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# Characterization of new types of stationary phases for fast liquid chromatographic applications

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ABSTRACT

The performance of a narrow bore silica based monolith column (5 cm × 2 mm) was compared to 5 cm long narrow bore (internal diameter ≤ 2.1 mm) columns, packed with shell particles (2.7 μm) and totally porous sub-2 μm particles (1.5 μm, 1.7 μm and 1.9 μm) in gradient and isocratic elution separations of steroids. The highest peak capacity could be achieved with the column packed with 1.5 μm totally porous particles. The columns packed with porous 1.7 μm and shell 2.7 μm particles showed very similar capacity. The monolith column provided the lowest capacity during gradient elution. The plate height (HETP) of the 2.7 μm Ascentis Express column was very similar to the HETP obtained with 1.5 μm and 1.7 μm totally porous particles. The Chromolith monolithic column displayed an efficiency that is comparable to that of columns packed with spherical particles having their diameter between 3 μm and 4 μm. A kinetic plot analysis is presented to compare the theoretical analysis speed of different separation media. At 200 bar, the monolith column provided the highest performance when the required plate number was higher than 5000 ($N > 5000$), however the efficiency drifted off faster in the range of $N < 5000$ than in the case of packed columns. If the possibility of maximum performance was utilized (1000 bar for sub-2 μm particles, 600 bar for shell particles and 200 bar for monolith column) the monolith column would provide the poorest efficiency, while the column, packed with 1.5 μm particles offered the shortest impedance time.



## 1. Introduction

Modern applications of chromatography now require faster and more efficient separations than those used in the past. Also in academic and industrial research the cutting of the separation time is in focus. The first priority for performing a fast separation is the selection of the appropriate stationary phase. Mobile phase optimization is also crucial to achieve the required selectivity and promote the short separation time but in this study we are focusing on column selection. The speed of analysis can be increased with different approaches.

One opportunity to get higher efficiency is to reduce the particle size. The particle size is a beneficial factor as it improves the rate of mass transfer and reduces the eddy diffusion effect, resulting smaller plate height and higher optimum linear velocity as the particle size is reduced. On sub-2 μm particles, due to the narrow peaks, sensitivity and separation are improved at the cost of pressure. It was proved that the analysis time could be reduced to a 1 min or 2 min interval without the loss of resolution and sensitivity [1,2]. Short columns, usually less than 50 mm in length, are run at high linear velocities giving high sample throughput. The inner diameter can be varied between 2 mm and 5 mm. The 4.6 mm inner diameter is a preferred size in high performance liquid chromatography (HPLC). HPLC systems offer about 400 bar at maximum performance. Flow resistance is inversely proportional to the square of particle size therefore in most cases the pressure limit of 400 bar was not enough to achieve a high-speed analysis. A new nomenclature has come about with the term ultra-high-pressure liquid chromatography (UHPLC). It was done so to describe the higher backpressure requirement. The first system for ultra-high-pressure separation was released in the year of 2004. The new hardware was able to work up to 1000 bar (15 000 psi) and the system was called ultra performance liquid chromatography (UPLC). The small particle size (less than 2 μm) and small column dimensions (length and inner diameter) resulted small peak broadening. Extra column effects are more significant for scaled down separations, therefore it is essential to minimize extra column dispersion. The inner diameters of the tubes, injector volume, detector volume and electronics must be changed to meet the requirements of small peak broadening.

Temperature in HPLC also offers a chance to cut the analysis time. High temperature reduces the viscosity of mobile phase and

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increases mass transfer. Analysis time can be shortened without the loss of resolution through column heating [3–6]. Systems with a maximum pressure capability of 400 bar can then be used with the sub 2 μm columns without over-pressuring the pump. Preheating of the mobile phase is essential to avoid brand broadening. This technique is sometimes called high temperature liquid chromatography (HTLC).

The third possibility to enhance the separation speed is the reduction of the intrinsic flow resistance by increasing the external porosity and the flow-through pore size of the packing. The monolith approach, originally initiated by the work of Hjertén et al. [7], Svec and Frechet [8], Horvath and coworkers [9], Tanaka and coworkers [10], which already lead to a number of well performing, commercially available polymeric and silica monolith columns [11,12]. Commercial silica monolith columns are the Chromolith (Merck KgGa, Darmstadt, Germany) and Onyx (Phenomenex, Torrance, CA, based upon technology licensed from Merck). These silica rods have a bimodal pore structure, which means that the columns possess a combination of very large internal surface area over which chemical adsorption can take place with high total porosity. However, within the skeletal structure of the rod there is a further network of mesopores, each 13 nm in diameter, which creates the large internal surface area. The flow resistance of a monolith column is similar to a column packed with 10 μm particles. The kinetic efficiency of monolith columns is comparable to columns packed with 3–5 μm particles. When monolith columns were introduced the commercially available columns were 5 cm and 10 cm long and 4.6 mm in diameter. The analysis time can be shortened with enhancing the flow rate of the mobile phase. Operations applying high flow rate cannot be used for mass spectrometric (MS) detection. This latter argument was the starting point of decreasing the monolith column dimensions. First the length was cut to 2.5 cm, after the inner diameter was decreased from 4.6 mm to 3 mm and 2 mm. Applying these narrow monolith columns, high-speed separation and MS detection can also be done with moderate flow. With the 2 mm internal diameter columns, the flow rates are typically under 1 ml/min in line with the requirements of electrospray ionization and with acceptable separation times. In addition, the smaller internal diameter columns save solvent for conventional LC–UV users due to smaller column volumes. Guiochon has presented in his review a practical insight about the principles and theory of monolith columns [13].

