Comparison of Reversed-Phase Nano LC Workflows Applicable for Routine Proteomics Analysis

Evert-Jan Sneekes, Marco Karsten, Wim Decrop, and Remco Swart,
Dionex Corporation, Amsterdam, The Netherlands

INTRODUCTION

Reversed-phase nano LC tandem MS has become the standard tool for protein identification studies. Current state-of-the-art nano LC pumps deliver continuous, direct mobile phase gradients at operating pressures that are compatible with UHPLC (~800 bar/11,500psi). On-line preconcentration techniques are typically applied to efficiently combine the low column flow rate of a few hundred nL/min with the relatively large injection volumes in the range of 5–20 µL.

Several nano LC preconcentration setups have been developed over the years, with column switching and the so-called vented column setup being the most popular. All these methods use a short reversed-phase trap column for preconcentration (and desalting) of sample components, often peptides. After switching the trap column in series with the nano LC column, the sample components are separated by the nano LC gradient.

This study compares various nano LC preconcentration setups for retention time precision, carryover, protein identification results, and sample throughput. In addition, the workflows are discussed in terms of their operating and maintenance convenience.

THEORY

In proteomics separation, strategies should offer the highest peak capacity possible and be MS compatible. Long columns packed with 2 µm C18 particles operated in a preconcentration mode fulfill both these requirements, by offering efficient separation, MS compatible solvents, and desalting capabilities. The separation of peptides is performed by RP gradients over the nano separation column, that generally encompass the following phases:

Loading the sample:	Isocratic at <3 % CH_3CN
Gradient separation:	From <3% to 45% CH_3CN
Wash:	Isocratic at >70% CH_3CN
Equilibration:	Isocratic at <3% CH_3CN

Small variation in solvent conditions and timing apply, but the above scheme allows good separation of tryptic digests.

Although the conditions at which the separations are performed are remarkably similar, there are big differences in fluidic setup that influence the way the conditions are applied to the column. Figure 1 shows the traditional column-switching preconcentration setup. The traditional method uses a trapping column that is switched in-line or off-line with the separation column depending on the stage of the analysis.

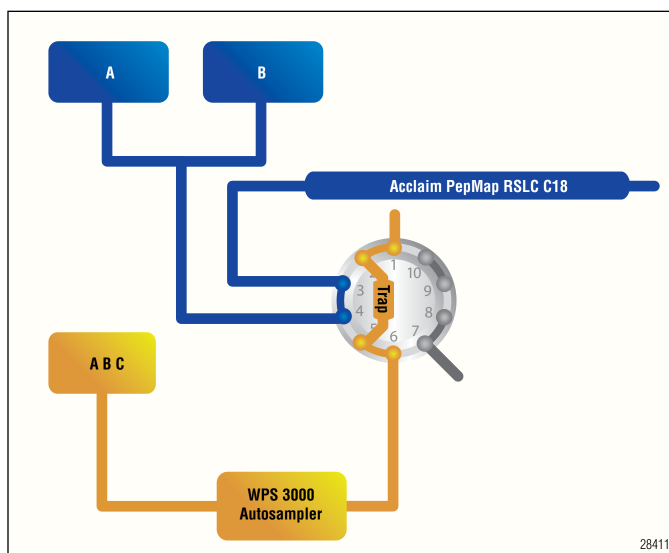

Figure 1. Traditional column switching setup. The loading pump is used to transfer the sample to the trap column and the gradient pump is used for the separation. The valve position depends on the stage of the separation, but flow over the nano column is always maintained.

This allows complete separation of the loading and the separation phase and makes it ideal for samples that require extensive washing. The washing and equilibration of the trap and separation column is performed with their respective pumps, offering more flexibility and higher throughput. This setup also features an uninterrupted flow and pressure on the nano column and MS spray interface.

The vented column setup has the trap and analytical column connected directly via a T-piece. This minimizes dead volume and even offers a possibility to move the column physically away from the switching valve and place it closer to the MS. The drawback is that during every run the nano column has to be pressurized and depressurized, adversely affecting column lifetime; with long columns packed with small particles this could mean pressure changes of up to 700 bar. The typical workaround is to program a controlled flow and pressure release, before switching the valve. This will add to the analysis time and reduce sample throughput.

