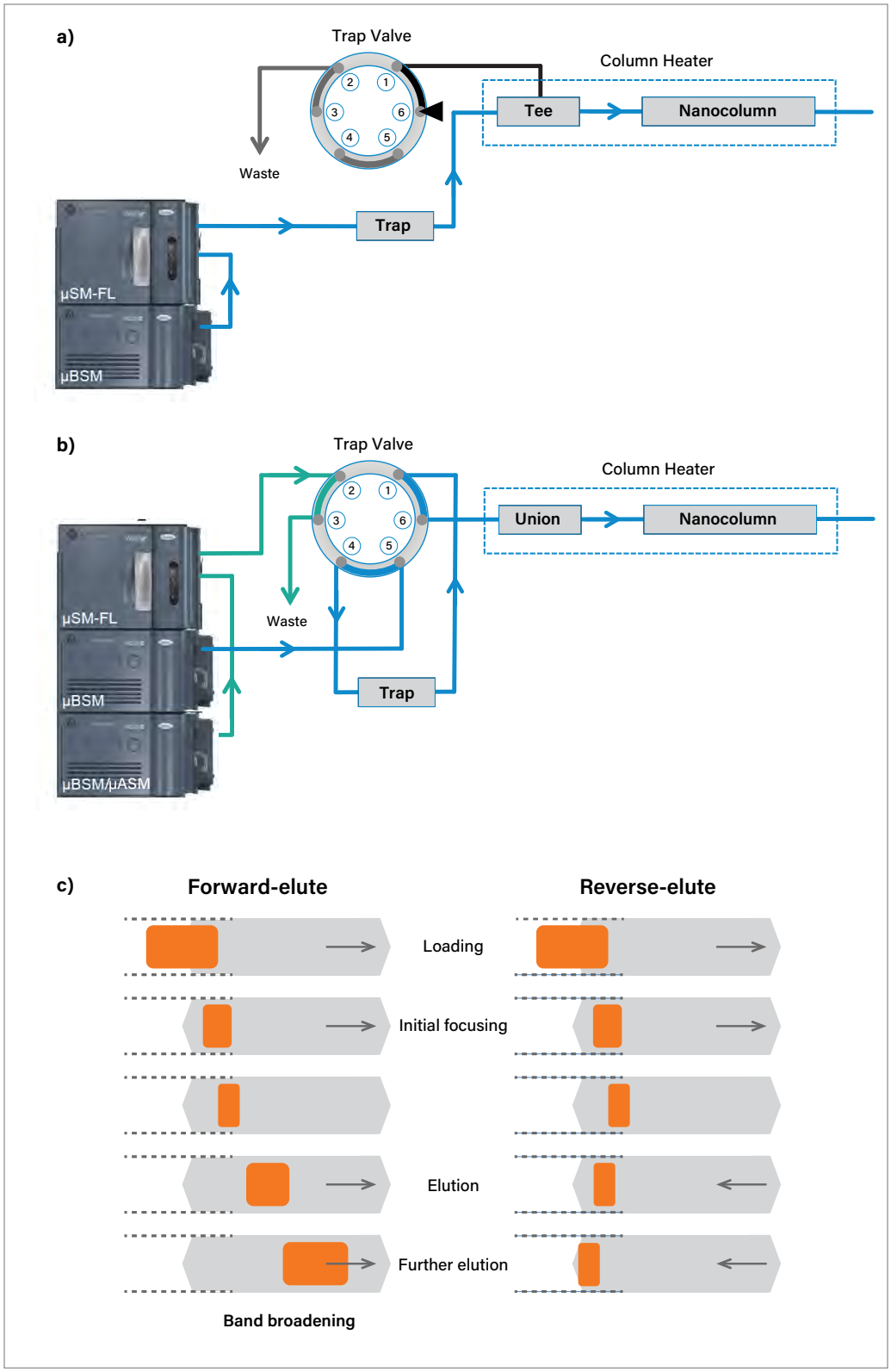


Figure 1. Two trapping configurations: a) forward-elute configuration with a single pump, and b) dual-pump reverse-elute configuration. Both diagrams show the valve positions and the flow directions during the elution/separation step. The schematic (c) shows what happens in the trap during the forward-elute and the reverse-elute method.

Another benefit of trapping is the ability to inject a large volume of sample into a nanocolumn for increased assay sensitivity. Figure 2 shows an example of the signal responses versus injection volume, in which two signature peptides were monitored after a complex protein digest sample was injected into a 150 µm x 100 mm iKey® Separation Device (0.76-µL column volume) with and without trapping. Peptide A eluted earlier than Peptide B. When the sample was directly injected into the iKey, the signal response for Peptide A did not proportionally increase with the increasing injection volume. The response linearity started to fail when 1 µL or more sample was injected. The maximum signal was reached with 5 µL of the sample injection, and a larger injection volume did not result in a greater signal. The response for Peptide B, on the other hand, kept increasing with the increasing injection volume although the linearity also started to fail when 2.5 µL or more sample was injected. In contrast, the trapping method greatly extended the injection volume range for both Peptides A and B. Ten microliters of the same sample could be injected while maintaining linearity, and more sample could be injected to achieve an extra signal response outside the linear range. In this example, the 15-µL injection gave a signal response 30% higher than the 10-µL injection for Peptide A. The maximum Peptide A response from the trapping method was more than 5 times greater than that from an equivalent direct injection method. The extended linear range and the increased maximum response resulting from trapping are consequences of both less volume overloading and the improved MS sensitivity from effective interference removal. These effects are especially beneficial to achieve high sensitivity for early eluting peptides.

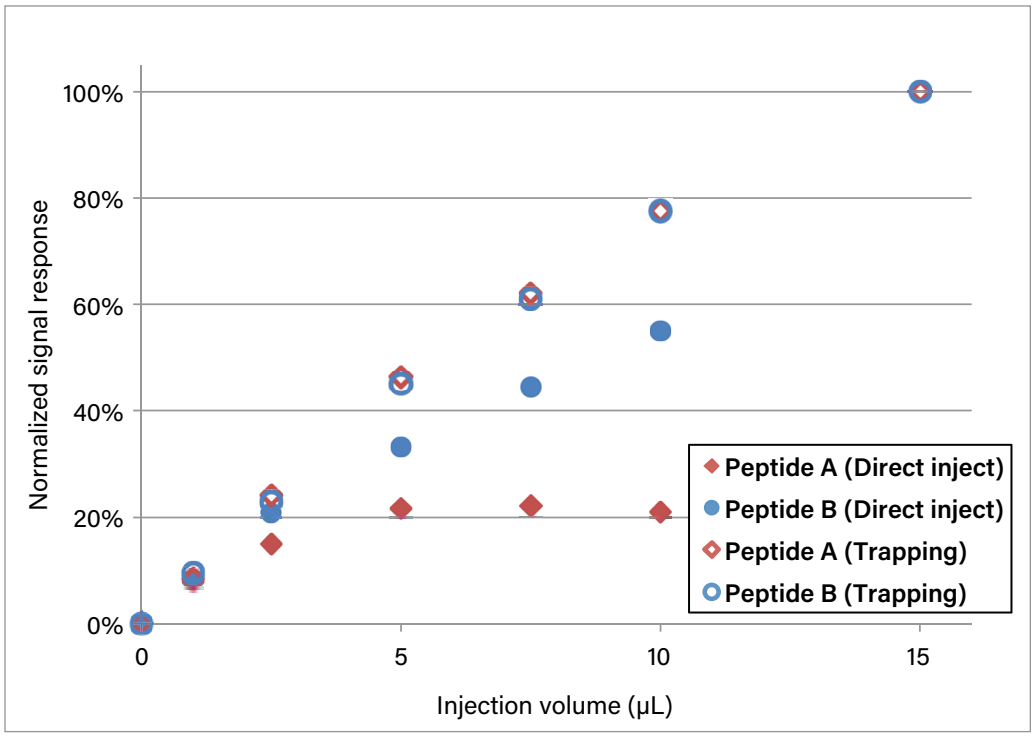


Figure 2. The effect of increasing injection volume on a direct injection method and a trap-and-elute method. A protein digest sample was injected into an HSS T3, 1.8 µm, 150 µm x 100 mm iKey Separation Device (p/n: 186007261) with and without trapping on a Symmetry® C_{18}, 5 µm, 300 µm x 50 mm Trap Column (p/n: 186007498) using an ACQUITY UPLC M-Class System/ ionKey® Source/Xevo® TQ-S Mass Spectrometer. Peptide A eluted earlier than Peptide B, and thus was more significantly influenced by the interference from the sample matrix.

