Abstract

The review highlights the fundamentals and the most prominent achievements in the field of high-performance liquid chromatography (HPLC) column development over a period of nearly 50 years. After a short introduction on the structure and function of HPLC columns, the first part treats the major steps and processes in the manufacture of a particle packed column: synthesis and control of particle morphology, sizing and size analysis, packing procedures and performance characterization. The next section is devoted to three subjects, which reflect the recent development and the main future directions of packed columns: minimum particle size of packing, totally porous vs. core/shell particles and column miniaturization. In the last section an analysis is given on an alternative to packed columns—monolithic columns, which have gained considerable attraction. The challenges are: improved packing design based on modeling and simulation for targeted applications, and enhanced robustness and reproducibility of monolithic columns. In the field of miniaturization, particularly in chip-based nano-LC systems, monoliths offer a great potential for the separation of complex mixtures e.g. in life science.

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1. Introduction

The consumption of analytical columns worldwide is estimated to be ca 2 million per year. If one assumes that each HPLC instrument requires five columns per year on average, one can easily estimate the number of HPLC instruments. These figures clearly indicate that a HPLC column is a widely used consumable; nevertheless, its impact on system performance is considerably higher than its cost contribution.

Only a minority of users, however, can imagine the complex structure of HPLC columns. Typically, an analytical column has an inner diameter (I.D.) of 4.6 mm and a length of 100 mm and is packed with 5 μm spherical particles. A simple calculation shows that such a column contains roughly 10 billion particles in a dense array taking into account that the column porosity is 40%.

It should be emphasized that it took more than 20 years before scientists and engineers were able to design and manufacture such columns in reproducible enough fashion to meet the stringent demands in quality control of modern analytical laboratories. It is evident that many innovative and experienced investigators have contributed to achieve these results.

This happened less in a planned concerted action but more in a frog-leap way with alternating technical and technological advances between equipment, e.g. high pressure pumps, sensitive UV-detectors, particle synthesis and characterization and corresponding column hardware design, until the idea was accepted, realized and integrated into a convincing technical approach.

The majority of the pioneers of modern liquid chromatography started from gas chromatography, while others had a background in inorganic, organic, physical or surface chemistry, in physics, or in chemical and mechanical engineering.

It took several decades before scientists invented, as an alternative to particle packed beds, columns made of a single monolithic block, composed of porous polymers or porous silica. The development of monoliths was born in the field of ceramics, where they were used as carriers in the field of catalytic conversion of automobile exhaust gases which were converted into non-toxic effluents such as water, nitrogen and carbon dioxide. Monoliths composed of high temperature stable oxides were manufactured as solid porous rods with regular channels with millimetre openings. The open structure of such monoliths enabled a fast reaction of the exhaust gas with the catalytic components deposited at the surface of the channels of the monoliths [1]. While such monoliths were made by extrusion of a paste composed of powdered and highly dispersed oxides and oxihydrates, attempts were made to generate monoliths with defined and controlled pore system via the sol–gel route using porogens. Pioneering work was done by Soga and Nakanishi at the Kyoto University, Japan [2,3], Tanaka at the Kyoto Institute of Technology [4] was the first who recognized the value of porous monolithic rods as columns for HPLC. The basic idea was to employ such monoliths as stable and reproducible columns enabling a much faster separation at lower pressure than particle packed beds were able to achieve. Simultaneously, Tennikova et al. synthesized rigid porous monoliths made of cross-linked polymers [5].

The development of porous microparticles and porous monoliths were one significant achievement among other drivers e.g. in instrumentation which provided the basis for the widespread application of HPLC in pharmaceutical and industrial analysis. The hyphenation of HPLC as a multidimensional technique coupled to mass spectrometric detection was another significant step towards solving complex analytical tasks. Looking forward one can assume that combining reliable equipment with sensitive detection is likely to become one of the most powerful analytical methods available for modern life science approaches such as proteomics, peptidomics, metabolomics. Yet this field is still in its infancy as compared to routine HPLC analysis in pharmaceutical industry, where the majority of today’s produced columns are applied and consumed.

This article will focus on aspects of column structure in connection with the expected chromatographic resolution and will critically examine the limitations and pitfalls in column design and development drawing on 50 years experience in this field. In addition, an attempt is made to compare and critically review
the two types of sorbents, namely packed particulate beds and monolithic structures applied to HPLC.

2. Particle packed columns

2.1. Structure and function of a high-performance liquid chromatography column

The selective separation of a complex mixture into individual species by column liquid chromatography requires a sufficiently large surface of the adsorbent particles with interactive surface sites to selectively retain analytes based on their chemical composition and structure. Usually a column packed with porous micron-sized particles (see Fig. 1) is applied in order to achieve this separation. In elution chromatography, the sample is transported by a convective flow through the packed column. The convective flow is generated by a pump (pressure driven mode). As the particles possess pores of the order of 10 nm, the internal surface carrying the molecular discriminators has to be reached by diffusion of the analytes. Diffusion of solutes in a liquid phase is usually three orders of magnitude slower than in the gas phase for small to medium sized molecules. In addition, diffusion into and out of tortuous pores (pore diffusion) reduces significantly the diffusivity as compared to diffusion in the liquid bulk phase. A central problem in HPLC is therefore to overcome the limitations of hindered mass transfer of solutes due to pore diffusion by providing sufficient access to the interactive surface sites. One way is to reduce the average particle size of the packing to minimize the diffusion path length by using micro particulate packings. Unfortunately, any reduction in particle size results in increased column pressure drop. Another alternative is to introduce a bimodal pore size distribution within the particles with mesopores to generate sufficient surface area and macropores to enhance the mass transfer kinetics. Those flow-through pores with sizes larger than 80 nm enable a convective flow within the particle.

The most powerful means, however, is to use an electrical field along the packed column (electrically driven chromatography in packed capillaries is called capillary electrophoresis or CEC). In the case of silica particles as a packing material, which carry a negative surface charge, and a buffer as an eluent, an electroosmotic flow (EOF) directed to the cathode is generated, which leads to a convective flow within the porous particles. As a result, the mass transfer kinetics are drastically enhanced, which is reflected in achieving large numbers of theoretical plates of such columns [7]. Although CEC became popular at the beginning of 1990s, the potential of this approach was hardly recognized. The theoretical treatment is still in its infancy and thus a basic understanding of the mass transfer and distribution equilibrium, particularly for charged analytes, is lacking [8–12].

2.2. How to make a high-performance liquid chromatography column?

A HPLC column is normally a stainless steel tube with 4.0 or 4.6 mm I.D. densely packed with micron size silica particles. The pathway from a mirror-finished tube to a packed HPLC column comprises a sequence of carefully controlled steps. The first is the synthesis and manufacturing of spherical particles (see Section 2.2.1). The second is the sizing and size classification (see Section 2.2.2). The surface functionalization usually involves several steps on its own. Next, a particle suspension is prepared, which is filtered at high flow-rate and increasing pressure through the column (column packing) (see Section 2.2.3). The column has to be flushed and conditioned and subjected to tests to assess the column performance and selectivity (see Section 2.2.4). Fig. 2 shows a scheme of the manufacturing process of an n-octadecyl bonded silica material of 5 μm average particle size [13].

2.2.1. Synthesis and particle formation

Classical LC is performed with irregularly shaped silica particles of 30–40 μm, 40–60 μm or larger sizes. These particles are obtained by consecutive grinding or milling of larger particles e.g. of silica xerogel lumps, followed by size classification, commonly performed with sieves of a given aperture. This holds for particles larger than 25 μm. Microparticulate packings are classified employing the air elutriation technique. In this way packings of 5–10 μm were made at the beginning of HPLC. There have been heavy scientific debates at the beginning of HPLC mainly at the annual Zlatkis Meetings in Houston, TX, USA, between 1969 and 1975, whether irregular or spherical particles would be the preferred packings of HPLC columns with respect to pressure drop, column stability and column performance. This discussion is still ongoing. To go deeper into such comparison, it must be said that not only the particle morphology must be taken into account but also the particle size distribution, the amount of fines and the type of packing procedure. A study under these aspects was performed by Verzele et al. [14]. In the period between 1970 and 1995 the columns with irregular particles were replaced by those packed with spherical particles at least for analytical columns. Spherical or spheroidal particles were produced from synthetic cross-linked polymers according to specific synthesis procedures. Packings
with irregular particles, in particular silica, are still on the
market.

At the beginning of the seventies no spherical silica particles
in the size range between 5 and 10 \( \mu \text{m} \) were available and
appropriate synthesis protocols had to be developed.

Several synthetic routes and processes can be employed to
generate spherical particles, which are specific for each material.
For the manufacture of spherical silica particles the following processes were employed:

1. subjectiong colloidal silica dispersions to gelling into a two phase system [15,16];
2. hydrolysis and polycondensation of polyethoxysiloxane (PES) to silica hydrogel beads under stirring in a two-phase
system [17–23];
3. agglutination of silica sols in presence of a polymer followed by calcination of the beads [24];
4. spray drying of silica sol suspensions [25];
5. formation and growing of silica nano particles in suspensions [26,27].

The silica sources were sodium silicates, stabilized silica sol suspensions and alkoxy silanes. In some cases templates, porogens and detergents were added to adjust and to control the pore structural parameters and to achieve the spherical morphology. Afterwards, the additives had to be thoroughly removed by washing after or the dried particles had to be subjected to a controlled calcination up to 600 °C to burn out the organic constituents.

The major goals of the manufacturing processes were to achieve a spherical morphology of the particles and simultaneously adjust and control the pore structural parameters such as the specific surface area, the specific pore volume or particle porosity and the average pore diameter.

The following values were reported for the most commonly used silica based packing materials employed as base materials for the separation of low molecular weight analytes (MW < 1000 Da):

- (i) specific surface area, \( a_s \) (BET): 100–400 m²/g;
- (ii) specific pore volume, \( v_p \): 0.4–1.0 mL/g;
- (iii) average pore diameter, \( p_d \): 6–50 nm.

Packings suitable for the separation of synthetic polymers and biopolymers possess larger pores of average diameter \( >50 \text{ nm} \). Correspondingly the specific surface area of these materials becomes smaller than 50 m²/g.