The concept of superficial or shell stationary phases, was introduced by Horvath et al. [14,15]. Horvath applied 50 μm glass bead particles covered with styrene-divinylbenzene based ion exchange resin. It became known as pellicular packing material. Later Kirkland presented, that 30–40 μm diameter superficially porous packings (1 μm phase thickness, 100 Å pores) provided much faster separations, compared with the large porous particles used earlier in liquid chromatography [16]. Later on the core diameter was reduced and the thickness of active layer was cut to 0.5 μm and was used for fast separation of peptides and proteins [17]. The most recent introduction of a superficially porous particle is the so-called fused-core particle [18,19]. Fused-core packing materials are commercially available in different diameters (2.7 μm and 5 μm). The 2.7 μm particles consist of a 1.7 μm nonporous core and a 0.5 μm porous silica layer, and the 5 μm particles consist of a 4.5 μm nonporous core and a 0.25 μm porous silica layer. Studies have proven [20] that the peak broadening is larger than we would think about the shorter diffusion path gives. It can be explained by the rough surface of particles in which the mass transfer rate is reduced through the outer stagnant liquid [21].

At practical work we can either choose a sub-2 μm column, monolith column or shell core column for high-speed separation. All types of columns have their own experimental barriers and the applied chromatographic systems also have limitations such as the allowable maximum operating pressure. The comparison of these different structured columns is not evident. The rate theory is well known but porous materials have different characteristic parameters compared to monolithic ones. Obtaining a clear insight about the separation speed and efficiency the kinetic plot method could be a useful tool [22–24].

In this study the effectiveness of sub-2 μm totally porous particles, porous silica layered solid core type (2.7 μm) particles and a monolith column were compared under isocratic and gradient elution conditions. The test analytes were steroids and a non-steroidal hormone bicalutamide (polar neutral compounds), which are used as a treatment in contraception, climax, prostatic hyperplasia, prostate cancer and hirsutism.

In our study the calculation of kinetic parameters in the case of the monolith column was related to 200 bar. The commercial rod columns are packaged in polytetrafluoroethylene (PTFE) or polyetheretherketone (PEEK) tubes that can hardly withstand pressures higher than 200 bar, in contrast with conventional columns that are packed in far stronger stainless steel tubes. This limits the velocity at which these columns can be used. In the literature we could not find any data for the pressure resistance of silica skeleton therefore we accepted this technical limitation of 200 bar. The conventional HPLC systems work up to 400 bar. At this pressure limit the performance of different columns was compared. The shell core particles are certified up to 600 bar, so calculations were also performed relating to this 600 bar limit. UPLC has 1000 bar as an upper pressure limit for columns packed with sub-2 μm particles. Our final calculation was related to 1000 bar for sub-2 μm particles, 600 bar for shell particles and 200 bar for the monolith column.

## 2. Experimental

### 2.1. Chemicals, column

Acetonitrile and methanol (gradient grade) were purchased from Merck (Darmstadt, Germany). For measurements water was prepared freshly using Milli-Q® equipment (Milli-Q gradient A10 by Millipore).

The test analytes and Rigevidone tablet (0.03 mg ethinylestradiol, 0.15 mg levonorgestrel/tablet) were produced by Gedeon Richter Plc (Budapest, Hungary). The purity of the analytes (by HPLC) was as follows: dienogest (17α-cyanomethyl-17β-hydroxy-estra-4,9(10)-diene-3-one) 99.8%, finasteride (N-tert-butyl-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide) 99.9%, gestodene (13-ethyl-17-hydroxy-18,19-dinor-17α-pregna-4,15-dien-20-yn-3-one) 99.7%, levonorgestrel (13-ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one, (−)) 99.9%, estradiol (estra-1,3,5(10)-triene-3,17-β-diol) 98.3%, ethinylestradiol (19-nor-17α-pregn-1,3,5(10)-trien-20-yn-3,17-diol) 99.9%, noretistherone acetate (17-acetoxy-19-nor-17-pregn-4-en-20-yn-3-one) 99.7%, tibolone (17-hydroxy-7α-methyl-19-nor-17α-pregn-5(10)-en-20-yn-3-one) 99.8% and bicalutamide (N-[4-cyano-3-(trifluoromethyl)phenyl]-3-(4-fluorophenyl)sulfonyl)-2-hydroxy-2-methyl propanamide, (±)) 99.7%.

