Achieving Improved Precision and Low Carryover in Parallel Reaction Monitoring Mass Spectrometry through Optimized Chromatographic Strategies with µPAC Neo Column

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Abstract

Purpose: Comparison was done between 50 cm µPAC Neo column from Thermo Fisher Scientific and packed bed columns sourced from an alternative vendor to assess the accuracy of quantitation in PRM-MS

Methods: Targeted quantitation using LC–MS/MS parallel reaction monitoring

Results: µPAC column significantly improved the accuracy of quantifying PRTC peptides with no carryover.

Introduction

Parallel reaction monitoring (PRM) stands as a pivotal approach across diverse domains of biomedical research, representing a targeted mass spectrometry technique instrumental for the simultaneous quantification and identification of specific analytes within intricate biological samples.[1] Microfabricated pillar array columns (µPAC™), an innovative separation technology, have garnered substantial attention in various realms of mass spectrometry. The design of µPAC columns features meticulously organized micrometer-sized silicon structures modified with C18, affording highly efficient separation capabilities coupled with low back pressure. In this study, the selection of the 50 cm µPAC Neo column from Thermo Fisher Scientific, in comparison with two packed bed columns sourced from the same alternative vendor, was undertaken to assess the accuracy of quantitation in PRM-MS.

Materials and methods

Sample Preparation

5, 10, 20, 40, or 80 fmol of Pierce™ Peptide Retention Time Calibration Mixture (P/N 8321) was spiked into 100 ng Pierce™ HeLa Protein Digest Standard (P/N 88328). 5 samples were analyzed by PRM. Fisher Scientific™ 0.1% Formic Acid in Water, Optima™ LC-MS grade (P/N LS118-212) was used as diluent.

Table 1. 50 cm µPAC Neo Column properties.

Parameter	50 cm µPAC Neo
Pillar shape	Cylindrical
Pillar Diameter (µm)	2.5
Interpillar distance (µm)	1.25
Channel Width (µm)	180
Channel depth (µm)	16
Length (cm)	50
Surface morphology	Core-shell
Porous layer thickness (nm)	300
Pore size range	100-300
Surface functionalization	C18 + HMDS
Flow rate range (nL/min)	100-750
Void volume (uL)	1.5
Maximum Pressure (bar)	450
Maximum Temperature (ºC)	60

Test Method(s)

50cm µPAC Neo column (P/N COL-NANO050NEOB) was applied to separate all spiked samples and they were tested in triplicates. Column properties are shown in Table 1. In chromatographic experiments, mobile phase “A” was water containing 0.1% formic acid and mobile phase “B” was 80/20 acetonitrile/water (v/v) consisting of 0.1% formic acid (by volume). A 39-min method running at 300 nL/min (LC gradient is shown in Table 2) was used to analyze tryptic peptides in PRM positive mode on Thermo Fisher Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. MS parameters are listed in Table 3.

Table 2. LC gradient

Time	Duration (min)	Flow (µL/min)	%B
0	0	0.3	1.0
0.1	0.1	0.3	4.0
20.1	20	0.3	22.5
27.6	7.5	0.3	35.0
30.1	2.5	0.3	45.0
31	0.9	0.3	99.0
39	8	0.3	99.0

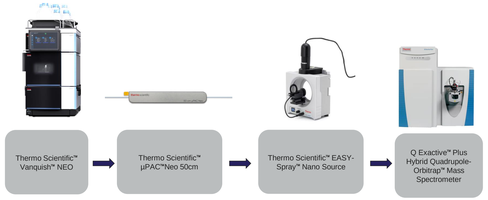
Table 3. MS method

Spray Voltage	1.8kV
MS1 Resolution	30K
m/z range	200-1200
RF lens %	50
AGC	1e6
Max IT	20 ms
HCD	30
PRM MS2 Resolution	15K
Isolation window	1.2
AGC	1e5
Max IT	20 ms

Data Analysis

Raw data were analyzed by Skyline 21.2.0.568.[2] y ions are used for quantitation, product ions from ion 3 and last ion-1 are selected. PRM acquisition method is checked and mass accuracy of 10 ppm is enforced for product ion identification.

Workflow



Results

Peak Area and RT stability comparison

The quantification process involved the utilization of the total peak area derived from the product ions of each PRTC peptide. In Figure 1, the product ion areas of peptide DIPVPKPK are delineated in (a), wherein the entirety of the peptide elution duration was considered for peak area calculation. Figures 1 (b) and (c) depict the peak areas of the DIPVPKPK across various concentrations of spiked PRTC peptides, revealing a discernibly diminished variation in peak area among triplicate experiments when employing the 50 cm µPAC Neo HPLC Column. The coefficient of variation (CV) for all 15 PRTC peptides is systematically computed and illustrated in Figure 1 (d). Notably, the implementation of the 50 cm µPAC Neo HPLC column yields significantly reduced CV values, indicative of enhanced reproducibility between columns. This improvement is attributed to the distinctive and well-organized stationary phase inherent to µPAC columns.

Figure 1. (a) Product ion areas of peptide DIPVPKPK. (b), (c) Peak areas of peptide DIPVPKPK at different levels of spiked PRTC peptides. (d) Violin boxplot of coefficient of variations (CV) from 15 PRTC peptides are calculated for each tested column. Red dots represent the average CVs in each column.