One has to take into account that these values decrease after the surface functionalization. The extent of decrease depends on the chemistry or the way the modification is performed (e.g. silanization, polymer coating, etc.).

Generally, a decision has to be made, whether the packing is employed for the separation of low molecular weight analytes (MW < 1000 Da) or for the separation of high molecular weight compounds (MW \( \gg 10,000 \) Da). As a rule of thumb, for efficient separation, the average pore diameter of an adsorbent particle should be four times larger than the hydrodynamic diameter of the analytes, in order to minimize hindered diffusion and to enhance mass transfer kinetics [28].

The major field of application is the resolution of low molecular weight fine chemicals and pharmaceuticals. Here, an average pore diameter of ca. 10–12 nm is sufficient, which results in a specific surface area of roughly 300 m²/g. An average pore diameter of 30 nm is needed to resolve analytes of a molecular weight between 20,000 and 50,000 Da. For polymers of larger molecular weight an average pore diameter of 50 or even 100 nm is required. The specific surface area then is notably lower.

However, in surface–solute interactions with biopolymers by ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), reversed-phase liquid chromatography (RPLC), etc., the magnitude of the specific surface is not the critical parameter as a result of the strong interactions between the biopolymer and the active sites of the surface. Studies on biopolymer separations with nonporous 2 \( \mu \text{m} \) particles and a specific surface area of only 5 m²/g in RPLC, HIC and IEC.
modes have demonstrated that fast and high-resolution separations can be achieved [29–32].

In terms of pore size and pore size distribution, two aspects have to be considered. First, a packing material should be free of micropores (pores < 2 nm). Micropores generate a high surface area, with a high adsorption potential. In addition, small pores lead to small mass transfer kinetics by slowing down the diffusion of solute molecules in the pores. The second issue is that the pores of packing should be highly interconnected to facilitate the mass transfer of analytes during the chromatographic separation process. The aspect of pore connectivity, which is well known in the design of heterogeneous catalysts, has received considerable attention during the last decade in the field of modelling and simulation of pore structures [33–35].

When discussing pore structural parameters and comparing different packing materials one should keep in mind that the specific surface area according to BET is determined with nitrogen – a small probe molecule in the gas phase at 77 K. HPLC takes place in a liquid phase and separates molecules, which typically are considerably larger than the nitrogen molecule. Thus the magnitude of the specific surface area according to BET, in such case, is not a proper measure of the accessibility of pores in the liquid phase. As a consequence, the effective surface area for specific interactions may be much smaller.

The same distinction holds for the characterization of the pore size, or more precisely the pore volume distribution of packings. Usually, the pore size distribution is derived from the desorption branch of the nitrogen sorption isotherm at 77 K by application of the Kelvin equation with appropriate corrections [36]. An alternative method, known as inverse size-exclusion chromatography (ISEC) using polymer standards [37–42] can be applied to overcome the drawbacks of gas sorption methods, since ISEC is based on liquid chromatographic measurements and more relevant specific surface areas and pore volume distribution values are obtained. This holds for both inorganic porous adsorbents with various surface modifications [43] as well as for swellable cross-linked organic polymers [44,45]. Various reviews [41,46] showed the applicability and reliability of the method, without taking of the pore connectivity and pore shape into account. Grimes et al. [42] recently achieved to estimate the pore connectivity.

The reported parameters can be expressed in relation to the unit of mass of adsorbent and packing. However, it is generally more relevant to refer to unit of column volume. Instead of using the bulk density of particles, the packing density is the most reliable parameter, i.e. the mass of particle being packed per unit volume of the column. The packing density ranges between 0.4 g/cm³ for highly porous particles with a specific pore volume larger then 1 g/cm³ up to 0.8 g/cm³ for low porosity particles having a specific pore volume of approximately 0.4 g/cm³. For example, highly ordered mesoporous silica of type MCM-41 (mobil composition of matter), first introduced by researchers from Mobil Oil Corp., USA, possess a high specific surface area in excess of 1000 m²/g and a high specific pore volume of larger than 1 cm³/g [47,48]. The bulk and packing density, however, is relatively low and thus the specific surface area per unit volume becomes by a factor of ~2 smaller then the value per unit mass. This becomes important when one compares different column packings. In consequence the pore structural parameters should be expressed per unit volume.

2.2.2. Sizing and size analysis

At the early application of HPLC, the packings of choice were irregularly shaped native silica particles operated with organic eluents in the so-called normal-phase chromatography mode. The first action was to mill and to grind larger silica xerogel particles to batches with the desired particle size. At this time no technical means were available to fractionate or size micro particulate packings. Sieving, as done traditionally, was not an efficient and economic process. Therefore, in the 1970s, based on their experience in air stream technology for aircrafts, Alpine (Augsburg, Germany) developed and introduced novel equipment for sizing based on the phenomenon of air elutriation. The core of the Alpine Zig-Zag siever was a metal wheel with zig-zag channels rotating at high speed, which gives a separation into coarse and fine particles. Removing particles at the upper and the lower end of the particle size distribution had to be repeated until a desired particle size distribution was achieved. The classification was accompanied by particle analysis to control the sizing process [49,50].

The smaller the particle – the higher the rotation speed of the wheel had to be. The particles were subjected to high abrasion forces while running through the channels of the siever and thus fines were formed, often adhering to the surface of larger particles. Based on the relationship between particle size and rotation speed the lower limit of sizing silica particles was ca. 2 µm. The sizing process usually resulted in substantial material losses, depending on how narrow the intended size distribution was. Losses of 50% (w/w) were common. In addition, the silica became polluted with iron; the latter had to be removed by an acid treatment of the sized material [13]. Apart from air elutriation [51], other methods can be applied [52] e.g. sedimentation of the dilute silica suspension in the counter current mode [53].

Particle size analysis is an essential tool to control the result of sizing. Nowadays, a number of effective technologies are available to assess the particles size distribution in the range between 1 and 10 µm. More than 50 different methods have been described in the literature for the analysis of particle shape and size distribution. They mainly differ in the applied measuring principle, the particle size range they can be used for, the information they are extracting from the particles and the necessary measuring time.

The most common measuring principles for the determination of particle size distributions of chromatographic packings are listed in Table 1. They can be grouped into five different categories according to their measuring principle: Size- and classification analysis, light scattering, sedimentation analysis, electronic measurements and microscopic principles.

The most common particle size analysis methods are collected and briefly described in Appendix A.

Each instrument is based on a given principal and measuring technique and thus provides a specific and varying average of the particle diameter – \( d_p \). The particle diameter can be expressed as the number average – \( d_{pn} \), the surface average – \( d_{ps} \), the weight
Table 1
The measuring principles for the determination of particle size distributions of chromatographic packings

<table>
<thead>
<tr>
<th>Method</th>
<th>Particle size range (μm)</th>
<th>Information from single particles or multitude</th>
<th>Form information</th>
<th>Separating method</th>
<th>System manufacturers (web address)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sieve analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry sieving</td>
<td>&gt;20</td>
<td>Single</td>
<td>No</td>
<td>Yes</td>
<td>Retsch (<a href="http://www.retsch.de">www.retsch.de</a>)</td>
</tr>
<tr>
<td>Air sieving</td>
<td>&gt;5</td>
<td>Single</td>
<td>No</td>
<td>Yes</td>
<td>Gilsonic (<a href="http://www.christison.com">www.christison.com</a>)</td>
</tr>
<tr>
<td>Sonic sieving</td>
<td>&gt;5</td>
<td>Single</td>
<td>No</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Light scattering</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laser diffraction</td>
<td>0.05–30</td>
<td>Multitude</td>
<td>No</td>
<td>No</td>
<td>Malvern (<a href="http://www.malvern.co.uk">www.malvern.co.uk</a>); Helos</td>
</tr>
<tr>
<td>Turbidimetric systems</td>
<td>0.05–30</td>
<td>Multitude</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Single-particle optical sensing (SPOS)</td>
<td>0.5–400</td>
<td>Single</td>
<td>No</td>
<td>Yes</td>
<td>Nicomp, Particle Sizing Systems (<a href="http://www.pssnicomp.com">www.pssnicomp.com</a>)</td>
</tr>
<tr>
<td>Light obscuration (AccuSizer)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sedimentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipette analysis</td>
<td>0.5–300</td>
<td>Multitude</td>
<td>No</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Sedimentation balance</td>
<td>0.5–300</td>
<td>Multitude</td>
<td>No</td>
<td>Yes</td>
<td>CPS instruments (<a href="http://www.cpsinstruments-eu.com">www.cpsinstruments-eu.com</a>)</td>
</tr>
<tr>
<td>Photo- or X-ray sedimentation</td>
<td>&gt;2</td>
<td>Multitude</td>
<td>No</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Centrifugal sedimentation</td>
<td>0.0005–50</td>
<td>Single</td>
<td>No</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Resistazone counters</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Electrical sensing zone (ESZ) method</td>
<td>0.4–1200</td>
<td>Single</td>
<td>No</td>
<td>No</td>
<td>Coulter Counter (<a href="http://www.beckman.com">www.beckman.com</a>)</td>
</tr>
<tr>
<td>Optical methods</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Light microscopy</td>
<td>0.5–250</td>
<td>Single</td>
<td>Yes</td>
<td>No</td>
<td>Image analysis: SigmaScan (<a href="http://www.spssscience.com">www.spssscience.com</a>)</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>0.001–10 (100)</td>
<td>Single</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Flow through photometry</td>
<td>2–9000</td>
<td>Single</td>
<td>Yes</td>
<td>Yes</td>
<td>Malvern (<a href="http://www.malvern.co.uk">www.malvern.co.uk</a>)</td>
</tr>
<tr>
<td>Flow cytometry (Sysmex)</td>
<td>0.7–160</td>
<td>Single</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Field flow fractionation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gravitational FFF</td>
<td>10–100</td>
<td>Multitude</td>
<td>No</td>
<td>Yes</td>
<td>Postnova analytics (<a href="http://www.postnova.com">www.postnova.com</a>)</td>
</tr>
<tr>
<td>Centrifugal sedimentation FFF</td>
<td>2–15</td>
<td>Multitude</td>
<td>No</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

average – $d_{pw}$ and the volume average – $d_{pv}$. For statistical reasons ranking of averages is as follows [54]:

$d_{pn} < d_{ps} < d_{pw} < d_{pv}$

The smallest value is the number average; the largest is the volume average in this series. Assuming that the weight of the spherical particle $w_i \approx s_i d_i^3 \approx n_i d_i^2$, where $s_i$ is the surface area of the spherical particle of diameter $d_i$ and $n_i$ is the number of the spherical particles, the weight average can be expressed by a surface average and the number average [18,55].