Ascentis Express C18 column (Supelco) with a particle size of 2.7 μm (50 mm × 2.1 mm) was purchased from Sigma–Aldrich Ltd., Budapest. Waters UPLC™ BEH C18 column with a particle size of 1.7 μm (50 mm × 2.1 mm) was purchased from Waters Ltd., Budapest. Grace Vision HT C18 column with a particle size of 1.5 μm (50 mm × 2.0 mm) was purchased from Lab-Comp Ltd., Budapest. Hypersil Gold C18 column (Thermo) with a particle size of 1.9 μm (50 mm × 2.1 mm) was purchased from Lab-Comp Ltd., Budapest. Chromolith FastGradient RP-18e column (50 mm × 2.0 mm) was purchased from Merck Ltd., Budapest.

### 2.2. Equipment, softwares

Throughout the measurements a Waters Acquity UPLC™ (ultra performance liquid chromatography) system with Empower software from Waters Ltd., Budapest, Hungary, and an Agilent 1200 RRLC (rapid resolution liquid chromatography) system with Chemstation software were employed. Calculation and data transferring to obtain the kinetic plots was achieved by using the Kinetic Method Plot Analyzer template (Gert Desmet, Vrije University Brussel, Belgium). Image-J (freeware image-processing software program developed at the National Institutes of Health) was used to determine the average domain size of the monolith column material. Solvent optimization was performed using Dry Lab 2000 Plus chromatography optimization software (Molnar-Institute Berlin, Germany).

### 2.3. Apparatus and methodology

The mobile phases were prepared by mixing appropriate amount of HPLC gradient grade acetonitrile and Milli-Q water. The mixtures were degassed by sonication for 5 min.

The stock solutions of reference standards were dissolved in methanol (1000 μg/ml). The solutions for the chromatographic runs were diluted from the stock solutions with acetonitrile/water 40/60 (v/v). The concentration of the test analytes was 10 μg/ml.

For the measurement of peak capacity, gradients with different time (5 min, 10 min, 15 min, 20 min and 25 min) were run from 10% to 80% acetonitrile. For this study 35 °C column temperature, 0.5 ml/min flow-rate, 0.5 μl injection and detection at 210 nm were applied.

The kinetic efficiency of the columns were determined with a mobile phase containing 40% acetonitrile, 35 °C column temperature, 0.5 μl injection and detection at 210 nm were applied. The flow-rate was varied from 0.05 ml/min up to 1.0 ml/min.

The measured plate numbers were corrected for extra-column volume and band broadening, which was measured by injecting levonorgestrel with a zero-dead-volume connector in place of the column. The plate heights for kinetic curves were calculated using the corrected plate counts.

### 2.4. Equations used for calculation

Peak capacity defines a measure of the column performance under gradient conditions [25–28]. Conditional peak capacity can be calculated with very simple formula, which uses the data of obtained chromatograms, such as retention times, average peak width and gradient duration time.

In this study we used the following equation to determine peak capacity

$$n_c^* = 1 + \frac{t_G}{w} \quad (1)$$

where $t_G$ is the gradient duration, and $w$ is the average peak width.

The column efficiency is mostly illustrated by the Van Deemter curves. Previously Desmet et al. [23,24] showed that it is very straightforward to map the kinetic performance of a given chromatographic support type by taking a representative set of the Van Deemter curve data and re-plotting them as $H^2/K_{V0}$ versus $K_{V0}/(uH)$ instead of $H$ versus $u$. The minimal analysis time can be calculated by simple rearranging the data in a measured Van Deemter curve and the value of the column permeability ($K_{V0}$). The following equations transform the linear velocity–plate height data into $t_0$ time versus plate number ($N$).

$$N = \frac{\Delta P}{\eta}\left(\frac{K_{V0}}{u_0 H}\right) \quad (2)$$

$$t_0 = \frac{\Delta P}{\eta}\left(\frac{K_{V0}}{u_0^2}\right) \quad (3)$$

$N$ is plate number, $\eta$ mobile phase viscosity, $\Delta P$ available pressure drop, $K_{V0}$ column permeability, $u_0$ linear velocity, $H$ plate height. The obtained values correspond directly to the minimal $t_0$ time needed in a column taken exactly long enough to yield a given number of theoretical plates. It is easy to combine the given $N$ value with the corresponding plate height value to obtain the corresponding column length.

The most common type of kinetic plot represents $t_0/N^2$ values as a function of $N$.

$$\frac{t_0}{N^2} = \frac{\eta}{\Delta P}\left(\frac{H^2}{K_{V0}}\right) = \left(\frac{\eta}{\Delta P}\right) E_0 = t_E \quad (4)$$

$E_0$ is defined as the separation impedance number [29]. The time required to obtain a certain resolution for a separation, with a specific pressure drop, is directly proportional to the separation impedance of the column. The lower the separation impedance, the better is the performance of the column. Plotting $t_0/N^2$ ratio as a function of $N$ and reversing the direction of the $N$ axis, the obtained impedance time plots still represent the same kinetic information as the plot of $t_0$ versus $N$, but regain the familiar view of a conventional Van Deemter curve.

