µ-Pillar Array Column – an Innovative Approach for the Separation and Characterisation of Complex Biological Samples

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Abstract

During the last few years, the µ-pillar array column has had an impact in low flow LC/MS applications. Here we will discuss this innovative approach to produce chromatographic columns, and the improvements this can offer for separation performance, reproducibility and robustness.

Introduction

Over the last decades, liquid chromatography (LC) has established itself as one of the most employed separation techniques in life sciences research, product development and quality control. The heart of the LC system is the separation column, where the sample compounds are separated from each other to facilitate the best possible detection and quantification.

One of the most visible developments in LC column technology is the continuous reduction of particle size. Many researchers will remember Waters introducing UPLC® or Ultra Performance Liquid Chromatography and the 1.7 µm fully porous silica particle columns [1], followed swiftly by other manufacturers, including Agilent, Phenomenex and Thermo Scientific, with their own sub-2 µm particle UHPLC (Ultra High Performance Liquid Chromatography) columns. In combination with the ongoing improvements in particle shape and purity, the smaller size allowed for more efficient separations with respect to resolution, speed and sensitivity. However, smaller particle size columns generate higher back pressures [2], requiring higher pump pressure capabilities. Traditional HPLC pumps provide an operating pressure of typically up to 6,000 psi or 420 bar, perfectly suited to run 3-5 µm particle columns. UHPLC pumps operate at significantly increased pressures, currently up to 22,000 psi or 1,500 bar [3]. Further UHPLC system optimisations, such as reduced gradient delay volumes, from solvent mixer to the head of the column, and extra-column volumes, from the column to the detector, allow the sub-2 µm particle columns to demonstrate their optimal performance.

An alternative development resulted in the solid core particle, also known as fused-core or superficially porous particle. These particles

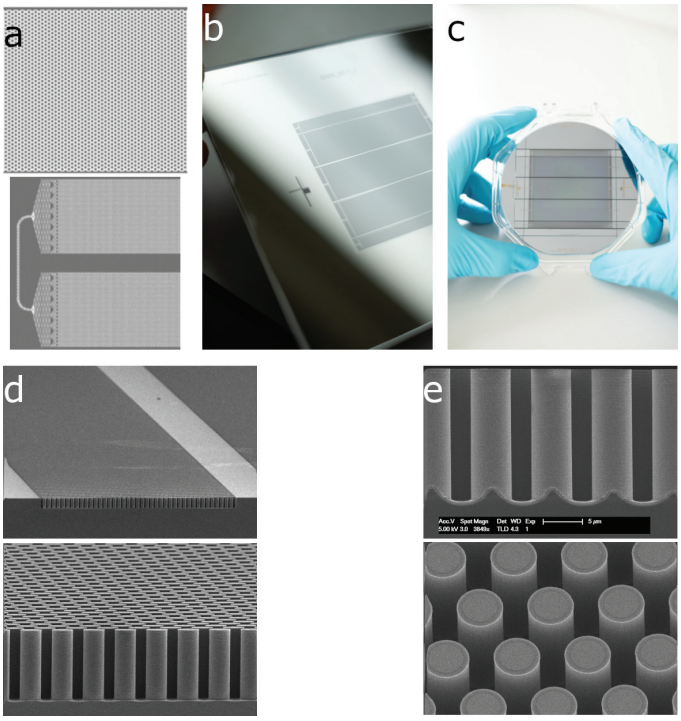

Figure 1. Overview of PharmaFluidics µ-pillar array (µPAC™) technology. (a) design of separation channel, (b) photomask, (c) silicon wafer, (d) µ-pillar bed after Deep Reactive Ion Etching (DRIE),(e) µ-pillars after porosification

are typically between 2-3 µm in diameter, with a non-porous core which is covered with a thin porous layer, providing increased separation efficiency and speed of analysis, without requiring as high a back

pressure to run as the sub-2 µm particles [4]. To take full advantage of the fused core particle columns, traditional HPLC systems may need optimisation of fluidic flow paths and volumes, but the required pump pressure is typically available.