Figure 2 shows two variations of the vented column setup. Figure 2 top uses a separate loading pump allowing higher loading flows for faster sample loading and washing. Figure 2 bottom uses the nano pump at elevated flow rate for loading.

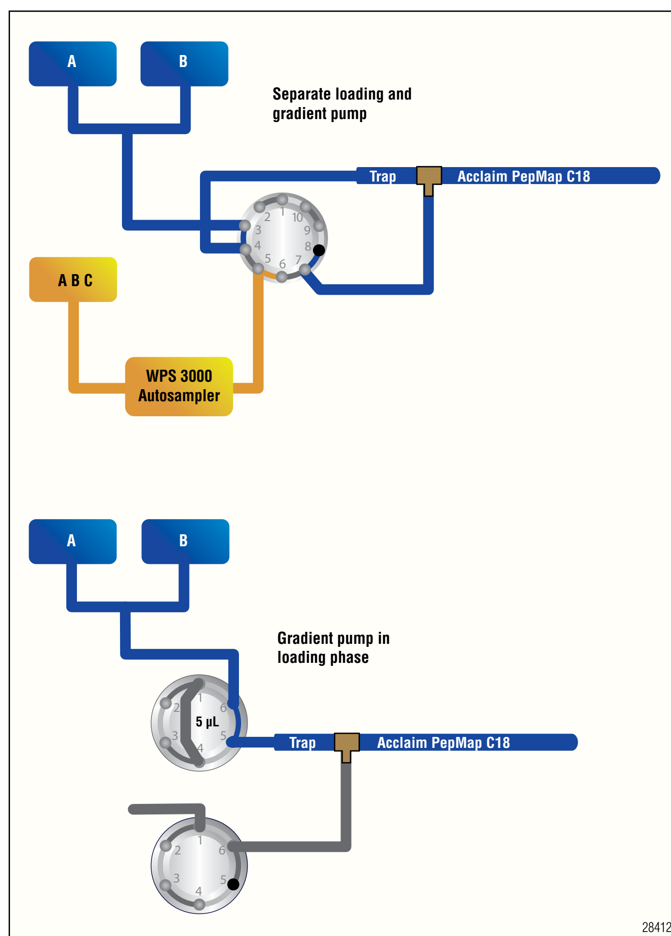

Figure 2 . Vented column setups, using separate loading and gradient pumps (top) or employing the gradient pump also in the loading phase (bottom).

EXPERIMENTAL

All experiments were performed using an UltiMate® 3000 RSLCnano system. This UHPLC compatible nano LC instrument is equipped with a high-pressure nano-flow gradient pump (Blue, solvents A and B) and a ternary low-pressure gradient pump (Gold, solvents A B C) that can be used as a loading pump depending on the experimental setup, e.g., Figure 1 and Figure 2 top.

All data were recorded by a UV detector and, where combined with ESI-MS detection, with a HCT iontrap from Bruker Daltonics.

RP Chromatography

Column:	75 µm i.d. × 150 and 500 mm Acclaim® PepMap™ C18 RSLC nano columns
Trap Column:	75 µm i.d. × 20 mm Acclaim PepMap C18 trap column
Mobile A:	Water, 0.05% TFA
Mobile B:	80% CH_3CN, 0.04% TFA
Loading Solvent:	Water, 0.05% TFA (when using the loading pump)
Flow Rate:	300 nL/min
Loading Flow Rate:	1 and 5 µL/min
Loading Time:	Varies with setup
Gradient:	4–55% B in 30 min, 5 min wash, 25 min equilibration
Samples:	Cytochrome C and Protein Mix Digest separations
Detection:	UV at 214 nm

RESULTS

Figure 3 shows the results for cytochrome separation on the traditional column switching, vented column with loading pump, and vented column with one pump. All setups are capable of performing the separation and all setups can do so reproducibly. Retention times and elution windows are comparable as well, indicating the dwell volume of the systems is not markedly different.

However, when looking in more detail, differences are observed. The use of a separate loading pump allows higher flow rates for the loading and equilibration phase. This results in equal run time for both column setups. When only one pump is used in the configuration, the flow during load and equilibration depends on the upper flow and pressure specifications of the pump. In this case it resulted in an increase in run time around 5 min.

Peak 7 and 8 often coelute; observing these as separate peaks is an indication of excellent column performance and minimal external column band broadening. Only in the traditional column switching setup are the peaks separated.

