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Efficiency of the new sub-2 μm core–shell (Kinetex™) column in practice, applied for small and large molecule separation

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ABSTRACT

At present sub-2 μm packed columns are very popular to accomplish rapid and efficient separations. Applying particles with shortened diffusion path to improve the efficiency of separation performs higher efficiency than it is possible with the totally porous particles having the same size. The advantages of sub-2 μm particles and shell particles are combined in the new Kinetex 1.7 μm particles. In this study a systematical evaluation of the efficiency and achievable analysis time obtained with 5 cm long narrow bore column packed with sub-2 μm core–shell particles (1.25 μm core diameter and 0.23 μm porous silica layer), and other type very efficient columns is presented. The efficiency of separation was investigated also for small pharmaceutical and large molecules (proteins). Van Deemter, Knox and kinetic plots are calculated. The results obtained with low molecular weight polar neutral analytes (272 g/mol, 875 g/mol), with a polypeptide (4.1 kDa) and with different sized proteins (18.8 kDa, 38.9 kDa and 66.3 kDa) are presented in this study. Moreover, particle size distribution, and average pore size (low-temperature nitrogen adsorption, LTNA) of the new very fine core–shell particles were investigated.

According to this study, increased flow rates can be applied on sub-2 μm core–shell columns to accomplish very fast separations without significant loss in efficiency. The new sub-2 μm shell particles offer very high efficiency both for small and large molecule separation.

1. Introduction

In liquid chromatography a revolutionary new period has been started with using sub-2 μm fully porous particles, monolith columns and shell particles.

On sub-2 μm particles, due to the narrow peaks, sensitivity and separation are improved at the cost of pressure. Smaller particles result in flatter van Deemter curves, allowing for higher flow rates while still maintaining near maximum efficiencies. It was demonstrated that the analysis time could be reduced to a 1- or 2-min interval without the loss of resolution and sensitivity [1,2]. Commercial HPLC instruments have a maximum operating pressure limit of 400 bar, leading to the common practice of using short columns packed with small particles to speed up analysis [3,4]. Knox and Saleem were the first to write about the compromise between speed and efficiency [5]. To overcome the pressure limitations of modern HPLC, the groups of Jorgenson [6,7] and Lee [8] manufactured dedicated instrumentation and columns to allow analysis at very high pressures. A new nomenclature was introduced with the term ultrahigh-pressure liquid chromatography (UHPLC). It describes the higher backpressure requirement ($p > 400$ bar).

Temperature in HPLC also offers a possibility to cut down the analysis time. Elevated temperature reduces the viscosity of mobile phase and thus increases mass transfer. The separation can be shortened without loss of resolution through column heating [9–12]. Preheating of the mobile phase is essential to avoid brand broadening. However, this strategy suffers of limitations such as the small number of stable packing materials at temperatures higher than 80 °C as well as the potential degradation of thermolabile compounds and the need to have a constant temperature along the chromatographic system. Therefore, until now, the pharmaceutical industry has not considered this approach routinely [13].

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. [14], Svec and co-workers [15], Horváth and co-workers [16], Tanaka and co-workers [17], which already lead to a number of well performing, commercially available polymeric and silica monolith columns [18,19]. The analysis time can be shortened with enhancing the flow rate of the mobile phase. The disadvantages for the monolithic columns are their limited stationary phase chemistries (commercially) and pressure limitation to 200 bar. Due to the low

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phase ratio, retention on monolithic columns is generally lower compared to packed columns.

The concept of superficial or shell stationary phases, was introduced by Horváth and co-workers [20,21]. Later Kirkland presented, that 30–40 μm diameter superficially porous packings provided much faster separations, compared with the large porous particles used earlier in liquid chromatography [22]. 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 [23]. Fused-core packing materials are commercially available in different particle diameters (2.7 μm and 5 μm). Studies have proven [24] that the peak broadening is larger than we would think 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 [25]. The most recent shell stationary phase was released in the year of 2009. This new Core–Shell™ technology is using sol–gel processing techniques that incorporate nano-structuring technology; a durable, homogeneous porous shell is grown on a solid silica core. Some recent studies have demonstrated advantages of new core–shell particles over sub-2 μm fully porous particles [26–29]. The new Kinetex column offers more efficient separation in the range of high linear velocities than the columns packed with sub-2 μm totally porous particles or other shell particles with thicker porous layer. When 5–25 cm long columns are used, the Kinetex column provides more favorable plate time values and offers very short analysis time [30].

The aim of our study was to make a critical evaluation of the practical possibilities of commercially available new column packed with sub-2 μm core–shell particles. The obtained results were evaluated in terms of gaining the separation speed as a function of desired plate count. Kinetic plots were constructed according to the method of Poppe [31] from the experimental van Deemter curves. The particle size distribution was estimated on the basis of scanning electron microscopic (SEM) measurements. Pore structure of sub-2 μm porous particles (1.7 μm Waters BEH C18), and core–shell particles (1.7 μm Kinetex C18) was compared on the basis of low-temperature nitrogen adsorption (LTNA). The test analytes were real life compounds of pharmaceutical interest, which are often analyzed in our practice. The results obtained with low molecular weight polar neutral analytes (272 g/mol, 875 g/mol) and with different sized proteins (4.1 kDa, 18.8 kDa, 38.9 kDa and 66.3 kDa) are presented in this study.

2. Experimental

2.1. Chemicals, columns

Acetonitrile, methanol (gradient grade) and trifluoroacetic acid (TFA) (Uvasol) 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 were polar neutral small pharmaceutical compounds and proteins. Estradiol (estra-1,3,5(10)-triene-3,17β-diol) was produced by Gedeon Richter Plc (Budapest, Hungary). Ivermectin (22,23-dihydroavermectin B_{1a}) was purchased from Bioastralis (Smitfield, Australia). A 4.1 kDa polypeptide, an 18.8 kDa protein, a 38.9 kDa protein and an IG1 antibody were produced by Gedeon Richter Plc (Budapest, Hungary). The 66.3 kDa bovine serum albumin (BSA) was purchased from Fluka (Sigma–Aldrich, Hungary).