PEPTIDE RETENTIVITY FOR COMMON NANOLC C_{18} STATIONARY PHASES

As previously mentioned, it is important to understand the overall retentivity of stationary phases when developing a successful trap-and-elute nanoLC-MS method. Yet, retentivity is dependent on many factors, such as the mode of retention, the kind of mobile phases, and the characteristics of the analytes. In this study, we have purposely narrowed the focus to proteomic peptide LC-MS analysis. Many such assays are based on reversed-phase separations using water and acetonitrile mobile phases modified with formic acid additive because of their excellent compatibility with nanoLC and MS ionization. The retentivity of several commercially available C_{18} stationary phases were experimentally measured for representative peptides using a similar gradient condition. The C_{18} stationary phases tested were: Waters BEH C_{18} 130Å, Waters HSS T3 100Å, Waters CSH™ C_{18} 100Å, Waters Symmetry C_{18} 100Å, and 'AP' C_{18} 100Å from Vendor T. Twelve peptides from the MassPREP™ Peptide Mixture (p/n: 186002337) and Quantitative Peptide Retention Standard (p/n: 186006555) were separated using 2.1 mm x 100 mm C_{18} columns packed with 5-µm particles held at 30 °C on an ACQUITY® UPLC® I-Class System interfaced with a Xevo TQ-S Mass Spectrometer. Each injection loaded 1 pmol of each peptide on the column. The obtained chromatograms showed that each C_{18}- stationary phase has unique retentivity (Figure 3). All peptides exhibited retention time shifts from one stationary phase to another, but some peptides showed more significant shifts than others. While it is uncommon for the elution order of the 12 peptides to change across the different C_{18} stationary phases, shifts in certain critical pairs were sometimes observed. Most notably, the elution order of Peptides 5 and 6 was different between stationary phases, and Peptides 9 and 10 swapped elution order when separated with 'AP' C_{18} versus all other stationary phases. These differences clearly illustrate the unique surface chemistries of different C_{18} reversed-phase stationary phases.

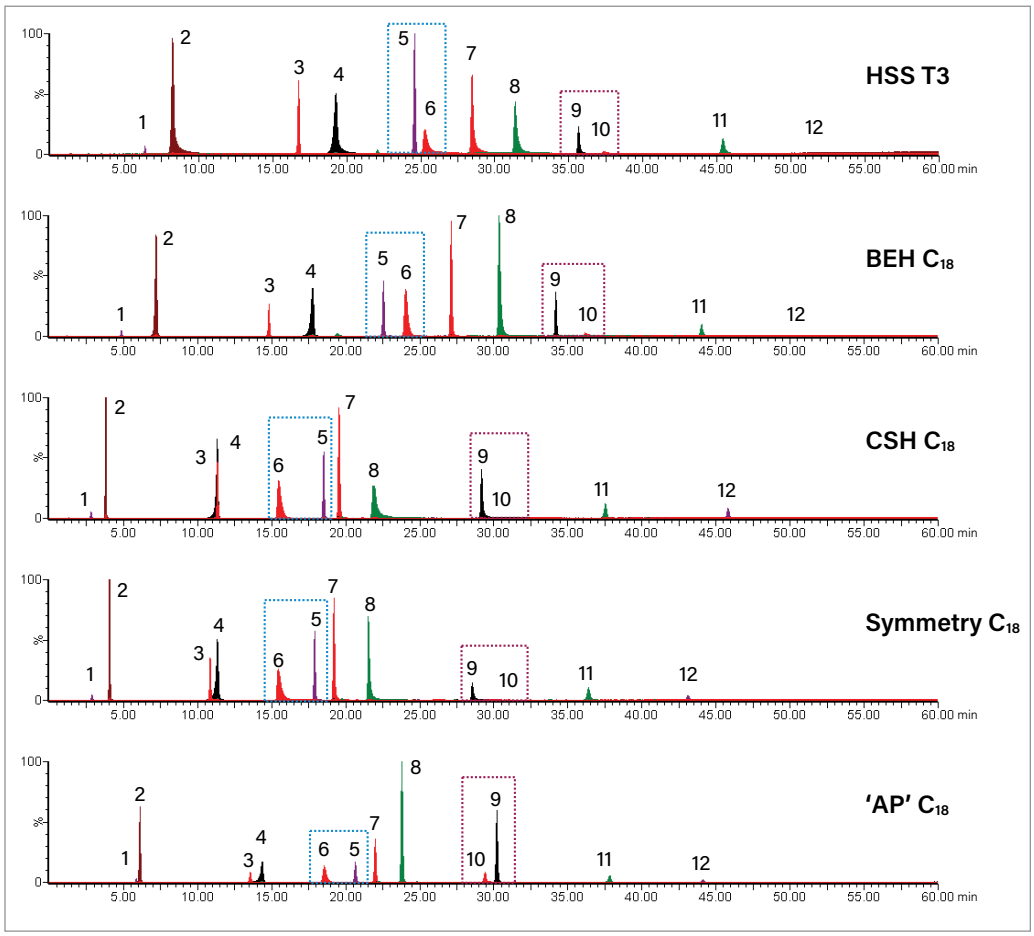


Figure 3. Example chromatograms acquired with commercially available 2.1 mm x 100 mm, 5 µm, C_{18} columns. The peptide mixture was separated with a water/acetonitrile gradient, each with 0.1% formic acid, running from 0 to 40% acetonitrile over 60 minutes, followed by a 6-minute washing at 90% acetonitrile. The flow rate was 0.2 mL/min. Monitored peptides were; DGYGK (1), RASG-1 (2), DTVGYGVK (3), angiotensin fragment 1-7 (4), DFVGYGVK (5), bradykinin (6), angiotensin II (7), angiotensin I (8), DFVGYGVKDFVGVGVK (9), renin substrate (10), enolase T35 (11), and enolase T37 (12).

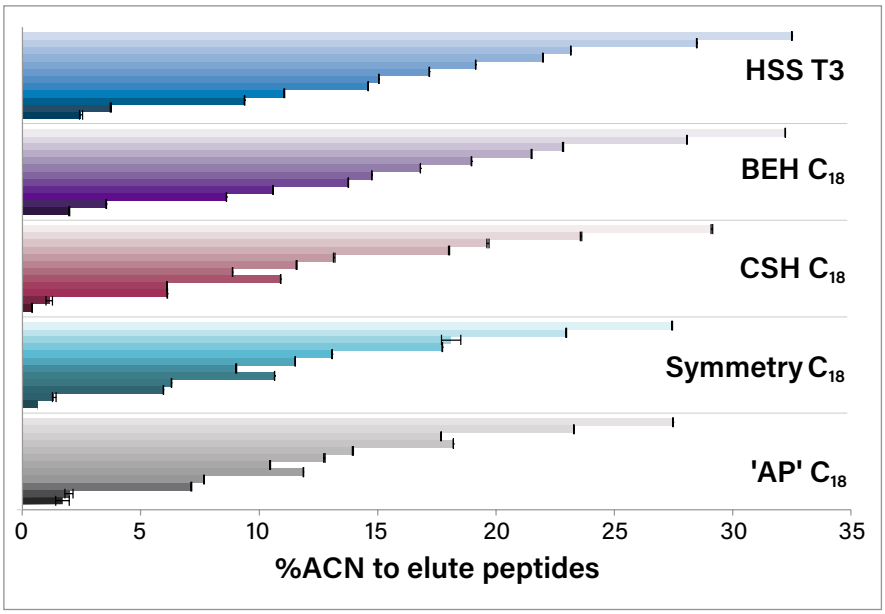


Figure 4. Retentivity of five C_{18} stationary phases. Each peptide retention was represented in terms of acetonitrile mobile phase composition that was required to elute the peptide from the column (% ACN). Error bars show the standard deviations from triplicate measurements.

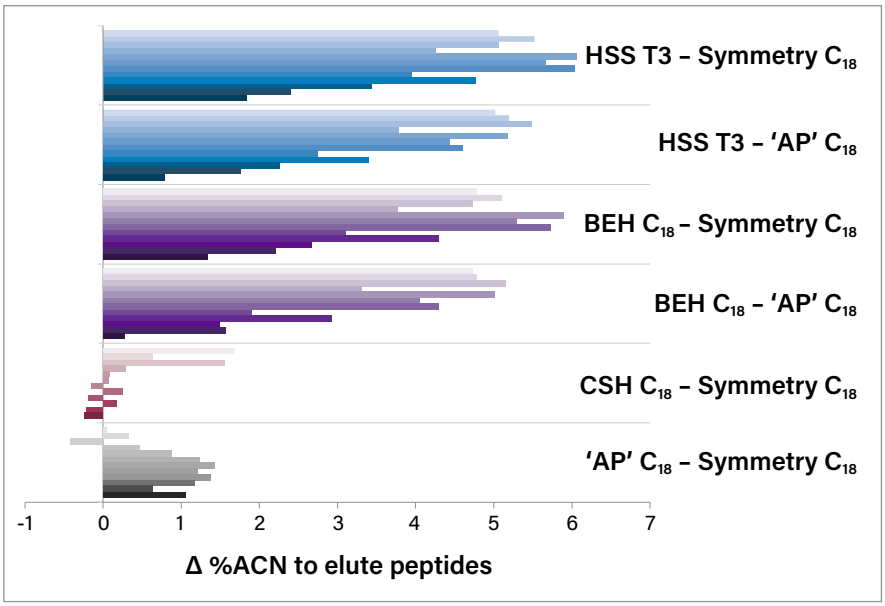


Figure 5. The retentivity differences of common C_{18} nanocolumn–trap pairs.

