The descriptive format of HPLC columns used to be unambiguous. The length and diameter of the cylindrical tube containing the bed specified the column dimensions, while the shape and diameter of the particle, its pore diameter and surface area adequately described the stationary phase. New developments including rectangular shaped columns and, in particular, monolithic stationary phases, mandate a new look at dimensions and morphology describing the separation column. This article aims to discuss trends and changes in HPLC column format.

Introduction

Given the challenge to discuss trends in column formats, one must first have an unambiguous understanding of the phrase column format and associated terminology (microbore, nanobore etc.). As no regulatory body yet exists to define these terms, an attempt is made here. Given these definitions, it will become easier to discuss and appreciate trends in HPLC column technology.

The format of an HPLC column includes the dimensional and morphological properties of the separation tube and stationary phase within; for example, one refers to column length and diameter of the tube, and particle size of the stationary phase. For theoretical and practical reasons the tube is always cylindrical. On the one hand, a cylindrical geometry simplifies models and calculations, while on the other hand cylindrical tubing from different materials and with a range of internal diameters (i.d.) is readily available. Format, however, is changing and columns with rectangular, square or other perimeter-shape conduits have appeared (e.g., on chips) and will soon become more widely used. In such instances, column diameter alone cannot describe the geometry of the column conduit.

The morphology of stationary phases is also changing. After the controversy surrounding irregularly shaped or spherical particles some years ago, chromatographers now use spherical particles simply characterized by diameter, pore size and volume and specific surface area. Particle diameter has reduced from 50 µm to 3 µm and a trend towards the use of still smaller particles (sub-2 µm) is emerging.

The recent commercial appearance of silica- and organic polymer–based monolithic HPLC columns makes the term particle size obsolete, or at least requires its redefinition. Tallarek and co-workers introduced a characteristic length for permeability and a characteristic length for diffusion as separate dimensions for a monolithic stationary phase.1

In addition, particle diameter, as an unambiguous size parameter for stationary phase, has also become blurred by the introduction of small-sized pellicular beads.2,3 In an approach to reduce the characteristic length for diffusion, a non-porous particle of ~3–4 µm diameter was covered with a porous layer of stationary phase of 0.5–1 µm depth, providing solute

<table>
<thead>
<tr>
<th>Description</th>
<th>Dimension</th>
<th>Approx. typical flow-rate (velocity 1–10 mm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open tubular liquid chromatography</td>
<td>&lt; 25 µm i.d.</td>
<td>&lt; 25 nL/min</td>
</tr>
<tr>
<td>Nanobore column HPLC</td>
<td>25 µm ≤ i.d. ≤ 100 µm</td>
<td>25–4000 nL/min</td>
</tr>
<tr>
<td>Capillary column HPLC</td>
<td>100 µm &lt; i.d. &lt; 1 mm</td>
<td>0.4–200 µL/min</td>
</tr>
<tr>
<td>Microbore column HPLC</td>
<td>1 mm ≤ i.d. ≤ 2.1 mm</td>
<td>50–1000 µL/min</td>
</tr>
<tr>
<td>Narrow(small)-bore column HPLC</td>
<td>2.1 mm &lt; i.d. &lt; 4 mm</td>
<td>0.3–3.0 mL/min</td>
</tr>
<tr>
<td>Normal-bore column HPLC</td>
<td>4 mm ≤ i.d. ≤ 5 mm</td>
<td>1.0–10.0 mL/min</td>
</tr>
<tr>
<td>Semipreparative column HPLC</td>
<td>5 mm &lt; i.d. ≤ 10 mm</td>
<td>5.0–40 mL/min</td>
</tr>
<tr>
<td>Preparative column HPLC</td>
<td>i.d. &gt; 10 mm</td>
<td>&gt; 20 mL/min</td>
</tr>
</tbody>
</table>
retention. In pellicular beads, retentive distances in the particle are decoupled from the hydraulic distances between particles and give rise to concurrent high efficiency and permeability — especially for high molecular weight substances.

Finally, retentive devices in which the packed zone is constructed by microfabrication techniques have been described and will demand other characteristic length parameters for a description of the hydraulics and dispersion of the column.