The new Kinetex core–shell columns packed with 2.6 μm shell particles (50 mm × 2.1 mm) and with 1.7 μm shell particles (50 mm × 2.1 mm) were obtained from GEN-Lab Ltd, Budapest. 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. Chromolith FastGradient RP-18e column (50 mm × 2.0 mm) was purchased from Merck Ltd., Budapest. All of the columns used in this study were new, no other experiments were performed on them.

2.2. Equipment, software

All measurements were performed using a Waters Acquity system equipped with binary solvent delivery pump, an auto-sampler and a photo diode array detector (Waters Ltd. Budapest, Hungary). The UPLC system had a 5 μl injection loop and a 500 nl flow cell (path length = 10 mm). A polyether ether ketone (PEEK) tube (15 cm × 0.1 mm) is located between the column outlet and the detector. The overall extra-column volume (V_{ext}) is 12 μl as measured from the injection seat of the auto-sampler to the detector cell at 1 ml/min. The measured dwell volume is 130 μl. Data acquisition with an 80 Hz data sampling rate and instrument control were performed by Empower 2 Software (Waters).

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). The non-linear curve fitting to van Deemter and Knox plots was performed using Statistica 9.0. Image-J (freeware image-processing software program developed at the National Institutes of Health) was used to determine the particle size and Statistica 9.0 (StatSoft Inc., USA) was used to determine the size distribution of column packing materials.

2.3. Apparatus and methodology

The mobile phase was prepared by mixing appropriate amounts of HPLC gradient grade acetonitrile and Milli-Q water. The mixture was degassed by sonication for 5 min. The isocratic mobile phase consisted of 48/52 (v/v%) acetonitrile/water for estradiol, 95/5 (v/v%) acetonitrile/water for ivermectin. The proteins were eluted with acetonitrile/water/TFA mobile phase. For the 4.1 kDa peptide 140/860/1, for the 18.8 kDa protein 440/560/1, for the 38.9 kDa protein 470/530/1 and for BSA (66.3 kDa) 610/390/1 ratio of acetonitrile/water/TFA was used.

The stock solutions of estradiol and ivermectin were set in acetonitrile (1000 μg/ml). The solutions for the chromatographic runs were diluted from the stock solutions with the mobile phase. In the case of proteins the stock solutions were set in water and then were diluted with the mobile phase. The concentration of the test solutions was 10 μg/ml.

The kinetic efficiency of the columns was determined with a mobile phase composition, which gave a range of retention factors (k) between 3.4 and 6.6 for estradiol and ivermectin on each stationary phase. For the proteins the mobile phases ensured a sufficient retention $k = 4–5$ in the low flow rate (F) range ($F < 0.3$ ml/min) and $k > 2$ at higher flow rates ($F > 0.3$ ml/min) on Kinetex and Waters BEH 1.7 μm packing. Differences in the retention factors (k) of course affect the shape of the obtained van Deemter type curves, as both the B- and C-terms of the equations, depend on analyte retention [32]. We have not made attempt to adjust the mobile phase composition to guarantee constant k for all analytes, because this would introduce additional variability in terms of viscosity and analyte diffusion coefficients, which would outweigh the minor effect of retention. The column temperature was set as 35 °C and for proteins we also applied 60 °C. The injected volume was 0.5 μl (partial loop with needle overfill mode), and UV detection at 215 nm (80 Hz) for estradiol and ivermectine and 280 nm for proteins were applied.

Since all experimental parameters, with the exception of the retention factor k, have been kept constant, these conditions can

be used to effectively compare the columns and the effect of solute properties on the performance characteristics. Moreover the effect of (analyte) solute mass and size (hydrodynamic diameter) on column efficiency was studied. The hydrodynamic diameter (D_H) of the proteins and the polypeptide were measured using a Nanosizer S unit (Malvern Instruments Ltd., Worecestershire UK). The measurements were performed with samples diluted in the appropriate mobile phase.

2.4. Equations used for calculation

The kinetic performance of different columns has been compared according to their van Deemter et al. plots [33]. The van Deemter equation explains that efficiency of the column varies with linear velocity.

$$H = A + \frac{B}{u} + Cu \tag{1}$$

where H is the HETP, u the chromatographic linear velocity of the mobile phase and A–C are constants. The position of the minimum on the HETP curve, and the optimum linear velocity, can be determined by the use of differential calculus. The optimum linear velocity occurs when the slope of the H versus u curve is zero, i.e. when $dH/du = 0$. This condition is satisfied when:

$$u_{opt} = \sqrt{\frac{B}{C}} \tag{2}$$

The value of H at the optimum linear velocity can be obtained by substituting the value of u given in Eq. (2) into Eq. (1).

$$H_{min} = A + 2\sqrt{BC} \tag{3}$$

The plate numbers of the columns were measured at a series of different flow rates to obtain the reduced plate height (h) versus reduced linear velocity (ν) plot. The calculation was performed according to Giddings [34]:

$$\nu = \frac{ud_p}{D_M} \tag{4}$$

The reduced plate height was calculated according to the next formula:

$$h = \frac{H}{d_p} \tag{5}$$

The h versus ν curves were fitted into the following equation:

$$h = a + \frac{b}{\nu} + c\nu \tag{6}$$

where a–c are constants.

Van Deemter type plots lack permeability considerations. Alternative approaches, mostly based on the kinetic principles first expounded by Giddings [35]. Later Poppe proposed the "Poppe plot" in which the plate time (t_0/N) is plotted against the plate number (N). This is a neat tool for visualizing the compromise between separation speed and efficiency [31]. N and t_0 can be calculated according to the following equations which have been introduced by Desmet et al. [36]:

$$N = \frac{\Delta P}{\eta}\left(\frac{K_{V0}}{uH}\right) \tag{7}$$

$$t_0 = \frac{\Delta P}{\eta}\left(\frac{K_{V0}}{u^2}\right) \tag{8}$$

where ΔP is the available pressure drop, K_{V0} the column permeability, η the mobile phase viscosity.