## 3. Results and discussion

### 3.1. Peak capacity

At first for a basic comparison peak capacity curves were measured for all five columns. The injected sample contained the nine model compounds (10 μg/ml) diluted with acetonitrile/water 40/60 (v/v). The measurements were carried out with the columns kept at a constant temperature of 35 °C. A flow-rate of 0.5 ml/min, injection volume of 0.5 μl and detection at 210 nm were applied. For the measurements a Waters UPLC and Agilent 1200 RRLC system was applied. Fig. 1 shows the obtained peak capacity curves of the different columns versus the gradient span.

The highest peak capacity could be achieved with the column packed with 1.5 μm totally porous particles. The columns packed with porous 1.7 μm and shell 2.7 μm particles show very similar capacity. The monolith column provides the smallest capacity during the gradient elution. Peak capacities of about 130–150 could be achieved in 25 min with the 5 cm long column packed with sub-2 μm totally porous or shell particles when steroid compounds are separated. The monolith column (50 mm × 2 mm) offers a peak capacity value of about 120 during a 25 min gradient span.

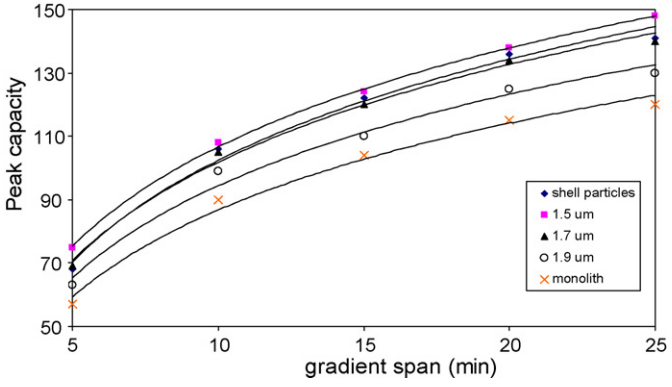


**Fig. 1.** Peak capacity curves (35 °C) of columns packed with 2.7 μm shell and sub-2 μm totally porous particles, and monolith column (5 cm long columns). Mobile phase: acetonitrile–water gradient, flow: 0.5 ml/min, injection: 0.5 μl, solute: mixture of 9 model compounds.

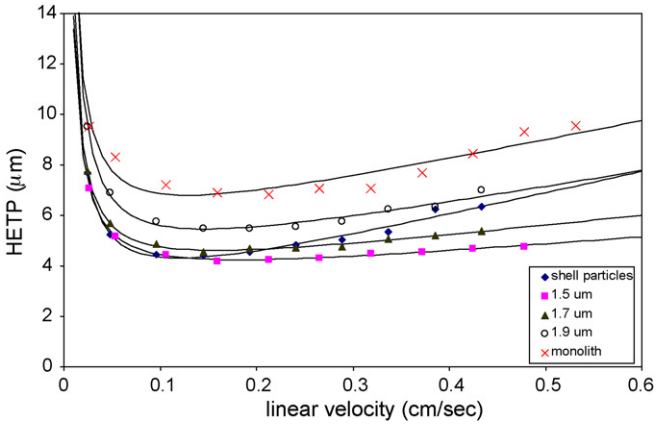


**Fig. 2.** Experimental Van Deemter plots of 2.7 μm shell and sub-2 μm totally porous particles and a monolith column (peak widths are corrected for the extra-column broadening). Mobile phase: 40% acetonitrile–60% water, temperature: 35 °C, injection: 0.5 μl, solute: levonorgestrel.

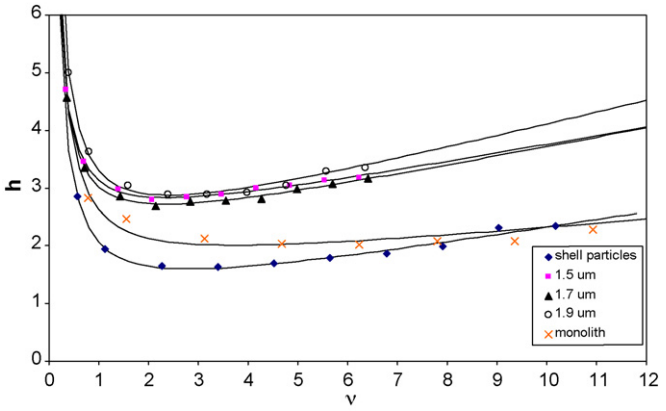


**Fig. 3.** Reduced plate heights of 2.7 μm shell and sub-2 μm totally porous particles and a monolith column. Mobile phase: 40% acetonitrile–60% water, temperature: 35 °C, injection: 0.5 μl, solute: levonorgestrel.

### 3.2. Column efficiency

The efficiencies of the five columns used in this study were measured at 35 °C by means of the Van Deemter plots. 0.5 μl of a 10 μg/ml levonorgestrel solution in acetonitrile/water 40/60 (v/v) was injected to acquire the data using the same solvent mixture as the eluent. This mobile phase composition gave the retention factor of levonorgestrel between 4 and 10 on each stationary phase. The flow-rate was varied from 0.05 ml/min up to 1.0 ml/min in the case of packed columns and from 0.05 ml/min up to 1.2 ml/min in the case of the monolith column. The measurements were carried out on a Waters Acquity UPLC system.

