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Facts and myths about columns packed with sub-3 μm and sub-2 μm particles

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ABSTRACT

Increasing the separating efficiency enhances the separation power. The most popular solution for improving chromatographic performance is to employ columns packed with small particle diameters (i.e., sub-2 μm particles) to induce a simultaneous improvement in efficiency, optimal velocity and mass transfer, albeit the cost of pressure. In this study a systematic evaluation of the possibilities and limitations of the separations obtained with 5 cm long narrow bore columns packed with 1.5–3.0 μm particles is presented. Several commercially available different sub-3 μm and sub-2 μm packed columns were evaluated by using van Deemter, Knox and kinetic plots. Theoretical Poppe plots were constructed for each column to compare their kinetic performance. Data are presented on different polar neutral real life analytes, to show that the separation time is not obviously shorter if the particle size is reduced. Comparison of low-molecular weight compounds (one steroid and one non-steroid hormone, with molecular weights lower than 500) and a high-molecular weight one (MW ~ 1000) was conducted. Same efficiency can be achieved with columns packed with 1.9–2.1 μm particles as with smaller particles. The column packed with 3 μm particles had the lowest reduced plate height minimum ($h = 2.2$) while the column with the smallest particles (1.5 μm) gave the highest reduced plate height minimum ($h \sim 3.0$). According to this study, the theoretically expected efficiency of very fine particles (diameter <2 μm) used in practice today is compromised. Investigation of this phenomenon is presented.

1. Introduction

Pharmaceutical industry is particularly interested in using rapid and efficient procedures for qualitative and quantitative analysis in order to cope with a large number of samples and to reduce the time required for delivery of results. Reducing analysis time and guarantying the quality of a separation in liquid chromatography (HPLC), require high kinetic efficiency. A general approach to increase the separation power is to enhance separating efficiency. The efficiency of a packed column can be described by the plate height model. There are several plate height models but van Deemter's is generally the accepted one [1]. The equation describes that efficiency varies with the linear velocity, and the nature of the second and third terms of the equation indicates a minimum value for plate height (HETP). In the third term of van Deemter equation, the particle size is squared and so the curve is steeper for larger particles at high linear velocities.

A recent solution for improving chromatographic performance is to employ columns packed with small particle diameters (i.e., sub-2 μm particles) to induce a simultaneous improvement in efficiency, optimal velocity and mass transfer. The main difficulty with using smaller diameter packings is that the pressure required to pump the mobile phase through the column, increases with the square of the particle diameter [2]. It is trendy to prepare conventional columns packed with very fine particles, in the 1–2 μm range, and operate them at high linear velocities. The small particle diameter and high linear velocity require very high inlet pressures. Most 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 [5] were the first to write about the compromise between speed and efficiency. 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. Numerous manufacturers since 2004 have introduced a new generation of columns packed with sub-2 μm porous particles [9,10] that generate reliable performance in comparison to those with conventional particle sizes [11]. In the meantime, analytical devices are able to handle pressures higher than 400 bar (such as the UPLC®) have been commercialized [12,13]. The term ultra-high pressure liquid chromatography, UHPLC, is used to describe the higher backpressure requirement (>400 bar) [14]. A critical aspect is the effect of frictional heating, which causes temperature gradients within the columns, which is significant for small particles at ultra-high pressure. The radial temperature gradient, due to the heat dissipation at the column wall,

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can cause significant loss in plate count [15,16]. Gritti and Guiochon [17] concluded that both longitudinal and radial temperature gradients are more significant when the column length is decreased. The radial temperature gradient can effectively lead to ~10% loss of efficiency when operating a 50 mm long column close to 1000 bar [18].

In recent times chromatographers' opinions differ about applying sub-2 μm particles. The adverse effects of ultra-high pressure are in focus today. On average the smaller the particle diameter is, the greater the difficulty in preparing a well-packed column bed is. Particle aggregation, frit blockage, particle fracture are all issues when high pressure is required to pack sub-2 μm particles [3]. Guo et al. [19] found that efficiency of sub-2 μm particles for small molecules is not as high as it was theoretically predicted and widely cited.

Sandra and coworkers [20] demonstrated that noteworthy differences in the optimal kinetic performance of a chromatographic system are observed compared to data for common test compounds and real life analytes. Data for test compounds do not reflect the performance attainable for pharmaceutical compounds and highlight the importance of using real life samples to perform kinetic evaluations. Guillarme et al. [21] reported that the molecular weight of the separated compounds is a critical parameter when the efficiency of a given separation is investigated. To determine the kinetic performance of columns Knox suggested such test compounds, which show ideal thermodynamic behavior, and they give well-shaped symmetrical peaks [22]. For this purpose, simple organic solutes with relative low-molecular masses, which have no strong interactive group, are expected to give the best results for column comparison.

The aim of our study was to make a critical evaluation of the practical possibilities and limitations of commercially available columns, packed with sub-2 μm particles, compared to columns packed with 2–3 μm particles. The advantages of 1.7 μm particles compared to 3.0, 3.5 and 5.0 μm particles are well documented [18,23] but according to our best knowledge the comparison of 5 cm long narrow bore columns packed with similar sized fine particles (1.5–2.5 μm) has not been investigated yet. 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 [24] from the experimental van Deemter curves. The test analytes were real life compounds, which are often analyzed in our practice. Ethinylestradiol is an orally bio-active estrogen used in almost all modern formulations of combined oral contraceptive pills, bicalutamide is an oral non-steroidal anti-androgen used in the treatment of prostate cancer and hirsutism and ivermectin which is a broad-spectrum antiparasitic medication. The results obtained with two low-molecular weight polar neutral analytes (MW = 296 and 430) and one high-molecular weight analyte (MW = 875) are presented in this study.