For the construction of kinetic plots, certain defining experimental parameters are used, including the maximum operating pressure (P), column reference length and flow resistance or permeability (K_V), temperature, mobile phase viscosity (η) and the diffusion coefficient of the solute in the mobile phase (D_M). Column particle sizes were obtained from the results of SEM measurements while maximum pressure was based on actual instrumental (UPLC) or column mechanical stability limitations (data obtained from column manufacturers). Column permeability was determined experimentally using the following relation:

$$K_{V0} = \frac{u\eta L}{\Delta P} \tag{9}$$

In which ΔP is the pressure drop over the column with length L, K_{V0} the column permeability, η the mobile phase viscosity and u the linear velocity. Viscosity values were calculated using equations derived by Chen and Horváth [37], and solute diffusion coefficients of a low molecular weight compound (estradiol) was calculated by using the Wilke–Chang equation [38]:

$$D_M = 7.4 \times 10^{-8}\frac{(\Psi M_S)^{0.5}T}{\eta V_A^{0.6}} \tag{10}$$

where Ψ is the solvent association factor, M_S the molecular weight of the mobile phase (g/mol), η its viscosity, T is the temperature, and V_A is the molar volume of the solute at its boiling point (cm^3/mol). V_A was estimated according to the group method of Schroder and Lebas [39].

2.5. Measurement of plate heights and column permeability

The influence on the apparent column efficiency of even a small extra-column volume of the instrument used is very important. The contribution of the extra-column volume can simply be considered as an additional constant to the eddy dispersion term, in the Van Deemter equation [40]. Extra-column effects are more significant for scaled down separations (column volume decreases) [41–43]. The extra-column variance depends on the nature and viscosity of the mobile phase and on the retention factor of the analyte.

The overall extra-column volume of our UPLC system was measured as 12 μl. This volume represents about 10% of the hold up volume of the compared 5 cm × 2.1 mm columns. Thus, for a fair column comparison it is necessary to correct the obtained plate heights for extra-column dispersion in case of the low molecular weight test analytes (estradiol and ivermectin). The extra-column band-spreading was negligible in the case of large analytes (proteins).

During the flow study, the flow rate of mobile phase was increased from 0.01 ml/min up to 1.2 ml/min. Three parallel injections were performed at each flow rate and the average peak width (2.35σ) at half peak height was used for the further calculation. The injection volume of 0.5 μl was applied (the injection mode was set as partial loop with needle overfill). The relative standard deviation of peak widths obtained with three repeated injections did not reach 5% for estradiol, ivermectin and 4.1 kDa peptide and it was under 10% when the 18.8, 38.9 and 66.3 kDa proteins were injected. The measured peak widths of estradiol and ivermectin were corrected for extra-column volume. It was measured by injecting the test solutes (estradiol and ivermectin) with a zero-dead-volume connector instead of the column at each flow rate and the same mobile phase, which was set during the flow study. The experimental HETP data were corrected for the contributions of the extra-column volume using the following equation:

$$H = L\frac{(t_h^r - t_h^f)^2 - (t_{h,a}^r - t_{h,a}^f)^2}{5.545(t_R - t_a)^2} \tag{11}$$

where t_h^r and t_h^f are the rear and front widths of the peak measured at half height, $t_{h,a}^r$ and $t_{h,a}^f$ are the rear and front widths of the peak

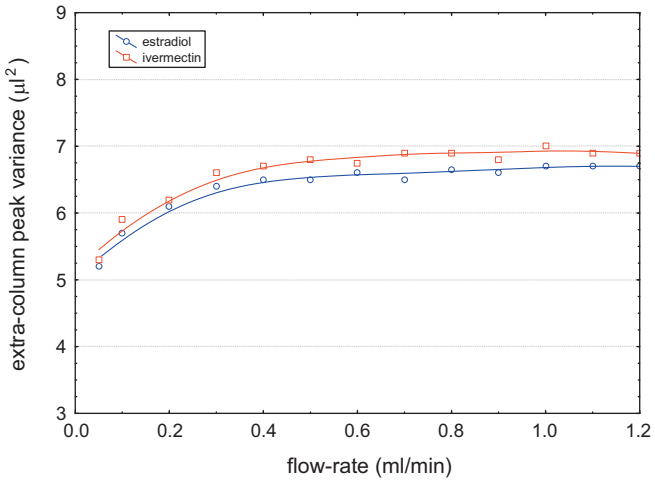

Fig. 1. Plot of extra-column variance versus mobile phase flow rate. Instrument: Waters Acquity UPLC system, mobile phase: 48% acetonitrile–52% water for estradiol, and 95% acetonitrile–5% water for ivermectin, temperature: 35 °C, injection: 0.5 μl. A zero-volume union was used in place of the column. Distance weighed least squares fitting are used.

measured at half height in absence of the column, t_R and t_a are the elution times (at peak apex) of the test compounds obtained with and without column, respectively.

The extra-column peak dispersion ($\sigma^2_{V,ext}$) was determined in μl^2 according to the next equation:

$$\sigma^2_{V,ext} = F^2\sigma^2_{ext} = F^2\frac{(t^r_{h,a} - t^f_{h,a})^2}{5.545} \quad (12)$$

where F is the flow rate (expressed in μl/min). The variance of the Acquity UPLC system was measured around 6–7 μl^2. The plots of μl^2 as a function of the flow rate are given in Fig. 1. The extra-column peak dispersion of the applied Acquity UPLC system causes an efficiency loss of about 25–35% for the Kinetex 1.7 μm (5 cm × 2.1 mm) column at the optimal linear velocity ($HETP_{min}$) when low molecular weight solutes are measured. The retention factors of the estradiol and ivermectin on the different columns varied between k = 3.4–6.6 (moderately and well retained compounds) and of the proteins it was measured as k = 4–5 in the low flow rate (F) range (F < 0.3 ml/min) and k > 2 at higher flow rates (F > 0.3 ml/min) on Kinetex and Waters BEH 1.7 μm packing.