Table 1. µ-Pillar specifications

	Separation length	µ-Pillar diameter	µ-Pillar height	Inter-pillar distance	Back pressure
µPAC™ 50	50 cm	5 µm	18 µm	2.5 µm	50 bar @ 300 nL/min
µPAC 200	200 cm	5 µm	18 µm	2.5 µm	100 bar @ 300 nL/min
µPAC™ capLC	50 cm	5 µm	28 µm	2.5 µm	90 bar @ 5 µL/min

Development of µ-pillar array columns

Over the last three decades, a novel approach to manufacture separation columns has surfaced. Inspired by the original proposals by the group of Fred Regnier in the 1990s [5,6], investigations began into developing micro-machined pillar array columns. The µ-pillars can be positioned in perfect order within the separation channel, and act as the separation backbone. The potential for column-to-column repeatability is significantly higher than with randomly packed columns, just as the possible improvements in separation efficiency, with a factor of 2-3 in comparison to traditional packed-bed columns [7].

Furthermore, the µ-pillar structure leads to reduced flow resistance. Both the pillar diameter and pillar to pillar distance can be tightly controlled, not only resulting in homogeneous separation paths, but also opening the opportunity to create structures that can operate at much reduced back pressures, in comparison to sub-2 µm particle columns [8]. This provides opportunities for much longer columns, with examples reaching up to 4 x 200 cm coupled together, operating below its maximum pressure of 350 bar [9].

Technical background of µ-pillar array columns

In contrast to the rather stochastic slurry packing process that is used for packing of particles that possess an inherent size distribution in traditional columns - resulting in a somewhat random distribution of the particles within the flow path - the backbone of the stationary phase in micro-pillar array columns is designed, much in the same way as electronic circuits in a microchip are designed. This design, in the form of a photomask is used to reproduce the same geometrical pattern over and over again on silicon wafers using light, in a process called photo lithography. In short, a silicon wafer, covered with an appropriate hard mask material layer, is first covered with a photoresist [10]. Shining light through the photomask projects the geometrical pattern on the photosensitive material resulting in a copy of the pattern after chemical development of the resist material. A dry etching step will subsequently remove the hard mask in those regions that are not protected by the remaining photoresist, making these regions accessible for the Deep Reactive Ion Etching (DRIE). This DRIE step removes silicon in the unprotected areas in a cyclic fashion, leaving perfectly vertical pillars and channel walls as defined by the photomask. In doing so, separation channels are formed that always contain the same number of pillars, are always located at the same well defined and perfectly ordered position, resulting in virtually identical copies of the same mask (typical variation both in position and dimensions ±50 nm) [11].

To achieve a higher loading capacity, the resulting micro structured wafers are subsequently rendered superficially porous using an electrochemical anodisation process in which the silicon wafer acts as the anode. Although the minimum plate height of about 5 µm hardly increases, the C-term increases significantly as the porous layer thickness increases [12]. With this in mind, a porous layer with a nominal thickness of 300 nm increases the loading capacity with a factor of 30 as compared to the non-porous case while keeping the C-term within acceptable levels [13]. After a few post-processing steps, the structured silicon wafer is anodically bonded to a glass wafer to obtain closed fluidic structures. As a last step, individual column chips are separated from the wafer-glass stacks by a process called dicing [14].

Individual µ-chips are transformed into chromatographic columns by first inserting and fixing the in- and outlet capillaries in the respective channels using a UV-curable gluing step, followed by an assembly step providing a protective housing and appropriate HPLC fittings. As a final step, the bonded phase is applied on the free surface of the pillars and the channel walls by coupling the bare backbones to a PharmaFluidics proprietary dedicated wet surface chemistry station.

The power of the µ-pillar array column technology lies in the fact that designs and realisations can be tuned to specific workflows based on the pillar shapes and diameters, pillar position and inter pillar distances and etching depth.

Application areas

In today's laboratories, the µ-pillar array columns have found their way especially into omics and related research. The flow regime of the µ-pillar array columns can be divided into two distinct ranges, nanoLC at 50-2000 nL/min and capillaryLC at 1-15 µL/min. With their extended lengths of up to 200 cm, the µ-pillar array columns match the requirements of omics applications that require highest sensitivity, resolution and reproducibility, and are ideal when only smallest sample volumes are available [15,16].

NanoLC is one of the gold standard separation techniques in proteomics allowing almost seamless connectivity to mass spectrometry (MS). Despite technological advances in MS detections and nanoLC systems, further developments of nanoLC columns has lacked a little. Connectivity to the nanoLC system has clearly improved, with the Thermo Scientific nanoViper connections as the prime example. Attempts to lower the volume from the column to the emitter have been pursued as well, e.g. PicoFrit® (New Objective) and EASY-Spray™ (Thermo Scientific), as well as chip-based separations like ionKey (Waters). But the traditional packed-bed separation channel has remained within all these formats, with the possible limitations as mentioned above.