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 were polar neutral compounds. Ethinylestradiol (19-nor-17α-pregn-1,3,5(10)-trien-20-yn-3,17-diol) and bicalutamid ((±)-N-/4-cyano-3-(trifluoromethyl)phenyl/-3-/(4-fluorophenyl)-sulphonyl/-2-hydroxy-methyl-propanamide) were produced by Gedeon Richter Plc (Budapest, Hungary). Ivermectin (22,23-dihydroavermectin B_{1a}) was purchased from Bioastralis (Smitfield, Australia). Structural information and molecular masses of the analytes used in the study are summarized in Fig. 1.

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. Column packed with 2.0 μm YMC UltraHT Pro C18 (50 mm × 2.0 mm) particles and Restek Pinnacle DB C18 1.9 μm (50 mm × 2.1 mm) columns were generous gift from Lab-Comp Ltd., Budapest. Shim-pack XR-ODS1 and Shim-pack XR-ODS2 columns with a particle size of 2.2 μm (50 mm × 2.0 mm) were purchased from Simkon Ltd., Budapest. Phenomenex Luna C18(2)-HST column packed with 2.5 μm particles (50 mm × 2.0 mm) and Gemini NX packed with 3.0 μm particles (50 mm × 2.0 mm) were purchased from GEN-Lab Ltd., Budapest. Thermo ODS Hypersil column packed with 3 μm particles (50 mm × 2.1 mm) and Hypersil Gold column packed with 1.9 μm particles (50 mm × 2.1 mm) were obtained from Bioszeparációs Technikai 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. Zorbax SB C18 column with a particle size of 1.8 μm (50 mm × 2.1 mm) was obtained from Kromat Ltd., Budapest. Fortis C18(2) 2.1 μm (50 mm × 2.1 mm) column was received from Lab-Comp Ltd. for testing as a demo column.

2.2. Equipment, software

All measurements were performed using a Waters Acquity system equipped with binary solvent delivery pump, an auto sampler, a photo-diode array detector. The UPLC system was purchased from Waters Ltd. Budapest, Hungary. The UPLC system had a 5 μl injection loop and a 500 nl flow cell (path length = 10 mm). For all experiments, instrument control was performed using Empower 2 (Waters) software.

Calculation and data transferring to obtain the kinetic plots were 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 MS Excel (Solver).

2.3. Apparatus and methodology

The mobile phase was prepared by mixing appropriate amount 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 acetonitrile/water for ethinylestradiol and bicalutamid elution, and 95/5 acetonitrile/water for ivermectin.

The stock solutions of reference standards were dissolved in acetonitrile (1000 μg/ml). The solutions for the chromatographic runs were diluted from the stock solutions 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 between 2 and 10 for the tested compounds on each stationary phase. Differences in the retention factors (k) affect the shape of the obtained van Deemter and Knox curves, as both the B- and C-terms of the equations depend on analyte retention [25]. No attempt was made to adjust the mobile phase composition to ensure 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. For ivermectin, a relatively high organic modifier concentration in the mobile phase (>90%) is necessary to keep the retention in the range of k = 1–10. The column temperature was set 35 °C, the injected volume was 1 μl, and UV detection at 220 nm (40 Hz) was 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 analyte properties on the performance characteristics.

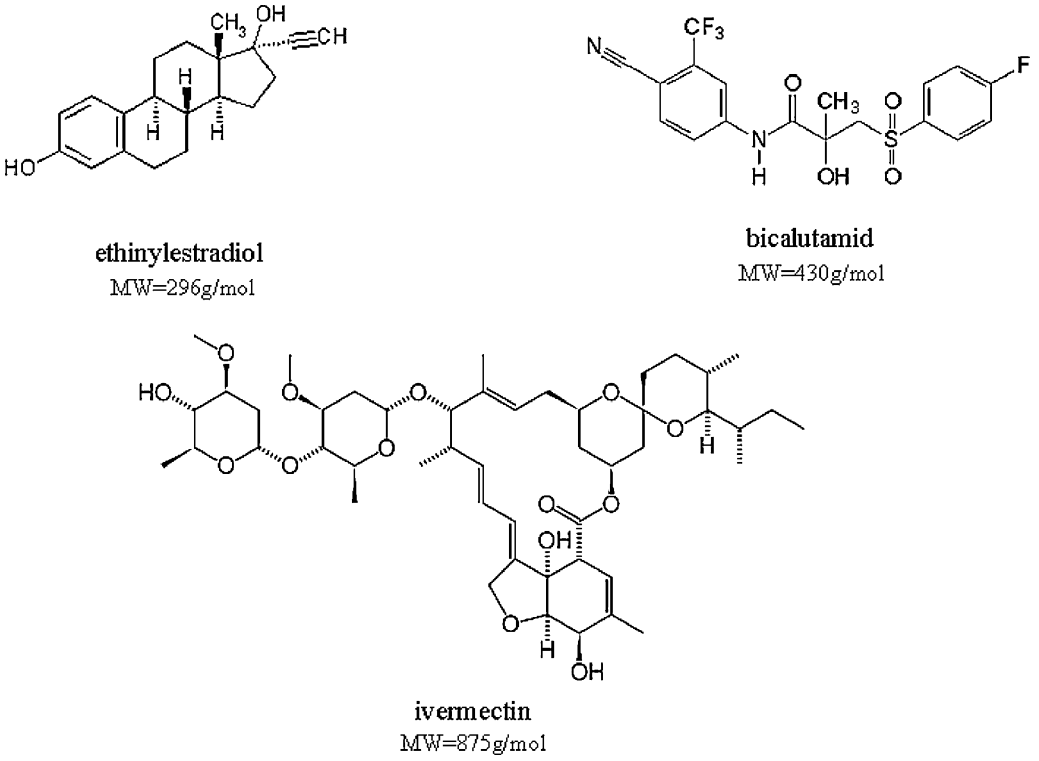

Fig. 1. Chemical structure and molar masses of the analytes employed in the current study.

During the flow study the linear velocity of mobile phase was increased from 0.03 cm/s up to about 0.5 cm/s. The measured plate numbers were corrected for extra-column volume and band broadening, which was measured by injecting ethinylestradiol with a zero-dead-volume connector instead of the column. The plate heights for kinetic curves were calculated using the corrected plate counts.

