Luna® 2.5 µm C18(2)-HST

Advantages of 2.5 µm for increasing the speed of analysis while maintaining high efficiency

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Part 1 – Theory

1.1 Abstract

Increasing HPLC Speed and Efficiency Without the Need for Ultra-High Pressures: New Columns Compatible With Existing and UHPLC Systems

The use of small particle HPLC columns to achieve very fast separations has garnered a great deal of attention recently. When one decreases the size of the column packing material in packed columns, one increases the overall column efficiency while decreasing the resistance to mass transfer. This, however, comes at the expense of an increase in backpressure. Several commercial sources of columns packed with materials that are sub 2 µm are now available. In order to use these columns at high linear velocities, however, pressures far exceeding that of conventional HPLC equipment are necessary. Furthermore, when these columns have been tested, their overall performance is significantly lower than predicted by theory. The lower performance is also accompanied by a higher resistance to mass transfer than would be expected, which leads to a further loss in performance when these columns are operated at very high linear velocities. We will demonstrate that we are able to obtain 80-90 % of the efficiency that current commercial sub 2 µm columns produce, at backpressures that are 30-50 % less, by using columns packed with 2.5 µm material. This significant reduction in backpressure allows these columns to be operated at pressures that are compatible with conventional HPLC equipment, which in turn allows for very high speed separations, on a wide variety of instrument platforms.

1.2 Introduction

As liquid chromatography continues to evolve, there is a growing desire to increase efficiency and decrease the analysis time of separations, thereby increasing throughput for the ever-growing number of samples. One method to increase the efficiency of the chromatographic column is to reduce the size of the packing material. The column's efficiency is inversely proportional to the packing material particle diameter, as shown in equation 1,[1].

$$N = \frac{L}{2.2 d_p} \qquad (1)$$

where N is the number of theoretical plates, L is the column length, d_p is the particle diameter, and 2.2 is the assumption for reduced plate height (h). In an effort to gain faster separation times the mobile phase linear velocity (flow rate) can be increased. When the size of the packing material is decreased, the resistance to mass transfer at high linear velocity is decreased according to the particle diameter squared. This relationship is shown in equation 2,[1-4]

$$H = 1.5 d_p + \frac{D_M}{\mu} + \frac{1}{6}\frac{d_p^2}{D_M}\mu \qquad (2)$$

where H is the height equivalent of a theoretical plate, D_M is the diffusion constant in the mobile phase, and µ is the linear velocity. The reduction in resistance to mass transfer that is obtained via using smaller particles allows the column to be run at increased linear velocities, with a lower efficiency loss than is experienced in columns that are packed with larger particles. The ability to both increase the overall efficiency, as well as maintain the efficiency at high linear velocities, allows one to maintain resolution

between critical pairs. The resolution between critical pairs, is proportional to the square root of efficiency, as is shown in equation 3,[3-4]

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k}{k+1}\right) \quad (3)$$

where R_s is the resolution, α is the column selectivity, and k is the retention factor. It is important to note from equation 3 that the selectivity and retention of the column have a much greater influence on resolution than that of efficiency, and these parameters are influenced by surface chemistry and particle carbon load, but not the size of the packing material.

There are drawbacks, however, to decreasing the size of the packing material. The first drawback being that as the size of the packing material is decreased, so are the interstitial particle spaces, assuming a well packed column. The smaller interstitial spaces lead to an increase in the resistance to flow. The increased resistance to flow means that the pressure that is required to generate a particular flow rate (linear velocity) will be increased as one decreases the size of the packing material. The pressure required to generate a particular linear velocity is inversely proportional to the particle diameter squared (d_p^2), as is shown in equation 4,[5]

$$\Delta P = \frac{\phi \eta L \mu}{d_p^2} \quad (4)$$

where ϕ is the flow resistance parameter, η is the mobile phase viscosity and ΔP is the pressure required to obtain linear velocity µ. The flow resistance parameter will be a function of the packing quality, and packing density of the column.

Recently, new HPLC instrumentation (Waters, Agilent, Jasco) has been introduced that allows operation at increased system pressures, that subsequently leads to the ability to utilize columns packed with smaller particle sized media. These higher-pressure instruments have been marketed with columns packed with media generally smaller than 2 µm in diameter. According to the theory, these smaller media should lead to a vast increase in the plates per meter that the columns generate.