This is where µ-pillar array columns can offer a significant step forward to further optimise the performance in low flow LC/MS. Taking full advantage of the previously described micro-machined lithographic manufacturing process and the tight control over the µ-pillar dimensions and position, the highest column to column reproducibility can be achieved. Figure 2 shows the reproducibility over seven µ-pillar array columns, separating from the same cytochrome c digest, with an average coefficient of variation of 0.63% over the nine peaks.

In addition, the µ-pillars are part of the original wafer where the separation channel is etched. In combination with the greatly reduced back pressures, as indicated in Table 1, this allows for a far higher number of sample injections than typically expected on a nanocolumn. Figure 3 shows a longevity experiment of µ-pillar array column, running for six months, performing the separation of

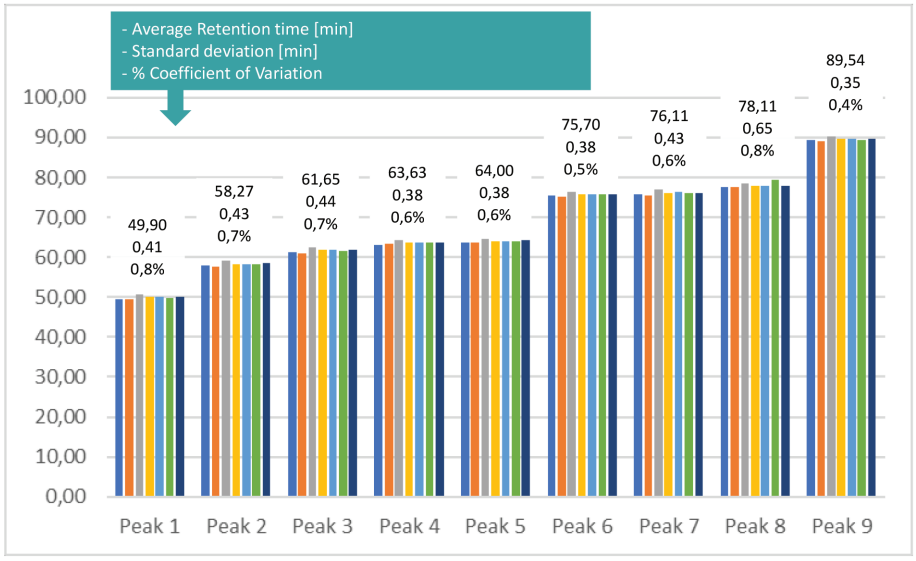

Figure 2. Column to column reproducibility over seven different µPAC™ columns, 500 fmol/µL of digested cytochrome c injected on each column.

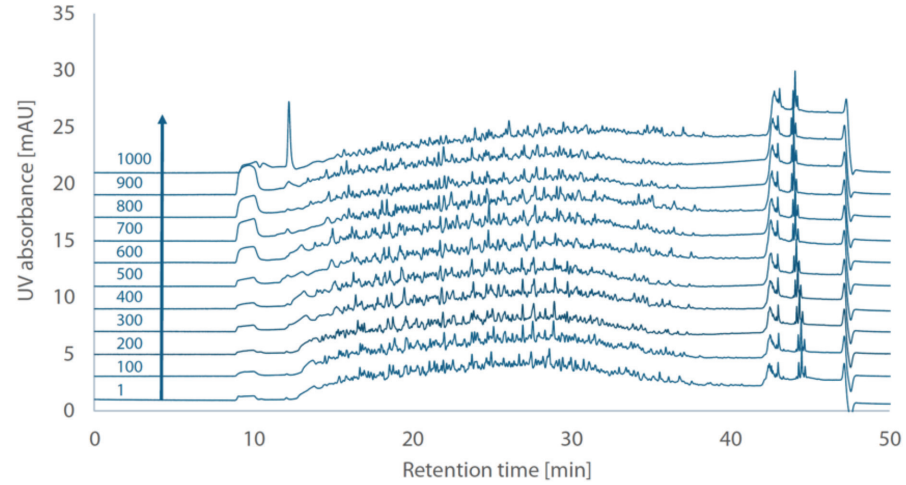

Figure 3. µ-pillar array column robustness. UV chromatograms obtained for the separation of 100 ng tryptic digested HeLa cells. Injections 1 to 1000 are displayed at a 100 injections interval. Injection volume 1 µL; flow rate 1 µL/min; gradient conditions 1-50% B in 30 min; mobile phase A H_2O+0.1% TFA / B 20% H_2O+80% ACN+0.1% TFA; column temperature 35°C; UV detection 214 nm.