The peptide retentivity of each stationary phase was estimated from the corrected retention time, derived from the measured peptide retention times and the void volume from each column, converted to the mobile-phase composition at the time of elution. Figure 4 presents these values in the form of the percent acetonitrile that was required to elute each peptide (% ACN) as measured for a range of different stationary phases. The overall peptide retentivity can be ranked from low to high as follows: Symmetry C_{18} < CSH C_{18} < 'AP' C_{18} < BEH C_{18} < HSS T3. 'AP' C_{18} showed a unique retentivity pattern compared to Waters C_{18} phases. It has retentivity similar to Symmetry C_{18}, the least retentive stationary phase, for strongly retained peptides.

On the other hand, its retentivity for weakly retained peptides was much stronger, somewhat comparable to that of the more retentive BEH C_{18} phase.

The collection of individual peptide data (Figure 4) provides a comprehensive overview of the retentivity characteristics of each C_{18} stationary phase. Figure 5 shows the retentivity differences between commonly used nanocolumn and trap combinations, which are more relevant in estimating the effectiveness of refocusing. Symmetry C_{18}, with its low general retentivity, was expected to be an effective trap stationary phase for both BEH C_{18} and HSS T3 nanocolumns. The retentivity differences for moderately retained peptides (4~8) and strongly retained peptides (9~12) from these pairs were all greater than 3% acetonitrile, suggesting that these peptides would benefit from effective refocusing. Weakly retained peptides (1~3) had smaller retentivity differences, as small as 1.3% ACN (BEH C_{18}–Symmetry C_{18}) and 1.8% ACN (HSS T3–Symmetry C_{18}). Thus, an HSS T3 column is expected to have a stronger refocusing effect compared with a BEH C_{18} column for weakly retained peptides. A CSH C_{18} column, despite its many advantages such as the unique selectivity and excellent peak shape,[7-9] would not benefit from pairing with a Symmetry C_{18} trap. The retentivity differences of 'AP' C_{18} and either HSS T3 or BEH C_{18} were significantly smaller for the most weakly retained peptide (0.8 and 0.3% ACN, respectively) but comparable for other peptides (>2.7% ACN). It was expected that an 'AP' C_{18} trap paired with a BEH C_{18} column would have almost no refocusing effect on polar peptides.

Conditions

LC system:	ACQUITY UPLC M-Class dual BSM setup with a fixed loop injector
Mass spectrometer:	Xevo TQ-S
Electrospray source:	Universal nanospray with a PicoTip fused silica emitter
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid
Weak needle wash:	Water + 0.1% TFA
Strong needle wash:	25:25:25:25 water/acetonitrile/ methanol/2-propanol + 0.2% formic acid
Trap column:	nanoEase™ M/Z Symmetry C_{18}, 5 µm, 180 µm x 20 mm (p/n: 186008821); 'AP' C_{18} trap cartridge from vendor T, 5 µm, 300 µm x 5 mm
Analytical nanocolumn:	nanoEase M/Z BEH C_{18}, 1.7 µm, 75 µm x 250 mm (p/n: 186008795); nanoEase M/Z HSS T3, 1.8 µm, 75 µm x 250 mm (p/n: 186008818); 'AP' C_{18} column from vendor T, 2 µm, 75 µm x 250 mm
Sample:	MassPREP Enolase Digest with Phosphopeptides (p/n: 186003286); 2 fmol/µL in water + 0.1% TFA
Injection:	3 µL partial loop injection from a 20 µL loop
Column temp.:	35 °C
Sample temp.:	10 °C
Trapping condition:	99.5:0.5 A/B, 15 µL/min x 2 min (optimized final condition)

Analysis gradient:

Time (min)	Flow rate (nL/min)	A	B
-	300	95	5
10	300	95	5
55	300	60	40
57	300	15	85
62	300	15	85
65	300	95	5
110	300	95	5

Capillary voltage:	~2.5 kV, fine-tuned with 100 fmol/µL GluFib
Source temp.:	100 °C
Dwell time:	54 ms 20 points across an 8 sec peak

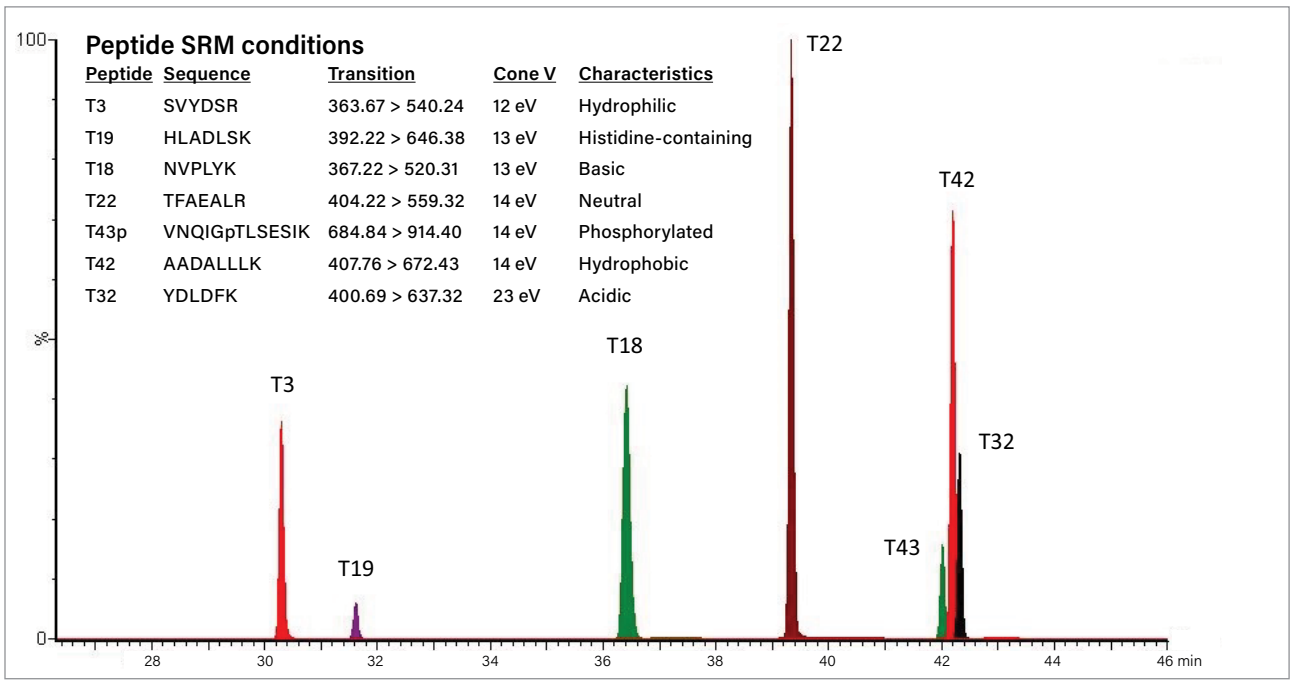


Figure 6. An example chromatogram separating the peptides using an HSS T3 column and a Symmetry C_{18} Trap.

NANOEASE M/Z

Waters introduced nanoEase M/Z Columns and Trap Columns for nano- and microflow UPLC-MS applications. nanoEase M/Z Columns and Traps are simple to install, reduce connection variability, and deliver superior chromatographic performance.

nanoEase M/Z Columns and Traps are equipped with ZenFit™ Connection Technology to streamline the installation of columns and traps. This reduces chromatographic inconsistencies caused by variations in the critical connections. ZenFit Connectors are easy-to-use, re-useable, fingertight fittings that can be reliably used even in demanding conditions (15,000 psi at 90 °C). ZenFit Connectors are compatible with most 10-32 threaded ports for 1/16" O.D. tubing, which are used in Waters UPLC and other manufacturers' equipment, such as Rheodyne® and VICI® valves.

In addition to being easy to install, the nanoEase M/Z Columns deliver the same high efficiency and selectivity as Waters ACQUITY UPLC M-Class Columns. With several available stationary phases for protein and peptide analysis, users can choose the columns that are most appropriate for their analysis needs.