Any discussion of trends in column format must therefore address these new developments. First, a clarification of nomenclature for cylindrical column formats is necessary and given in Table 1. This choice of nomenclature may seem arbitrary but it serves to clarify the terminology used in this discussion.

In an editorial for this same journal in 1999, I argued that the chasm between theoretical and practical performance of sub-microbore HPLC columns was still too large to allow the wider use of capillary-bore HPLC columns. Crossing this chasm was regarded as feasible if practical and instrumental requirements were met such as rigorous conservation of performance when reducing column i.d.; robustness in practical usage coupled with ability to solve real-world problems; and, especially, commercial availability. This was anticipated to take some considerable time.

How can one be so wrong? Merely four years later, an array of modules, systems and columns for capillary and nanoflow HPLC have become commercially available, meeting the putative requirements formulated in 1999.6–8

What has changed? One major factor has been the overwhelming growth of LC–MS in drug discovery and development. Technological advances in mass analysers providing more structural information at high sensitivity are one reason, as is the requirement to obtain maximum information from continuously dwindling sample amounts. Particularly in the field of proteomics, minute quantities of protein samples are available, mandating a reduction in column dimensions into sub-millimetre i.d. A fortunate circumstance has been that the sensitivity of ESI-MS detection is not compromised by concomitant reduction in peak volume and solvent flow-rate. In fact, reduction of solvent flow-rate is a mandatory requirement for high sensitivity in nano-electrospray ionization.

Instrument manufacturers have surmounted the technical challenges to deliver sub-microlitre/min flow-rates and sub-microlitre volume samples. The issue of minimizing extra-column band spreading is addressed by the use of very narrow i.d. connection capillaries. Physics also helps here as volume dispersion in capillaries reduces with the sixth power of the diameter, whilst in packed columns it reduces with the square of the diameter.

### Table 2: Results of H-u curve fit according to the simplified van Deemter equation.

<table>
<thead>
<tr>
<th>Solute</th>
<th>k’</th>
<th>Min. H/Velocity</th>
<th>Slope (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetophenone</td>
<td>0.28</td>
<td>6.2 µm/1.04 mm/s</td>
<td>2.24</td>
</tr>
<tr>
<td>Methyl benzoate</td>
<td>0.38</td>
<td>6.2 µm/1.11 mm/s</td>
<td>2.15</td>
</tr>
<tr>
<td>Hexyl paraben</td>
<td>0.55</td>
<td>6.6 µm/0.85 mm/s</td>
<td>2.77</td>
</tr>
<tr>
<td>Heptyl paraben</td>
<td>0.65</td>
<td>6.9 µm/0.91 mm/s</td>
<td>2.73</td>
</tr>
<tr>
<td>Anthracene</td>
<td>0.84</td>
<td>7.1 µm/1.56 mm/s</td>
<td>1.84</td>
</tr>
</tbody>
</table>

From the plot of velocity versus pressure, a column resistance factor of ~350 was derived.

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**Figure 1:** H-u curve for a capillary HPLC column. Format 150 × 0.5 mm, Zorbax XDB-C18, 3.5 µm. Solvent water/acetonitrile 30/70; temperature ambient; sample volume 0.05 µL, solutes indicated in the graph. Agilent Capillary HPLC system with prototype 80 nL flowcell.

**Figure 2:** Column test of Zorbax 300SB-C18, 150 × 0.3 mm, 3.5 µm on the Agilent Capillary HPLC system with prototype 80 nL flowcell. Solvent, water/acetonitrile 50/50, 4 µL/min. Injection volume 0.1 µL. Sample constituents see figure and Table 3. Temperature ambient.