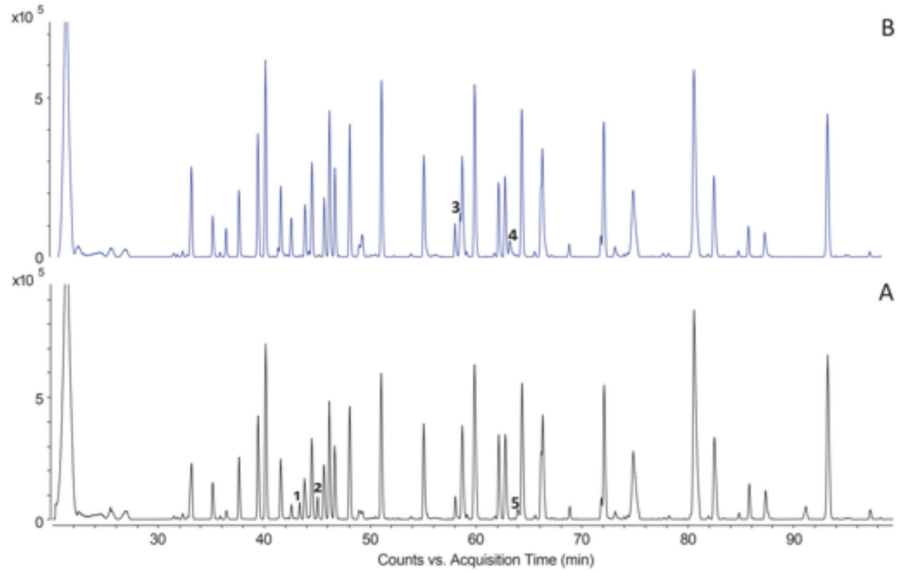

Figure 4. LC-MS total compound chromatogram of the tryptic digest of a Remicade original drug (bottom trace) and a candidate biosimilar (upper trace). Distinct differences in the chromatograms are labelled 1-5.

1000 HeLa digests, each followed by a blank and a cytochrome c digest injection, which results in a total number of 3526 injections on a single µ-pillar array column. The HeLa digest separation was performed in a 30 minute gradient, 60 minute cycle time, with a flow rate of 1 µL/min. The mobile phases A and B were prepared at the start of the experiment, and not refreshed for the duration of the experiment. Despite the degradation of the mobile phases, the repeatability of the HeLa digest separation is outstanding [17].

Moreover, with the increasing importance of protein-based drugs, proteomics workflows are finding their way into pharmaceutical and biotech laboratories, with peptide mapping becoming an essential part in the discovery and development of therapeutic monoclonal antibody (mAb) or antibody-drug conjugate (ADC) targets. These molecules are large (approximately 150 kDa) and heterogeneous, differing in post-translational modifications, amino acid structures and higher order structures [18]. With larger numbers of original mAb drugs running out of patent, biosimilar products are expected to become available. As the name implies, they will have to be highly similar but not identical. To characterise and monitor this similarity, highly sensitive and high resolution LC/MS are required. Figure 4 shows an example of the comparison of the original Remicade drug versus a candidate biosimilar, using a tryptic digest peptide map and clearly showing five distinct differences in the total compound chromatograms.

But µ-pillar array columns are not only restricted to peptide mapping applications. Metabolomics and lipidomics researchers are also performing more sample restricted experiments. Although nanoLC/MS might not always be their first choice, despite the sensitivity that can be achieved, reduced column IDs are being investigated. Promising results have been achieved at low microliter per minute flow rates, typically performed using 300 µm ID columns [19]. However, these columns would be packed with the same stationary phase as their nanoLC counterparts, with the same challenges as described above.