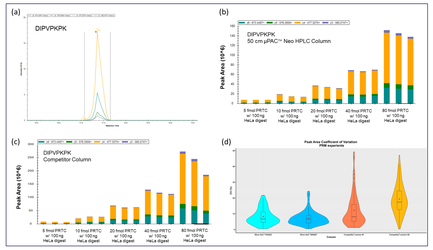
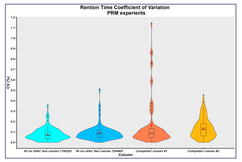


Figure 2. Coefficient of variation of retention time for two 50 cm µPAC Neo columns and two columns from the same competitor.



The 50 cm µPAC Neo columns exhibit notably low mean CVs in Figure 2 , denoted by the red dots with values below 0.5% and more focused CV distributions. In contrast, the CVs of retention times obtained from competitor’s columns exhibit greater instability among triplicates and higher variability, even though these two columns has identical dimensions and are produced by the same competitor. This stability is of importance in ensuring the accurate quantification of the spiked peptides.

Calibration Curve

For the quantification of spiked PRTC peptides in the HeLa sample, a targeted PRM experiment was employed, with the GISNEGQNASIK peptide chosen for calibration curve comparison (Figure 3). Across all calibration curves, the Y-axis represents the theoretical ratios (1, 2, 4, 8, 16) of spiked PRTC peptides using 5 fmol as a reference, while the X-axis signifies the experimental ratios of spiked PRTC peptides. Linear equations and corresponding R^2 values are provided on the graphs, with a slope closer to 1 indicating a greater accuracy of experimental ratios to theoretical values.

Figure 3 (a) and (b) depict results obtained from the 50 cm µPAC Neo columns, revealing a strong correlation between experimental and theoretical ratios. The slopes for these two columns are 0.877 and 0.9377, respectively, demonstrating a proximity to 1 when compared to the slopes of competitor's columns displayed in Figure 3 (c) and (d). This underscores the capability of 50 cm µPAC Neo columns to accurately represent the theoretical quantitation of spiked peptides or peptides of interest.

Figure 3. Calibration curve of GISNEGQNASIK peptide from two 50 cm µPAC Neo columns (a), (b) and two columns from the same competitor (c), (d).

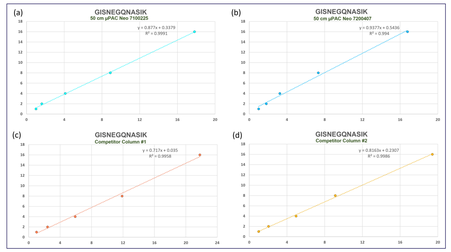
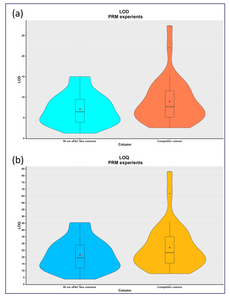


Figure 4, All PRTC peptides’ LOD and LOQ for two 50 cm µPAC Neo columns and two columns from the same competitor.

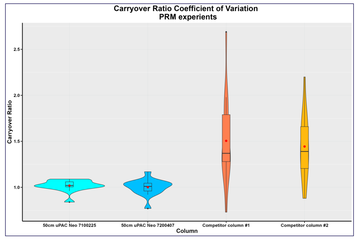


Limits of detection (LOD) and quantification (LOQ), defined as the lowest concentration of the analyte reliably detectable and quantifiable, serves as another pivotal criterion. As illustrated in Figure 4, the mean values of LOD and LOQ are considerably lower for 50 cm µPAC Neo columns, and the distribution is more condensed in comparison to alternative columns. This attests to the superior sensitivity and precision achieved with the 50 cm µPAC Neo columns in this experimental context.

Carryover testing

Additionally, an assessment of carryover for each PRTC peptide was conducted through a sequential procedure involving the analysis of 80 fmol PRTC-spiked samples, followed by a blank run for cleaning, and subsequent examination of 5 fmol PRTC-spiked samples. The carryover ratio was calculated as the peak area of the 5 fmol sample following the 80 fmol spiked sample, divided by the peak area of the initial 5 fmol spiked sample, as illustrated in Figure 5. Remarkably, the mean ratio observed with the 50 cm µPAC Neo columns equated to 1, indicative of minimal carryover. Conversely, columns from competitors demonstrated a mean ratio of 1.5, with the maximum carryover ratio exceeding 2.5. This substantial disparity underscores a heightened level of carryover in competitor's columns, posing a potential detriment to the accuracy of quantitation.

Figure 5. Carryover ratio for two 50 cm µPAC Neo columns and two columns from the same competitor.



Conclusions

Performance: The 50 cm µPAC Neo HPLC columns outperformed competitors, showing superior reproducibility, precision in calibration curves, and minimized carryover.

Sensitivity: They demonstrated lower limits of detection and quantification, showcasing heightened sensitivity.

Reliability: These findings establish the 50 cm µPAC Neo columns as a superior choice for accurate and reliable quantitative analyses in complex biological samples.

References

1. Rauniyar, N. Parallel Reaction Monitoring: A Targeted Experiment Performed Using High Resolution and High Mass Accuracy Mass Spectrometry. Int. J. Mol. Sci. 2015, 16, 28566-28581.
2. Stacy D. Sherrod, Matthew V. Myers, Ming Li, Jeremy S. Myers, Kristin L. Carpenter, Brendan MacLean, Michael J. MacCoss, Daniel C. Liebler, and Amy-Joan L. Ham Journal of Proteome Research 2012 11 (6), 3467-3479

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