The particle size distribution can be presented as a cumulative distribution or as a differential distribution. There are three different average values: the mean, the median and the mode [55]. Table 2 gives an example of certain characteristic values of silica packing. In HPLC, the volume average of the particle size is the most informative one, because it refers to the volume of the column. However, the number average is also useful to visualize the fine particle and their distribution.

There has been much discussion on how narrow a particle size distribution should be to generate the most stable packing and the highest column performance. As a rule of thumb the ratio of the $d_{p90}$ value (average value at 90% of the cumulative distribution) to the $d_{p10}$ value (average value at 10% of the cumulative distribution) should amount from 1.5 to 2.0 for particles in the range of 3–7 μm (analytical HPLC columns) [56].

2.2.3. Packing procedures

In the early stages of LC, columns were obtained by dry packing 30–40 μm particles into glass columns using a methodology, that involved repeated damping (mechanically tapping the column or lifting it up in the air and letting it drop) of the packed column. [57]. The first HPLC columns were also dry-packed into stainless steel columns with microparticulate packings by Huber and Kraak at the University of Amsterdam by mechanically pushing the particles into the column blank with a rod.

Enormous efforts were undertaken to develop an efficient packing procedure for micro particulate packings in stainless steel columns [58]. The most common one is the slurry technique: a dilute suspension of the particles is forced under high pressure and high flow-rate into the column. The column end contains a porous frit, which retains the particles. When 4.6 mm I.D. 250 mm long columns are packed with 5 μm particles, the back pressure may rise up to 500 bar at flow rates in excess of 10 mL/min. Such procedure would require a high pressure pump and a properly designed stainless steel reservoir.

The steps are as follows [59]:

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Table 2
Summary of a particle size measurement (volume and number statistics), performed with Particle Sizing Systems, Santa Barbara, California, U.S.A. (data supplied by Dr. F. Krebs, Merck KGaA, Darmstadt)

<table>
<thead>
<tr>
<th>Volume statistics (geometric) 6.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculations from 0.96 to 33.50%</td>
</tr>
<tr>
<td>Volume 100.0% Coinc. connected 195,770</td>
</tr>
<tr>
<td>Mean 8.267 μm SD 0.091</td>
</tr>
<tr>
<td>Median 8.236 μm</td>
</tr>
<tr>
<td>Mean/median ratio 1.004 Variance 0.0082</td>
</tr>
<tr>
<td>Mode 8.089 μm Skewness:-4.625e-001 left skewed</td>
</tr>
<tr>
<td>Size (μm) 6.067 6.475 8.236 10.74 11.54</td>
</tr>
<tr>
<td>d_{p90}/d_{p10} 1.66</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number statistics (geometric) 6.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculations from 0.96 to 33.50%</td>
</tr>
<tr>
<td>Number 100.0% Coinc. connected 195,770</td>
</tr>
<tr>
<td>Mean 6.240 μm SD 0.221</td>
</tr>
<tr>
<td>Median 7.138 μm</td>
</tr>
<tr>
<td>Mean/median ratio 0.874 Variance 0.049</td>
</tr>
<tr>
<td>Mode 7.341 μm Skewness:-2.010e-001 left skewed</td>
</tr>
<tr>
<td>Size (μm) 1.545 2.865 7.138 9.344 10.09</td>
</tr>
<tr>
<td>d_{p90}/d_{p10} 3.26</td>
</tr>
</tbody>
</table>

(i) preparation of a stable suspension of highly dispersed particles;
(ii) filling of the column at high flow rates of >10 mL/min and high pressures up to 500 bar employing a stainless steel reservoir;
(iii) washing the column and conditioning with the eluent.

As a rule of thumb the packing pressure should always be significantly higher than the working pressure. A stable column has a porosity between 35 and 45%. This value does not correspond to the densest packing order, which is 26% for a hexagonally close packed bed [60]. However, the packing density varies close to the wall as compared to the center. Inhomogeneities in the packing due to wall effects are assumed for approximately 20 particle diameters. Such packing inhomogeneities can result in channel formation and voiding effects, diminishing the expected column performance. Most experience was gained on the packing of 3–10 μm particles in stainless steel columns. It took column manufacturers approximately 10 years to develop procedures to reproducibly pack analytical columns of 4 and 4.6 mm I.D., respectively. The main outcome is discussed in the paper of Kirkland and DeStefano [61]: ‘A very important aspect identified was that the particles must be totally suspended and not aggregated in the slurry liquid to be used for the packing process. Higher packing pressures generally were found favourable for both performance and stability. This high-pressure operation requires good strength in particles, which is characteristic of porous silica micro spheres used in the test; irregular particles were deficient in this regard. The low viscosity slurry method was identified as superior over high-density techniques for packing silica micro spheres, and the constant pressure packing method invariably was favoured over the constant flow rate.

It was found that thin screens for capturing the packing at column inlets and outlets produced the highest column efficiency. However, this material is less useful for preparing long-term stable columns, and thin porous frits generally are preferred for this purpose. There was no performance advantage found for packing the column in an upwards vs. downwards direction, but the downwards approach is more convenient.”

Particles larger than 10 μm could be packed by the dynamic axial compression technique in preparative columns of 50 mm inner diameter and larger. In general, there exist two methods to fill columns in preparative scale: this is dry packing and slurry packing (filtration technique) methods. Even though the dry filling method is easy to perform, the slurry method gives, especially for smaller particles, more efficient and more reproducible columns [62]. For extended stability and reproducibility of the packing processes in the preparative mode, new techniques were developed, such as dynamically axial compression [63–67] and radial compression [68–73]. These methods allow one to change the packed bed volume, through solving the instability problem in the packed bed, which is due to the formation of channels, voids and inhomogeneous dense packing formed by slurry packing process.

The packing of microbore columns (I.D. between 1 and 3 mm) and of fused silica capillaries (I.D. between 200 and 20 μm I.D.) requires different conditions and extended experience. Different column packing techniques could be applied, such as: dry packing [74], high-pressure slurry packing [75], packing using supercritical carbon dioxide [76], electrokinetic packing [77], and packing with centripetal forces [78]. Though different packing methods allows one to obtain a stable capillary column, the preparation of robust frits is not easy. The most common technique is the fusing of silica particles [79,80]. Although
silica particles can be fused together, the general observation was that after such a treatment the column performance had decreased, still leaving the problem of the frit preparation in the capillary columns an unresolved issue.

Particular effort is required to pack columns with particles smaller than 5 μm. The adhesion forces between the particles become much larger then for particles with \( d_p > 5 \) μm and thus stable suspension have to be prepared with highly dispersed individual particles before slurry packing.

A number of research projects related to the characterization of column bed structure involved the application of techniques such as NMR imaging [81–84], pulsed-field-gradient NMR spectroscopy [85–87], hydrodynamic chromatography [88], and visual monitoring of elution bands in glass columns [89].

2.2.4. Evaluation of the column performance

In general there are three major criteria in characterizing a HPLC column:

(a) The hydrodynamic properties expressed by the column pressure vs. flow dependency provide an insight into the flow behaviour. From these data the column permeability can be calculated and compared with the expected value based on the average particle diameter and the column dimensions.

(b) The kinetic properties of the column expressing the mass transfer kinetics of analytes are a measure of the peak dispersion of a column. The peak dispersion is characterized by the theoretical plate height (\( H \)) and the number of theoretical plates (\( N \)). A more detailed diagnosis of the kinetic performance is based on the dependency of the plate height (\( H \)) as the function of the linear velocity (\( u \)) of the eluent.

(c) The thermodynamic properties are expressed by the retention coefficients and the selectivity coefficient of test solutes under constant conditions.

2.2.4.1. Hydrodynamic properties. The column pressure drop \( \Delta p \) is proportional to the viscosity \( \eta \) of the eluent, the column length (\( L \)) and the linear velocity of the eluent \( u \) and inversely proportional to the average particle diameter (\( d_p \)) of the packing squared (Eq. (1)):

\[
\Delta p = \frac{\Phi \eta L u}{d_p^2}
\]  

(1)

where \( \Phi \) is the column resistance factor, which varies between 500 and 1000 for a well packed column [90]. The operating pressures of reversed phase columns of 4 mm I.D. and 100 mm length usually range between 50 and 100 bar. Changes of the column backpressure are commonly a strong indicator that the column bed structure alters and the column performance declines. The column pressure drop becomes significant when the particles of the packing are smaller than 2 μm (see Section 2.3.1). The column pressure is important for particles of all sizes, as it plays a central role in maximizing plates/time or optimizing the performance of a given column. There are many review articles, which discuss the critical role of pressure in both using columns and evaluating their performance [91,92].