Of course this method contributes to some uncertainty while the extra-column band broadening depends on the pressure (compressibility of solvent) and without a column it is not possible to predict this effect. We assume that this relatively minor effect does not cause an important error in estimated column efficiency since all tested columns were compared with the same way, of extra-column volume correction. The plate heights for kinetic curves were calculated using the corrected plate numbers.

The pressure drop characteristics of the columns were examined by subtracting the pressure drop of connection tubes ΔP_{ext} from the total pressure drop ΔP_{tot} obtained with the system to yield the effective column pressure ΔP_{col} (=$\Delta P_{tot} \subseteq \Delta P_{ext}$). Then this value was used to Darcy's law to obtain the column permeability. The extra-column pressure drop was measured with a zero-dead-volume connector instead of the column at each flow rate and the same mobile phase, which was set during the flow study.

3. Results and discussion

3.1. Column efficiency for small molecules

3.1.1. Flow study

At first the kinetic properties of the investigated new sub-2 μm Kinetex core–shell column were evaluated at the temperature of 35 °C by means of their van Deemter plots. A small amount of solutes (10 μg/ml) diluted with mobile phase was injected to obtain the data. Estradiol was eluted with acetonitrile/water 48/52 (v/v), ivermectin was eluted with acetonitrile/water 95/5 (v/v). The injection volume of 0.5 μl was applied. The constants of the H–u curves (A, B and C) were obtained by fitting experimental data to the van Deemter equation (Eq. (1)) using the least square optimization method. A comparison between the A, B, C terms, optimum linear velocity and minimum plate heights for each of the test analytes is presented in Table 1.

A, B, and C are constants determined by the magnitude of band broadening due to eddy dispersion, longitudinal diffusion, and resistance to mass transfer, respectively [44,45]. The constant A depends on the quality of the column packing and on the contribution of slow mass transfer across the moving stream (short and long range trans-channel dispersion). Therefore it is necessary to mention that all the columns, which were tested and compared in this study, came from different providers and thus, both the quality of packing and particle size distribution could have an effect on efficiency.

The B-term increases with solute retention as more less time is available for diffusion to take place and the stationary phase (surface diffusion). B- and C-terms of the van Deemter equation depend on analyte retention.

It can be obviously seen and have to be emphasized, that smaller than 3 μm ($HETP_{min}$ = 2.6 μm) plate height was obtained with the 1.7 μm core–shell column for estradiol. In our previous study for estradiol we obtained $HETP_{min}$ > 3 μm values with Kinetex 2.6 μm columns [30]. So decreasing the diameter of Kinetex particles and the thick of the porous layer (from 0.35 μm to 0.23 μm) is manifested in significantly increased kinetic efficiency. The smallest plate height obtained with 2.6 μm Kinetex column for estradiol was 3.2 μm and it is 2.6 μm when the 1.7 μm Kinetex column is used. Consequently cutting the particle size and maintaining the ratio of the core diameter and the particle diameter (ρ = 0.73) of Kinetex material yielded roughly 20% gain in plate height. Moreover, the smaller particle diameter resulted a significant shift in the linear velocity optimum towards the higher values. In the case of estradiol the optimum linear velocity obtained with 2.6 μm was 0.24 cm/s while the 1.7 μm particles gave the minimum plate height value at 0.37 cm/s. The similar tendency was observed when ivermectin was injected. The optimum in linear velocity was shifted from 0.08 cm/s to 0.13 cm/s. If we compare the results to a same size fully porous particle (Waters Acquity BEH C18, 1.7 μm, 50 mm × 2.1 mm column) the shift in optimum linear velocity is more serious. In the case of the fully porous 1.7 μm particles the optimum linear velocity was evaluated as 0.14 cm/s for estradiol and 0.05 cm/s for

Table 1
Summary of fitted van Deemter constants (A, B, C), optimal linear velocity and minimum plate heights. (k: retention factor).

Kinetex C18, 1.7 μm (50 mm × 2.1 mm)	k	A	B	C	u_{opt} (cm/s)	$HETP_{min}$ (μm)
Analyte						
Estradiol (MW = 272)	5.5	2.199	0.073	0.522	0.37	2.6
Ivermectin (MW = 875)	5.2	3.584	0.044	2.466	0.13	4.3

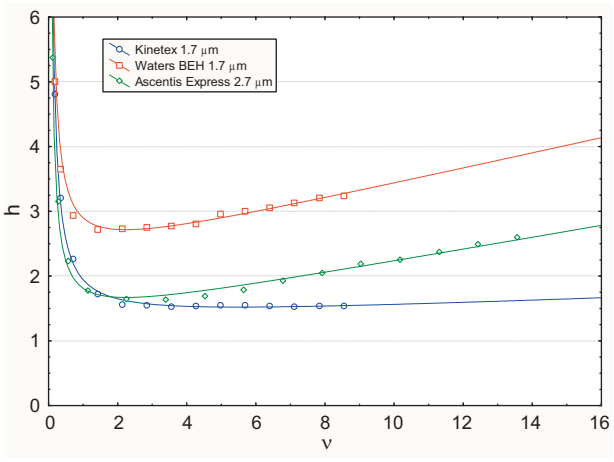

Fig. 2. Experimental h–ν plots of 1.7 μm core–shell (Kinetex C18, 5 cm × 2.1 mm), 1.7 μm fully porous (Waters BEH C18, 5 cm × 2.1 mm) and 2.7 μm fused-core (Ascentis Express C18, 5 cm × 2.1 mm) columns (peak widths were corrected for the extra-column broadening). Mobile phase: acetonitrile/water 48/52 (v/v), temperature: 35 °C, injection: 0.5 μl, test analyte: estradiol.

ivermectin [30]. Using the 1.7 μm core–shell packing instead of the fully porous 1.7 μm particles results a 2.6 times improvement in optimal linear velocity for both test analyte.

