PharmaFluidics
The micro-Chip Chromatography Company

Technical Note

µPAC™ LC, a new era in column-to-column reproducibility

KEYWORDS

Micro Pillar Array Column, µPAC™, Microfabrication, Capillary LC, Low flow LC, RPLC-MS/MS, Retention time variation, Gradient separation, Column performance, Tryptic digest

Introduction

The importance of establishing robust and reliable analytical methods is of paramount importance in today's life science research and the (bio)pharmaceutical industry. To meet the required quality specifications imposed by authorities, analytical methods that have proven to yield consistent results are essential. Time and resource consuming processes where these methods need to be re-evaluated and validated, should be avoided at all cost [1]. Liquid chromatography, either coupled with UV detection or mass spectrometry has a prominent position within biomarker discovery and quality control workflows. Among other factors, the quality of the LC column has a significant impact on the data reproducibility and method robustness. LC columns are typically fabricated by packing spherical silica particles into a cylindrical column. Even though column technology has improved enormously in the past decades, batch-to-batch repeatability is still a critical issue that can have a serious impact on LC workflow robustness. Aside batch-to-batch variations (particle size distribution and chemical composition) of the silica material, the packing process itself introduces a certain degree of heterogeneity, preventing the fabrication of multiple LC columns with an identical stationary phase backbone morphology.

By using an entirely different LC column fabrication process, where nanometer precision 2D designs are transferred onto silicon wafers and transformed into a uniform array of superficially porous silicon pillars, PharmaFluidics brings an extremely robust alternative to the LC column market, the micro Pillar Array Column format (µPAC™). Apart from eliminating virtually any column to column variability, precise positioning of these 5 µm diameter silicon pillars creates a stationary phase support that introduces minimal dispersion (or dilution of the samples) into the separation process [2, 3]. These columns can also be operated at LC pump pressures that are significantly lower than what is needed to operate the current state-of-the-art in packed bed capillary flow LC columns (sub 2 µm particles), hereby reducing the shear force on LC pump components and positively affecting their lifetime.

Experimental

LC	
LC system	Thermo Scientific™ Ultimate 3000 LC system
Analytical columns	PharmaFluidics µPAC™ capLC C18 – 5 µm pillars - 28 µm x 1000 µm x 500 mm
	Packed bed A: 2 µm C18 particles – 300 µm x 150 mm
	Packed bed B: 3 µm C18 particles - 300 µm x 150 mm
Mobile phase	A: Water (100%) with 0,1% (v/v) TFA B: Water/acetonitrile (20/80) with 0,1% (v/v) TFA
Loading buffer	Water/acetonitrile (99/1) with 0,1% (v/v) TFA
Flow rate	10 µL/min
Gradient profiles	non-linear 1-35% B in 17,5 min gradient
Temperature	50 °C
Sample	2 pmol/µL Cytochrome C digest
Injection	1 µL Full loop injection
Detection	UV 214 nm - 45 nL flow cell - 2.5 Hz

Table 1. Experimental conditions

The main goal of this study is to compare the column-to-column reproducibility of the µPAC™ capLC column format to state-of-the art commercially available packed bed column alternatives. The basic performance characteristics of the µPAC™ capLC have already been discussed in previous technical and application notes [4-6]. Reversed phase capillary LC analysis of a protein tryptic digest (Cytochrome C digest) is performed on a series of columns (three column types, n=3), and column-to-column reproducibility is compared in terms of retention, efficiency and peak shape.

Results and discussion

Retention

The most critical parameter when comparing column-to-column reproducibility is the retention time that is achieved for the different compounds in a sample, tryptic peptides in the current situation. UV traces obtained for the separation of a Cytochrome C tryptic digest sample on a number of different columns are shown in figures 1, 2 and 3 respectively. 8 peptide peaks that were successfully separated and that were clearly distinguishable for all columns have been selected for analysis. As some of the tryptic peptides could not be completely resolved on the packed bed columns, these have been omitted from the analysis. Results for all columns have been summarized in table 2 and relative variation in retention time for all columns was plotted in figure 4. A clear improvement in reproducibility can be achieved when working with the microfabricated µPAC™ column format. Whereas state-of-the art packed bed alternatives show absolute retention time variation values in the order of 5 to 12s on average, sub second variation (0.95s on average) is achieved with the µPAC™ column format. This results in a relative variation in retention time of 0.24 (%CV), compared to 0.62 and 2.02 for the packed bed alternatives, which is up to three times more reproducible. Because of the unique stationary phase backbone format, clear benefits towards workflow robustness and data consistency between different columns can be achieved with the µPAC™ column. Aside offering an extremely robust and reproducible solution for standardized analytical procedures, LC based analytical strategies that rely on the transfer of accurate retention times can have great benefit from using this new column format.