It is generally accepted that a well packed column is a column that has a reduced plate height (plate height normalized for the size of the packing material) of between 2.2 and 2.5. For most conventional commercial HPLC columns, one expects columns having a 1.7 - 1.8 µm material to generate 222,000 – 267,000 plates per meter. The efficiencies that are being observed experimentally, however, are significantly lower than these predicted values.

This report compares the efficiency and backpressure commonly observed for sub 2 µm materials, as well as the new 2.5 µm Luna® material. The data will illustrate that the performance of sub 2 µm materials is much lower than expected for columns packed with these materials, and subsequently Phenomenex has been able to achieve the same efficiency or very close to the same efficiency as sub 2 µm materials, with a 2.5 µm material, at one half to two thirds the backpressure. Since columns packed with the 2.5 µm media have lower backpressure, it means these 2.5 µm columns may be operated at pressures that are traditionally used in HPLC instruments, leading to a much greater flexibility in instrumentation, ultimately leading to greater method portability across laboratories and instrument platforms.

Part 2 – Set Up

2.1 Experimental

All LC separations were performed on systems equipped with a binary pump, variable wavelength detector and micro flow cell system [Jasco X-LC system (Easton, MD) or an Agilent 1100 HPLC (Wilmington, DE)]. All HPLC grade solvents were purchased from EMD (Gibbstown, NJ) and used as received unless otherwise noted. The reverse phase column test mixture was purchased from Phenomenex (Torrance, CA) and contained the following test probes, Uracil (0.01 mg/mL), Acetophenone (0.22 mg/mL), Benzene (9.42 mg/mL), Toluene (9.42 mg/mL), and Naphthalene (9.42 mg/mL). For all work done on the X-LC system, this mixture was diluted by a factor of ten using acetonitrile. For individual run conditions please refer to the figure captions.

All columns were purchased from the individual manufacturers and used as received.
Zorbax® SB-C18 1.8 µm 50 x 2.1 mm columns from Agilent Technologies (Wilmington, DE).
Hypersil GOLD™ C18 1.9 µm 50 x 2.1 mm from Thermo Electron Corporation (Waltham, MA).
ACQUITY UPLC™ BEH C18 1.7 µm 50 x 2.1 mm columns from Waters Corporation (Milford, MA).
Luna® 2.5 µm C18(2)-HST 50 x 2.0 mm columns from Phenomenex Inc. (Torrance, CA).