2.2.4.2. Peak dispersion. The first researchers who systematically studied peak dispersion phenomena were Martin and Synge [93], van Deemter et al. [94] and Giddings [95,96]. The treatment of the mass transfer processes and the distribution equilibria between the mobile and stationary phase in a column lead to equations which link the theoretical plate height as the decisive column performance parameter to the properties of the LC systems such as linear velocity of eluent, diffusion coefficient of analyte, retention coefficient of analyte, column porosity, etc.

van Deemter proposed an equation, which described the column performance as a function of the linear velocity for a packed column in gas chromatography coated with a stationary liquid layer. Similar equations, however, with other terms were derived for LC by numerous researchers (see Table 3). It became common practice to refer to all \( H \) vs. \( u \) plots collectively as van Deemter plots. A minimum in the plate height vs. linear velocity curve is observed where the column performance is the highest. Knox suggested a three term equation to describe the dependency of the theoretical plate height \( H \) of a column as a function

<table>
<thead>
<tr>
<th>Model</th>
<th>Limiting case for large ( v ) (velocity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>van Deemter et al. [94]</td>
<td>( h = A + \frac{B}{v} + Cv )</td>
</tr>
<tr>
<td>Giddings [96]</td>
<td>( h = \frac{1}{1/A + 1/Eu} + \frac{B}{v} + Cv )</td>
</tr>
<tr>
<td>Snyder (1969)</td>
<td>( h = \frac{1}{A} + \frac{B}{v} + Cv )</td>
</tr>
<tr>
<td>Huber and Hulsman (1976)</td>
<td>( h = A + \frac{B}{v} + Cv )</td>
</tr>
<tr>
<td>Kennedy and Knox [128]</td>
<td>( h = A + \frac{B}{v} + Cv )</td>
</tr>
<tr>
<td>Horvath and Lin (1978)</td>
<td>( h = A + \frac{B}{v} + F )</td>
</tr>
<tr>
<td>van Krefeld and van den Hoed (1978); Afeyan et. al (1990)</td>
<td>( h = A + \frac{B}{v} + DCv )</td>
</tr>
<tr>
<td>Yang et al. (1992)</td>
<td>( h = A + D )</td>
</tr>
</tbody>
</table>
of the linear velocity of the eluent by Eq. (2):

\[ H_{\text{total}} = \frac{L}{N} = A u^{1/3} + \frac{B}{u} + C u \]  

(2)

where \( H_{\text{total}} \) is the total theoretical plate height of a column; \( u \), is the linear velocity of the eluent and \( A \), \( B \) and \( C \) are constants.

The \( A \)-term corresponds to the convective dispersion by flow through the tortuous column bed, the \( B \)-term expresses the dispersion due to longitudinal molecular diffusion and the \( C \)-term is a measure of the equilibration of the analyte between the stationary and mobile phase in a column. \( H_{\text{total}} \) vs. \( u \) is dominated at the left hand side of the minimum by \( B \) and at the right hand side by the term \( C \) at higher linear velocities. Well packed columns have the approximate values of \( A < 1 \), \( B \approx 2 \) and \( C < 0.1 \). [98].

It is useful to convert \( H \) and \( u \) to dimensionless parameters by Eqs. (3) and (4):

\[ h = \frac{H}{d_p} \text{(reduced theoretical plate height)} \]  

(3)

\[ v = \frac{u d_p}{D_m} \text{(reduced linear velocity)} \]  

(4)

where \( D_m \) is the diffusion coefficient of the analyte in the mobile phase.

The application of reduced parameter is quite common for chemical engineers comparing the performance of columns in unit operations such as distillation, extraction, adsorption. Typical reduced parameters are: the Peclet number (Eq. (5)), the Sherwood number (Eq. (6)) and the Reynolds number (Eq. (7)):

\[ Pe = \frac{d_p u}{D_m} \text{(Peclet number)} \]  

(5)

\[ Sh = \frac{k_d d_p}{v_m} \text{(Sherwood number)} \]  

(6)

\[ Re = \frac{u d_p}{v_m} \text{(Reynolds number)} \]  

(7)

where \( d_p \) is the average particle size; \( u \), linear velocity of the eluent; \( k_d \), partial mass transport coefficient in the flowing medium, and \( v_m \) is the kinetic viscosity of the mobile phase [99]. The Peclet number is then identical with the reduced velocity (see Eqs. (4) and (5)).

The particular advantage of this approach is the ability to compare the performance of columns packed with particles of different sizes.

Unger et al. [100] demonstrated experimentally that the course of the reduced plate height-reduced linear velocity plots are similar for all types of packings independent of whether they are irregular or spherical, for silicas and aluminas of widely differing average particle diameter.

A more detailed analysis of the \( H \) vs. \( u \) curves published in the LC literature is often not possible for the following reasons: Firstly, the plate height is commonly measured by simple approximations and not accurately by the method of statistical moments. Secondly, the measurements were performed on instruments, which had substantial dead volumes, and no corrections were performed to correct the total plate height with respect to the extra-column contributions. Needless to say, plate height measurements should be performed at isocratic conditions. A recent investigation including a number of commercial columns packed with micron size particles has indicated that the impact of these terms in assessing the column performance has to be re-investigated [101]. Still, the usual aim in column design is the need to make \( C \)-term of \( H \) vs. \( u \) curve as small as possible, what would allow a fast and efficient separation, even at higher linear velocities, where \( v \gg 10 \).

The optimum linear velocity is \( v_{\text{opt}} \) was found to be between 2 and 5 with reduced plate heights between 2 and 5. In other words, at the best conditions the plate height values correspond to twice the average particle diameter. \( H \) value have been reported which are smaller than 2 [102].

The current trend in column development is to make the analysis faster and more sensitive with respect to detection. One alternative is to reduce the particle size on the expense of column pressure drop using classical packing materials. The other alternative is to change the particle design or to apply electrically driven chromatography such as CEC. We will treat this issue in more detail in the next section.

A more advanced concept on the characterization of the column performance was pioneered by Poppe [91], which was further developed by Tanaka et al. [103] and Gzil et al. [104]. Poppe introduced the so-called kinetic plots: plate time \((\log(t_0 N))\) as a function of the number of theoretical plates \( N \), where \( t_0 \) is the elution time of a non-retained compound. In order to assess the limits of column performance as a function of particle size, column pressure drop, etc. Tanaka et al. [103] applied kinetic plots to compare monolithic silica columns with particle packed columns. The experimentally obtained kinetic plots clearly indicated the advantage of monolithic column structures over particle packed columns with respect to maximum column performance.

The increase of the plate numbers of a HPLC column or the number of plates per unit time of the LC system, however, is only one side of the coin and very much resembles the situation in HPLC at the middle 1970s. It should be remembered that the chromatographic resolution \( R_s \) is mainly governed by the selectivity of the phase system (stationary and mobile phase) rather than by the column performance. Thus the message is to develop highly selective stationary phases with acceptable column performance and column stability. As the emphasis of this article is not directed to stationary phase development, the reader is referred to numerous literature sources [105–110].

2.3. Selected topics

2.3.1. The ultimate minimum particle size in high-performance liquid chromatography – fiction and facts

The subject of minimum particle size in HPLC was already discussed in depth at the advent of HPLC by several researchers. In 1975 Halasz stated in a paper: “At a column pressure of approximately 500 bar, the temperature of the eluent may increase up to 35 °C. Temperature and viscosity gradients exist in axial and radial directions inside the column. For routine work the particle size should be 5 μm < d_p < 3 μm. The mini-
The major advantages in using columns with sub 2-μm particles were the gain in higher column efficiency, short analysis time by raising the flow-rate and high detection sensitivity, particularly when working with mass spectrometry as detector/separator.

However, columns packed with such particles need a special LC system. Shortening the connecting tubes and reducing the tube diameter should minimize extra column effects. To reduce frictional heating and minimize temperature effect the column diameter should be reduced to 2 mm or smaller. Columns should be short in length and possess frits with the desired porosity to retain the particles. The injection volume should be small as well as the volume of the detector cell, in case UV detection is applied. A source of information is an article by Giesche et al. [114], who studied the packing technology, column bed structure and chromatographic performance of columns packed with nonporous 1–2 μm size reversed phase silicas.

Employing 2 μm nonporous C18 bonded silica particles plate height values between 5 and 8 μm were generated at linear eluent velocities between 2 and 6 mm/s. At a flow-rate of 2.5 mL/min the column pressure drop was 500 bar (column dimensions 53 × 4.6 mm). Fig. 3 shows the corresponding plate height–linear flow velocity curves of analytes with retention coefficients between \( k' = 0.39 \) and 2.99 for such a column. To keep extra-column effects to a minimum, the injection volume was 0.6 μL, the volume of the detector cell 0.3 μL and the time constant of the UV detector <60 ms. Such columns enabled ultra-fast separations of analytes in less than 60 s [115]. Fig. 4 exhibits an electron scanning micrograph of such particles. As the particles were totally non-porous, the specific surface area per milliliter of column volume was in the order of approximately 5 m²/mL. Consequently, the mass loadability was by a factor of 100 lower than that of a common analytical column. To achieve the same retention of analytes in the reversed phase mode as compared to a 4.6 mm I.D. analytical column packed with 5 μm particles, the water content of the eluent had to raised from 40/60 to 80/20 water/acetonitrile volumetric ratio. The column was extremely mechanically stable up to packing pressures of 2000 bar because the particles were non-porous.

MacNair et al. [116] demonstrated that such particles could also be packed into fused silica capillary columns generating several thousand plates per meter of column length at elevated pressure in excess of 1000 bar.

With the breakthrough of coupling HPLC with mass spectrometric detection the way was open to connect columns packed with porous sub-2 μm particles with an LC instrument designed for higher pressures and coupled to MS. The result was a substantial increase in sensitivity and sample throughput (Acquity UPLC system of Waters) [117,118].

An essential element of the Acquity UPLC system are Water’s novel XTerra columns based on the concept of particle hybrid technology [119].

The term hybrid stands for porous inorganic/organic particles with an inorganic skeleton and a bonded organic moiety distributed in the bulk phase as well as at the surface. The first attempts to synthesize silica-organic hybrid-particles were made by two routes [120]:

(i) hydrolyzing a tetraalkoxysilane and an organotrialkoxysilane to a poly(organoalkoxysiloxane);
(ii) adding an organotrialkoxysilane to a poly(alkoxysiloxane).

Both intermediate products were subjected to complete hydrolysis and condensation at a two-phase system under vigorous stirring, whereby spherical organo-silica particles were formed.
The particular feature of Waters novel hybrid technology was the use of ethane bridged silanes (e.g. (bistriethoxysilyl)ethane) and tetraethoxysilane to form a bridged poly(ethoxysiloxane). The bridged ethylsiloxane silica (called BEH) was synthesized as porous sub 2 μm particles with a narrow particle size distribution. Due to the ethyl bridge the particles display a high mechanical stability and an enhanced pH stability in the pH range between 1 and 12. XTerra materials and columns are available with different kinds of organic moieties and surface chemistries [121].

In order to avoid the application of high pressure Advanced Materials Technology, Chadds Ford, PA, USA have recently introduced particles with a solid core and a porous shell, as explained in Section 2.3.2.