Reproducibility is excellent for all setups, typically below 0.1% RSD for all peaks when looking at consecutive injections.

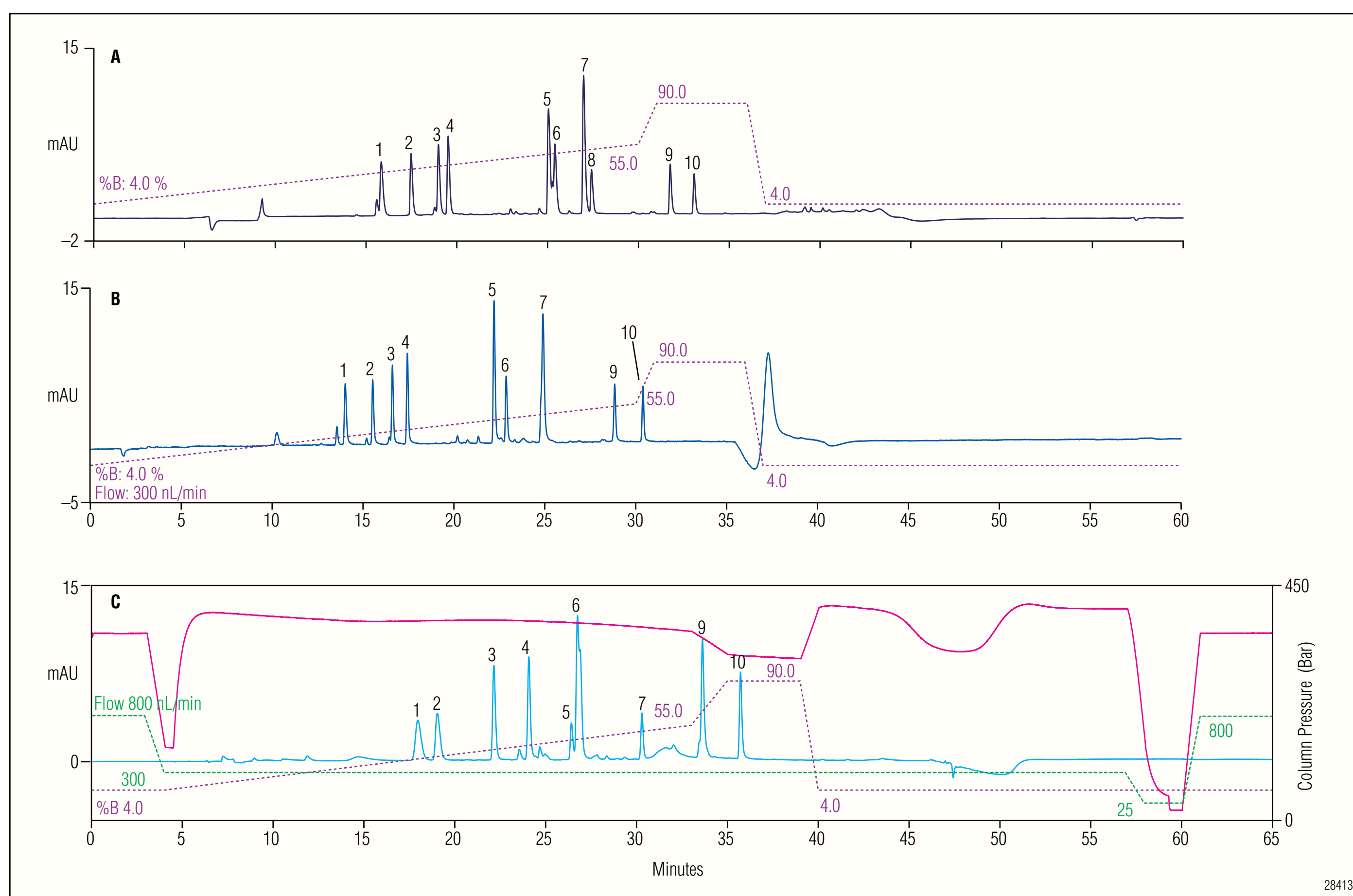

Figure 3. Comparison of cytochrome C separation under standard conditions of A) traditional (Figure 1), B) vented, two pumps (Figure 2 top), C) vented, single pump (Figure 2 bottom) setups. Panel C shows as an inlay of the column pressure as a result of flow variation and gradient formation during the run.