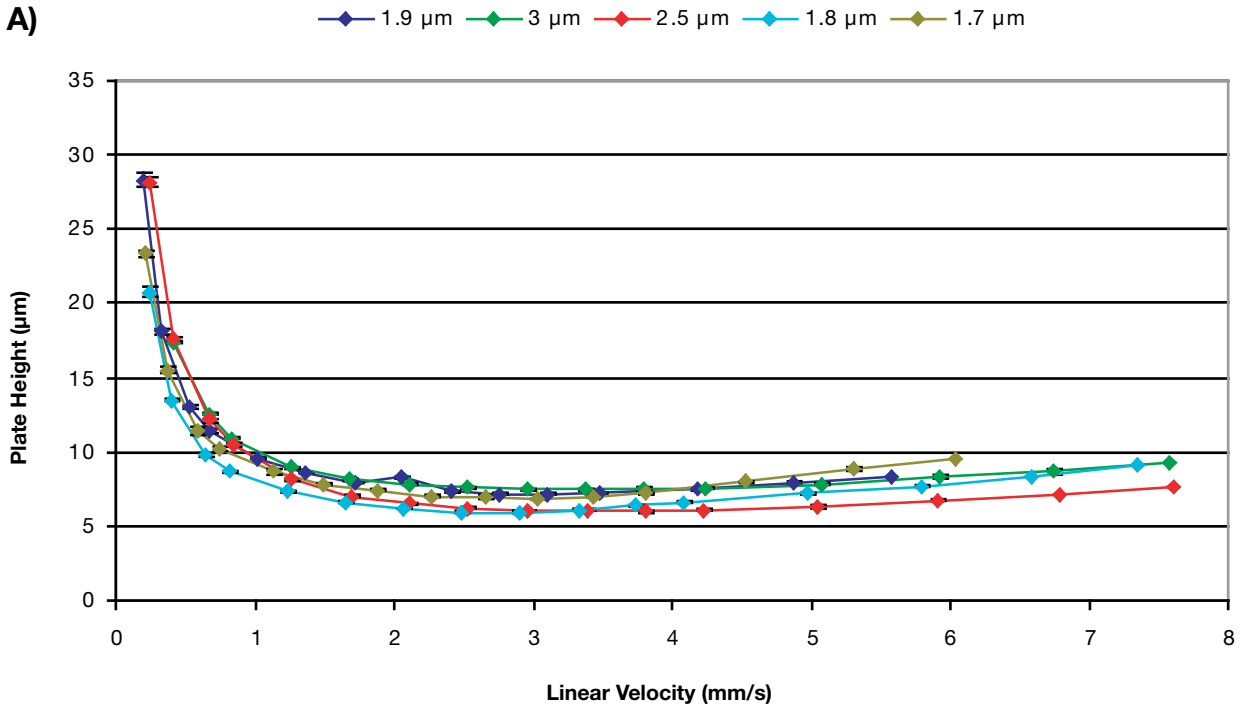
It should be noted that all efficiency and asymmetry data shown herein was based upon calculations done either by ChemStation software, or by EZChrom Elite software (Agilent Technologies). All efforts were made to ensure system suitability across instrument platforms, and results were verified between different independent laboratories.