A promising alternative to using submicron particles is to employ them in CEC. In this case, the electrical field generates the flow and plate heights of 1–2 d p have been generated. Porous silica beads with an average particle diameter between 0.2 and 3 μm, n-octyl surface functionalized have been studied in 100 μm I.D. fused silica capillaries in CEC [122].

Thus there is still a need for fundamental studies on CEC to elucidate the retention mechanism for polar and charged analytes and to design appropriate systems. Furthermore, CEC is much more complex than pressure driven LC with regard to two phenomena: (i) the EOF is not constant but varies as a function of many system- and operational parameters and (ii) retention and selectivity in CEC is changing when the field strength alters [123].

2.3.2. Totally porous vs. core/shell particles

The development of efficient packing materials in HPLC was never a straightforward approach. There were false starts and intermediate solutions, which in the long run proved to be unsuccessful. An example was the introduction of porous layer beads. Before totally porous particles were applied in HPLC, porous layer beads were introduced as packings based on the pioneering work of Horvath and co-workers [124,125]. The objective was to enhance the mass transfer kinetics of solutes by reducing the diffusion path length. Due to the large particle diameter the columns generated relatively low backpressure and were dry packed by damping. Although the specific surface area was considerably larger as compared to nonporous 2 μm particles the mass loadability was limited. Such products are still commercially available and employed as packings in precolumns.

The porous layer bead particles consisted of an impermeable core of 30–40 μm diameter and a porous silica shell of approximately 1 μm thickness. They were operated in the normal-phase LC mode. The porous layer contained mesopores and the specific surface areas ranged between 1 and 30 m²/g [126]. Chromatographic tests of commercial products in normal-phase chromatography gave plate height values in the range of 1–4 mm at a linear velocity of 2.5 cm/s. The measured plate is mainly determined by the A-Term, which represents the packing quality of the column bed. At higher velocities, the C term of plate height curves was relatively small and this enabled one to obtain efficient and fast separations [126].

An investigation was subsequently made of the impact of the estimated thickness of the porous layer d s in the range between 0.5 < d s < 0.9 μm at constant particle size and constant pore diameter of the porous layer on the theoretical plate height of selected solutes in normal-phase chromatography. A relationship of \( H = d_s^{1.4} \) was obtained which is to be expected [127]. Comparative column performance studies of porous layer beads and totally porous particles were carried out by Kennedy and Knox [128] at the same time.

Recently, Advanced Materials Technology has introduced a novel type of a column known as Halo HPLC column, which is based on a fused core particle technology developed by Kirkland. The 2.7 μm silica particles are composed of a solid core of 1.7 μm thickness surrounded by a porous layer of 0.5 μm. The particles possess a specific surface area of 150 m²/g and an average pore diameter of 9 nm. They are available with a variety of bonded reversed phase chemistry [129]. The column I.D. is 2.1, 3.0 and 4.6 mm, respectively. Columns packed with such particles show low mass transfer resistance values at high linear velocities enabling very fast separations of low molecular weight analytes in less than one minute at column back pressures of approximately 300 bar.

Recently Gritty and Guiochon [101] performed a comparative study on the performance of micron size RP silica columns including the Halo column at a wide range of the mobile phase velocities. The Halo column performed best for low molecular compounds (e.g. naphthalene), but is not as good as the other studied columns using insulin as solute. The relatively high C-term of the Halo column at high flow rates was attributed to the roughness of the external surface of the Halo particles which might generate a high film mass transfer resistance.

2.3.3. Column miniaturization: from meso to micro to nano – where is the end?

Column miniaturization has been under discussion for many years, particularly since 1980 when fused silica capillaries of I.D. in 100 μm range were first manufactured. They were first applied in gas chromatography with liquid stationary phase coatings inside. It is beyond the scope of this article to provide an in depth survey of micro bore and capillary columns in HPLC [92,130]. The main idea to reduce the column diameter was to minimize the dilution of the sample to be resolved and to achieve higher peak heights as compared to the 4.6 mm I.D. columns, while maintaining the column efficiency.

The next step in I.D. diminution was to introduce Microbore stainless steel columns of 1–2 mm I.D. Such columns can be still operated with a conventional HPLC system. Systematic studies on the packing procedure and the efficiency of such columns revealed that indeed the same column performance could be achieved as compared to 4 mm I.D. columns using the same packing materials. The critical issue is the column hardware and the packing process. The stainless steel tubings should be stable to high pressures and contain a mirror finish of the inside wall. The packing pressure is commonly above 1000 bar. The optimum packing pressure is dependant on the type of silica packing.
Roumliotis et al. [131] increased packing pressures to 2000 bar and the silicas were still mechanically stable. To achieve the expected column performance several factors (injection volume, detection volume, connecting tubes) have to be considered to minimize the contributions of extra-column effects to the total peak dispersion. It should be emphasized that 1 mm bore columns require a very low peak broadening system, minimizing extra-column-volume contribution otherwise the plate number are adversely effected. Table 4 provides a survey of HPLC columns of varying I.D. and the corresponding flow-rates [132].

Viewing Table 4 it becomes evident, that the operation of micro and nano columns require low volume flow-rates for which classical HPLC instruments that run with columns of 1.0–4.6 mm I.D. are not designed. Consequently, a micro-LC and nano-LC system, respectively, is required to match the flow-rate in the microliter to nanoliter/min range. Such systems are not only operated under isocratic conditions, but also under gradient elution conditions. Rapp and Tallarek have reviewed the state-of-art in the generation and control of micro- and nanoliter flow rates with special emphasis on feasibility, automation, delay times and dead volumes [133]. In addition, the volume of detector cells has to be reduced.

The range of 300–20 μm column I.D. is covered by fused silica capillaries coated outside with a poly(ethyleneimine) layer. They can be operated as open tubes and packed tubes. The smallest I.D. capillary columns applied had an I.D. of 20 μm [134].

For the operation of capillary columns, special equipment is required. One has to make sure that for the range of 10–300 μm column I.D. the approximately typical flow rate (linear eluent velocities between 1 and 10 mm/s) is in the range from 10 nL/min to 100 μL/min and the pressure limit up to 5000 psi [135]. Various injection systems and detectors are applicable. Also one needs special frits and column connections. The packing of fused silica capillaries is achieved by slurry packing, but a miniaturized packing device should be employed.

Another important aspect using micro and nano-LC columns relates to the mass loadability of the column, which is a measure of the column capacity [136,137]. Analytical columns are commonly operated in the linear range where the retention coefficient of an analyte and the plate number \(N\) is constant and independent of the sample size. Further increase of the sample size leads to a diminution of the retention coefficient \(k\) and a drastic decrease of column plate number \(N\). At further increase of the sample size, the column is overloaded. The linear range, where analytical columns could be operated without overload was arbitrarily defined as the linear capacity corresponding to the sample mass per gram of the stationary phase causing a 10% decrease of \(k\) and a 50% decrease of \(N\) for a given solute [57]. However, the mass loadability or column capacity is affected by a number of the parameters such as the composition and type of eluent, the type of stationary phase (e.g. the chain length of an alkylbonded silica), the type of solute (ionic, neutral, basic, acidic) and its molecular weight. As a rule of thumb, the mass loadability for reversed phase C18 column is about 2 mg of sample per gram of stationary phase for a low molecular weight solute. It accounts to about 10 mg/g of stationary phase for peptides and increases to about 100 mg/g of stationary phase for proteins on an ion-exchange column. McCalley et al. [138,139] and Buckenmeier et al. [140] have studied over-loading effects for various solutes on a large number of columns (reversed phase silicas, polymeric columns) and came to much more refined conclusions.

By decreasing the column I.D. the mass of packing or stationary phase decreases proportionally too (see Table 4). In other words, a packed 100 μm I.D. column contains ca. 0.13 mg of packing and the mass loadability is estimated to be 0.01 μg.

The lowest I.D. of capillary columns is reported to be 10 and 20 μm. The latter were packed with 0.8 μm reversed-phase silica particles [134]. The capillary columns were employed in ultrahigh-throughput proteomics using fast RPLC separations with electrospray ionization (ESI) MS/MS. Also monolithic C18 bonded silica columns were manufactured and tested for the same purpose by the same group [141].

There are a number of inherent problems associated with the manufacture and operation of these capillary columns:

(a) packing of capillaries with particulate materials or/and manufacture of stable and homogeneous monolithic columns;
(b) selection of appropriate frit systems in case of particle packed capillaries to avoid significant extra-column volume contributions;
(c) connection to the mass spectrometer.

To overcome these problems a microfluidic chip was designed by Agilent Technologies, Germany to be used at nano- and

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Table 4: A survey of HPLC columns of varying I.D. and the corresponding flow-rates

<table>
<thead>
<tr>
<th>Column I.D. (μm)</th>
<th>Flow rate (μL/min)</th>
<th>Column volume (μL)</th>
<th>Mass of silica per column (mg)</th>
<th>Mass loadability per column (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4600</td>
<td>1000</td>
<td>1660</td>
<td>1100</td>
<td>110</td>
</tr>
<tr>
<td>4000</td>
<td>760</td>
<td>1260</td>
<td>830</td>
<td>83</td>
</tr>
<tr>
<td>2000</td>
<td>190</td>
<td>310</td>
<td>210</td>
<td>21</td>
</tr>
<tr>
<td>1000</td>
<td>47</td>
<td>80</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>300</td>
<td>4.3</td>
<td>7</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>100</td>
<td>0.5</td>
<td>0.8</td>
<td>0.5</td>
<td>0.05</td>
</tr>
<tr>
<td>50</td>
<td>0.125</td>
<td>0.2</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>10</td>
<td>0.005</td>
<td>0.08</td>
<td>0.05</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Assumptions: column dimensions, \(L = 100\) mm, total column porosity \(\varepsilon_t = 0.7\), skeleton porosity \(\varepsilon_s = 0.3\), silica skeleton density 2.2 g/cm³. A mass loadability of 0.1 mg/g of stationary phase was assumed (reproduced by the permission of authors [132]).
3. Monolithic columns

3.1. The basic idea and the pioneers

As compared to particle bed columns, monolithic columns represent a single piece made of porous cross-linked polymer or porous silica. Monoliths are made in different formats as porous rods, generated in thin capillaries or made as thin membranes or disks.