Fig. 2 shows the obtained HETPs (micrometer) versus the linear velocity. The fitting parameters of $A$, $B$, $C$, the calculated $u_{opt}$, and $HETP_{min}$ values are reported in Table 1.

The plate height of the 2.7 μm Ascentis Express column is very similar to the HETP of 1.5 μm and 1.7 μm totally porous particles. The Chromolith monolithic column displayed an efficiency that is comparable to that of columns packed with spherical particles with diameter between 3 μm and 4 μm.

The C term for shell particles and monolith columns is significantly higher than for sub-2 μm particles. In the case of shell particles it can be explained by the rough surface of particles in which the mass transfer rate is reduced through the outer stagnant liquid [20].

The optimum of mobile phase velocity is the highest when the sub-2 μm packed columns ($u_{opt} \approx 0.15$–$0.18$ cm/s) are used. In the case of shell particles the low optimum linear velocity might also be the consequence of the rough surface of shell particles [21].

From the experimental Van Deemter plots the reduced plate heights were also calculated. There is no clear-cut definition how to calculate the reduced plate height of monolith phases. On the theoretical front, Vervoort et al. [30] developed a structural model of the monolith and calculated the influence of the average size of the domains on the column efficiency. Minakuchi et al. [10] calculated the reduced plate height on the basis of the domain size, $d_{dom}$, with $h = H/d_{dom}$. Scanning electron microscopic (SEM) images and Image-J (image-processing software) were used to determine the average domain size of the Chromolith FastGradient RP-18e material in this study. The domain size was evaluated as Tanaka and coworkers [10] suggested. The sum of the size of a through-pore and the size of the neighboring silica skeleton measured along two lines perpendicular to each other. An average domain size of $d_{dom} = 3.38$ μm was obtained for the Chromolith column.

Fig. 3 shows the reduced HETPs ($h = H/d_p$ and $H/d_d$), with $H = A + B/u + Cu$, $d_p$ the particle size and $d_d$ the domain size, versus reduced velocity ($\nu = ud_p/D_M$ and $ud_d/D_M$) with $D_M$ the diffusion coefficient in the mobile phase.

The column of 2.7 μm shell particles has a reduced plate height minimum of approximately $h = 1.6$ in this study. The lowest reduced plate height ever reported of columns packed with shell particles (HALO) is about $h = 1.4$ [18]. The monolith column provided a reduced plate at the optimum flow rate of $h = 2.0$. Columns of the sub-2 μm particles showed higher reduced plate values. The three columns packed with totally porous particles display a very similar ($h \approx 2.8$) reduced plate height. The very small reduced plate height of the column packed with shell particles can be explained by the shorter diffusion path and by the very narrow particle size distribution [20,21,31].

### 3.3. Kinetic plots

The permeability of the five columns was assessed from the plot of the experimental column pressure ($P$) versus flow-rate (Fig. 4).

**Table 1**
Van Deemter parameters, $u_{opt}$, $HETP_{min}$ and $h_{min}$ values of levonorgestrel on the five columns at 35 °C.

| Parameters | Ascentis Express C18 (2.7 μm) | Grace Vision C18 (1.5 μm) | Acquity BEH C18 (1.7 μm) | Hypersil Gold C18 (1.9 μm) | Chromolith FastGradient RP-18e |
|---|---|---|---|---|---|
| $k$ (retention) | 7.02 | 8.57 | 6.05 | 7.70 | 4.52 |
| $A$ | 2.17 | 3.11 | 3.27 | 3.50 | 4.77 |
| $B$ | 0.13 | 0.10 | 0.11 | 0.14 | 0.13 |
| $C$ | 8.95 | 3.11 | 4.27 | 6.72 | 7.96 |
| $u_{opt}$ (cm/s) | 0.12 | 0.18 | 0.16 | 0.15 | 0.13 |
| $HETP_{min}$ (μm) | 4.31 | 4.24 | 4.62 | 5.46 | 6.80 |
| $h_{min}$ | 1.60 | 2.83 | 2.72 | 2.87 | 2.01 |

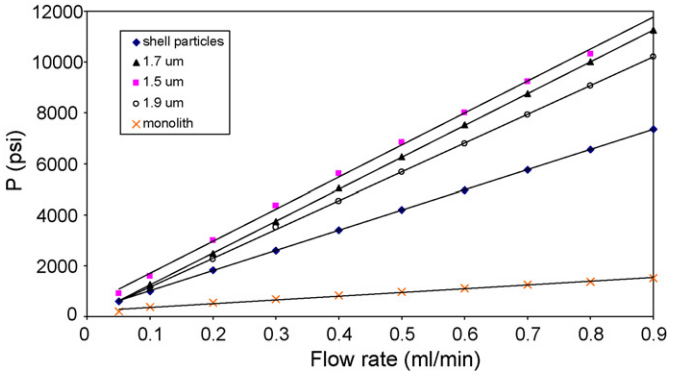


**Fig. 4.** Column permeability of columns packed with 2.7 μm shell and sub-2 μm totally porous particles, and a monolith column (5 cm long columns). Mobile phase: 40% acetonitrile–60% water, temperature: 35 °C.