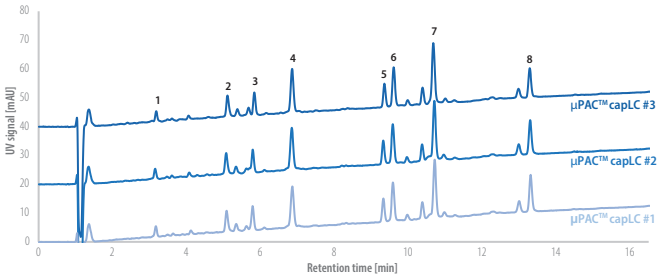

Figure 1. UV chromatograms obtained for the separation of a Cytochrome C tryptic digest (2 pmol) on 3 different µPAC™ capLC columns. Flow rate 10 µL/min, column temperature 50°C, gradient 1-35%B in 17.5 min, A: 100% Water 0.1%TFA, B: 80% Acetonitrile - 20% Water -0.1%TFA.

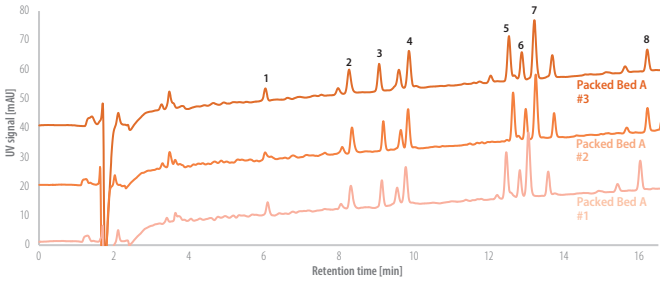

Figure 2. UV chromatograms obtained for the separation of a Cytochrome C tryptic digest (2 pmol) on 3 different packed bed columns from vendor (A: 2 µm particle diameter – 300 µm column diameter – 150 mm column length). Flow rate 10 µL/min, column temperature 50°C, gradient 1-35%B in 17.5 min, A: 100% Water 0.1%TFA, B: 80% Acetonitrile - 20% Water - 0.1%TFA.

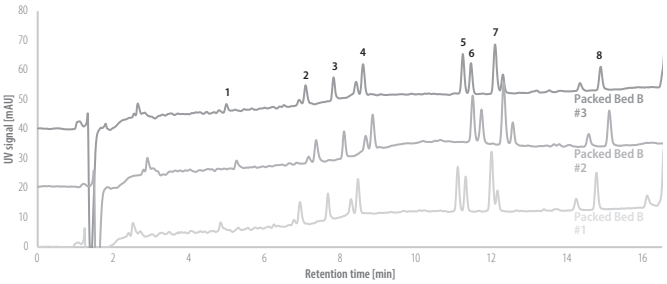

Figure 3. UV chromatograms obtained for the separation of a Cytochrome C tryptic digest (2 pmol) on 3 different packed bed columns from vendor B (3 µm particle diameter – 300 µm column diameter – 150 mm column length). Flow rate 10 µL/min, column temperature 50°C, gradient 1-35%B in 17.5 min, A: 100% Water 0.1%TFA, B: 80% Acetonitrile - 20% Water - 0.1%TFA.