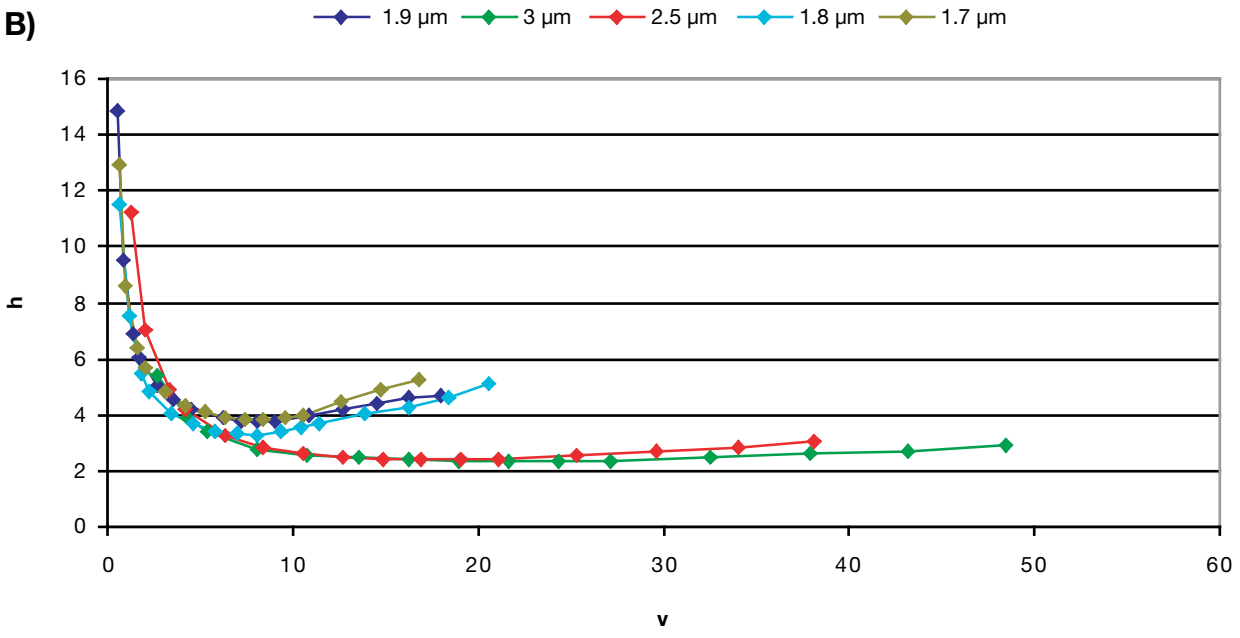
Part 3 – Results

3.1 Results and Discussion

As the packing material size decreases (***Equation 1***) there should be an increase in the efficiency (theoretical plate height reduction). ***Equation 2*** also indicates there should be a lowered resistance to mass transfer with the smaller particles. We therefore examined the plots of plate height vs. linear velocity (Van Deemter plots) for columns packed with media ranging from 3 µm to 1.7 µm. These Van Deemter plots are shown in ***Figure 1A***. Not only were the sub 2 µm packed columns no better than the 2.5 µm packed columns, but in many cases the sub 2 µm performance was on par with conventional 3 µm packed columns. In an effort to ascertain the overall column quality, the efficiency results from ***Figure 1A*** were normalized with respect to particle size (Knox plot, ***Figure 1B***). If columns were of equal relative performance, then the curves in 1B would all overlay each other. As seen in ***Figure 1B***, the relative performance is not the same and the curves for the sub 2 µm columns are significantly higher (worse) than either the 2.5 µm or the 3 µm columns.

Figure 1: A) Plate height vs. linear velocity % for 50 x 2 mm columns packed with various packing materials. Mobile phases were adjusted between 60-65 % acetonitrile in water in order to match retention factors for naphthalene. Injection volumes of 0.2 µL were used and detection was performed at 254 nm, B) Knox analysis of the plate height vs. linear velocity data presented in A. The diffusion constant in the mobile phase was assumed to be 5 x 10^{-5} cm^2 s^{-1} for all cases.





In ***Figure 2***, the expected performance (blue bar) is plotted along with the actual performance (red bar) measured on three different columns (averaged for three runs each). The expected performance was calculated based upon a reduced plate height of 2.2, which is not uncommon for 3 and 5 µm particles. Figure 2 shows the performance obtained from columns packed with sub 2 µm columns is consistently lower than expected from these size media. These poorer results are consistent with values reported in the literature.[6-8]

Figure 2: Theoretical efficiency expected as well as the actual efficiency that was obtained on columns with decreasing particle size. All column were 50 x 2 mm, and the theoretical efficiency is based upon a reduced plate height of 2.2. The actual efficiency numbers represent at least 3 runs on 3 different columns.