When one critically judges the progress made with particle packed columns in HPLC over the last 50 years and consider the problems caused by the assembly of particles in a column it becomes immediately obvious, that the search for alternatives is a logical consequence. Cross-linked polymers are ideally suited to be synthesized in a confined space. Thus, the initial attempts were made with polyurethane foams to be applied in size exclusion chromatography and in gas chromatography [142–144]. Hjerten was the first one who developed continuous polymer beds based on polyacrylamide for the fast separation of biopolymers [145]. Tennikova and Svec synthesized poly(glycidyl methacrylate ethylene dimethacrylate) polymers as disks called macroporous polymer membranes [5] which were commercialized later by BIA Separations, Lubljana, Slovenia under the trade name Convective Interaction Media (CIM). Svec and Frechet [146] extended the family of continuous polymer beds by poly(styrene divinylbenzene) copolymers. Later on Premstaller et al. [147] extended the approach to prepare functionalized polymeric capillaries for the separation of nucleic acids. Buchmeiser et al. [148] introduced the ring-opening metathesis polymerization (abbreviated as ROMP) as an alternative synthetic route to free radical polymerization which enabled the syntheses of materials with a controlled pore structure and surface functionality.

Polysaccharides such as agarose, cellulose and cross-linked dextran were also employed as such [149,150] or used by supports in layered stacks and rolled layer [151,152]. Polysaccharides suffer from weak mechanical stability.

Parallel to the development of cross-linked polymers, Soga and Nakanishi made the fundamental studies [2,3,153] to synthesize continuous beds made of porous silica. The specific feature was that the monoliths contained a discrete bimodal pore size distribution made by large flow through pores in the micrometer pore size range and smaller diffusive pores (meso pores) in the nm size range. Tanaka immediately recognized the added value of the so-called “silica rods” as monolithic columns in HPLC and made the pioneering investigations in close collaboration with Nakanishi and co-workers.

Based on this concept Merck, Darmstadt, Germany, introduced monolithic columns as 4.6 mm columns and as 100 μm I.D. capillaries.

The major goals of applying monolithic columns in HPLC were to achieve high-speed separations; low column backpressure and fast mass transfer kinetics [154].

3.2. Monolithic silica columns

3.2.1. Formation processes and pore structure control of silica monoliths

The starting silica sources are tetramethoxysilane, tetraethoxysilane or n-alkytrialkoxyxilanes which are subjected to acid catalyzed hydrolysis and condensation in presence of water soluble polymers such as polyethylene glycols and polyacrylic acid and surfactants as additives. The multicomponent solution converts into a sol–gel system by a nucleation and growth mechanism in which small fractions of a finely dispersed phase grow in size (see Fig. 5a) being limited by a thermally activated diffusion process. A second process, called spinodal decomposition, takes place leading to a co-continuous domain structure, which remains stable over an extended period of time (see Fig. 5b). The gel morphology is controlled by the kinetics of two competitive processes: the domain coarsening and the structure freezing by the sol–gel transition. The resulting gels are aged and a solvent exchange is performed to tailor the pore structure. The macroporous gel domains are filled with the polymer, which has been burned out by calcination after drying. The mesopore structure and mesopore size is adjusted by hydrothermal treatment conditions. In this way the process enables to generate two continuous pore systems and to adjust and control the pore size, and porosity of macro pores and mesopores independently [2–4,153,156–171].

The manufacturing process of monolithic silica rods with 4.6 mm I.D. comprises the following consecutive steps: preparation of the starting sol, phase separation and gelation, aging and drying. After drying the rods are cladded with poly(ether ether ketone) (PEEK). Surface functionalization is performed in situ. The product is called Chromolith Performance and marketed by Merck, Darmstadt, Germany.

In case of fused silica capillaries the formation of the monolithic structure occurs within the capillary. Fig. 6 shows an electron scanning micrograph of the cross section of a 100 μm I.D. capillary.

The bimodal pore structure of a Chromolith column is characterized by a distinct bimodal pore structure: macro pores of 2 μm in diameter and meso pores with an average pore diameter of approximately 13 nm. The total porosity of the monolithic column amounts to 80% and higher, the larger proportion accounts for the macro pores. The mesopores generate a specific surface area of approximately 300 m²/g.

Silica monoliths (rods) of 4.6 mm I.D. size were characterized by the classical pore structure analysis such as nitrogen sorption at 77 K, mercury intrusion, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). As a result the mesopore volume and macro pore volume distribution were assessed to detect the size of diffusive pore and the size of flow through pores. Tanaka and co-worker introduced the parameter of domain size as a measure being the sum of the thickness of the silica skeleton and the flow through pore diameter. These authors stated that large ratios of flow through pores to the skeleton size and a high porosity lead to high column efficiencies per unit pressure drop [158].
Fig. 5. (a) Nucleation and growth (diffusion limited) of silica-based monoliths, where dispersed domains with sharp interfaces grow by diffusion-controlled kinetics (reproduced by the permission of author [155]). (b) Spinoidal decomposition (spontaneous) of silica-based monoliths, where interconnected domains with diffuse interfaces grow exponentially with time (reproduced by the permission of author [155]).

Unger et al. [172] examined the column performance and hydrodynamic properties of a series of native silica monoliths of 4.6 mm I.D. with graded macropore size between 1.9 and 6 μm at constant mesopore size of 12 nm. They also determined the pore connectivity of the mesopores using the model of Meyers et al. [173,174]. They found, that the pore connectivity $n_T$ had a tendency to decrease with increasing macropore diameter. Increasing macropore size lead also to increased theoretical plate heights at $u_{\text{optimum}}$ of the plate height linear velocity dependencies. The column pressure drop vs. volume flow-rate dependencies were lowest for the highest macropore diameter and increased with smaller flow-through pores.

Tallarek and co-workers introduced the equivalent sphere dimension as a parameter to compare monolithic silica columns and microparticulate packed silica columns [175–177]. They claimed that silica monoliths have to be described by two characteristic lengths: a characteristic length for the band dispersion $d_{\text{disp}}$ derived from the C-term of the $H$ vs. $u$ curve and a particle diameter $d_{\text{perm}}$ representing the hydraulic permeability. As a result of this analysis applying insulin and angiotensin as analytes on a C18 modified monolith they found that $d_{\text{disp}}$ was 3.9 and 2.5 μm, respectively, and $d_{\text{perm}}$ 15.6 μm. In other words the C18 bonded silica monolithic showed a column performance equivalent to approximately 3 μm packed column and a column permeability comparable to approximately 15 μm packed column.

The structure of silica monoliths and the flexibility to tailor the pore structure and the column format have initiated a number of systematic studies to model and to simulate the pore structure by the pore network model and to compare the results of model analysis with particle packed columns [178,179]. Liapis et al. stated [179]: “The results of this work indicate that since in monoliths the size of through-pores could be controlled independently from the size of the skeletons, then if one could construct monolith structures having (a) relatively large through-pores with high through-pore connectivity that can provide high flow-rates at low pressure drops and (b) small-sized skeletons with mesopores having an appropriate pore size distribution (mesopores having diameters that are relatively large when compared with the diameter of the diffusing solute) and high pore connectivity, $n_T$, the following positive results, which are necessary for obtaining efficient separations, could be realized: (i) the value of the pore diffusion coefficient, $D_{mp}$, of the solute would be large, (ii) the diffusion path length in the skeletons would be short, (iii) the diffusion velocity, $v_D$, would be high, and (iv) the diffusion response time, $t_{\text{drt}}$, would be small. Monoliths with such pore structures could provide more efficient separations with respect to (a) dynamic adsorptive capacity and (b) required...
pressure drop for a given flow-rate, than columns packed with porous particles."

The classical methods in characterizing the pore structure of monoliths, particular those in the capillary format have two major disadvantages: the conditions at which the materials are characterized do not resemble those applied in liquid phase adsorption processes as HPLC. Secondly, the amounts of stationary phase in monolithic silica capillaries are too small to be applied to classical characterization methods.

For these reasons we have focused on ISEC as an in situ characterization method [42]. ISEC is a well-known procedure [37–41]. We have improved the approach by including the parallel pore model (PPM) and the pore network model (PNM) to enable the calculation of several characteristic parameters from ISEC experiments. It should be emphasized in this context that the calculations are also valid for particle packed columns [42]: “The PPM and PNM proposed in this work are able to determine the void fractions of the macropores and silica skeleton, the pore connectivity of the mesopores, as well as the pore number distribution (PND) and pore volume distribution (PVD) of the mesopores. The results indicate that the mesoporous structure of all materials studied is well connected as evidenced by the similarities between the PVDs calculated with the PPM and the PNM, and by the high pore connectivity values obtained from the PNM. Due to the fact that the proposed models can predict the existence of the second inflection point in the exclusion curves, the proposed models could be more applicable than other models for ISEC characterization of chromatographic columns with small diameter macropores (interstitial pores) and/or large macropore (interstitial pore) void fractions. It should be noted that the PNM can always be applied without the use of the PPM, since the PPM is an idealization that considers an infinitely connected porous medium and for materials having a low (<6) pore connectivity the PPM would force the PVD to a lower average diameter and larger distribution width as opposed to properly accounting for the network effects present in the real porous medium.”

The logical next step is to use the results of modelling and simulation and to connect them to chromatographic performance parameters such as plate height, chromatographic resolution or peak capacity and column capacity. This allows one to optimize the parameters of the pore structure of monoliths with respect to certain target parameters provided one has the necessary know-how in the synthesis to achieve the desired values of parameters.

Key parameters in modelling and simulation of monoliths are [180]:

(i) $\Delta P$: column back pressure (linked to the volumetric flow rate, $Q_v$);
(ii) $r_{m}$: analyte characteristic molecular radius;
(iii) $K_{eq}$: analyte equilibrium adsorption constant;
(iv) $\varepsilon_t$: total column void fraction;
(v) $\varepsilon_b$: through-pore (macropore) void fraction;
(vi) $\varepsilon_p$: skeleton-pore (mesopore) void fraction;
(vii) $D_0$: skeleton diameter (characteristic transverse dimension) of the monolith;
(viii) $d_{mac}$: nominal diameter of the through-pores;
(ix) $d_{mes}$: nominal diameter of the skeleton-pores.