Further enhancement can be seen in the mass-transfer characteristic. In the case of estradiol the obtained C-term of 1.7 μm core–shell Kinetex material is lower with a factor of about 1.25 compared to the 2.6 μm Kinetex packing and 5.9 times lower compared to the same sized (1.7 μm) fully porous particles (the results of 2.6 μm Kinetex and 1.7 μm Waters BEH columns can be seen in our previous study [30]). When ivermectin is injected the improvement in mass-transfer properties is more advantageous. The C term obtained with 1.7 μm Kinetex is 2.6 times lower than it was achieved with 2.6 μm Kinetex and 7.7 times lower than it was obtained with the 1.7 μm totally porous packing. These results are in agreement with theoretical expectations. Kaczmarski and Guiochon state that the mass-transfer resistance of shell particles exhibit much lower plate heights for large molecular size compounds than do fully porous particles, this advantage increasing with decreasing thickness of the shell, and thus the improvement in efficiency is more serious when larger molecular weight solutes are investigated [46].

The reduced plate height versus reduced linear velocity plots, generally provide a comparison between different columns, which should be independent of the particle size [47]. Fig. 2 shows the experimental h–ν curves of estradiol (MW = 272) obtained on different type 5 cm long narrow bore columns, and the fitted a, b, c constants, optimum linear velocity and minimum reduced plate heights are reported in Table 2.

A poorly packed column gives a high value for a (2.5–5), while the well-packed column has a low value of a (0.5–2.5). The Kinetex 1.7 μm 5 cm × 2.1 mm column gave an a value of 1.3 which is equal with the value was obtained with Ascentis Express column and better than Acquity BEH's. Previously for the 2.6 μm, 5 cm × 2.1 mm Kinetex column we obtained an a value of 1.8, while the wider 3.0 and 4.6 mm internal diameter Kinetex columns (2.6 μm) performed significantly lower a values [30].

Table 2
Summary of fitted constants (a, b, c), optimal reduced linear velocity (ν) and minimum reduced plate heights (h). The data of 2.7 μm Ascentis Express and 1.7 μm Waters Acquity BEH columns were taken from our previous study [30].

Column	a	b	c	ν_{opt}	h_{min}
Analyte: estradiol (MW = 272)					
1.7 μm Kinetex core–shell	1.293	0.636	0.021	5.5	1.5
2.7 μm Ascentis Express fused-core	1.250	0.474	0.094	2.2	1.7
1.7 μm Waters Acquity BEH	2.483	0.360	0.079	2.1	2.7

The b term accounts for the longitudinal diffusion and significantly depends on the solute retention [48,49]. The c term expresses the effect of mass-transfer resistance in both stagnant mobile and stationary phases and is important for good performance especially at high-reduced velocities. An acceptable value of c for an efficient packing material is under 0.1 [47]. All of the tested columns gave c values under 0.1. The 1.7 μm Kinetex column performed approximately 3.7 times lower c value that the column packed with same sized fully porous particles. Plate height models are written as the amount of four different contributions such as (1) reduced longitudinal diffusion, (2) eddy dispersion, (3) the external film mass transfer and (4) the transparticle mass-transfer resistance. The transparticle mass-transfer resistance for shell particles was first derived by Kaczmarski and Guiochon [46]. According to this theory the intraparticle diffusity depends on the ratio (ρ) of the diameter of the solid core to that of the particle in a core–shell particle. The ratio of solid core and particle diameter for Kinetex 1.7 μm material is $\rho = 0.73$ according to the vendor, Gritti determined this ratio as $\rho = 0.72$ [28]. If we follow this theory, approximately 2 times larger diffusivity can be expected than in the case of totally porous particles of the same size (1.7 μm). According to our measurements the Acquity BEH column packed with 1.7 μm totally porous particles gave 3.8 times higher c term than it was obtained with the Kinetex 1.7 μm core–shell column. The difference in expected c term can probably be explained with the differences of particle shape and external surface properties. Another cause could also be that the mass-transfer resistance is mainly controlled by the external film mass-transfer resistance and not by the transparticle mass transfer. The c term obtained with 2.7 μm shell type Ascentis Express column is comparable to the c value of fully porous sub-2 μm columns. Consequently decreasing the particle diameter of the core–shell particles (from 2.7 and 2.6 μm to 1.7 μm) offers significant advantages in column efficiency.

For a well-packed column (packed with fully porous particles) the minimum reduced plate height normally is in the range of 2–2.5 [4]. The column of 2.7 μm shell particles had a reduced plate height minimum of approximately $h = 1.7$ in this study. The lowest reduced plate height ever reported for the 2.7 μm fused-core column (Halo or Ascentis Express) is $h = 1.4$ [24]. The Kinetex 1.7 μm narrow bore column performed the value of $h = 1.5$. Gritti reported an h_{min} value of 1.1 for 2.6 μm Kinetex wide-bore column (10 cm × 4.6 mm) for anthracene [27], and in our previous study we reported $h = 1.2$, 1.3 and 1.9 for the 2.1 mm, 3.0 mm and 4.6 mm internal diameter Kinetex 2.6 μm columns [30].

3.1.2. Separation speed, kinetic plots

To compare the theoretical separation speed, kinetic plots of different 5 cm long narrow bore columns were constructed. Columns with different permeability and efficiency such as column packed with sub-2 μm totally porous particles, packed with sub-2 μm core–shell particles and packed with sub-3 μm shell particles moreover a monolithic column with low intrinsic flow resistance were compared.

The permeability of the compared columns was assessed from the experimental column pressure (P). Column permeability data 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 (according to Eqs. (7) and (8)).