Again, µ-pillar array columns can be used here as well. For instance, sample complexity in lipidomics is quite considerable, with the LIPID MAPS Structure Database consisting of just under 45000 unique lipid structures. With µ-pillar array column lengths of 200 cm, these columns are ideal to take on this complexity, as is demonstrated in Figure 5 where a human blood plasma lipid extract was analysed in a 60 minute gradient [20]. The upper trace

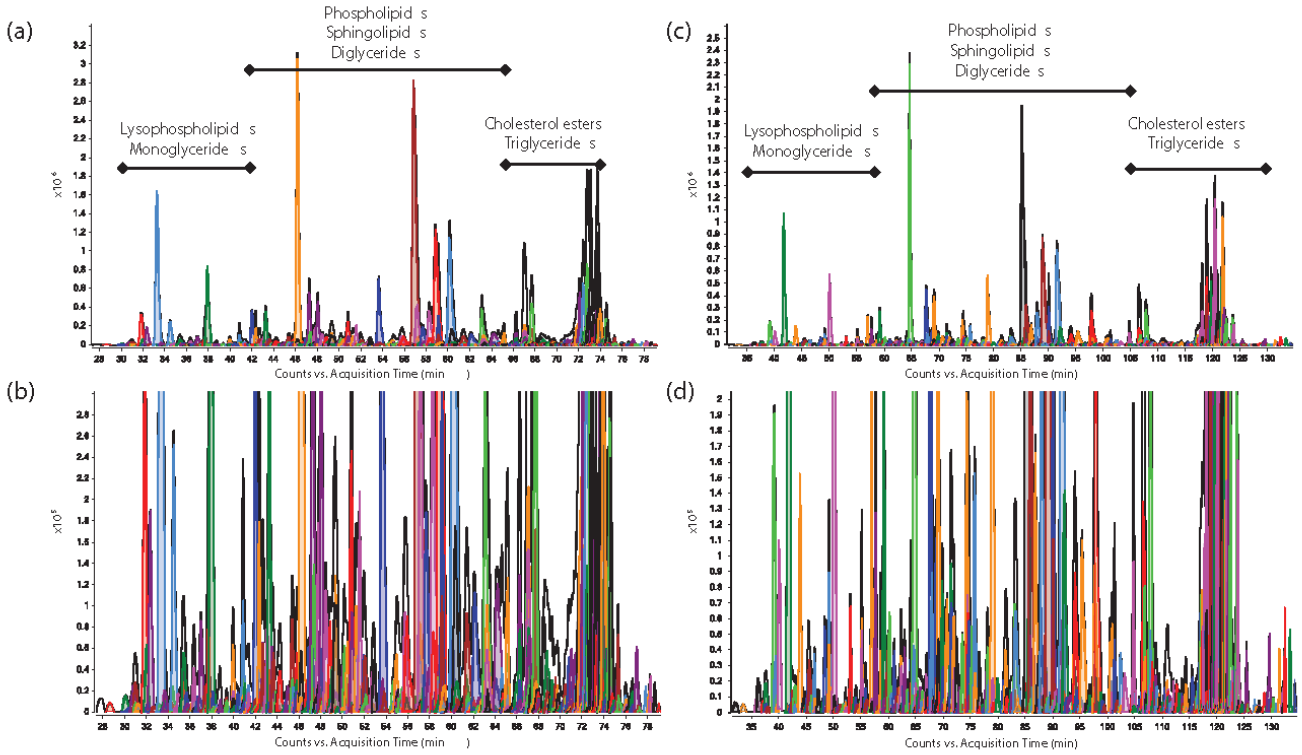

Figure 5. LC-MS compound chromatogram at different scaling, obtained in positive electrospray ionisation mode for a human plasma lipid extract. (a) and (b zoomed) 60 min gradient, (c) and (d zoomed) 120 min gradient.

shows the LC-MS compound chromatogram, demonstrating not only the complexity of the sample, but also the high separation efficiency obtained by the µ-pillar array column. The major lipid classes are nicely distinguishable within the chromatogram.

Conclusion

µ-Pillar array columns can offer tremendous steps forward in LC/MS applications. The novel approach of the structure allows for much more flexibility in the design of the separation channel, promising up- or downscaling of chromatographic methods. With the perfectly ordered positioning of the µ-pillars, and the tight control over the dimensions, much more homogeneous flow paths within the column are achievable, minimising peak broadening effects and improving injection to injection reproducibility. With the reduced back pressures, even at column lengths of 200 cm, column robustness can be increased significantly, allowing more sample injections, as demonstrated with the HeLa digest experiment.

With the development of products for increasing flow rate ranges, the µ-pillar array columns will continue to become available for more liquid chromatography applications. Currently, they are available for up to 15 µL/min, an inviting flow rate for researchers working with limited sample amounts. But the promise of µ-pillars is likely to be upscaled to higher flow rates as well.

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