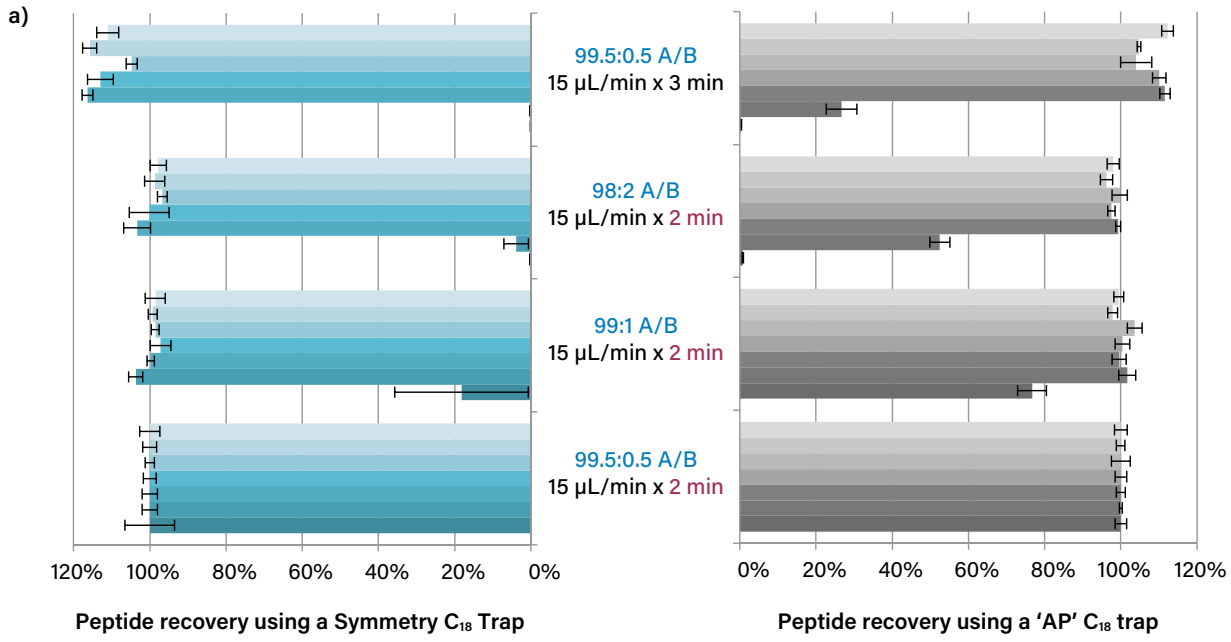
OPTIMIZING TRAPPING CONDITIONS

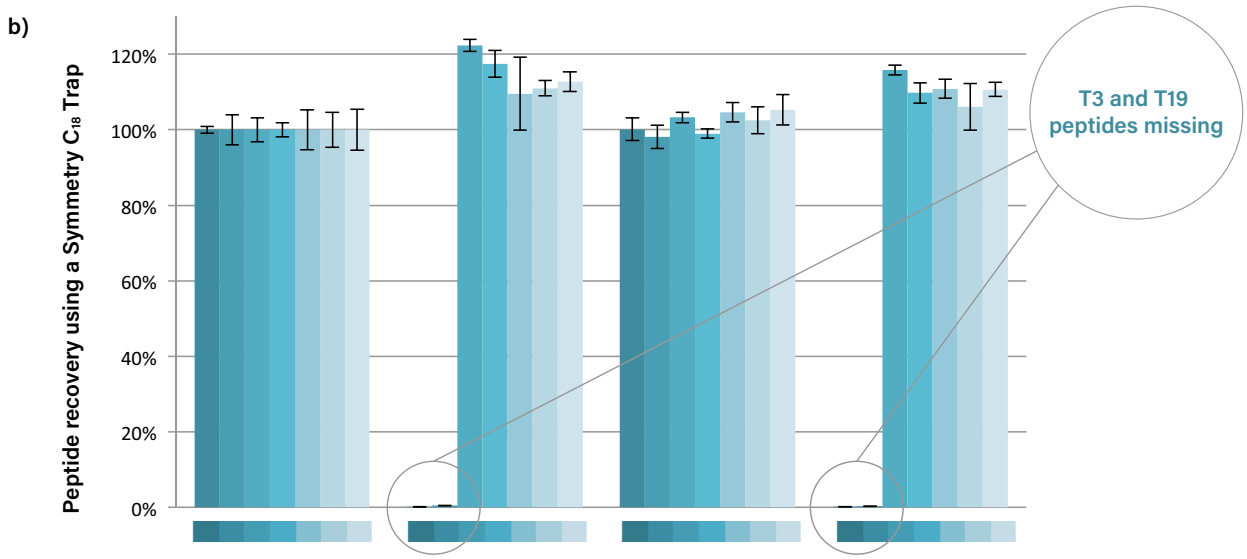
Because of the selective nature of the trapping process, it is not uncommon that some peptides are lost during trapping. Peptide recovery is largely dependent on the retention, and thus can be experimentally controlled by parameters that influence peptide retention. For example, column retentivity and solvent strength should be selected so that the trap retains the species of interest while discarding unwanted ones. The trapping volume should be large enough so that the sample within the injection loop reaches the trap while effectively washing off salts and ionic species, but small enough not to flush out weakly retained peptides from the trap column. Trapping mobile-phase composition and eluent volume are therefore effective tools for adjusting trapping capabilities. We discussed that it is desirable to select a trap column that has a lower retentivity than the nanocolumn to maximize the chromatographic efficiency through refocusing.

The downside of having a less retentive trap, however, is that it is more likely to lose weakly retained peptides during trapping. It is thus important to optimize the other trapping conditions to compensate for the less retentive trap.

Several trapping conditions were tested on a Symmetry C_{18} Trap and an 'AP' C_{18} trap to monitor the recovery of each peptide. Trapping at 15 µL/min of 99.5:0.5 A/B flow for 2 minutes gave the best overall recovery for both traps, and was selected as the optimized trapping condition. Figure 7 shows the peptide recovery calculated from the average peptide peak areas from triplicate injections of the peptide mix standard, which was then normalized to the recovery values acquired with the optimized condition. Details of the separation conditions can be found in the Conditions section. The data showed that the Symmetry C_{18} Trap did not effectively capture early eluting peptides T3 and T19 when used with aggressive trapping conditions. For example, their recovery dropped significantly when the trapping mobile phase contained even slightly more acetonitrile than the optimized condition (i.e., 99:1 or 98:2 A/B compared to 99.5:0.5 A/B). Trapping with a mobile phase that had less acetonitrile (99.9:0.1 A/B), on the other hand, resulted in the same recovery as the optimized condition (data not shown for brevity). The 'AP' C_{18} trap showed a similar trend of losing early eluting peptides with aggressive trapping conditions, but had slightly better recovery than the Symmetry C_{18} Trap. Examples can be found with the higher recoveries of 'AP' C_{18} for T3 at 99:1 A/B and T19 at 98:2 A/B. The recoveries for well-retained peptides were equivalent for both traps. This slight recovery difference is in agreement with the peptide retentivity results shown in Figure 4. 'AP' C_{18} had stronger retentivity for weakly retained peptides than Symmetry C_{18}, while its retentivity for moderately or strongly retained peptides was equivalent. Besides the mobile-phase composition, the trapping volume also influences recovery (Figure 7b). At the same mobile phase composition of 99.5:0.5 A/B, the recoveries of weakly retained peptides decrease with increasing trapping volume. This is due to the weakly retained peptides being flushed out of the trap, by way of isocratic elution. Changing the trapping flow rate while maintaining the volume, on the other hand, did not make a measurable difference.

The strong retentivity of 'AP' C_{18} is, in theory, beneficial because it allows the use of more aggressive trapping conditions to remove ionic matrix contaminants more effectively without losing polar peptides. This benefit, however, is too subtle to make a significant impact in real applications, and thus is often not realized. The results shown here indicate that the composition of the trapping mobile phase and the trapping volume are more significant and predictable parameters for optimizing the peptide retention on a trap.





Composition	99.5:0.5 A/B			
Flow Rate	15 µL/min		10 µL/min	
Time	2 min	3 min	3 min	4 min
Volume	**30 µL**	**45 µL**	**30 µL**	**40 µL**

Figure 7. Peptide recoveries from various trapping conditions using a) a Symmetry C_{18} Trap and an HSS T3 column vs. a 'AP' C_{18} trap and column pair. The recovery of a Symmetry C_{18} Trap with a BEH C_{18} column was equivalent to the Symmetry C_{18} Trap and the HSS T3 column pair, and thus is not presented. Data b) shows the peptide recoveries using a Symmetry C_{18} Trap and an HSS T3 column with varying trapping flow rates and times. Each bar represents the normalized peptide recovery calculated from the average peak areas. The error bars represent the standard deviation from triplicate injections. The peptides are presented with different shades of the color in the order of their approximate elution: T3 (darkest), T19, T18, T22, T43p, T42, and T32 (lightest).

CHROMATOGRAPHIC PERFORMANCE OF NANOCOLUMN–TRAP PAIRS

Importance of high chromatographic resolution

Chromatographic performance can be measured in various ways. The most straightforward metric is the width of the peaks. A sharp peak, either from isocratic or gradient elution, can be translated in terms of more commonly used performance indicators, such as a high plate count (N), high resolution (R_s), or large peak capacity (P_C). Each of these metrics represents the ability to distinguish a unique chromatographic peak from neighboring peaks, which is the basis for all chromatographic analysis and quantitation. With the use of mass spectrometry detection, however, the requirement to achieve baseline peak separation has become less stringent. The ability to identify multiple analytes from coeluting peaks using their *m/z* values has relieved the burden of separating all peaks with baseline resolution, and thus greatly advanced the analysis capability for complex samples that generate hundreds of peaks in a single chromatographic run. The increased sample complexity, in turn, brought forth new challenges for LC-MS assays that necessitated increased chromatographic performance. One example is matrix effects.[10] When the analyte of interest co-elutes with one or more other species from the sample, the ionization efficiency of the analyte can be influenced by the co-eluting species. Competition within the ionization process from this situation often leads to suppressed ion yields and decreases in MS signal intensity. Alternatively, a matrix effect can affect the ionization process to produce signal enhancement. In general, matrix effects depend on the nature of the co-eluting species and can be difficult to predict. This uncertainty creates a risk to the assay robustness by preventing reliable quantitation. Because it is difficult to investigate the root cause and the extent of matrix effects, it is most desirable to create an LC method which minimizes co-elution. To make things more complex, the co-eluting peaks are more difficult to separate when the relative abundances of analytes are significantly different. In order to completely separate a low abundance species from a large neighboring peak, chromatographic resolution must be greater than that required to separate peaks of similar height (Figure 8). In addition, an increase in peak width results in a smaller peak height which in turn makes peak detection more difficult. For all these reasons, the importance of chromatographic resolving power should not be underestimated.