Fig. 3 shows the calculated isocratic Poppe plots of estradiol on the compared columns at the maximum applicable pressure for each column to represent the utilization of maximum performance (UHPLC application). The data for maximum pressure were

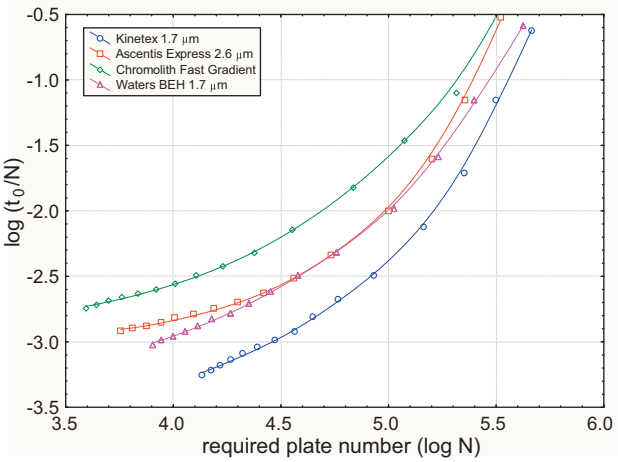

Fig. 3. Poppe plots of estradiol. Experiments were conducted on 5 cm long narrow bore columns in 48/52 ACN/H_2O, η = 0.85 cPoise, at 35 °C. Available max. pressure: 200 bar for Chromolith column, 600 bar for Ascentis Express and 1000 bar for Waters BEH and Kinetex 1.7 μm columns.

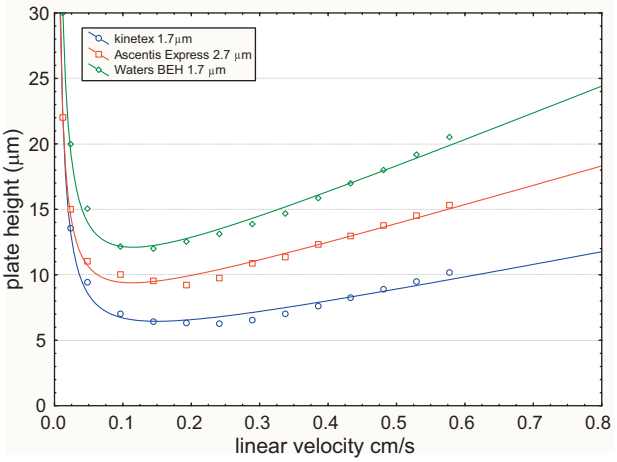

Fig. 4. Experimental Van Deemter plots of 1.7 μm core–shell (Kinetex C18, 5 cm × 2.1 mm), 2.7 μm fused-core (Ascentis Express C18, 5 cm × 2.1 mm) and 1.7 μm fully porous (Waters BEH C18, 5 cm × 2.1 mm) columns. Mobile phase: acetonitrile/water/TFA 140/860/1, temperature: 35 °C, injection: 0.5 μl, test analyte: 4.1 kDa polypeptide.

obtained from the column manufacturers: 1000 bar for Waters BEH and Kinetex columns, 600 bar for Ascentis Express column and 200 bar for the Chromolith column. These plots represent the theoretical separation speed when the maximum performance of an UHPLC system is utilized (p_{max} = 1000 bar).

The resulting curves, one for each column, demonstrate the maximum speed obtainable at a given required plate number (N) and also demonstrate the effect of the choice of column (stationary phase type; totally porous particles, shell particles, monolith column). Please note that the plate times depend on the maximum allowable pressure drop, which is different for the tested columns.

It can be obviously seen that the Kinetex 1.7 μm column provides the most favorable plate time values and offers the shortest analysis time practically in the whole plate count range. The column packed with totally porous 1.7 μm particles (Waters BEH) and the column packed with 2.7 μm shell particles offer very similar separation time. The monolithic column gives the highest plate time values in the full plate number range, thus performs the longest analysis. We can conclude that due to the very small plate height values obtained with the 1.7 μm Kinetex column, it outperforms and provides more efficient separation than the column packed with the same size totally porous particles and other shell type columns packed with larger particles (2.7 μm).

When column performance is evaluated it is practical to compare the column lengths for given plate numbers (figure is not shown). If the separation requires a plate number of N = 100,000 it can be achieved approximately on a 47 cm long Kinetex 1.7 μm column, a 49 cm long Ascentis Express column, a 50 cm long Acquity column, or on a 83 cm long Chromolith narrow bore column. We can conclude that for similar efficiency shorter columns can be applied if the Kinetex 1.7 μm column is chosen. Thus faster analysis time can be performed on 1.7 μm core–shell particles than on totally porous 1.7 μm particles or larger sized fused-core particles. The Ascentis Express and Acquity BEH columns offer very similar separation speed, while the analysis requires the most time when the Chromolith column is used.

3.2. Column efficiency for peptide

A small amount of 4.1 kDa polypeptide (10 μg/ml) diluted with mobile phase was injected to obtain the data. The polypeptide was eluted with acetonitrile/water/TFA 140/860/1 mobile phase. The injection volume was set as 0.5 μl. Fig. 4 shows the obtained H–u plots. The constants of the curves (A, B and C) were obtained by fitting experimental data to the van Deemter equation (Eq. (1)) using the least square optimization method. A comparison between the A, B, C terms, optimum linear velocity and minimum plate heights of each column is presented in Table 3.

The minimum plate height obtained with 1.7 μm core–shell particles $HETP_{min}$ = 6.3 μm is about 2 times lower than the $HETP_{min}$ value obtained with the column packed of same size fully porous particles ($HETP_{min}$ = 12.1 μm). The Ascentis Express column performed a $HETP_{min}$ = 9.2 μm in this comparison. The optimum in linear velocity was measured as 0.15 cm/s in the case of Kinetex 1.7 μm column, 0.12 cm/s for Ascentis Express column and 0.11 cm/s for Waters BEH column. The 1.7 μm Kinetex column performed approximately 2 times lower C value that the column packed with same sized fully porous particles. It is in good agreement with the theory of Kaczmarski and Guiochon [46].

The major advance of 1.7 μm core–shell particles is the approximately 50% decrease in plate heights compared to fully porous 1.7 μm particles. The permeability of the Kinetex 1.7 μm and Waters 1.7 μm column is approximately the same, thus faster separation can be expected on the core–shell column when peptides are eluted. Fig. 5 shows the plate time versus required plate number plots (isocratic Poppe plot) to present that significantly shorter analysis time can be expected on Kinetex 1.7 μm column than on Waters BEH or on Ascentis Express column in the case

Table 3
Summary of fitted van Deemter constants (A, B, C), optimal linear velocity and minimum plate heights. k (retention factor) of a 4.1 kDa polypeptide. .