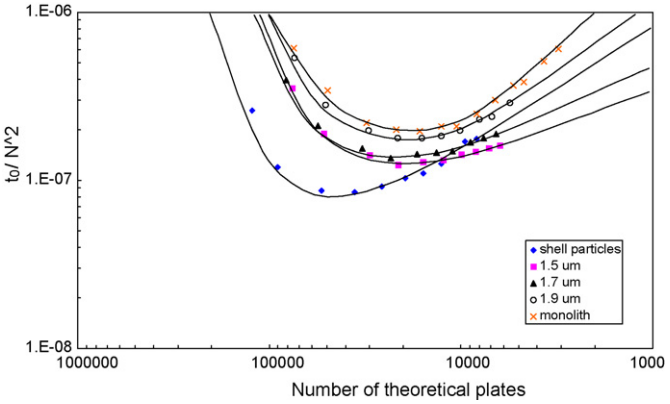


**Fig. 7.** Impedance time plots with a maximum available pressure drop of 200 bar for monolith column and 600 bar for packed columns.

Column permeability data, which were used for the calculations of kinetic plots, were corrected with system pressure drop (extra column pressure drop).

The data in a measured Van Deemter curve and the value of the column permeability were used to calculate the kinetic plots. The obtained $t_0/N^2$ values versus the required plate number ($N$) curves are shown in Figs. 5–8. All of the kinetic plots in Fig. 5 are related to 200 bar (2900 psi) pressure drop. The commercial monolith rod columns are packaged in PEEK tubes, which are certified up to 200 bar. At 200 bar the monolith column presents the best performance if the required plate number is higher than 5000 ($N > 5000$). On the other hand at the ascending part (related to C-term) of the kinetic curve, the monolith column displays the steepest slope (lowest efficiency). If the separation demands only $N < 5000$ plate numbers, it is feasible with the column packed with 1.5 μm porous particles within shorter analysis than with the monolith column.

In Fig. 6 the kinetic plot of monolith column is related to 200 bar (2900 psi) pressure drop while the plots of packed columns are related to a maximum available pressure drop of 400 bar (5800 psi). The advantage of the shell particles against porous particles and monolith column can be demonstrated clearly with these impedance time plots. Much shorter impedance time can be achieved with 2.7 μm shell particles than with porous particles or monolith columns. However, the impedance time of Ascentis Express column increases faster with decreasing required plate numbers. At 400 bar, which is the practical limit of a conventional HPLC system, the shell core column provided the best results.

Other comparison can be seen in Fig. 7. In this comparison the upper pressure limit of the column packed with shell particles was applied for packed columns as a maximum available pressure drop. At 600 bar, the performance of monolith column and the sub-2 μm stationary phases is changed. The monolith column ($\Delta P_{max}$ = 200 bar) provides similar efficiency as the column packed with 1.9 μm porous particles ($\Delta P_{max}$ = 600 bar). At 600 bar, which is the practical limit of fused core columns, the most favorable material is the fused core one.

At 1000 bar, which is the practical limit of UPLC, the sub-2 μm stationary phases gave similar performance and are comparable with the shall core one at 600 bar although the columns packed with 1.5 μm and 1.7 μm porous particles performed slightly

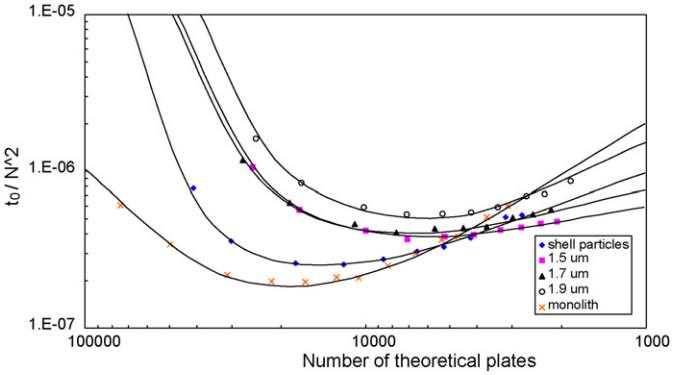


**Fig. 5.** Impedance time plots with a maximum available pressure drop of 200 bar.

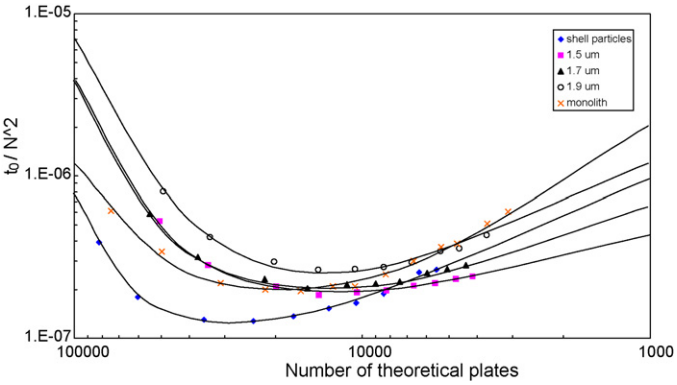


**Fig. 6.** Impedance time plots with a maximum available pressure drop of 200 bar for monolith column and 400 bar for packed columns.