Table 1. Peak Results for the Cytochrome C Separations in Figure 3						
	Traditional		Vented, Two Pumps		Vented, One Pump	
Peak	Ret.Time (min)	PWHH (sec)	Ret. Time (min)	PWHH (sec)	Ret. Time (min)	PWHH (sec)
1	15.867	9.25	14.02	7.51	17.987	20.55
2	17.508	7.95	15.533	6.31	19.06	16.15
3	19.025	7.24	16.62	5.92	22.147	9.81
4	19.558	7.65	17.44	6.35	24.087	9.39
5	25.05	8.16	22.207	6.78	26.433	8.29
6	25.4	7.08	22.873	6.65	26.767	17.65
7	26.983	7.83	24.9	8.8	30.3	8.14
8	27.425	7.15	—	—	—	—
9	31.767	6.78	28.847	6.67	33.627	9.37
10	33.083	6.66	30.4	6.31	35.727	8.59

Dead Volume from Connections

Often vented column setups are reported to have better performance because the trap and separation are close together. This is not caused by the vented setup, but by the dwell volume between the columns. To evaluate this effect the traditional setup was compared to a vented column setup with a low pressure (200 bar) PEEK™ T-piece and a UHPLC compatible (800 bar) Ti T-piece.

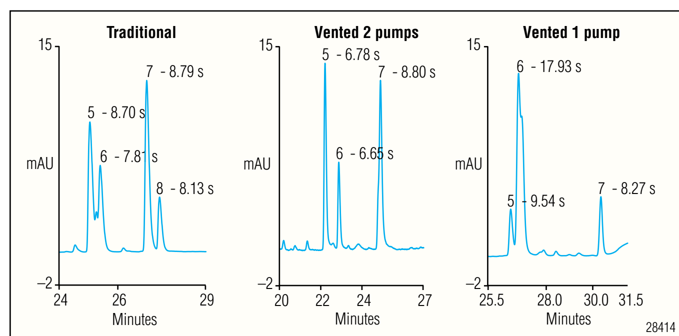

Figure 4. Effect of peak width between trap and nano column with a valve with 150 µm boring, low pressure PEEK T-piece, and high pressure T-piece with 250 µm boring.

Figure 4 shows that the UHPLC-compatible T-piece has significantly broader peaks. Although the PEEK T-piece shows nice peak widths, it does not allow higher pressure. Therefore, the traditional column switching setup for UHPLC nano applications (e.g., longer columns) is preferred. It offers good separation performance with high backpressure capabilities.

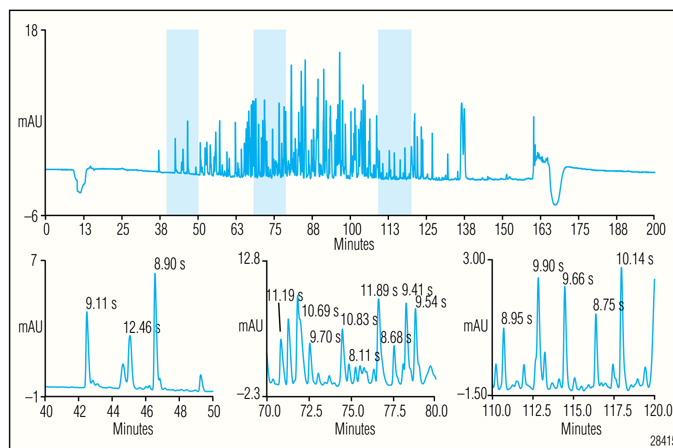

Figure 5. Protein Mix Digest (tryptic digest of six proteins) separation on a 75 µm × 500 mm long Acclaim PepMap RSLC column in the traditional column swithing method. The smaller chromatograms show portions of the run with Peak Width Half Height in seconds.

The application of longer columns in proteomics is one of the easiest methods to increase peak capacity. Figure 5 demonstrates that the longer column (500 mm) can separate more complex samples with the same efficiency as the 150 mm column separates a cytochrome C sample.

Carryover

The loading pump used in this research has three solvent channels, and only in the traditional setup does it allow washing the trap column simultaneously with equilibrating the nano column. In a trapping experiment, the trap column is off line with the loading flow during the separation. At this time the system can be flushed with high organic solvent. Typically the trap column is switched back in line with the loading flow just before the end of the run, but by switching back earlier the trap column can be flushed with an organic solvent with higher elution strength and at a higher flow rate (i.e., more column volumes in the same time). The only care to be taken is to have sufficient equilibration time before the next sample is injected. Figure 6 shows schematically how this wash procedure is applied to the trap column.