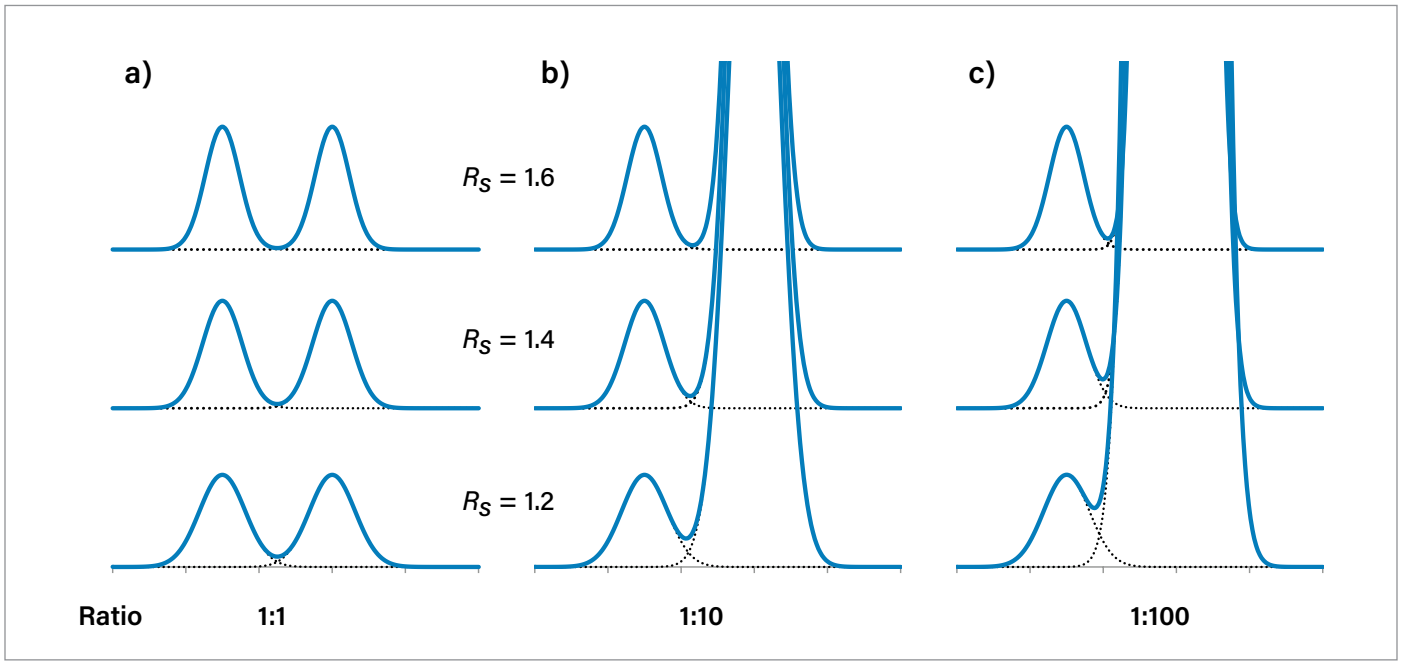


Figure 8. Simulated chromatograms illustrating the required minimum resolution to separate two neighboring peaks of different peak height ratios. (a) Two peaks of the same height, (b) two peaks with a 1:10 height ratio, and (c) two peaks with a 1:100 height ratio. Resolution (R_s) values are 1.6, 1.4, and 1.2 from top to bottom. In order to distinguish two peaks of equal height with no more than 10% of the peak height being overlapped, the separation must have a minimum resolution of 1.2. This value increases to 1.4 for two peaks with a 1:10 height ratio, and to 1.6 for a 1:100 ratio. This means that more chromatographic separation power is required to reliably quantify the low abundance peptides.

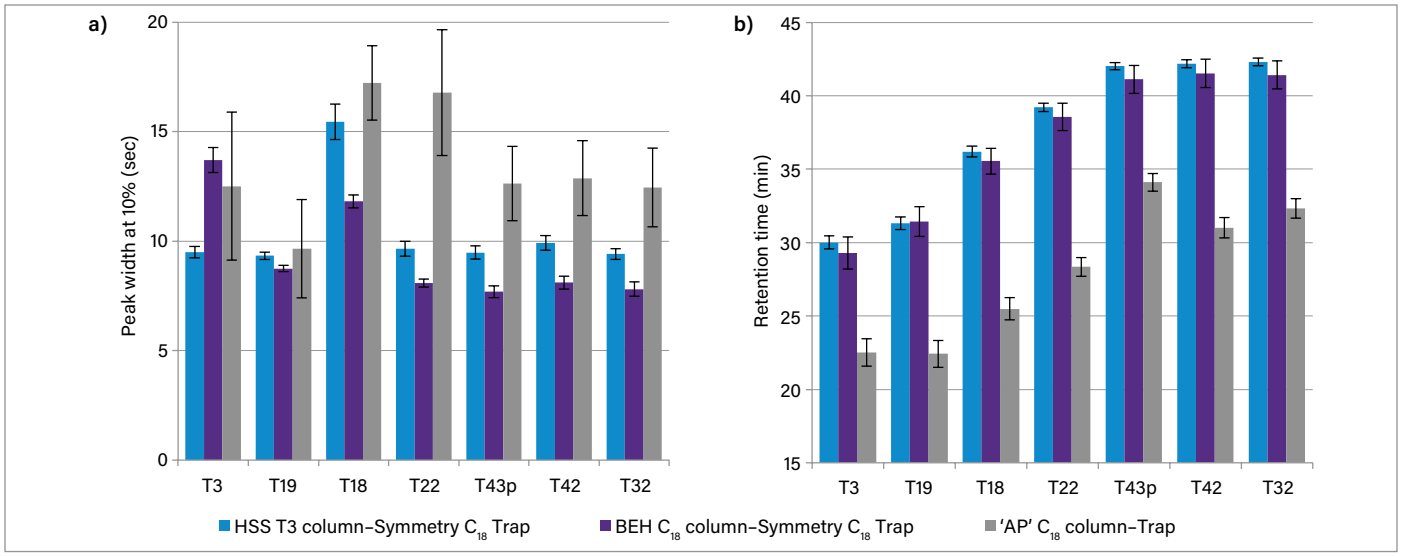


Figure 9. Chromatographic performance of commercially available trap–nanocolumn pairs. The charts show a) peptide peak widths, and b) retention times of HSS T3 columns–Symmetry C_{18} Traps (n = 4, blue), BEH C_{18} columns–Symmetry C_{18} Traps (n = 4, purple), and 'AP' C_{18} columns and traps (n = 5, grey). Bars represent the average measurements from n pairs, and the error bars denote their standard deviations.

Chromatographic performance and reproducibility of common nanocolumn-trap pairs

To this end, we tested the chromatographic capabilities of several commercially available nanocolumns and traps as applied to separations of enolase tryptic digest. The nanocolumn-trap pairings tested were: nanoEase M/Z HSS T3 Column–Symmetry C_{18} Trap (n = 4), nanoEase M/Z BEH C_{18} Column–Symmetry C_{18} Trap (n = 4), and 'AP' C_{18} nanocolumn–trap from vendor T (n = 5), where n denotes the number of pairs tested. Each pair was tested by repeatedly injecting the proteolytic peptide standard after the system was conditioned and equilibrated. The average peak widths and retention times from four consecutive injections were determined for each column-trap pair, and they were again averaged to calculate the average performance of the pairing. The selective reaction monitoring (SRM) dwell time was set to acquire 20 data points across 8 seconds, ensuring that small differences in peak widths could be accurately measured. The smallest peak width acquired during the test was 7.5 seconds, equivalent to 18 points across the peak width. Experimental details and the characteristics of the peptides monitored in this experiment can be found in the Conditions section.

The retention times and peak widths at 10% peak height (PW10%) for each nanocolumn-trap pair are shown in Figure 9. The HSS T3 column–Symmetry C_{18} Trap pairs showed consistent peak widths for all peptides except for T18, a basic peptide. The BEH C_{18} column–Symmetry C_{18} Trap pairs had the narrowest peaks for the late-eluting peptides while showing comparably wide peaks for the hydrophilic peptide T3. The 'AP' C_{18} column and trap pairs exhibited the poorest chromatographic performance of all, showing the widest peaks for most peptides.