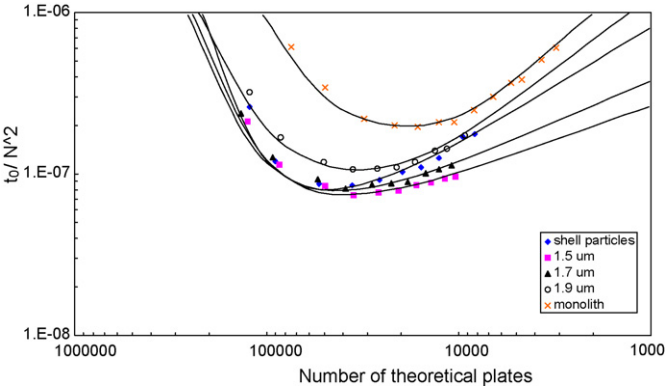


**Fig. 8.** Impedance time plots with a maximum available pressure drop of 200 bar for the monolith column, 600 bar for the column packed with shell particles and 1000 bar for the columns packed with sub-2 μm particles.

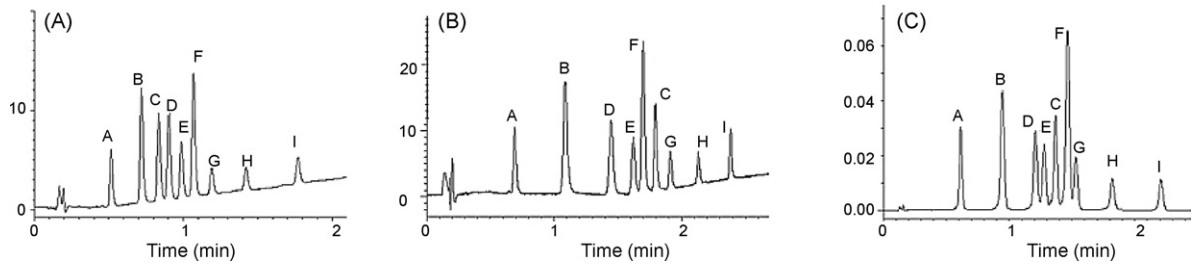


**Fig. 9.** Chromatograms of model sample obtained with the column packed with shell particles (A), the column packed with sub-2 μm totally porous particles (B) and the monolith column (C). Compounds: (A) dienogest, (B) estradiol, (C) finasteride, (D) ethinylestradiol, (E) gestodene, (F) bicalutamide, (G) levonorgestrel, (H) tibolone and (I) noretistherone-acetate. Chromatographic conditions: (A) Ascentis Express C18 2.7 μm (50 mm × 2.1 mm) column, mobile phase: acetonitrile–water gradient elution (35–60% AcN, in 2.0 min), flow: 0.5 ml/min, column temperature: 20 °C, injection volume: 2 μl, detection: 210 nm. (B) Acquity UPLC BEH C18 1.7 μm (50 mm × 2.1 mm) column, mobile phase: acetonitrile–water gradient elution (25–80% AcN, in 2.8 min), flow: 0.6 ml/min, column temperature: 65 °C, injection volume: 2 μl, detection: 210 nm. (C) Chromolith FastGradient RP-18e (50 mm × 2.0 mm) column, mobile phase: acetonitrile–water gradient elution (20–55% AcN, in 3.0 min), flow: 1.1 ml/min, column temperature: 58 °C, injection volume: 2 μl, detection: 210 nm.

shorter impedance time than the fused core column. In Fig. 8a comparison can be seen which presents the kinetic plots at the maximum available pressure drop for each column (200 bar for the monolith column, 600 bar for the column packed with shell particles and 1000 bar for sub-2 μm porous particles). In this comparison the monolith column seems to be not the best choice. If the possibility of maximum performance of an UPLC system is utilized, the monolith column provides the poorest efficiency, but if the performance of the HPLC pump is limited (<400 bar) the monolith column rivals with the columns packed with sub-2 μm or shell particles. At low pressure the monolith column is the most advantageous choice, however, if the linear velocity is increased over 0.4 cm/s, the mass transfer kinetic is unfavorable and the peak broadening is significant. The impedance time of the shell core and the monolith column increases faster with decreasing required plate numbers, comparing to sub-2 μm particles.

### *3.4. Fast separation of steroids on different types of stationary phases, representative chromatograms (gradient elution)*

The nine model compounds can be separated with sufficient resolution on all of the investigated columns within 2–3 min. Using the same mobile phase (acetonitrile–water) the analysis time and eluting order depend on the selectivity of stationary phases and on temperature. In this study we have not intended to compare the selectivity of the phases, we investigated only the efficiency of the columns, however optimizing a binary or ternary mobile phase composition is very useful to get the optimum separation, especially in the case of steroid analysis. In this paper we emphasize the performance of stationary phases. We intend to deal with mobile phase optimization for ultra fast separation in a future paper.