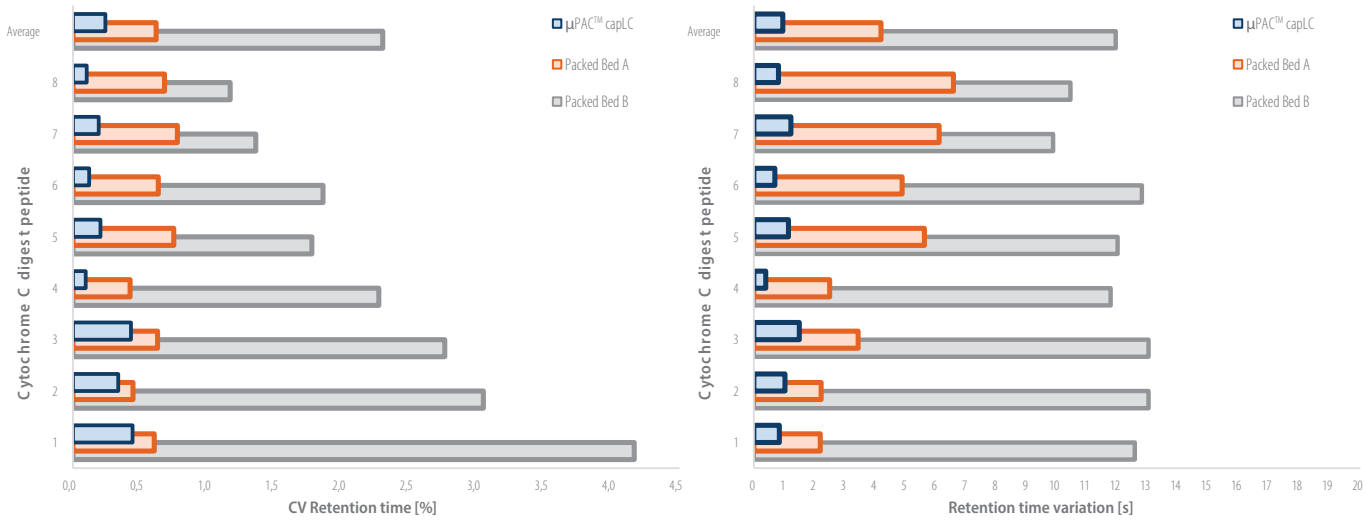

Figure 4. Comparison of the inter column retention time variation observed for 8 selected tryptic peptides for all column types evaluated a) expressed in absolute values - s, b) expressed in relative values - %CV.

Retention time [min]																		
Peak n°	µPAC™ 1	µPAC™ 2	µPAC™ 3	Average	Stdev	%CV	PB A 1	PB A 2	PB A 3	Average	Stdev	%CV	PB B 1	PB B 2	PB B 3	Average	Stdev	%CV
1	3,17	3,15	3,18	3,17	0,01	0,44	6,093	6,027	6,033	6,05	0,04	0,60	4,84	5,253	4,987	5,03	0,21	4,16
2	5,09	5,07	5,11	5,09	0,02	0,34	8,307	8,333	8,26	8,30	0,04	0,45	6,933	7,36	7,08	7,12	0,22	3,04
3	5,79	5,79	5,83	5,80	0,03	0,43	9,133	9,173	9,06	9,12	0,06	0,63	7,673	8,1	7,82	7,86	0,22	2,76
4	6,86	6,85	6,85	6,85	0,01	0,09	9,773	9,833	9,853	9,82	0,04	0,42	8,467	8,853	8,6	8,64	0,20	2,27
5	9,32	9,32	9,35	9,33	0,02	0,20	12,447	12,633	12,52	12,53	0,09	0,75	11,1	11,493	11,233	11,28	0,20	1,77
6	9,58	9,58	9,60	9,59	0,01	0,12	12,807	12,967	12,86	12,88	0,08	0,63	11,307	11,727	11,453	11,50	0,21	1,85
7	10,71	10,70	10,67	10,70	0,02	0,19	13,04	13,233	13,193	13,16	0,10	0,77	11,993	12,313	12,087	12,13	0,16	1,36
8	13,31	13,29	13,28	13,29	0,01	0,10	16,02	16,213	16,207	16,15	0,11	0,68	14,767	15,107	14,873	14,92	0,17	1,17
					0,02	**0,24**					**0,07**	**0,62**					**0,20**	**2,03**

Table 2. Retention time values obtained for 8 selected tryptic peptides from a Cytochrome C digest. Data from 3 column types (n=3 for each) is shown. µPAC™ capLC column: Blue, µPAC™ 1-3 , Packed bed vendor A: Orange, PB A 1-3 , Packed bed vendor B: Grey, PB B 1-3.

Column efficiency

After selecting the desired stationary phase chemistry, the first selection criterion for choosing an appropriate LC column is often the separation efficiency that is needed. Separation efficiency is inversely related to the peak width and has a great impact on the resolution that can be achieved for a given sample. The sharper the peaks, the more compounds can be successfully identified and quantified. It is evident that column performance should also be as consistent as possible. Table 3 summarizes the separation efficiency (and its consistency between columns) that was achieved for the separation of a tryptic digest sample. An average peak width of 0.13 min (measured at base) was obtained for the µPAC™ capLC column, whereas this was 0.15 min for both packed bed alternatives, resulting in respective peak capacity values of 136, 115 and 120. In addition to the superior separation performance, the relative variation between columns was also significantly lower for the µPAC format (2.3 versus 5.8 and 4.6% CV).