2.4. Equations used for calculation

The kinetic performance of different columns has been compared mostly in terms of their van Deemter plots [26]. The van Deemter equation describes that efficiency varies with linear velocity.

$$H = Ad_p + \left(\frac{BD_M}{u}\right) + C\left(\frac{d_p^2 u}{D_M}\right) \quad (1)$$

where H is the HETP, d_p the particle size of the column packing material, u the linear velocity of the mobile phase, D_M the analyte diffusion coefficient 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:

$$u_{opt} = \frac{D_M}{d_p}\sqrt{\frac{B}{C}} \quad (2)$$

Accordingly the optimum linear velocity is inversely related to the particle size, and directly proportionate to the analyte diffusion coefficient.

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).

$$H_{min} = d_p(A + \sqrt{BC}) \quad (3)$$

The plate numbers of the columns were measured at a sequence of different flow rates to obtain the reduced plate height (h) versus reduced linear velocity (ν) plot. The calculation was achieved according to Horvath and Lin [27]:

$$\nu = \frac{ud_p}{D_M} \quad (4)$$

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

$$h = \frac{H}{d_p} \quad (5)$$

The h versus ν curves were fitted into Knox equation:

$$h = A\nu^{1/3} + \left(\frac{B}{\nu}\right) + C\nu \quad (6)$$

Such plots as van Deemter and Knox lack permeability considerations. Alternative approaches, mostly based on the kinetic principles first expounded by Giddings [28] have been used with some success by various authors [5,24,29]. In the "Poppe plot" the plate time (t_0/N) is plotted against the plate number (N). This is an elegant tool for visualizing the compromise between separation efficiency and speed [24]. With the help of Poppe plots, it is possible to calculate the best plate number that can be reached with a certain maximum allowable pressure. N and t_0 can be calculated according to the following equations:

$$N = \frac{\Delta P}{\eta}\left(\frac{K_{\nu_0}}{uH}\right) \quad (7)$$

$$t_0 = \frac{\Delta P}{\eta}\left(\frac{K_{\nu_0}}{u^2}\right) \quad (8)$$

where ΔP is the available pressure drop, K_{ν_0} the column permeability, η the mobile phase viscosity.

Later Desmet et al. [25,30] 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_{v_0} versus K_{v_0}/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_{v_0}).

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

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

in which ΔP is the pressure drop over the column with length L, K_{v_0} the column permeability, η the mobile phase viscosity and u the linear velocity. Viscosity values were calculated using equations derived by Chen and Horváth [31], and analyte diffusion coefficients were calculated using the Wilke–Chang equation [32].

3. Results and discussion

3.1. van Deemter and Knox plots (flow study)

At first the kinetic properties of the investigated columns were assessed at the temperature of 35 °C by means of the van Deemter plots. A small amount of analytes (10 μg/ml) diluted with mobile phase was injected to acquire the data. Ethinylestradiol and bicalutamide were eluted with acetonitrile/water 48/52 (v/v), ivermectin was eluted with acetonitrile/water 95/5 (v/v). The injection volume of 1 μl was applied. Fig. 2 shows the obtained HETPs (micrometer) versus the linear velocity (cm/s). 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 among the A, B, and C terms, optimum linear velocity and minimum plate heights for each of the columns is presented in Table 1.

It can be obviously seen that the obtained results significantly depend on the test analyte. A, B, and C are system constants determined by the magnitude of band broadening due to eddy diffusion, longitudinal diffusion, and resistance to mass transfer, respectively [33,34]. A depends on both the quality of the column packing and the contribution of slow mass transfer across the moving stream. The obtained values of the coefficients A, B and C for the different columns, can be accounted for by the minor contribution of several effects (packing characteristics and the combined effects of frictional heating and high pressures) on the velocity-dependence of the plate height. B- and C-terms of the equation depend on analyte retention. The B-term is expected to increase with analyte retention as more time is available for diffusion to take place in the mobile phase. The obtained A terms for ethinylestradiol and bicalutamide are quite similar but for ivermectin they are significantly different. The larger the molecular weight of the analyte is the higher the obtained C-term is in the fitted van Deemter curves. When ivermectin is investigated the values of C-term is 2–7 times higher compared to values obtained with ethinylestradiol and bicalutamide. It can be probably explained with the diffusion coefficient of the analytes (D_M) and with the variance in pore structure of the different particles. If we follow the theory we would expect that smaller particles perform lower plate heights and higher optimum linear velocity. According to this study this is not so evident especially not in the case of ivermectin. The differences of efficiency in the range of 1.5–2.2 μm particles are not as significant as in the range of 2.2–3.0 μm. The smallest particles (1.5 μm) do not perform as much higher efficiency compared to the column packed with 1.7–2.1 μm particles as we expected due to the theory. Moreover in the case of ivermectin a surprising result can be seen, that the column packed with the smallest particle (1.5 μm) performs higher minimum plate height than the columns packed with larger particles (1.7, 1.8, 2.1 and 2.2 μm). Another amazing phenomenon is that the optimal linear velocity does not increasing significantly when the particle size is smaller than 2.1 μm. The columns packed with 1.5, 1.7, 1.8, 1.9, 2.0 and 2.1 μm provide practically the same optimal velocity, however favorable mass transfer characteristics should translate into a shift to the optimal velocity to higher values when the particle size is reduced. When the particle size was reduced to 1.5 μm from 2.1 μm, we did not observe sig-