Column	k	A	B	C	u_{opt} (cm/s)	$HETP_{min}$ (μm)
Analyte: 4.1 kDa polypeptide						
Kinetex C18, 1.7 μm (5 cm × 2.1 mm)	5.8	3.427	0.227	10.063	0.15	6.3
Ascentis Express C18, 2.7 μm (5 cm × 2.1 mm)	6.0	5.952	0.205	15.152	0.12	9.2
Waters BEH C18, 1.7 μm (5 cm × 2.1 mm)	6.2	7.300	0.276	20.963	0.11	12.1

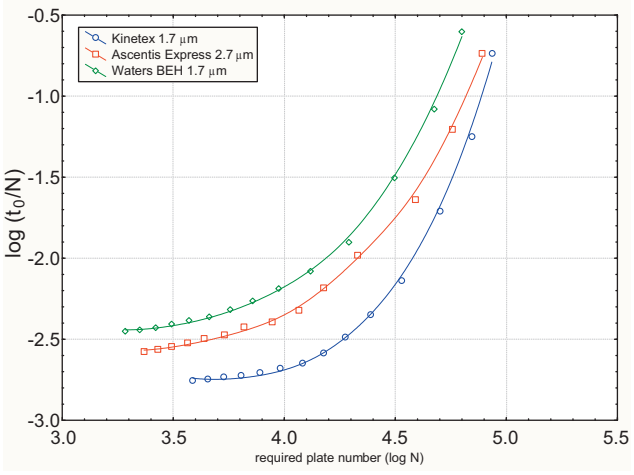

Fig. 5. Poppe plots of 4.1 kDa polypeptide. Experiments were conducted on 5 cm long narrow bore columns in acetonitrile/water/TFA 140/860/1, η = 0.99 cPoise, at 35 °C. Available max. pressure: 600 bar for Ascentis Express and 1000 bar for Waters BEH and Kinetex 1.7 μm columns.

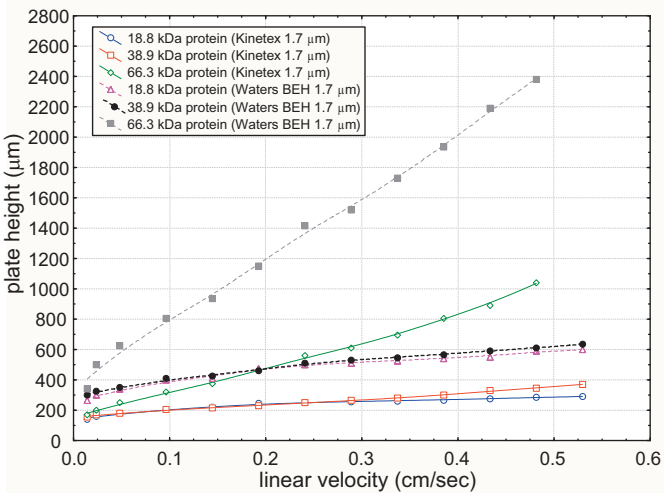

Fig. 6. Experimental *H–u* curves of 1.7 μm core–shell (Kinetex C18, 5 cm × 2.1 mm) and 1.7 μm fully porous (Waters BEH C18, 5 cm × 2.1 mm) columns. Mobile phase: 440/560/1 (for the 18.8 kDa protein), 470/530/1 (for the 38.9 kDa protein) and 610/390/1 (for BSA, 66.3 kDa) ratio of acetonitrile/water/TFA, temperature: 60 °C, injection: 0.5 μl, test analytes: proteins.

of peptide separation. The permeability of the compared columns was assessed from the experimental column pressure (P). Column permeability data 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 (according to Eqs. (7) and (8)). According to Fig. 5 it is obvious that the Kinetex column outperforms the other two columns in the plate count range of N = 1000–100,000.

3.3. Column efficiency for proteins, comparing 1.7 μm core–shell and fully porous particles

The *H–u* curves were obtained on the new sub-2 μm Kinetex core–shell column and on Waters BEH column with three different size proteins (18.8 kDa, 38.9 kDa and 66.3 kDa). A significant degree of peak tailing was unavoidable at ambient or low column temperature therefore elevated temperature was used for the estimation of column efficiency. At 60 °C each three protein eluted with relatively symmetric Gaussian peak shape. Fig. 6 shows the experimental *H–u* curves of Kinetex 1.7 μm and Waters BEH column.

As the weight (and size) of the protein is larger, the slope of the *H–u* curves becomes steeper. Considering that the particle size of the two packing is similar and the Kinetex column gives approximately 2 times higher plate counts, therefore significantly faster analysis can be expected with Kinetex column than with the Waters BEH column in the case of large proteins.

It is necessary to mention that the nominal pore size of the two packing is about 10 nm and 13 nm, thus these columns are not completely suitable for large protein separation and some size exclusion effect is inevitable. The hydrodynamic diameter (D_H) of the proteins and the polypeptide were measured using a Nanosizer S unit (Malvern Instruments Ltd., Worecestershire UK). The measurements were performed with samples diluted in the appropriate mobile phase. The results based on dynamic light scattering are summarized in Table 4.

Compounds with lower molecular mass than 5000 g/mol can be separated with high efficiency (HETP < 10 μm) at high linear velocity in isocratic elution mode with the new sub-2 μm core–shell column. We can conclude that Kinetex 1.7 μm material offers the possibility for a fast isocratic separation of peptides.

Nitrogen adsorption/desorption isotherm was measured at 77 K, using a Quantachrome Nova 2000E computer controlled apparatus. Samples were outgassed at 120 °C for 24 h. The average pore size estimated from the Brunauer–Emmett–Teller (BET) isotherm parameters [50] were obtained as 9.3 nm for Kinetex 1.7 μm packing and 13.9 nm for Waters BEH 1.7 μm material. Hence the average pore size of the Waters BEH particles is about 1.5 times larger than that of the Kinetex 1.7 μm particles moreover the pore size distribution is also different. The micropore volume was determined as 0.018 cm^3/g for Kinetex 1.7 μm particles and 0.040 cm^3/g for Waters BEH 1.7 μm particles, while the total pore volume of Kinetex material was estimated as 0.19 cm^3/g, and it was calculated as 0.46 cm^3/g for Waters BEH. The listed differences in pore structure may explain that the same size core–shell particles provides merely 1.8–2.5 times lower plate height values than the totally porous particles, however according to the theory we should expect larger differences in plate count – more advantage – for large analytes. Producing sub-2 μm core–shell particles with larger pore size (e.g. 30 nm – which is widespread for general protein separation) should manifest the full advantage of core–shell sub-2 μm particles for protein separations.