Another more pragmatic means is to experimentally measure chromatographic data such as plate height vs. linear velocity curves and resolution parameters and to connect the findings with the pore structural characteristics of the monolithic columns [181].

Even though the potential of using monolithic silica columns for peptide and protein separation was recognized [171,182–184], the pore structure of the monoliths needed to be adapted to the peptidic analytes to ensure a rapid mass transfer and a high accessibility of the stationary surface [181].

Numerous theoretical models proved this assumption of high porosity, high homogeneity and small-sized skeletons with mesopores large enough not to hinder the passage of the molecules in and out of the pore [178,179,185–187]. First, it was proven by a series of theoretical calculations, that a perfectly ordered flow-through pore network, instead of traditionally used packed bed columns, would gain efficiency in LC separation [188,189]. It was shown as well that a considered monolithic micro-structure column would allow to perform $N > 100,000$ plate separations in a few hundreds of seconds [190]. The further theoretical analysis led to the conclusion that the large porosity supports can always potentially yield shorter analysis times or larger plate numbers than small porosity supports but need sub micrometer feature sizes to actually achieve this [185,186]. If a strong improvement of the structural homogeneity (assuming constant domain size conditions) or a degree of the domain size (assuming constant homogeneity conditions) would be achieved by monolithic column synthesis, such a support would have an applicable potential, where no existing chromatographic supports seems to be able to operate [191].

### 3.2.2. Chromatographic properties

Major chromatographic features of monolithic silica columns arise from the large through-pore size/skeleton size ratios and high porosities, resulting in high permeability and large number of theoretical plates per unit pressure drop. High permeability and small diffusion path length provided by the presence of large through-pores and relatively small-sized skeletons resulted in the lower plate height and the lower pressure drop with monolithic silica columns compared with a particle-packed column, making faster separations possible with current instrumentation. Fig. 7 displays the potential of applying the monolithic silica column in gradient separation. For the detailed survey on articles touching the relationship between structural properties of silica monoliths and their chromatographic performance and selectivity see Table 5.

### 3.3. Polymer-based monolithic columns

#### 3.3.1. Synthesis approaches and characterization

As compared to the formation of silica monoliths the formation of porous cross-linked polymeric monoliths has a wide variety of facets in terms of starting monomers, co-monomers, initiators, solvents and porogens.
Table 5
Survey on important research articles connecting the structural properties with the chromatographic properties of silica monoliths

<table>
<thead>
<tr>
<th>Properties</th>
<th>Subject and specific features in brief</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast separation property due to the low-pressure drop across the column</td>
<td>Monolithic silica rod columns can be prepared with independent control of the mesopores and the through-pores (macropores) [2–4,153,156–172]. As a result, chromatographic columns with higher total porosity and bigger through-pore sizes as compared to particulate ones are obtained [4,158,169,192–194,175]. This enables one to perform faster separations [139,172,176,195–198,182,199–206].</td>
</tr>
<tr>
<td>High-efficiency property due to the fast mass transfer kinetics and high binding capacity of monolithic silica columns</td>
<td>Monolithic silica columns show high efficiency on the basis of the small-sized silica skeletons providing sufficient surface area for the separation and fast mass transfer kinetics due to the large-sized through-pores. Such monolithic silica columns were used as a robust and efficient separation medium for the peptide mapping by reversed-phase HPLC [206], separation of biological macromolecules [198,182] as well as separation of low molecular weight analytes [207,187,208–210].</td>
</tr>
</tbody>
</table>

Applications

- Analysis of low molecular weight analytes
- High throughput analysis of drugs and metabolites
- Separation of environmentally relevant substances and food additives
- Separation of enantiomers
- Separation of complex biological samples
- Separation of complex biological samples in multidimensional HPLC

The syntheses of macroporous cross-linked polymers, their structural characterization and their application in size-exclusion chromatography (SEC) is described in depth in a survey article by Seidl et al. [239]. The most in depth monograph in this field was published by Svec et al. [240]. The tailoring of the morphology of polymeric monoliths is shown for methacrylate-ester based monoliths by Eltink et al. [241].

Typically, the pore texture of polymeric monoliths can be described as an assembly of fused micro globules with graded densities. The interstices generate a macroporous system with pore diameters in excess of 100 nm. The reported specific surface area is much smaller than those of monolithic silicas (<50 m²/g). It seems that smaller pores (mesopores and micropores) are often present to a minor degree, their appearance depends on the solvent and the swelling properties of the material.

One major advantage of polymeric monoliths over silica monoliths is the fact that the surface functionality can be generated and controlled by the use of appropriate co-monomers. Thus hydrophobic, hydrophilic, polar and charged surfaces can be obtained. Also polymeric monoliths are manufactured in a wide variety of column formats as thin membranes, disks, capillary columns and large bore preparative columns.

3.3.2. Chromatographic properties

For the detailed survey on the chromatographic properties of polymeric monoliths, we refer to selected reading [242–245]. In general, polymeric-based monolithic columns, concerning their chromatographic properties, are applied in two areas:

(i) as monolithic capillaries with hydrophobic and other functionalities as capillaries in the separation of peptides and proteins, particular in the proteomics field [246–249];
(ii) as disks, rods and other formats in the isolation and purification of biopolymers. Poly(methylmethacrylate) monoliths...
Table 6
Structural and chromatographic parameters of monolithic silica columns Chromolith\textsuperscript{®} Performance (RP 18e) and microparticulate silica columns (C18 reversed phase)

<table>
<thead>
<tr>
<th>Property</th>
<th>Monolithic silicas (chromolith)</th>
<th>Microparticulate silicas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pore structural data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pore modality</td>
<td>Bimodal</td>
<td>Bimodal</td>
</tr>
<tr>
<td>Mesopore diameter</td>
<td>13 nm</td>
<td>7–30 nm</td>
</tr>
<tr>
<td>Column porosity and specific pore volume, respectively</td>
<td>Column porosity &gt;80%, and pore volume approximately 1 cm(^3)/g</td>
<td>Specific pore volume of the particles 0.3–1.1 cm(^3)/g; column porosity depends on the packing density of particles and can be in the range of 40–60%</td>
</tr>
<tr>
<td>Macropore diameter</td>
<td>2 (\mu)m (flow through pores)</td>
<td>Interstitial pores (pore size approximately 40% of average particle size)</td>
</tr>
<tr>
<td>Column macroporosity</td>
<td>Approximately 55%</td>
<td>35–50% (interstitial column porosity)</td>
</tr>
<tr>
<td>Specific surface area</td>
<td>300 m(^2)/g</td>
<td>70–510 m(^2)/g</td>
</tr>
<tr>
<td>Chromatographic parameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column format ((L); I.D.)</td>
<td>100 mm; 4.6 mm</td>
<td>100–250 mm, 4–4.6 mm</td>
</tr>
<tr>
<td>Column hardware</td>
<td>PEEK</td>
<td>Usually stainless steel</td>
</tr>
<tr>
<td>Column pressure drop</td>
<td>&lt;20 bar at 1 mL/min</td>
<td>40–150 bar at 1 mL/min</td>
</tr>
<tr>
<td>Volume flow-rate range</td>
<td>0.01 mL/min–10 mL/min</td>
<td>0.01 mL/min–5 mL/min</td>
</tr>
<tr>
<td>Plate height values\textsuperscript{a}</td>
<td>Approximately 12 (\mu)m</td>
<td>&gt;9 (\mu)m</td>
</tr>
<tr>
<td>Plate number values\textsuperscript{a}</td>
<td>Approximately 80,000 N/m</td>
<td>30,000–110,000 N/m</td>
</tr>
</tbody>
</table>

*Comparison data on commonly used C18 phases, of average particle size from 4 to 10 \(\mu\)m.\textsuperscript{a} Values are given for a neutral compound.*

4. Comparison of the structure and performance of particle packed and monolithic columns

The column performance characteristics of poly (methylmethacrylate) monolithic capillaries under the aspects of kinetic plots was studied in detail by Eeltink et al.\textsuperscript{[241]}. The column performance of a 5 \(\mu\)m C 18 bonded silica column was compared with a monolithic C 18 bonded silica column in Reversed Phase HPLC under isocratic conditions. The particle packed column showed a plate height \(H\) of 10–15 \(\mu\)m at a linear velocity of 1 mm/s. The \(H\) vs. \(u\) curve of the monolithic column followed the same course but remained nearly parallel to the abscissa up to a linear velocity of 7 mm/s. At this high velocity the packed column could not be operated due to the high column back pressure. The monolithic RP column showed a column back pressure which was three to five times lower than the particle packed column\textsuperscript{[169]}. A fundamental examination on the chromatographic performance of monolithic silica columns as compared to particle packed columns was made by Leinweber et al.\textsuperscript{[175]}. The band dispersion characteristics of insulin and angiotensin II on monolithic C18 bonded silica columns were examined. From the \(H\) vs. \(u\) curves the mechanical and non-mechanical contribution of the dispersion to the total plate height was analyzed. The superposition of the reduced plate height – reduced velocity curves indicated that the mass transfer properties of the monolithic columns in the mesopores corresponded to that of packed columns with an equivalent dispersion particle diameter of 3 \(\mu\)m.