**Peaks:** 1 = thiourea, 2 = dimethylphthalate, 3 = diethylphthalate, 4 = biphenyl, 5 = o-terphenyl.
New Column Formats

A plethora of columns with dimensions ranging from 0.075–1 mm i.d., lengths of 20–250 mm and filled with the plurality of stationary phases found in narrow-bore and normal-bore columns is commercially offered. Therefore, it is challenging to verify the criteria of uncompromised performance and robustness of capillary and nanobore HPLC columns. In Figure 1, an H-u curve is depicted for a 150 × 0.5 mm column packed with ZORBAX XDB C18, 3.5 µm (Agilent Technologies, Wilmington, Delaware, USA). The data was obtained on an Agilent capillary HPLC system (Agilent Technologies, Waldbronn, Germany) in which a prototype small-volume flowcell (80 nL) was installed. After regression of the data (see Table 2) to the simplified form of the van Deemter equation,

\[ H = A + B/u + C \]  

[1]

interesting conclusions can be drawn. The minimum value of the height equivalent to a theoretical plate (HETP) (~2 dp) and the velocity at minimum (1 mm/s) as well as the slope of the H-u curve (2 ms) are close to the values expected from theory for an HPLC column of wider i.d. Therefore, the column performance was measured directly without compromising efficiency by external band spreading in the system. These results also demonstrate that a narrow column can be as efficient as a normal-bore HPLC column. The value of the column resistance factor is significantly lower than for a wider i.d. column, indicating a higher permeability.

Driving the column i.d. down further to 0.3 mm is an even greater challenge to the system with regard to extra-column band spreading. This is illustrated in Figures 2 and 3, and Table 3. In Figure 2, a column packed with Zorbax 300SB–C18, 3.5 µm, 150 × 0.3 mm was tested in the same system as used in Figure 1. In Figure 3, a similar column using an Eksigent NanoLC System (Livermore, California, USA). The Eksigent NanoLC System uses microfluidic flow control technology to deliver flow-rates of 20 nL/min to 10 µL/min without flow splitting. The system provides precise, low-dispersion injection and uses fully dispersed UV absorbance detection at 200–380 nm with a linear array detector possessing a microfabricated 45 nL UV flowcell.

Though the test conditions are not identical, it can be concluded safely that in the standard capillary HPLC system (Figure 2), the column efficiency suffers by extra-column dispersion. In the Eksigent Nano LC System the column again
demonstrates a good match to theoretical expectations (Figure 3). This result reiterates that capillary dimensions for HPLC columns don’t impact the separation efficiency provided the column is operated under proper conditions.

Is further reduction still possible? Can one do meaningful chromatography with columns ≤ 100 µm i.d.? Actually one has to deal with this question in reverse. In 1994 Mann and Wilm described the nano-electrospray ionization technique. Originally intended as an off-line or flow injection inlet system for MS, attempts were soon made to place an HPLC column in front of the interface. The main hurdle was meeting the flow-rate requirement for nano-electrospray ionization (0.1–1.0 µL/min). This requirement dictates that the column i.d. should be in the 50–100 µm range.

Again, fortunate circumstances helped the realization. The stage was prepared for packing of HPLC columns ≤ 100 µm i.d. by capillary electrochromatography column-preparation procedures that were established in the mid-1990s. It also appeared that the instrumentation concepts, developed to drive capillary-column HPLC, could be adapted to the lower flow-rates required by nanobore HPLC. The use of such columns is

<table>
<thead>
<tr>
<th>Peak (in elution order)</th>
<th>Plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiourea</td>
<td>6617</td>
</tr>
<tr>
<td>Dimethylphthalate</td>
<td>7506</td>
</tr>
<tr>
<td>Diethylphthalate</td>
<td>7992</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>12 102</td>
</tr>
<tr>
<td>o-Terphenyl</td>
<td>14 969</td>
</tr>
</tbody>
</table>

Table 3: Column test of Zorbax 300SB C18, 150 × 0.3 mm, 3.5 µm.
demonstrated in the high-efficiency separation of a complex mixture of peptides from the tryptic digestion of eight proteins (Figure 4).

Approximately 50–60 peaks are found in the chromatogram and in combination with the automated MSn capabilities of the ion trap mass spectrometer, the proteins could be identified via database searches using the MS/MS spectra of the separated peptides.