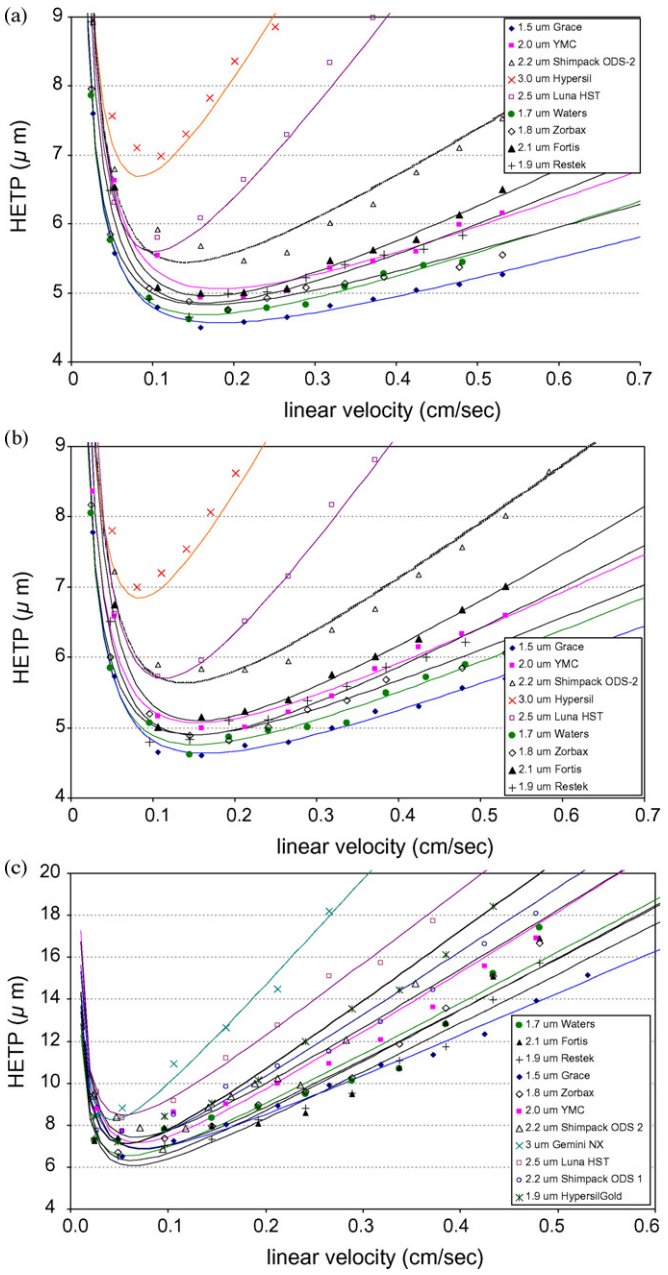

Fig. 2. Flow curve comparison of commercially available sub-3 μm and sub-2 μm packed columns obtained with (a) ethinylestradiol, (b) bicalutamide and (c) ivermectin. Experiments were conducted on 5 cm long narrow bore columns in 48/52 ACN/H_2O (a and b) and 95/5 ACN/H_2O (c) at 35 °C.

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

Column	*A*	*B*	*C*	μ_{opt} (cm/s)	$HETP_{min}$ (μm)
Analyte: ethinylestradiol					
1.5 μm Grace Vision HT C18	3.371	0.110	3.265	0.183	4.567
1.7 μm Waters BEH C18	3.391	0.105	3.984	0.163	4.686
1.8 μm Zorbax C18	3.616	0.102	3.610	0.168	4.830
1.9 μm Restek Pinnacle C18	3.193	0.136	5.072	0.164	4.856
2.0 μm YMC UHT Pro C18	3.422	0.149	4.489	0.182	5.060
2.1 μm Fortis C18	2.997	0.163	5.870	0.167	4.956
2.2 μm Shimpack XR ODS-2	3.347	0.148	7.481	0.141	5.450
2.5 μm Luna C18 HST	2.354	0.165	16.008	0.102	5.604
3.0 μm Hypersil ODS	3.162	0.147	21.127	0.084	6.691
Analyte: bicalutamide					
1.5 μm Grace Vision HT C18	3.185	0.119	4.416	0.164	4.635
1.7 μm Waters BEH C18	3.278	0.112	4.872	0.153	4.758
1.8 μm Zorbax C18	3.415	0.112	4.941	0.151	4.903
1.9 μm Restek Pinnacle C18	2.995	0.145	6.271	0.152	4.900
2.0 μm YMC UHT Pro C18	3.337	0.134	5.619	0.154	5.071
2.1 μm Fortis C18	2.853	0.175	7.204	0.156	5.097
2.2 μm Shimpack XR ODS-2	3.244	0.165	8.679	0.138	5.641
2.5 μm Luna C18 HST	2.901	0.214	16.861	0.113	5.701
3.0 μm Hypersil ODS	3.174	0.152	22.097	0.083	6.845
Analyte: ivermectin					
1.5 μm Grace Vision HT C18	3.881	0.112	20.376	0.074	6.905
1.7 μm Waters BEH C18	3.644	0.084	24.913	0.059	6.535
1.8 μm Zorbax C18	3.212	0.133	24.922	0.073	6.853
1.9 μm Restek Pinnacle C18	3.050	0.096	23.930	0.063	6.076
1.9 μm Hypersil Gold C18	3.300	0.107	34.257	0.056	7.123
2.0 μm YMC UHT Pro C18	3.119	0.139	29.566	0.068	7.167
2.1 μm Fortis C18	3.137	0.100	25.266	0.063	6.316
2.2 μm Shimpack XR ODS-1	3.674	0.097	30.920	0.056	7.133
2.2 μm Shimpack XR ODS-2	3.560	0.129	28.894	0.067	7.419
2.5 μm Luna C18 HST	4.542	0.107	36.050	0.055	8.472
3.0 μm Gemini NX	3.846	0.093	51.904	0.042	8.250

nificant improvement in efficiency although the efficiency should have been inversely proportional to the particle size. In the case of 3.0, 2.5 and 2.2 μm particles, the observed efficiency is in good agreement with the theory. It is necessary to emphasize that all the columns, which were compared came from different providers and thus, both the quality of packing and particle size distribution could have an effect on efficiency.