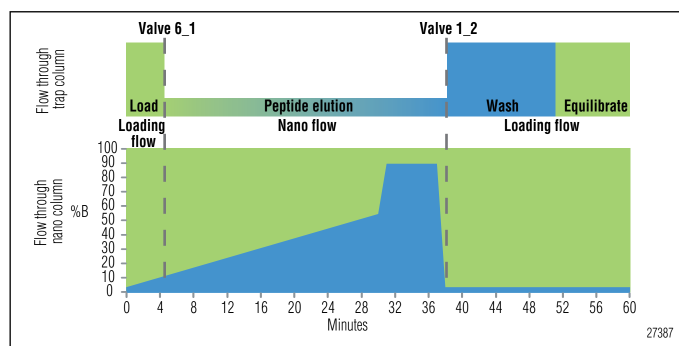

Figure 6. Solvent composition over the trapping column related to the gradient. Solvent A in green, solvent B in blue.

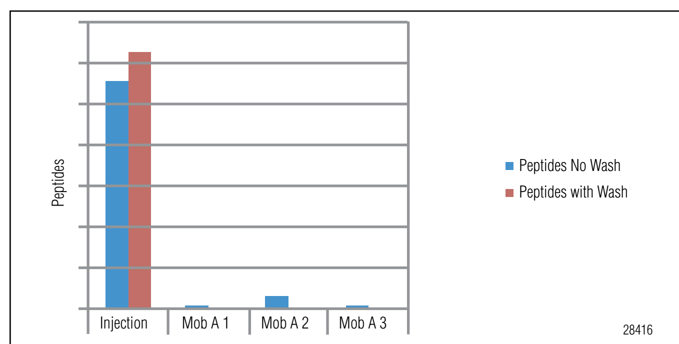

Figure 7. Results of the application of the high flow washing of the trapping column for the packed C18 and monolithic columns. The short gradients were used for both columns. Runs without wash are shown in blue and runs with application of the trap column wash step are shown in red.

Applying this simple step has no effect on run time or separation performance, but immediately reduces the measured carryover on both the packed C18 and monolithic columns. As can be seen in Figure 7, no peptides are detected in the Mob A injections (sampler + trap + column) when the wash procedure is applied. Here the wash solvent applied had the same composition (80% CH_3CN, 0.04% TFA) as the elution solvent. Of course for more complex or more sticky samples, the elution strength of the solvent can be increased by using stronger elution agents (e.g., isopropanol). This was also tested and showed the same reduction in carryover as with 80% CH_3CN (data not shown), but for the chosen measurement conditions the CH_3CN wash was sufficient.

CONCLUSION

- The most critical element in nano LC separation performance was found to be the dead volume between the trap column and the nano LC separation column.
- Current switching valves offer an internal volume that will not affect the peak width compared to a vented column setup. Lowering the internal volume will be at the expense of nano UHPLC compatibility.
- The ability to decouple loading and nano flow offers advantages for carryover reduction. The stronger wash solvents in combination with the higher flow capabilities have been demonstrated to reduce column carryover.
- The choice between vented and nonvented column setups is largely dependent on experimental parameters, such as column pressure, dead volume, sample cleanliness, carryover, and effect of spray interruptions.
- The RSLCnano platform allows the flexibility to perform all three evaluated setups without additional intstrumental requirements.

PepMap is a trademark and Acclaim and UltiMate are registered trademarks of Dionex Corporation.
PEEK is a trademark of Victrex PLC.

Passion. Power. Productivity.

Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America
U.S./Canada (847) 295-7500

South America
Brazil (55) 11 3731 5140

Europe
Austria (43) 1 616 51 25 Benelux (31) 20 683 9768; (32) 3 353 4294
Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10 Germany (49) 6126 991 0
Ireland (353) 1 644 0064 Italy (39) 02 51 62 1267 Sweden (46) 8 473 3380
Switzerland (41) 62 205 9966 United Kingdom (44) 1276 691722

Asia Pacific
Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 2764 2735
Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190
Taiwan (886) 2 8751 6655

www.dionex.com

LPN 2785-01 3/11

©2011 Dionex Corporation