The 'AP' C_{18} pairs produced peaks for well-retained peptides that were on average 20% or 40% wider in comparison to HSS T3 or BEH C_{18} columns paired with Symmetry C_{18} Traps, respectively. We observed that a few 'AP' C_{18} pairs produced narrow peaks for early-eluting T3 and T19, which were comparable to peaks from either of the Waters column-trap pairings. None of the well-retained peptide peaks from the 'AP' C_{18} pairs, on the other hand, was narrower than the Waters pairs. It was also noted that the peak widths for the 'AP' C_{18} pairs were highly variable. The peak width relative standard deviations (RSDs) from the 'AP' C_{18} pair ranged from 10 to 27%. In contrast, the HSS T3 or BEH C_{18} columns paired with Symmetry C_{18} Traps showed superior peak width reproducibility with RSDs ranging from only 2 to 5%. The 'AP' C_{18} pairs also showed unusually wide peaks for T22, a neutral peptide, in addition to the basic peptide, T18, suggesting the presence of a unique surface interaction mechanism that is different from BEH C_{18} and HSS T3.

Retention times proved to be equally interesting to compare, seeing how they reflect both the retentivity of the nanocolumn stationary phases and the fluidic volumes of the traps and nanocolumns. The 'AP' C_{18} pair had shorter retention times versus HSS T3 or BEH C_{18} pairs. As expected from the C_{18} retentivity comparison (Figure 4), the low retentivity of the 'AP' C_{18} stationary phase is the primary reason for the short

retention times. The slightly stronger retentivity of HSS T3 compared with BEH C_{18} is reflected in the observed retention time differences. It should also be noted that the HSS T3 columns showed the most reproducible retention times, with RSDs less than 1.5%.

Effect of retentivity on chromatographic performance

The nanocolumns and traps were cross-paired with each other to better understand the effects of retentivity on separation performance. To avoid confounding this study with product variability, we chose to use the best performing nanocolumns and traps from the above tests for further investigations. Figure 10 shows the chromatographic performance of each cross-pair in terms of average PW10% from four replicate injections on a single column–trap pair. In summary, Symmetry C_{18} Traps showed superior chromatographic performance in all pairings. For moderately and strongly retained peptides, the 'AP' C_{18} trap gave about 15% wider peaks when paired with the HSS T3 or the BEH C_{18} column and 20% wider peaks when paired with the 'AP' C_{18} column. The peak width difference was more noticeable for weakly retained peptides: the 'AP' C_{18} trap had about 20 to 130% wider T3 and T19 peaks than the Symmetry C_{18} Trap when paired with the HSS T3 or the BEH C_{18} nanocolumn. It was also noted that the T22 peak width decreased significantly when the 'AP' C_{18} column was paired with the Symmetry C_{18} Trap instead of the 'AP' C_{18} trap. The wide T22 peak was earlier ascribed to a secondary interaction on the 'AP' C_{18} stationary phase, which appears to be mitigated by using the Symmetry C_{18} Trap.

Some peak width differences between the cross-paired subjects can be explained by considering the retentivity of each stationary phase. For example, 'AP' C_{18} had a strong retentivity for hydrophilic compounds which was not so different from the retentivity of BEH C_{18} or HSS T3 (Figure 5). This small retentivity difference prevented the effective refocusing of weakly retained peptides, and thus resulted in broad T3 and T19 peaks. The lack of refocusing was more obvious with the BEH C_{18} column paired with the 'AP' C_{18} trap, resulting in the 30-second-wide T3 peak. On the other hand, the strong hydrophilic retentivity of 'AP' C_{18} may be acceptable as a column stationary phase only when it is paired with the less retentive Symmetry C_{18} Trap. The narrow T3 and T19 peaks from the 'AP' C_{18} column–Symmetry C_{18} Trap pair (7.9 and 7.5 second peak widths, respectively) are due to the favorably large retentivity difference for hydrophilic peptides. It is in the logical order to ask how large retentivity difference is required for effective refocusing. One clue can be found by comparing the retentivity difference (Figure 5) with the refocusing of peptide peaks from various column-trap pairs in Figure 10. With this, we empirically estimate that a retentivity difference greater than about 1 to 1.5% ACN is sufficient to achieve effective refocusing.

It can be noted that the peak widths for strongly retained peptides (T22 through T32) were all similar to each other for any given column-trap pair in Figure 10. This observation may be due to the large retentivity difference as described above. Some pairs, however, cannot be explained using the retentivity difference only. They exhibited somewhat narrow peaks although their retentivity differences were not large enough for effective refocusing. It is believed that another factor that contributed to this effect was the strong retention of peptides to the trap stationary phase during trapping. As reviewed in the earlier section, strongly retained peptides are less likely to travel through the packed bed of the trap, and are thereby less susceptible to band broadening. Peptides T22 through T32 all showed strong retention to the tested trap stationary phases, and they are expected to experience much less band broadening compared to the less retentive peptides such as T3 and T19. Another example can be found from the 'AP' C_{18} pair that showed relatively small band broadening for T3 and T19 compared to other pairs with the Symmetry C_{18} Trap.

Effects of device dispersion on chromatographic performance

There are other peak width differences that cannot be explained by retentivity alone. For example, while a given column-trap pair showed consistent peak widths for strongly retained peptides, these peak width values were significantly different between the pairings. This difference is obvious when comparing the average peak widths for well-retained peptides, T43p to T32, from various pairings (Table 1). With the same column, the Symmetry C_{18} Traps provided narrower peaks than 'AP' C_{18} traps. For example, the 'AP' C_{18} column showed an average peak width of 11.2 seconds when paired with the 'AP' C_{18} trap. The same column provided much narrower peaks (9.5 seconds on average) when paired with the Symmetry C_{18} Trap. A similar improvement in resolution can be found with other columns. We speculate that this difference was caused by the dispersion contributions from the traps. A trap can contribute to the overall dispersion in various ways. One of the dispersion sources is the quality of the packed bed. When an analyte band passes through a packed bed, the non-uniform nature of the flow paths creates a variance in path lengths. This effect is called eddy diffusion, and it constitutes a significant source of band broadening. Table 1 shows that the well-retained peptide peaks acquired using 'AP' C_{18} traps are 8 to 17% wider

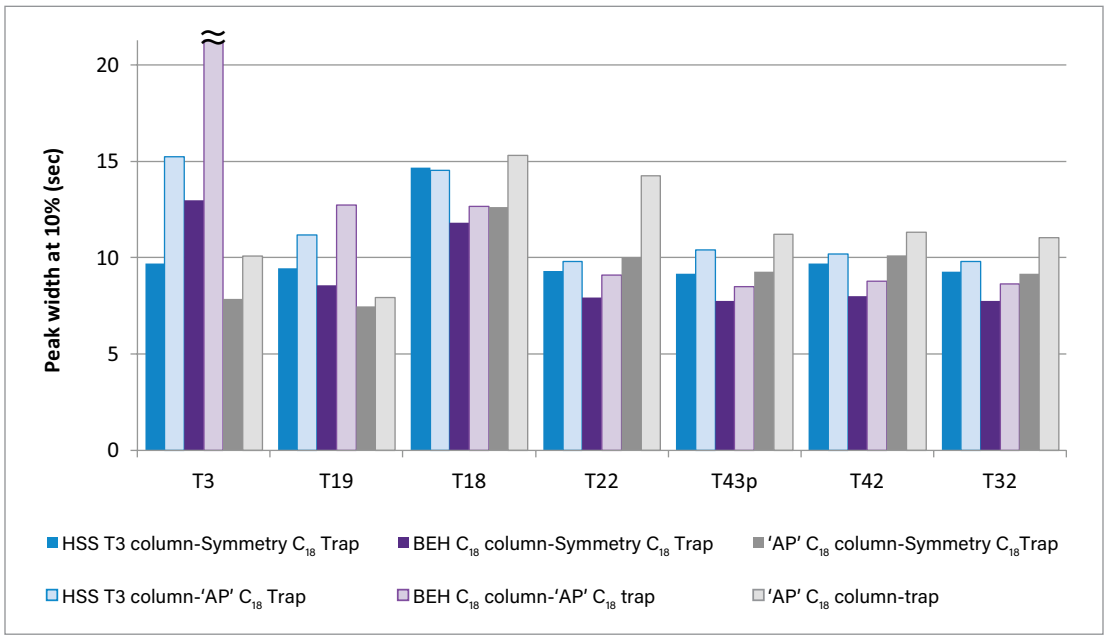


Figure 10. Chromatographic performance of column-trap cross-pairs. Each bar represents the average peak width (PW10%) from four replicate injections on a single nanocolumn-trap pair. Note that the BEH C_{18} column–'AP' C_{18} trap pair showed the wide T3 peak.

Table 1. Peak widths and peak capacity values from each cross-pairing.