In generally the Knox plots provide a comparison among different columns, which should be independent of the particle size [22]. A poorly packed column has a high value of *A* (2.5–5), and a well-packed column has a low value of *A* (0.5–1.5). In the case of low ν values, the second contribution (B/ν) is predominant whereas the third term $C\nu$ becomes predominant in the case of high ν values. *B* accounts for the longitudinal diffusion and depends on the solute retention. Generally a *B* value of 2 is admitted by many authors, but depending on the retention factor of the compound, *B* could attain values up to 5 [35,36]. The *C* term expresses the effect of mass transfer resistance in both stagnant mobile and stationary phases and is critical for good performance especially at high-reduced velocities. A satisfactory value of *C* for an efficient packing material is around 0.1–0.2 [22]. For a well-packed column the minimum reduced plate height normally is in the range of 2–2.5 [3]. It can be seen in this study that the minimum reduced plate heights obtained with sub-2 μm particles are higher than the plate heights obtained with columns packed with 2–3 μm particles (Fig. 3 and Table 2). The lowest reduced plate height is measured for the 2.5 and 3 μm columns ($h_{min} \sim 2.2$–2.3), while the highest value was obtained for the column packed with the smallest (1.5 μm) particles ($h_{min} \sim 3.0$). This suggests that the columns packed with larger particles (2.5–3.0 μm) have a more ideal packed bed. The minimum reduced plate height measured for Waters BEH 1.7 μm column ($h_{min} = 2.75$) is in good agreement with the results of previous reports [37–39]. The obtained reduced plate heights and Knox plots prove the previous results, that the smaller the particle diameter is the greater the difficulty in preparing a well-packed column bed is [3,15,19]. Another possible explanation of the efficiency loss may be the formation of frictional heat effect at high pressure [15–17,40].

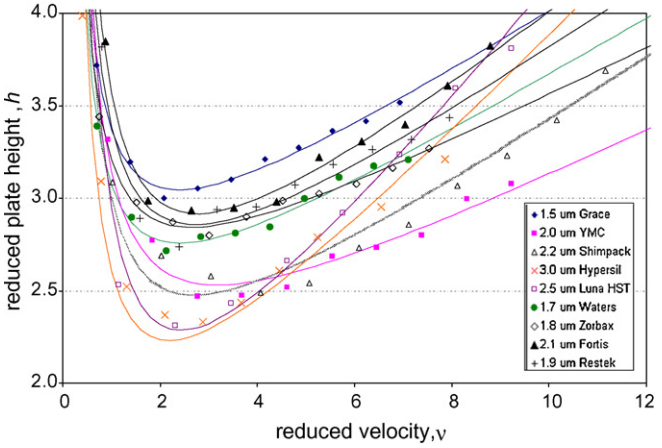

Fig. 3. Knox curves of commercially available sub-3 μm and sub-2 μm packed columns obtained with ethinylestradiol. Experiments were conducted on 5 cm long narrow bore columns in 48/52 ACN/H_2O at 35 °C, $D_M = 1.15 \times 10^{-5}$ cm^2/s.

An expressive theoretical comparison is displayed in Fig. 4. These figures show the differences (Δ) between the experimental and theoretically predicted *H*–*u* curves and show a tendency of deviation from theoretical values. The smaller the particle size is the higher the deviation is. If the same quality of packing is supposed for the 1.5, 1.7, 1.8, 1.9, 2.0, and 2.2 μm columns as for the really well-packed 3 μm column, and we consider only the particle size difference between the columns, than the theoretical van Deemter plots can be calculated easily according to Eq. (1). It can be seen that, when the particle size is 2.2 μm, the theory and experimental data of plate heights are in good correlation. When the particle size is lower than 2.2 μm the theoretically expected efficiency fails in practice. The difference between the theoretical and experimental efficiency increases as the particle size is reduced. In the case of 1.5 μm Grace column, the efficiency loss expressed in minimum plate height is more than 1 μm.

3.2. Kinetic plots (Poppe plots)

Usually the kinetic characteristics of different columns have been compared in terms of the van Deemter curves. However, these plots do not consider the column permeability and they do not tell one which particle design and what column format to choose for a particular separation [30]. To address this issue, Poppe proposed the "Poppe plot" wherein the plate time (t_0/N) is plotted against the

Table 2
Summary of fitted Knox constants (*A*, *B*, and *C*), optimal reduced linear velocity (ν) and minimum reduced plate heights (h).

Column	*A*	*B*	*C*	υ_{opt}	h_{min}
Analyte: ethinylestradiol					
1.5 μm Grace Vision HT C18	2.247	0.953	0.167	2.389	3.045
1.7 μm Waters BEH C18	1.995	0.915	0.159	2.403	2.756
1.8 μm Zorbax C18	2.135	0.937	0.133	2.650	2.842
1.9 μm Restek Pinnacle C18	1.878	1.326	0.181	2.710	2.857
2.0 μm YMC UHT Pro C18	1.711	1.300	0.129	3.174	2.530
2.1 μm Fortis C18	1.763	1.592	0.208	2.764	2.816
2.2 μm Shimpack XR ODS-2	1.522	1.284	0.178	2.688	2.477
2.5 μm Luna C18 HST	0.707	1.912	0.326	2.422	2.286
3.0 μm Hypersil ODS	1.054	1.281	0.270	2.178	2.230