Peak width [min]																		
Peak n°	µPAC™ 1	µPAC™ 2	µPAC™ 3	Average	Stdev	%CV	PB A 1	PB A 2	PB A 3	Average	Stdev	%CV	PB B 1	PB B 2	PB B 3	Average	Stdev	%CV
1	0,11	0,11	0,10	0,11	0,01	5,41	0,14	0,14	0,15	0,14	0,01	4,03	0,13	0,15	0,13	0,14	0,01	8,45
2	0,13	0,13	0,13	0,13	0,00	0,00	0,17	0,16	0,18	0,17	0,01	5,88	0,15	0,16	0,15	0,15	0,01	3,77
3	0,12	0,12	0,11	0,12	0,01	4,95	0,14	0,13	0,15	0,14	0,01	7,14	0,12	0,13	0,13	0,13	0,01	4,56
4	0,15	0,15	0,14	0,15	0,01	3,94	0,17	0,15	0,16	0,16	0,01	6,25	0,14	0,15	0,15	0,15	0,01	3,94
5	0,12	0,12	0,12	0,12	0,00	0,00	0,15	0,14	0,15	0,15	0,01	3,94	0,14	0,15	0,14	0,14	0,01	4,03
6	0,13	0,13	0,13	0,13	0,00	0,00	0,14	0,13	0,15	0,14	0,01	7,14	0,13	0,15	0,13	0,14	0,01	8,45
7	0,13	0,14	0,14	0,14	0,01	4,22	0,16	0,15	0,17	0,16	0,01	6,25	0,15	0,16	0,16	0,16	0,01	3,69
8	0,14	0,14	0,14	0,14	0,00	0,00	0,14	0,14	0,15	0,14	0,01	4,03	0,15	0,16	0,15	0,15	0,01	3,77
				0,13	**0,00**	**2,32**				**0,15**	**0,01**	**5,80**				**0,15**	**0,01**	**4,60**

Table 3. Peak width (4σ) values obtained for 8 selected tryptic peptides from a Cytochrome C digest. Data from 3 column types (n=3 for each) is shown. µPAC™ capLC column: Blue, µPAC™ 1-3 , Packed bed vendor A: Orange, PB A 1-3 , Packed bed vendor B: Grey, PB B 1-3.

Peak symmetry

Peak symmetry is also an important chromatographic parameter that should be considered when comparing different column types or looking at column-to--column reproducibility. Bad symmetry values (caused by either peak tailing or fronting) can have multiple causes, and in low flow LC often arise from poor

column installation. Other common sources of peak asymmetry are sample overloading and secondary interaction mechanisms. Symmetrical peaks are more favorable as this positively affects detection sensitivity and separation resolution. Table 4 summarizes the asymmetry values (EP) that were obtained for all selected peptides. In general, asymmetry values between 0.8 and 1.2 can be considered as good, with 1 being perfectly symmetrical (peaks that show fronting have values lower than 1, peaks that show tailing have values higher than 1). Fair symmetry values were obtained on all three column types, with an average value of 1.08 for the µPAC™ column and 1.10 and 1.22 for the packed bed alternatives.

Asymmetry [EP]																		
Peak n°	µPAC™ 1	µPAC™ 2	µPAC™ 3	Average	Stdev	%CV	PB A 1	PB A 2	PB A 3	Average	Stdev	%CV	PB B 1	PB B 2	PB B 3	Average	Stdev	%CV
1	0,84	1,05	1,15	1,01	0,16	15,61	0,94	1,19	0,85	0,99	0,18	17,73	n.a.	1,83	1,34	1,59	0,35	21,86
2	1,11	1,13	1,25	1,16	0,08	6,51	1,28	1,4	1,18	1,29	0,11	8,56	1,16	1,41	n.a.	1,29	0,18	13,76
3	1,04	n.a.	1,15	1,10	0,08	7,10	1,02	1,09	1,05	1,05	0,04	3,33	1,1	1,15	n.a.	1,13	0,04	3,14
4	1,04	1,04	1,08	1,05	0,02	2,19	n.a.	1,05	1,06	1,06	0,01	0,67	1,06	1,09	n.a.	1,08	0,02	1,97
5	1,11	1,10	1,05	1,09	0,03	2,96	1,72	0,99	0,98	1,23	0,42	34,50	1,11	n.a.	1,14	1,13	0,02	1,89
6	1,14	1,07	1,11	1,11	0,04	3,17	1,16	1,07	1,04	1,09	0,06	5,73	1,12	n.a.	1,04	1,08	0,06	5,24
7	1,04	n.a.	1,04	1,04	0,00	0,00	1,07	1,09	1,1	1,09	0,02	1,41	n.a.	1,17	1,3	1,24	0,09	7,44
8	1,07	1,14	1,11	1,11	0,04	3,17	0,96	1,05	1,03	1,01	0,05	4,66	1,13	1,18	1,3	1,20	0,09	7,26
				1,08	**0,05**	**5,09**				**1,10**	**0,10**	**8,41**				**1,21**	**0,07**	**5,81**