Column	Trap	Peak width at 10% height (PW10%)							Average PW10% (T43p–T32)	P_c, 10%
		T3	T19	T18	T22	T43p	T42	T32		
HSS T3	Symmetry C_{18}	9.7	9.4	14.7	9.3	9.2	9.7	9.3	9.4	289
HSS T3	'AP' C_{18}	15.2	11.2	14.5	9.8	10.4	10.2	9.8	10.1	267
BEH C_{18}	Symmetry C_{18}	13.0	8.6	11.8	7.9	7.7	8.0	7.7	7.8	346
BEH C_{18}	'AP' C_{18}	30.1	12.7	12.7	9.1	8.5	8.8	8.7	8.6	313
'AP' C_{18}	Symmetry C_{18}	7.9	7.5	12.6	10.0	9.3	10.1	9.2	9.5	284
'AP' C_{18}	'AP' C_{18}	10.1	7.9	15.3	14.3	11.2	11.3	11.0	11.2	242

than those obtained with Symmetry C_{18} Traps. This difference strongly suggests the superior dispersion characteristics of Symmetry C_{18} Traps, which may come from their packing quality. Another clue for the better packing quality is the pair-to-pair reproducibility shown in Figure 9, where the peak width reproducibility with the Symmetry C_{18} Traps was much higher than that with the 'AP' C_{18} traps (5% vs. 27% RSD). A similar argument can be made about the nanocolumns. With the same trap, the peak widths for strongly retained peptides were smallest with the BEH C_{18} column and the largest with the 'AP' C_{18} column.

Practical implications of high chromatographic performance

The chromatographic peak width data can be converted into a more representative indicator of resolving power, peak capacity (P_C), to compare the chromatographic performance of the nanocolumn-trap pairs in a more universal way.

Peak capacity can be experimentally calculated using the following equation,

$$P_c = 1 + \frac{t_{gr}}{w}$$

where t_{gr} is the gradient length and w is the peak width. We used the average peak widths measured from peptides T43p, T42, and T32 (Table 1). The BEH C_{18} nanocolumn–Symmetry C_{18} Trap pair showed a 43% greater peak capacity than the 'AP' C_{18} pair (346 vs. 242) for well-retained peptides. Meanwhile, the HSS T3-Symmetry C_{18} pair had a peak capacity of 289, which is 19% higher than the 'AP' C_{18} pair. These improvements are quite remarkable, because one alternative method to improve the chromatographic efficiency, or the peak capacity, is to use a long column with a long gradient. Trying to increase the peak capacity by using a longer column, however, has practical limitations. A longer column produces a higher back pressure at the same flow rate. A longer column also requires a longer gradient and thus a longer run time.

Even after accepting all these limitations, the gain in peak capacity by increasing the column length will have a diminishing return. The peak capacity is expected to increase in proportion to the square root of the isocratic plate count (N),[11] which is proportional to the length of the column. This expectation has been experimentally verified, wherein a P_c increase of 40% was obtained when column length was doubled.[12,13] These data along with the theoretical estimation predicts that the BEH C_{18} nanocolumn–Symmetry C_{18} Trap pair would perform similarly to an 'AP' C_{18} pair that has a nanocolumn of twice the length. Clearly it is more beneficial to select an optimal column-trap pair than to increase the column length in order to achieve a higher peak capacity.

EFFECTS OF INJECTION VOLUME AND THE SAMPLE MATRIX

One benefit of trap-and-elute chromatography is the ability to inject a large volume of sample without compromising chromatographic efficiency. As reviewed in the earlier section (Figure 2), the direct injection method has a practical limit on the allowable injection volume in proportion to the column volume. While trap-and-elute methods can accommodate larger injection volumes than the equivalent direct-inject methods, therefore improving the assay sensitivity, the maximum injection volume should be experimentally determined because each analyte in the sample might interact differently with the trap. Typically a weakly retained analyte has a lower maximum injection volume because it tends to keep traveling through the trap during trapping and may eventually exit from the trap.

Figure 11 and Table 2 show the relationships of the peak area versus the injection volume for the tested column-trap pairs, along with the changes in peak width. Duplicate injections were made at each injection volume using an optimized trapping condition (99.5:0.5 A/B at 15 µL/min for 2 minutes). For easy comparison, each peak area was normalized to the average peak areas from 10-µL injections. The 'AP' C_{18} trap, as expected from its high retentivity for hydrophilic peptides, did not lose peptides even when a large volume of sample was injected. The Symmetry C_{18} Traps also showed no peptide loss despite its lower retentivity relative to the 'AP' C_{18} trap. This again underlines that the trapping efficiency depends not just on the retentivity of the trap stationary phase, but also on the trapping conditions. In this example, the optimized trapping condition achieved the maximum recovery for peptides of a wide retention range (Figure 7) and, at the same time, did not lose any peptides when a large volume of sample was injected (Figure 11). It was also noted that the measured peak widths from all three column-trap pairs did not correlate with the injection volume, confirming the robustness of the optimized trapping method toward the increased injection volume. The Symmetry C_{18} Trap paired either with a HSS T3 or a BEH C_{18} column showed excellent peak width reproducibility throughout the tested injection volume range of 1 to 20 µL (RSD 1 to 4%).

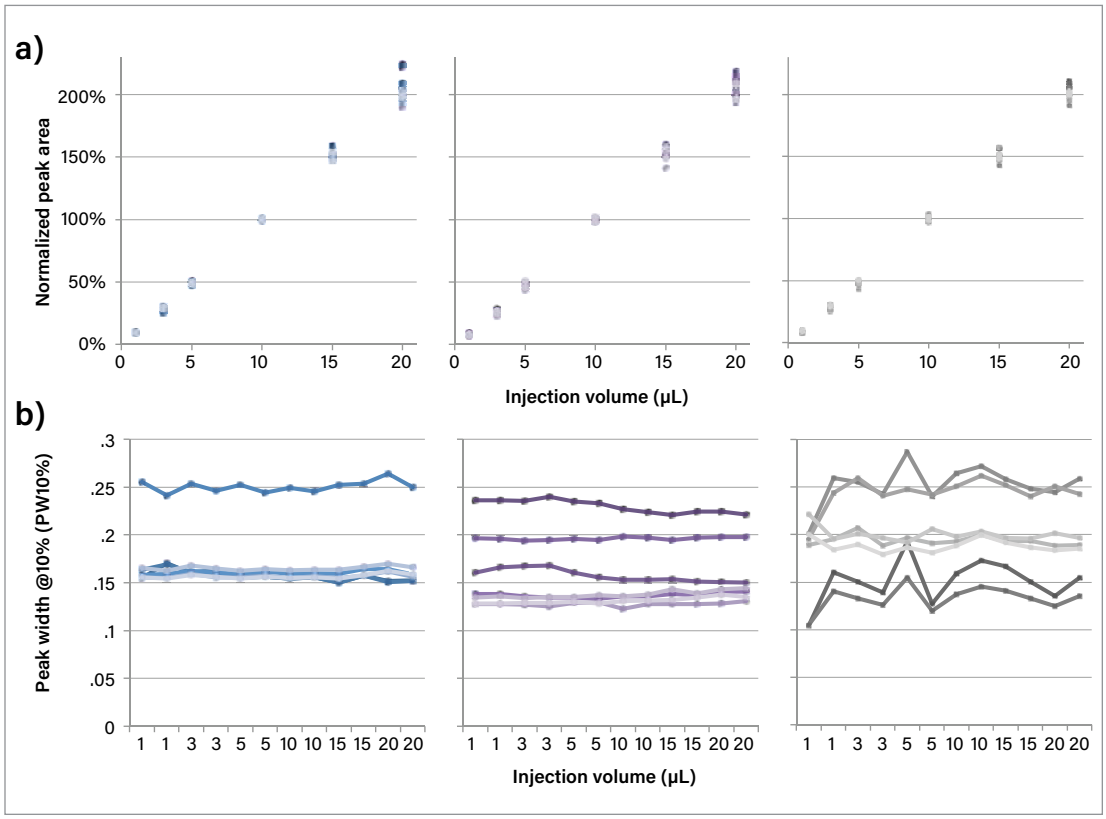


Figure 11. Chromatographic responses vs. injection volume in, a) normalized peak area, and b) peak width (PW10%). Duplicate injections were made at each injection volume using the optimized trapping condition. Each peak area was normalized against the average peak areas acquired from 10-µL injections. The peptide peak widths from 12 consecutive injections were displayed in reference to the injection volume. Blue data points represent results acquired from the HSS T3 column–Symmetry C_{18} Trap pair, purple points from the BEH C_{18} column–Symmetry C_{18} Trap pair, and grey points from the 'AP' C_{18} column–trap pair. The peptides are presented with different shades of the color in the order of their approximate elution: T3 (darkest), T19, T18, T22, T43p, T42, and T32 (lightest).

Table 2. Injection linearity and peak width consistency.