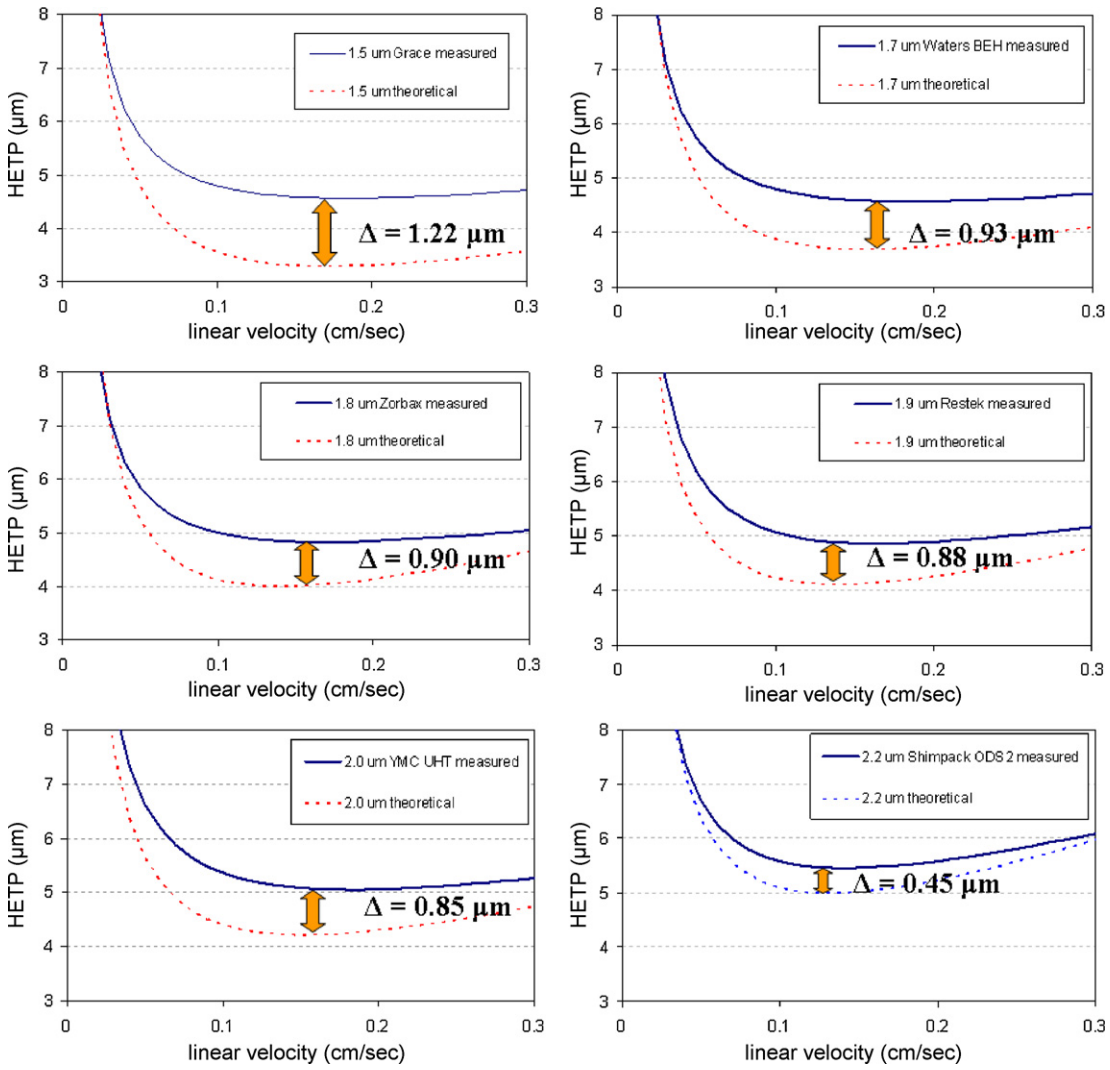

Fig. 4. Theoretical van Deemter plots of the 5 cm long narrow bore columns. The theoretical estimation of the plots was calculated according to Eq. (1) by replacing the particle size of 3 μm to 1.5, 1.7, 1.8, 1,9, 2.0 and 2.2 μm, and keeping the *A*, *B*, and *C* values of the 3 μm column.

plate number (*N*). This is a neat tool for visualizing the compromise between separation efficiency and speed [24].

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. 5 shows the calculated isocratic Poppe plots on the compared columns at maximum applicable pressure for each column to represent the utilization of maximum performance (the data for maximum pressure were obtained from the column manufacturers). These plots represent the theoretical separation speed when the maximum performance of the UPLC system is utilized (P_{max} = 1000 bar).

The resulting curves, one for each column (particle size), demonstrate the maximum speed obtainable at a given required plate number (*N*) and also demonstrate the effect of the choice of column (particle size). Generally, the smaller the particle is, the smaller the plate number is [24]. Please note that the plate times depend on the maximum allowable pressure drop, which is different for the tested columns. A column packed with larger particles can offer faster separation if it has a stationary phase with stronger mechanical stability (higher P_{max}) than the column packed with smaller particles.

In the case of ethinylestradiol (Fig. 5a) the 2.1 μm Fortis, 1.9 μm Restek and the 1.7 μm Waters column provide practically the same plate time values and offer the shortest analysis time if the separation requires 5000–100,000 plate counts. The 1.5 μm Grace column is only useful if very low plate numbers ($N < 4000$) are required. It can be beneficial when very short columns (L = 2–3 cm) are applied and only a small number of analytes are planned to be separated. It is surprising that the 2.1 μm Fortis column outperforms other columns, which are packed with smaller particles (2.0 μm YMC and 1.8 μm Zorbax). If the separation demands higher than 100,000 plate counts the Hypersil column packed with 3.0 μm particles can give a good chance for the shortest analysis. Theoretically the 100,000 plate counts can be reached with a 102 cm long Hypersil 3 μm column (0.022 cm/s). If the column length is a critical factor, the most favorable choices are the 1.7 μm Waters, 1.9 μm Restek and 2.1 μm Fortis packing, because these materials can perform the 100,000 plate numbers with the shortest columns (74–77 cm long columns at 0.029 cm/s linear mobile phase velocity). The sate of the art column packing technology cannot allow packing such long columns. The only way to get these high plate counts is to connect the columns in sequence. However applying these column lengths the analysis time increases significantly, the separation becomes to be impractical, and on the other hand the coupling of the columns in series