Three examples are presented for steroid separation using a fused core column (Ascentis Express C18 2.7 μm, 50 mm × 2.1 mm), a sub-2 μm packed column (Waters UPLC™ BEH C18 1.7 μm, 50 mm × 2.1 mm) and a narrow bore monolith column (Chromolith FastGradient RP-18e 50 mm × 2 mm). Throughout the measurements a Waters Acquity UPLC™ system was employed.

Initial four input experiments (gradient time—column temperature model) were performed to optimize the separation. Linear gradients with 6 min and 18 min (0.5 ml/min) at 20 °C and 60 °C column temperature were run. DryLab software was used to predict the optimal solvent ratio, gradient program, flow rate and temperature, which condition would give the most favorable separation. On each medium it was possible to achieve a suitable separation within 2–2.5 min (Fig. 9).

### *3.5. Practical example: sub-1 minute separation of ethinylestradiol and levonorgestrel from tablet sample (isocratic elution)*

This example presents very fast isocratic separations of ethinylestradiol and levonorgestrel from Rigevidon tablet, using a sub-2 μm packed column, a fused core column and a monolith column. The active substances were extracted from the tablets with acetonitrile–water solvent mixture then were sonicated, centrifuged and diluted. The injected sample solution contained 8 μg/ml ethinylestradiol and 40 μg/ml levonorgestrel. As seen in Fig. 10, baseline separation was achieved on each stationary phase within 1 min.

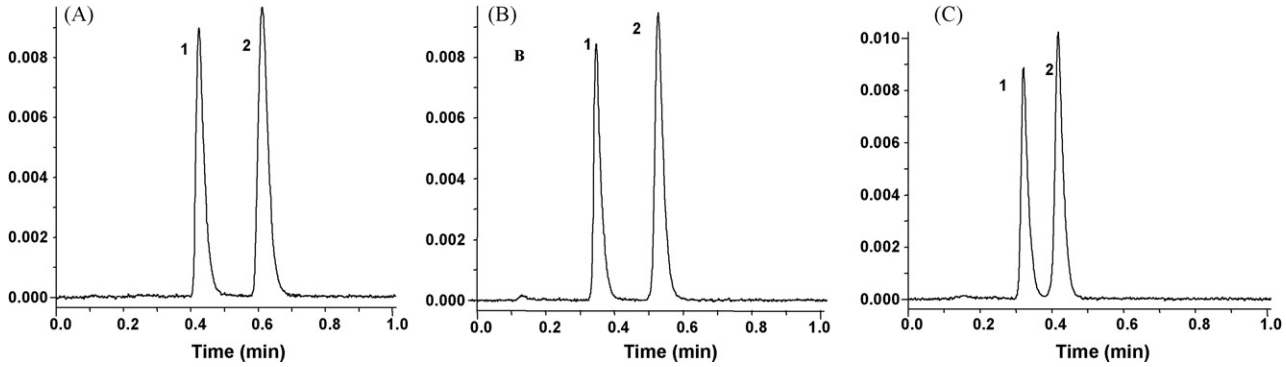


**Fig. 10.** Chromatograms of the extract of Rigevidon tablet, obtained with the column packed with shell particles (A), the column packed with sub-2 μm totally porous particles (B) and the monolith column (C). Compounds: (1) ethinylestradiol and (2) levonorgestrel. Chromatographic conditions: mobile phase: acetonitrile–water 50–50 (v/v), flow: 0.8 ml/min, column temperature: 50 °C, injection volume: 0.5 μl, detection: 220 nm. Columns: (A) Ascentis Express C18 2.7 μm (50 mm × 2.1 mm), (B) Acquity UPLC BEH C18 1.7 μm (50 mm × 2.1 mm), (C) Chromolith FastGradient RP-18e (50 mm × 2.0 mm) column.

## 4. Conclusion

Peak capacities of about 130–150 could be achieved in 25 min with the 5 cm long narrow bore columns packed with sub-2 μm totally porous or shell particles when steroid compounds are separated. The monolith column (50 mm × 2.0 mm) offered a peak capacity value of about 120 during a 25 min gradient span.

The Ascentis Express column packed with 2.7 μm fused-core particles (solid cores and 0.5 μm thick porous shell with 9 nm pore diameter) offer a really high separation power with modest operating pressure. The performance achieved under both gradient and isocratic condition, is comparable to those obtained with totally porous sub-2 μm particles. If the possibility of maximum performance is utilized, the monolith column provides the poorest efficiency, but if the performance of the HPLC pump is limited (<400 bar) the monolith column rivals with the columns packed with sub-2 μm or shell particles. At 200 bar, the monolith column provided the highest performance if the required plate number was higher than 5000 ($N > 5000$), however the efficiency drops off faster with decreasing required plate numbers than in the case of columns packed with sub-2 μm totally porous particles.

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