Table 3. Asymmetry factor (EP) values obtained for 8 selected tryptic peptides from a Cytochrome C digest. Data from 3 column types (n=3 for each) is shown. µPAC™ capLC column: Blue, µPAC™ 1-3 , Packed bed vendor A: Orange, PB A 1-3 , Packed bed vendor B: Grey, PB B 1-3. Peptide peaks where no value could be calculated are listed as n.a., insufficient resolution with adjacent peptide peaks prevents correct calculation of the asymmetry factor.

Conclusion

The superior fabrication robustness of microfabricated LC columns (µPAC™) is demonstrated in this technical note. Whereas state-of-the-art LC columns that have been packed with the same batch of silica material show retention time variation values in the order of 5 to 12s, sub second variation was achieved with three different µPAC™ capLC columns. With values down to 0.24% CV on three different µPAC™ columns, this approaches what can be achieved on just a single conventional packed bed column, highlighting the unique potential for standardizing analytical procedures. In addition to the inter column retention time consistency, excellent and consistent separation performance is demonstrated for tryptic digest samples, generating highly symmetrical peptide peaks.

Key features of the µPAC™ capLC column

Flow rate flexibility

1 to 15 µL/min – corresponding column backpressures of respectively 19 and 300 bar – maximum operating pressure is 350 bar.

Column robustness

Each column has been manufactured by etching channels out of a solid piece of silicon and contains no particles nor frits.

Column to column reproducibility

Each column is manufactured using the same lithographic mask, making every column identical.

Separation performance

Peak capacity values above 200 can be obtained with short (30-90 min) gradient separations.

References

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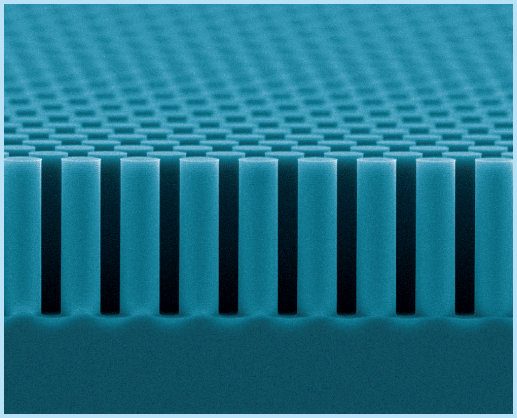
[4] Assessment of capillary flow HPLC column performance. PharmaFluidics Technical note 7

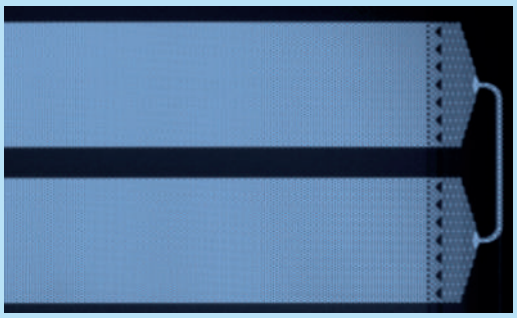
[5] Bottom-up proteomics profiling at capillary LC flow rates. PharmaFluidics Application note

[6] High throughput, routine and comprehensive proteome analysis using a micro Pillar Array Column (µPAC™) based capillary-flow LC-MS workflow. PharmaFluidics Application note

µPAC™ driven separations – Better by Design

Conventionally LC columns are fabricated by stacking (packed beds) or depositing (monoliths) material into a capillary. PharmaFluidics' µPAC™ technology (micro Pillar Array Column) is unique in its kind as it is built upon the precise micromachining of designed chromatographic separation beds into silicon. This approach brings along three crucial and unique characteristics:





Perfect Order.

µPAC™ beds are designed with a high degree of order, eliminating heterogeneous flow paths otherwise present in conventional columns (so called Eddy dispersion). Flow through µPAC™ columns adds very little dispersion to the overall separation. As a result, peaks remain sharper and sensitivity is increased.

High Permeability.

µPAC™s operate at moderate pressures, typically lower than 300 bar. Separation channels with exceptional length (50 cm to 200 cm) are therefore possible. These are folded onto a small footprint by a interconnecting concatenating bed segments.

Solid Backbone.

The micromachined backbone of the separation bed forms a rigid structure that is not influenced by pressure. There are no obstructions by touching surfaces, and there is no risk for perturbations by pressure fluctuations.

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