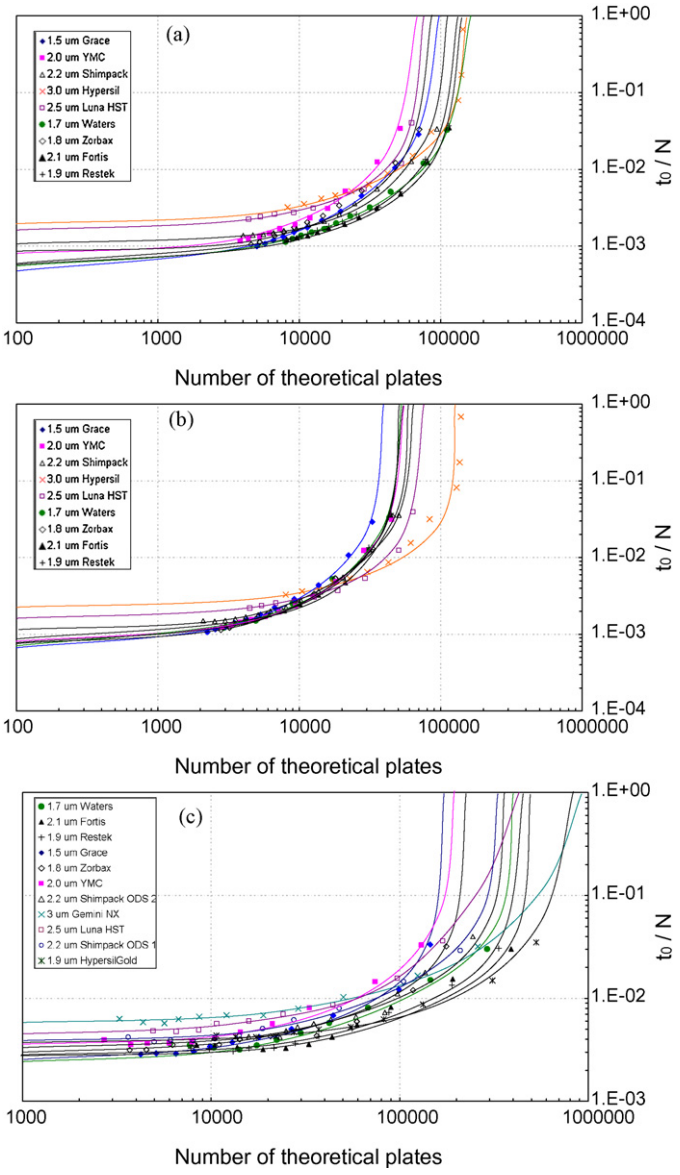

Fig. 5. Poppe plots of commercially available sub-3 μm and sub-2 μm packed columns obtained with (a) ethinylestradiol, (b) bicalutamide and (c) ivermectin. Experiments were conducted on 5 cm long narrow bore columns in 48/52 ACN/H_2O (a and b) η = 0.85 cP, and 95/5 ACN/H_2O (c) η = 0.34 cP at 35 °C. Available max. pressure: 1000 bar for Waters BEH, Restek Pinnacle, Fortis and Hypersil Gold columns, 830 bar for Grace Vision column, 700 bar for Shimpack XR-ODS2 column, 600 bar for Zorbax column, 500 bar for YMC UHT Pro column and 400 bar for Shimpack XR-ODS1, Luna HST, Gemini NX and Hypersil columns.

would probably causes lower efficiency compared to the calculated result.

When bicalutamide is investigated (Fig. 5b), the obtained Poppe plots are in good agreement with the theoretically expected curves. All curves cross over in a plate number range of around 3000–20,000. On the right side of these crossover points the columns packed with greater particles provide smaller plate time. On the left side, the smaller particles produce faster separations. If the analysis requires N < 2500 plate counts it can be achieved within the shortest time with the 1.5 μm Grace column. If higher than 30,000 plate numbers are demanded the 3.0 μm Hypersil column performs the shortest separation. It is necessary to mention that if 5 cm long columns are applied, the obtained plate numbers can be expected in the range of N = 5000–10,000, and in this range all column packed with sub-2 μm particles offer practically the same plate times. If one needs an ultra-fast separation (e.g. $t_0 \sim$ 10–20 s), the gain in plate count by reducing particle size is negligible in the range of 1.5–2.0 μm.

The obtained curves for ivermectin (Fig. 5c) – which is the largest one (MW = 875) – show different behaviors compared to the smaller analytes. The Hypersil column packed with 3 μm particles gives faster separation only when extremely high plate numbers are required (N > 800,000). In the range of N = 10,000–70,000 the 2.1 μm Fortis and 1.9 μm Restek columns provide the fastest analysis. If the separation requires a plate number of N < 10,000 (which is typical in the case of 5 cm long narrow bore columns) the analysis can be achieved within practically the same time with the 1.5 μm Grace, 1.7 μm Waters, 1.9 μm Restek and 2.1 μm Fortis columns. An interesting result was obtained with the same particle size prepared by two manufacturers (1.9 μm Restek and Hypersil Gold). The two columns give significantly different efficiency. The Hypersil Gold column offers much faster separation if the required plate numbers are higher than 80,000 while the Restek column is better for ultra-fast separations (short columns).

The performance in kinetic plot representation is elevated for ivermectin compared to the other two compounds. Ivermectin has been eluted with 95% ACN, a mobile phase with very low viscosity (η = 0.34 cP at 35 °C) while ethinylestradiol and bicalutamide have been eluted with 48% ACN (η = 0.85 cP at 35 °C). The low viscosity of the mobile phase applied for ivermectin elution probably explains this phenomenon mentioned above (according to Eqs. (7) and (8)).

We can draw the conclusion that the 1.7 μm Waters, 1.9 μm Restek and 2.1 μm Fortis columns offer practically the same efficiency and separation speed when 5 cm long narrow bore columns are applied. The Zorbax 1.8 μm column presents higher plate times (slower separation) compared to the above-mentioned columns in the case of each three analytes because of its relatively low maximum available pressure drop (P_{max} = 600 bar). The efficiency of the 1.5 μm Grace column is more comparable to the 1.7 μm Waters, 1.9 μm Restek and 2.1 μm Fortis columns'. The maximum allowable pressure of the Grace column is 830 bar which is weaker than the other columns' (1000 bar). This is why the 1.5 μm Grace column can be advantageous only when the required plate count is relatively low. The benefit provided by a higher pressure limit (i.e. 1000 bar) of the columns can be promising for column development.