Recent advances which point to the future direction of column format miniaturization have been described by Yin et al. who presented initial results of a chip-based reversed-phase column coupled to MS. The channel in the chip has a width of 75 µm, a depth of 50 µm and an overall length of 40 mm. A sketch of the chip with a photograph of the bed structure is given in Figure 5.

The separation column was packed with ZORBAX 300SB-C18 5 µm particles and a typical separation obtained from this column is shown in Figure 6. The chip column is contained in a high-pressure rotary valve allowing delivery of solvent or sample to the separation channel. Particles at the outlet side are kept in place by the so-called “keystone” effect.

The separation achieved is remarkable, bearing in mind the short length of the separation bed. Effectively, separations similar to those obtained with nano-bore HPLC columns can be obtained on a chip.

It may seem that the end of this development has not yet been reached. However, one may anticipate that it will become increasingly difficult to form a packed bed in conduits where the diameter, or width and depth, are just 5–10 times larger than the particle diameter. This is an area of current research in separation technology. The monolithic or microstructure column bed approaches may eventually be more promising for this kind of separation device.

New Stationary-Phase Morphologies

The rapid emergence of monolithic-type HPLC columns, both in normal-bore and capillary-bore sizes, is changing the dimension format of stationary phases. In a monolithic bed structure it is much less evident as to what is regarded as a hydraulically relevant dimension or the diffusion relevant distance. This picture is complicated by several different approaches towards the goal of creating a monolithic bed structure (silica- or organic polymer-based). As mentioned before, pellicular-type or non-porous particles also decouple the characteristic hydraulic and solute transport distances in the stationary phase.

Tallarek and Leinweber recently illustrated this situation (Figure 7) for silica-based particles and monoliths. These authors have concluded that the equivalent particle size for permeability of a silica-based monolith was ~11 µm while the equivalent particle size for dispersion would be a totally porous particle of 2 µm. This leads to a significant improvement of the separation impedance (plates per unit time and pressure).

Tanaka and coworkers have elegantly verified this conclusion. They took the theoretical approach by Poppe that establishes performance limits for particulate columns and
verified the model by comparison of the practical performance of a particulate and a monolithic column. The results are demonstrated in Figure 8.

According to Poppe’s calculation, for particulate columns there cannot be a better plate number than that limited by the 400 bar diagonal line. The particulate column that was used by Tanaka indeed shows very good agreement with this theoretical prediction. The data points for the monolithic columns appeared in the theoretically forbidden area on the right of the diagonal. The obvious reason for this observation is that the column resistance factor, assumed in the calculation, does not apply for the monolith and is in truth a factor of 3–4 lower. These fundamental results demonstrate the benefit of the silica-based monoliths.

The silica monoliths are presently offered commercially in normal-bore chromatography i.d. but it can be anticipated that they will also soon become available in capillary-bore and nanobore formats.

In an alternative approach, Bonn and Huber have demonstrated the effectiveness of a polystyrene/divinylbenzene monolith in capillary-bore format. Columns based upon their technology are now commercially available from LC Packings. An illustration of the separation abilities of this column is given in Figure 9.

Conclusion

Quite obviously the reserved view on practical usage of sub-1 mm i.d. HPLC columns, projected in the editorial \(^5\) in 1999, seems inappropriate in 2003. Both instrumental and column technology have developed into the low-µm i.d. region spurred by the requirements of bioanalytical separations, especially those of proteomics. The descriptive dimensions of columns, their geometry and morphology are changing, requiring a clearer description of new formats. It can be anticipated that with the trend to further diameter reduction, monolithic column technology will start to dominate the stationary phases. In very narrow i.d. capillaries or chips it will become increasingly difficult to manipulate and control particles. Additionally, in this small scale, alternative solvent drive methods will become important (electric field assistance).

For this author, however, one aspect stands out as a major factor, which makes HPLC such a strong separation technique, and that is its scalability according to operational demands. From very narrow i.d. capillaries to centimetres-wide preparative columns, HPLC provides a uniform mechanism that allows easy adaptation of parameters to separation problems and, therefore, requires much less effort than other techniques.

The upcoming HPLC 2003 meeting will show plenty of examples of this view.

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References


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