	Injection linearity (average R^2)	RSD of the peptide peak widths (n = 12)						
		T3	T19	T18	T22	T43p	T42	T32
HSS T3 column–Symmetry C_{18} Trap	0.9988	3.4%	3.7%	2.4%	1.3%	1.3%	1.4%	1.5%
BEH C_{18} column–Symmetry C_{18} Trap	0.9983	2.9%	4.3%	0.7%	1.7%	1.6%	2.7%	2.3%
'AP' C_{18} column-trap pair	0.9991	15.1%	9.7%	8.3%	6.9%	2.9%	3.8%	3.5%

This consistency can be explained by the efficient refocusing effect that comes from the sufficient retentivity difference between the trap and column stationary phases. The T3 and T19 peak widths were slightly more variable than the peak widths of the well-retained peptides, which could be understood with the above-mentioned band broadening effect in the trap. The 'AP' C_{18} trap and column, on the other hand, had a much larger variation in peak widths which could be due to poor packing quality as noted before (RSD 3 to 15%).

Another potential adverse effect from the injection comes from the matrix components in the sample. Typical biological samples, such as serum or plasma, contain many endogenous proteins and phospholipids. Due to their complex structures and the presence of multiple reactive sites, these matrix components tend to bind strongly to the column. Their adsorption not only adversely affects chromatographic performance but also increases column backpressure. Because nanocolumns have small column volumes, nanoLC suffers more from these adverse

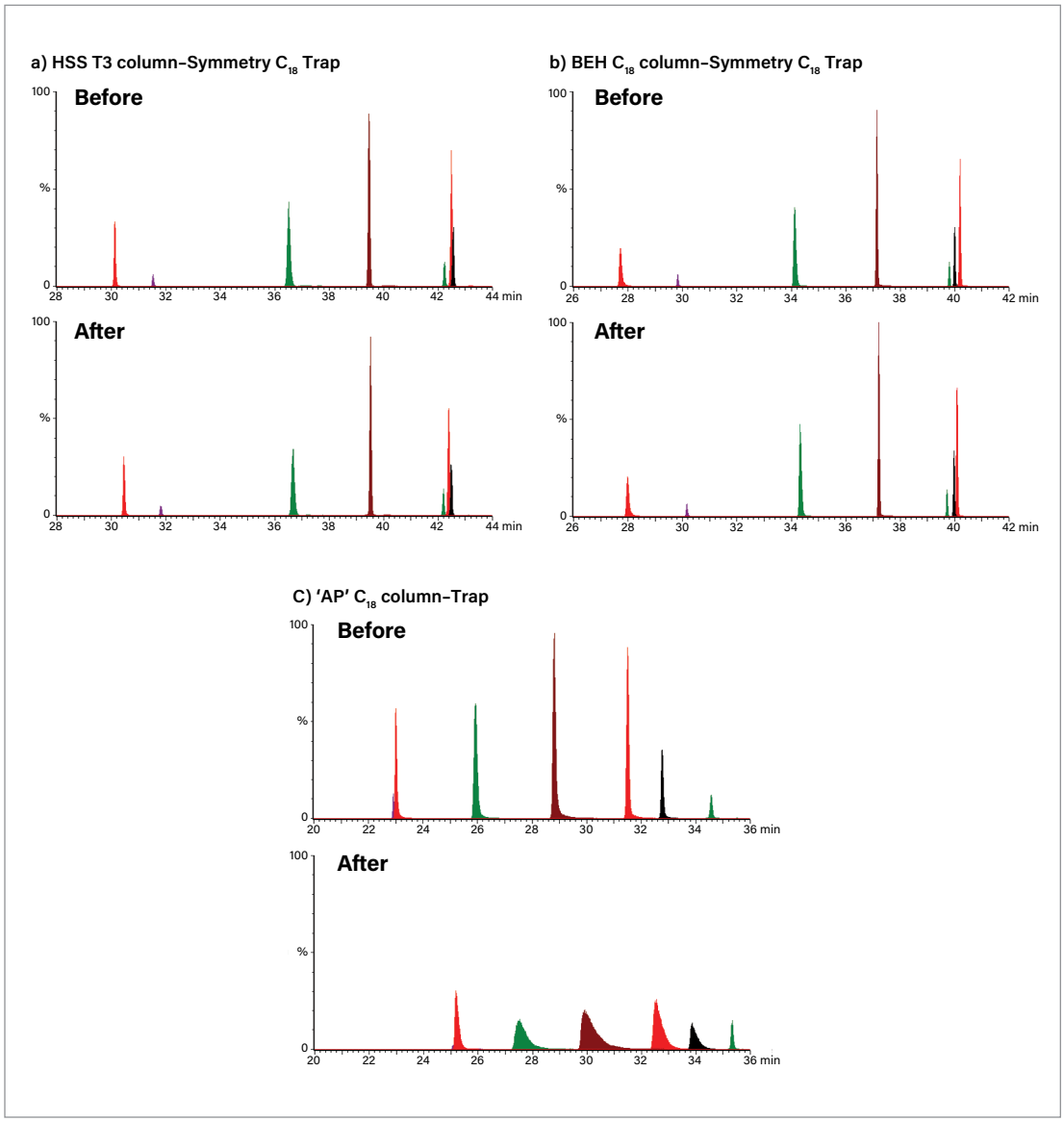


Figure 12. Example chromatograms showing the effects of injecting a crashed plasma sample on a) the HSS T3 column-Symmetry C_{18} Trap pair, b) the BEH C_{18} column–Symmetry C_{18} Trap pair, and c) the 'AP' C_{18} column–trap pair. Each panel shows the peptide separation chromatograms acquired before the charging and at the third injection after the charge.

effects than larger scale LC does. Figure 12 illustrates the effect of injecting a large volume of 'dirty' sample. Each nanocolumn-trap was charged with five consecutive 10-µL injections of crashed rat plasma supernatant (1:2 plasma/acetonitrile with 0.1% formic acid, v/v) between peptide standard separations.

With the Symmetry C_{18} Traps, peak widths and retention times did not change before and after the plasma charge. There was a slight decrease in signal intensity directly after the plasma charge, but the signal intensity returned to the original level after a couple of additional injections and runs. This could be attributed to a temporary ion suppression effect from the sample matrix. The behavior of the 'AP' C_{18} trap was different from that of the Symmetry C_{18} Trap. After being charged with the injection of crashed plasma, the 'AP' C_{18} trap showed significant peak broadening. The peptide standard peaks broadened by 10 to 400% and remained that way for the next several runs. Peak widths returned to the original level only after the trap and the nanocolumn had been thoroughly washed with the mobile phase with a high organic content (15:85 A/B) and re-equilibrated to the initial condition.

CONCLUSIONS

This study reviewed the performance of several commercially-available traps and nanocolumns in multiple aspects, and rationalized the findings with theory. From the data and discussions, we can draw a few practical recommendations in creating a successful trap-and-elute chromatographic method. First, the method development process should start with the selection of an appropriate nanocolumn and a trap, based on the retentivity of stationary phases. The retentivity of each individual stationary phase is important, but the ability to pair the nanocolumn and the trap based on their retentivity difference unlocks the possibility to tune an assay for certain target analytes. For example, a large retentivity difference between the nanocolumn and the trap, such as with the HSS T3 column and the Symmetry C_{18} Trap, provides good chromatographic performance for peptides with vastly different retention. Therefore, this pair is highly recommended for non-targeted assays for proteomic peptide samples.

A BEH C_{18} column and a Symmetry C_{18} Trap pair, on the other hand, has a sufficiently large retentivity difference for moderately-to-strongly-retained peptides while having a comparatively small retentivity difference for early eluting peptides. This pair is thus not recommended for analyzing hydrophilic peptides, but is very well suited for analyzing most other peptides with unsurpassed resolution, thanks to the superior efficiency of BEH C_{18} nanocolumns. Example uses of a BEH C_{18} nanocolumn paired with a Symmetry C_{18} Trap include targeted quantitation analyses that require maximum sensitivity for known analytes.

Second, the trapping flow conditions should be carefully checked to make sure that no analytes are lost during the analysis. Parameters to consider include the mobile-phase composition and the volume of the trapping solvent. Using a trap with a more retentive stationary phase helps to minimize the analyte loss, but often the trapping flow conditions offer more flexible and useful tools for increasing the peptide recovery than changing the retentivity of the trap stationary phase. If the injection volume or the sample matrix will be changed during the analysis, their influences on the recovery and the chromatographic performance should also be checked beforehand. Finally, the dispersion contributions from the trap and the nanocolumn have a significant effect on the chromatographic resolution and assay reproducibility. Undesirable high dispersion can result from a non-optimal hardware design or from manufacturing variability.

In summary, this work emphasizes the importance of selecting an appropriate nanocolumn and trap when developing a trap-and-elute nanoLC method. Waters nanoEase M/Z Nanocolumns and Traps, with an optimum selection of stationary phases and unsurpassed chromatographic resolution, enable users to successfully implement a robust and high-resolution trap-and-elute nanoLC method for challenging analyses.

- HSS T3 column–Symmetry C_{18} trap: recommended for non-targeted assays.
- BEH C_{18} column–Symmetry C_{18} trap: recommended for targeted analysis for maximum sensitivity.

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