3.3. Practical example: impurity profiling of ethinylestradiol containing tablet (isocratic elution); comparison of column efficiency

This example presents a fast isocratic separation of ethinylestradiol impurities and degradation products from spiked tablets, using 1.7 μm Waters, 1.9 μm Restek and 2.1 μm Fortis columns. The substances were extracted from the tablet with acetonitrile–water solvent mixture then were sonicated, centrifuged and diluted with the mobile phase. The chromatographic conditions were the same except for the mobile phase. The strength of the mobile phase was adjusted to ensure constant retention (k) for all analytes on each column. The isocratic mobile phase consisted of 30/70 acetonitrile/water for Waters 1.7 μm column, 32/68 acetonitrile/water for Restek 1.9 μm column and 35/65 acetonitrile/water for Fortis 2.1 μm column. The flow rate was set as 0.7 ml/min. The columns were thermostated at 35 °C, the injected volume was 1 μl, and UV detection at 220 nm (40 Hz) was applied. The obtained chromatograms show very similar efficiency (Fig. 6). This example also supports our result that reducing the size of particles below 2.1 μm is not as significant as it can be expected. The investigated three columns nearly perform the same separation. It is necessary to mention that the suitability of a separation mainly depends on the selectivity of the separation media. In this study we have not intended to compare the selec-

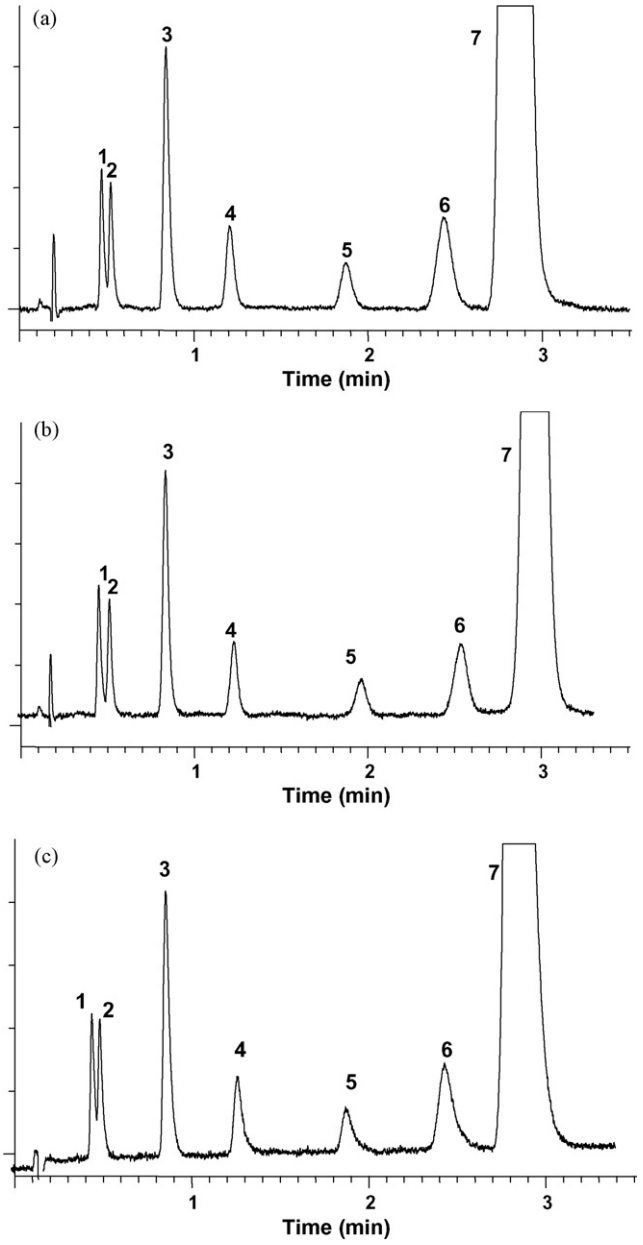

Fig. 6. Chromatograms of ethinylestradiol containing spiked tablet samples, obtained with (a) Acquity UPLC BEH C18 1.7 μm (50 mm × 2.1 mm), (b) Restek Pinnacle C18 1.9 μm (50 mm × 2.1 mm) and (c) Fortis C18 2.1 μm (50 mm × 2.1 mm) columns. Chromatographic conditions: mobile phase: acetonitrile–water 30–70 (v/v) (a), acetonitrile–water 32–68 (v/v) (b), acetonitrile–water 35–65 (v/v) (c), flow: 0.7 ml/min, column temperature: 35 °C, injection volume: 1 μl, detection: 220 nm. Peaks: 6-α-OH-ethinylestradiol (1), 6-β-OH-ethinylestradiol (2), 6-keto-OH-ethinylestradiol (3), 16-keto-OH-ethinylestradiol (4), estradiol (5), 9,11-dehydro-ethinylestradiol (6) and ethinylestradiol (7).

tivity of the phases, we investigated only the efficiency of the columns.

4. Conclusion

During this study we have obtained such data that well proves, that the efficiency of sub-2 μm particles is not as high as it was theoretically predicted earlier. The difference between the theoretical and experimental column efficiency increases as the particle size is reduced. Data were presented on different polar neutral real life analytes, which showed that the separation time is not obviously shorter if the particle size is reduced. Similar efficiency can be achieved with columns packed with 1.9–2.1 μm particles as with smaller particles (1.5–1.8 μm). If the particle size is 2.5 μm or larger, the theory and experimental data of plate heights were in good correlation.

The obtained results significantly depend on the test analytes. The measured van Deemter and Poppe curves for ivermectin – which is the largest test analyte in this study – show different behaviors, compared to the smaller analytes. When ivermectin is investigated the values of *C*-term is 2–7 times higher compared to values obtained with ethinylestradiol and bicalutamide.

The 1.7 μm Waters Acquity BEH C18, 1.9 μm Restek Pinnacle C18 and 2.1 μm Fortis C18 columns gave practically the same efficiency and separation speed when 5 cm long narrow bore columns were applied. The benefit provided by a higher pressure limit (mechanical stability) of the columns can be promising for column development in the future.

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