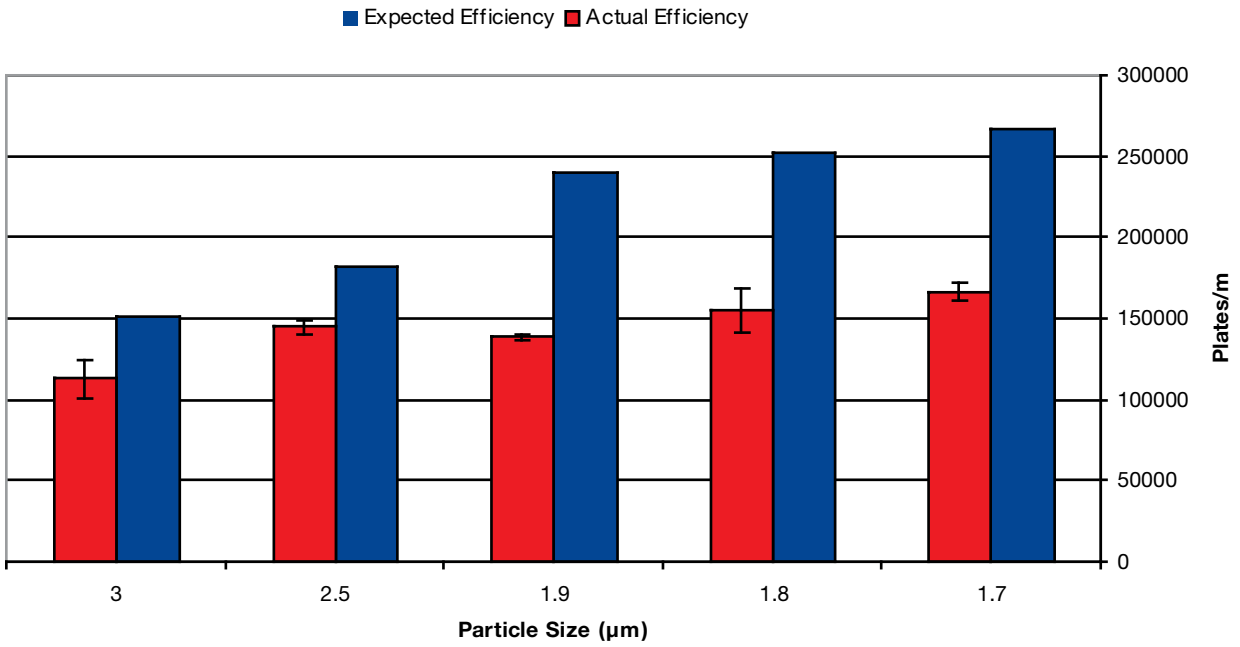
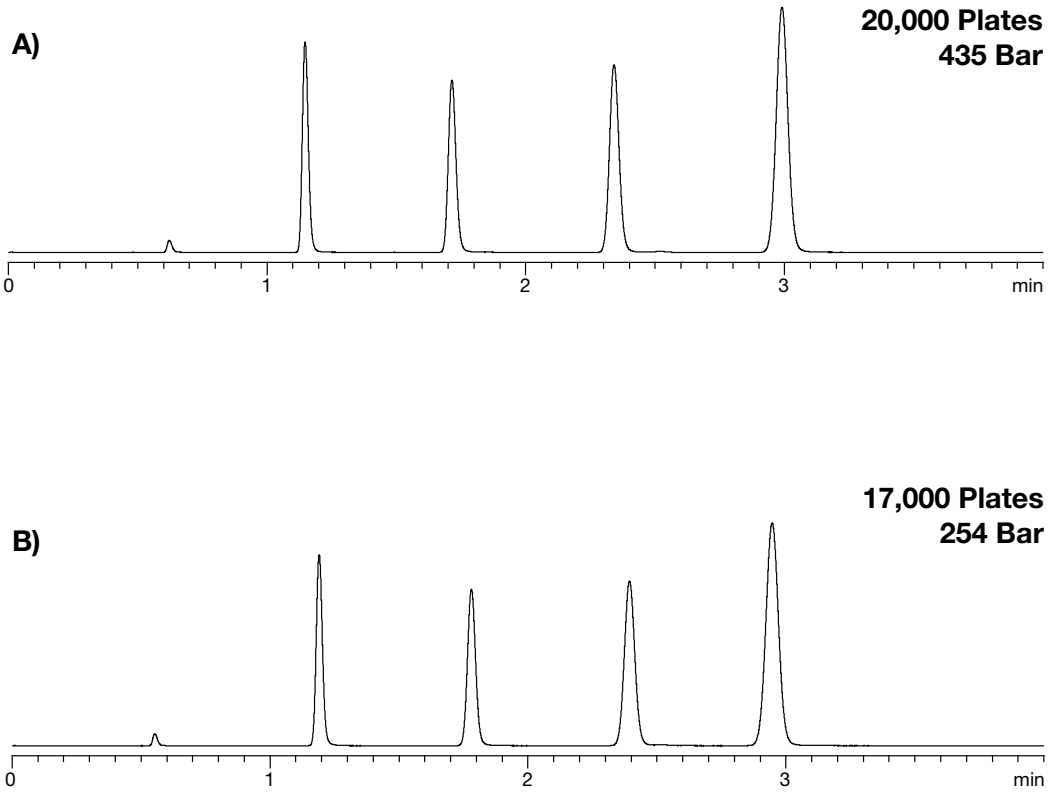