3.4. Particle size distribution of Kinetex 1.7 μm packing material

SEM images and Image-J (image-processing software) were used to show the roughness of the particles and to determine the particle size distribution of the Kinetex sub-2 μm material (Fig. 7). SEM pictures of the Kinetex 2.6 μm particles were already presented in our previous study [30].

Table 4
Summary of molecular weight (MW), hydrodynamic diameter (D_H) and plate heights (H) obtained with Kinetex C18, 1.7 μm, 5 cm × 2.1 mm column.

Analyte MW (Da)	Analyte D_H (nm)	H (μm) at 0.2 cm/s	H (μm) at 0.3 cm/s	H (μm) at 0.45 cm/s
4100	0.7	6.4	6.6	8.2
18800	2.2	244	255	273
38900	5.4	232	265	329
663000	7.1	463	610	893

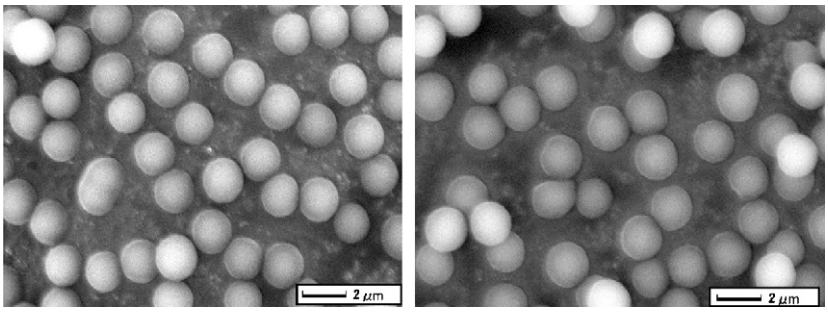

Fig. 7. Scanning electron microscopic images of sub-2 μm Kinetex particles.

Table 5
Particle size distribution (on the basis of SEM measurements) of 1.7 μm Kinetex particles. For the comparison data of 2.6 μm Kinetex, 2.7 μm Ascentis Express and 1.7 μm Waters BEH were taken from our previous paper [30].

Column	d90/10	Mean	RSD%
1.7 μm Kinetex core–shell	1.32	1.85	6.95
2.6 μm Kinetex core–shell	1.15	2.50	5.94
2.7 μm Ascentis Express fused-core	1.16	2.72	5.30
1.7 μm Waters Acquity BEH	1.53	1.81	15.79

For the calculation of particle size distribution 300 individual particles were considered. The diameters of the particles were measured along the horizontal direction. The mean diameter of the sub-2 μm Kinetex particles was determined as 1.85 μm (RSD = 6.95) against the nominal 1.7 μm (Table 5).

It can be seen that the Kinetex sub-2 μm particles have smooth surface (much softer than Ascentis Express's or Halo's). The nature of the particle surface, its smoothness or degree of roughness affects considerably the film mass-transfer kinetics, hence the column efficiency. While the film mass transfer is nearly negligible with ultra-smooth particles. Another observation is that the particle size distribution of 1.7 μm Kinetex is significantly wider than the 2.6 μm Kinetex particles and some irregular shaped particles can be observed on the SEM images however the distribution of 1.7 μm Kinetex particles is more advantageous than the 1.7 μm Waters BEH particles. According to our previous study in which the SEM images of 2.6 μm Kinetex particles are presented, the 2.6 μm Kinetex particles are more spherical than the sub-2 μm Kinetex particles [30].

4. Conclusion

In this study a systematical evaluation of the potential of the separations obtained with 5 cm long narrow bore columns packed with new 1.7 μm shell particles (1.25 μm core diameter and 0.23 μm porous silica layer, Kinetex™), and with other very efficient 5 cm long narrow bore columns was presented.

The new sub-2 μm Kinetex column performs a very flat *C* term both for small and large molecules. Lower than 3 μm plate height can be expected for low molecular weight test analytes with the new sub-2 μm Kinetex column. The narrow bore Kinetex column performed a minimum reduced plate height value of $h = 1.5$. Cutting the particle size from 2.6 μm to 1.7 μm and maintaining the ratio of the core diameter and the particle diameter ($\rho = 0.73$) of Kinetex material yielded roughly 20% gain in plate height. Moreover, the smaller particle diameter resulted a significant shift in the linear velocity optimum towards the higher values.

When peptides are separated, the major advantage of 1.7 μm core–shell particles is the approximately 50% improvement in plate heights compared to fully porous 1.7 μm particles. Compounds with lower molecular weight than 5000 g/mol can be separated with high efficiency (HETP < 10 μm) at high linear velocity in isocratic elution mode. We can conclude that Kinetex 1.7 μm material offers the possibility for a fast isocratic separation of peptides.

For very large test analytes (proteins) the sub-2 μm Kinetex column offers approximately 2 times lower plate counts than columns packed with same size fully porous particles, therefore significantly faster analysis can be expected with the new Kinetex column than with other columns packed with sub-2 μm particles in the case of large proteins.

It is necessary to emphasize that in the case of very efficient columns such as the sub-2 μm Kinetex, the extra-column variance of the commercially available LC systems with very low dispersion (<10 μl^2) is not negligible. The extra-column peak dispersion of the UPLC system applied in this study causes an efficiency loss of about 25–35% for the Kinetex 1.7 μm (5 cm × 2.1 mm) column at the optimal linear velocity ($HETP_{min}$) when low molecular weight analytes are separated.

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