Table 7
Comparison of pore structural and operational parameters of monolithic polymeric columns and research monolithic silica columns

<table>
<thead>
<tr>
<th>Property</th>
<th>Cross-linked polymeric monoliths</th>
<th>Silica monoliths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pore modality</td>
<td>Unimodal (broad)</td>
<td>Bimodal (macro + meso)</td>
</tr>
<tr>
<td>Macropore diameter</td>
<td>0.05–10.0 (\mu)m</td>
<td>1–10 (\mu)m</td>
</tr>
<tr>
<td>Macroporosity</td>
<td>10–95%</td>
<td>40–70%</td>
</tr>
<tr>
<td>Total column porosity</td>
<td>Low-density and high density monoliths</td>
<td>0.8–0.9</td>
</tr>
<tr>
<td>Pore morphology</td>
<td>Globular structure</td>
<td>Spongy and worm-like structure</td>
</tr>
<tr>
<td>Surface functionality</td>
<td>Adjusted by functional co-monomers</td>
<td>Introduced by grafting from and grafting on surface modification</td>
</tr>
<tr>
<td>Column format</td>
<td>Preparative size to capillary formats</td>
<td>Analytical, microbore, capillary formats</td>
</tr>
<tr>
<td>Column pressure drop</td>
<td>Distinct higher column back pressure as compared to monolithic silica columns</td>
<td>1/3–1/5 as compared to 5 (\mu)m packed columns</td>
</tr>
<tr>
<td>Linear velocity range</td>
<td>1–7 mm/s and higher</td>
<td>1–7 mm/s and higher</td>
</tr>
<tr>
<td>Plate height and plate numbers</td>
<td>(H = 5–10 \mu)m at optimum (u)</td>
<td>(H = 5–10 \mu)m at optimum (u)</td>
</tr>
<tr>
<td>pH range for application</td>
<td>Acidic to strong alkaline</td>
<td>Acidic to pH 8</td>
</tr>
<tr>
<td>Typical application areas</td>
<td>Separation of peptides and proteins (analytical) Isolation and purification of biopolymers</td>
<td>Separation of low molecular weight compounds and peptides</td>
</tr>
</tbody>
</table>
The through pore system of the monolithic column controlling the hydraulic permeability was translated into an interstitial pore system of a packed bed. The comparison resulted in an equivalent permeability particle diameter of 15 μm.

Tanaka et al. [169] applied the kinetic plots (log \( t_0/N \) vs. \( N \)) as a measure to demonstrate the superior performance of monolithic silica capillary columns vs. microparticle packed beds in the capillary format.

In a more fundamental study Gzil et al. [185] treated the impact of variable bed porosity on the chromatographic performance parameters. They defined limiting cases for separations requiring small plate number and high plate numbers and linked this parameter to the optimum external porosity.

Tables 6 and 7 provide a comparison of the structural and chromatographic properties of monolithic silica columns and microparticulate silica columns (Table 6) and the characteristics of polymer based and monolithic silicas (Table 7).

5. Conclusion – where are we now and where are we going?

5.1. Where are the needs and where are the alternatives?

The success of HPLC as a widely accepted separation technique and platform at analytical scale, preparative scale and process scale was a result of an interdisciplinary and integrated research and development combined with a rapid technology transfer into effective, versatile robust and reliable systems. The central and most essential part of an HPLC system is the separation column. More than 20 years of academic, technical and engineering knowledge and experience have been invested into the manufacture of selective, efficient, reproducible and robust columns. Most of the achievements made are based on experimental and technical experience knowing the needs of the users and the demands in the respective application areas rather then on a solid fundamental theoretical basis.

Contrary to many predictions and expectations, 4 and 4.6 mm I.D. columns packed with microparticulate C18 bonded silicas dominate the market. There is a slight tendency to the use of miniaturized column formats [250,251]. This means that microparticulate packed C18 columns will maintain its leading position as benchmark columns in the future. The high surface area and the relatively high mass loadability make them most suitable as working horses in HPLC technology.

Miniaturized columns such as micro bore and fused silica capillaries cover only a minor part in application. They are mostly applied in research laboratories. The reasons for this fact are that firstly one needs a miniaturized HPLC equipment (at least for fused silica capillaries) and secondly, experience and skills are required to operate such systems in an optimum way.

Monolithic columns are still the major subject of extensive research. Monolithic columns as compared to particulate ones have the ability and potential of designing an optimum structure, which will lead to designed columns with optimum performance and selectivity for the various facets of application areas. Apart from this option monolithic columns offer a high robustness and easier maintenance than packed columns with frits.

Most of the modelling and simulation activities are seen in the field of monolithic silicas. There is still an unexplored potential in the fundamental understanding and design of polymer-based monoliths.

The results obtained on columns packed with 2 μm spherical particles hyphenated to mass spectrometers as second separators and detector will meet the current demands on sensitive and fast high resolution separations in pharmaceutical and chemical analysis.

The future challenges and highest demands on liquid based separation techniques will originate in life science applications. On the top is the search for biomarkers for diagnostic purposes and the development of therapeutics. Related to that is the isolation and purification of biotech products such as recombinant proteins and vaccines by bio-processing.

The sample mixtures in bio fluids from human sources, animals and plants are extremely complex with regard to chemical composition and structure. They contain a large number of chemical entities and cover a large range of abundance ratio.

The resolution of such complex mixtures can be only accomplished by using multidimensional LC systems with selective phase systems in combination with powerful detection system as e.g. mass spectrometers. Selectivity is the key target parameter and can be achieved by a design of novel materials with superior mass transfer properties and advanced surface functionalities.

The potential of packing and stationary phase design and development has not been explored to the full extent.

Other powerful separation techniques based on electro-driven systems are still in their infancy and promise a high potential of selective molecular recognition for polar and charged analytes.

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The authors are grateful to Dr. J. J. Kirkland, Dr. R. Ditz and Professor K. Sing for critical comments and suggestions to improve the manuscript as well as to Merck KGaA, Darmstadt, Germany for financial, material, and intellectual support for this work.

Appendix A. Survey on the most common methods of particle size analysis of HPLC packings

A.1. Sieve and classification analysis

One of the oldest and easiest methods to determine the particle size distribution is the sieve analysis. Because of its ease of use this method is widely applied in official methods, e.g. many pharmacopoeias (USP, Ph.Eur.). The mesh width of sieves, used for sieve analysis, is standardized according to DIN-methods (DIN 4188) or US-standard methods (ASTM E11-70/E 161-70). The most serious drawback of sieve analysis is the application range, which is to particles >20 μm. For the analysis of finer powders the sieve analysis has to be combined with other methods.
A.2. Light scattering

When light hits a collective of particles it is partly absorbed and partly diffracted. The angle and the intensity of the diffracted light are depending on the size of the particle. At a constant wavelength large particles diffract the light into small scattering angles and small particles with low intensity into high scattering angles. The intensity of the diffracted light can be used for quantification. Several systems with different geometric design of the equipment have been developed over the last few years. Most of the systems use lasers as the source of focused light. As light scattering is a technique, which is applied to a multitude of particles, the biggest problems stems from the deconvolution of the obtained light pattern.

When comparing particle size distributions it is very important to know which instrument is used, because the recorded values can be considerably affected by differences in optics design, software algorithms and sample preparation and concentration [252–254].

A.3. Sedimentation

The sedimentation of a particle in a liquid is dependent on its size, density and form in accordance with Stoke’s law (Eq. (8)):

\[ d_p = \sqrt{\frac{18\eta}{(\rho - \rho_d)g}} \sqrt{\frac{h}{t}} \]  

(8)

g, gravitation; \( h \), falling height; \( t \), falling time; \( \eta \), viscosity; \( (\rho - \rho_d) \), difference in density between the particle and the dispersion solvent.

For particles as small as 0.5 \( \mu \)m the measurement can be done under the earth’s gravity. One of the oldest instruments for sedimentation analysis is the ANDREASEN-pipette, where at definite times samples are withdrawn from a sedimenting suspension and the mass fraction is determined by weighing. Modern systems are working with light or X-ray detection of the particles passing by the analysis window. To speed up analysis time the sample cell itself can be moved at a given rate passing the detector (wide-angle scanning photo-sedimentometer).

Some prerequisites must be fulfilled for sedimentation analysis: the sedimentation liquid has to be totally inert and must have a density, which is lower than the particle density. No convection within the liquid should take place due to temperature or density gradients. As a high particle concentration can cause density gradients, the particle concentration should be below 1%. For systems working with the earth’s gravitation the minimum particle diameter is around 1 \( \mu \)m because of the Brownian motion, which disturbs the measurement of smaller particles.

Sedimentation analysis gives relatively high resolution results and is used for a wide range of materials, including the determination of agglomerates [255].

A.4. Electronic measurements

The change of the electrical resistance is measured in an electro stream counter. The system most commonly used is the Coulter Counter. The particles of interest have to be suspended in a conducting electrolyte and are passed through an orifice, which is situated between two electrodes. For the conducting electrolyte the same prerequisites have to be fulfilled as for the suspension liquid in sedimentation analysis. In most cases sodium chloride solutions at low concentrations are used. The particles within the suspension liquid are forced through the small orifice by means of a controlled vacuum. On either side of the orifice two electrodes are mounted. Due to the presence of a particle within the inspection zone, which exhibits a high electrical field, the resistance of the electrolyte changes, generating a voltage pulse the amplitude of which is assumed to be proportional to the volume of the particles.

A.5. Microscopic methods

Microscopic methods are characterized by a particular disadvantage and one great advantage. The disadvantage is given by the fact that only a small population of particles is used for the determination of the particle size distribution. Before the programs of computer-automated image analysis became available the determination of the particles size distribution was very tedious and laborious since it involved direct comparison with sets of reference circles (the reticule, engraved on the eyepiece of the microscope). Even with the newest powerful image analysis programs hundreds of digitized images may be required to get enough information to establish statistical significance for the particle size determination.

The biggest advantage, which all image analysis systems have in common, is the shape information obtained from single particles. Only by using microscopic techniques is it possible to judge the quality of a chromatographic sorbent in terms of its physical uniformity. The most valuable information obtained from microscopy is the determination of fines in the micrometer and sub-micron range attached to particles (Fig. 8A) and the identification of miss-formed and broken particles (Fig. 8B).

A.6. Field flow fractionation

The combination of two different forces is used in the field flow fractionation technique (FFF), where a gravitational (GFFF) or centrifugal field is applied perpendicular to a flow direction. The particles are placed in an empty capillary and moved by the liquid flow in one direction. Due to the applied field the particles are forced according to their size towards a wall of the capillary. The particles are driven by the mobile phase towards the capillary outlet at different velocities depending on their size. The particle size distribution is recorded by an UV-detector placed at the end of the capillary. With this instrumental setup it is possible to determine the particle size distribution of chromatographic sorbents either of inorganic or organic nature in the particle size range from 1 to 100 \( \mu \)m [256–258]. The method is supposed to be accurate, fast and inexpensive, as standard HPLC equipment can be used for the measurement. Only recently first attempts have been made to set up a standardless method for the measurement of silica particles. The method
size-distribution profiles without calibration of the system [259].

allows the direct conversion of fractograms into quantitative,
size-distribution profiles without calibration of the system [259].

References