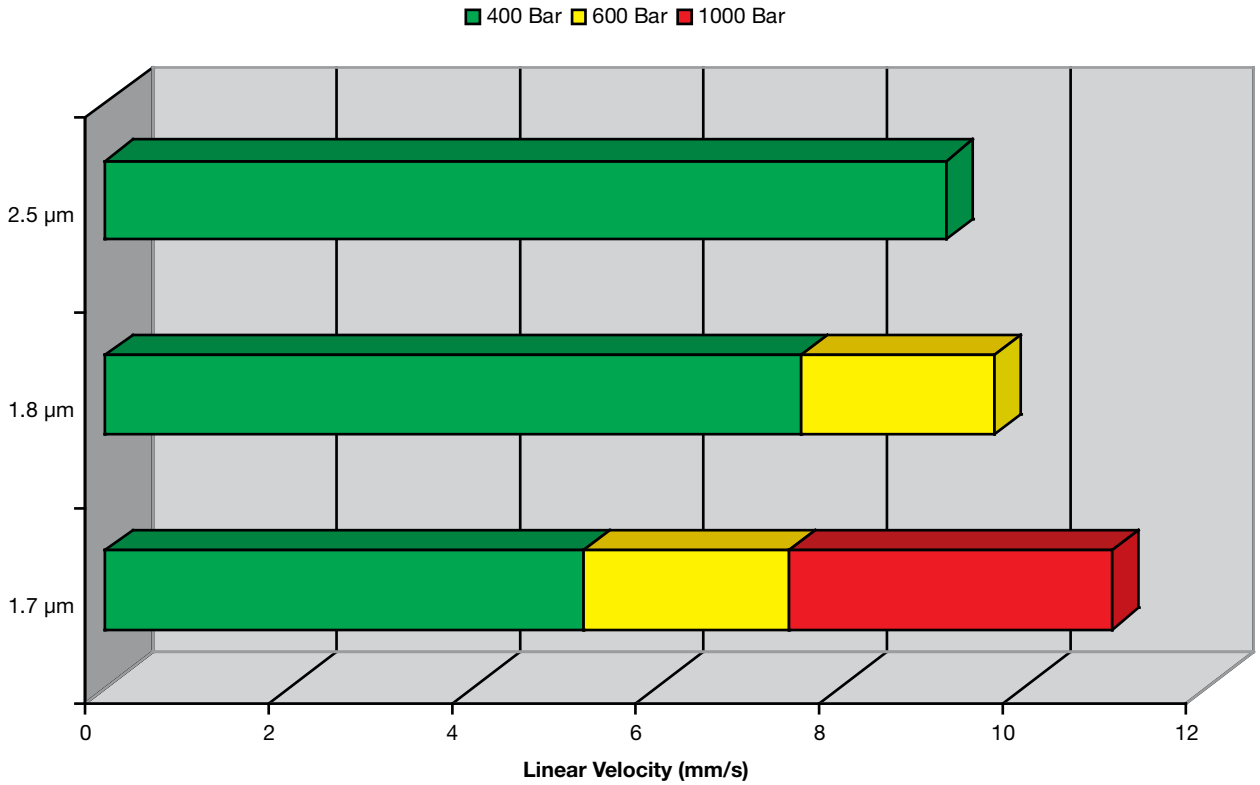
Figure 3 illustrates results obtained with a 100 x 2.1 mm column packed with a 1.7 µm material and a 100 x 2.0 mm column packed with a 2.5 µm material. The 1.7 µm column demonstrated a fifteen percent increase in efficiency; however, this column required almost 45 percent more backpressure to operate at the same linear velocity as the 2.5 µm column. If increased efficiency is a benefit and column backpressure is considered a major drawback in method transfer or portability, there is a low rate of return on the overall column performance in terms of the backpressure investment.

Figure 3: Isocratic test separations run on 100 x 2 mm columns packed with A) a 1.7 µm material and B) a 2.5 µm material. The mobile phase was 65:35 acetonitrile:water (v/v), a 0.3 µL injection was performed and UV detection was at 254 nm. The peaks in order of elution were uracil, acetophenone, benzene, toluene, and naphthalene. The flow rate on both columns was 0.35 mL/min at 25 °C.



Equation 4 shows the dependence of backpressure with respect to particle size. Due to the increased backpressure from the smaller particle, the ability to operate the column at higher linear velocities will be limited to the HPLC system pressure limit. In ***Figure 4***, the linear velocities obtained for columns packed with 2.5 – 1.7 µm media are shown. The color-coding depicts the type of HPLC system that is capable of generating the necessary backpressure to obtain the different linear velocities. In the case of the 2.5 µm column, a linear velocity of 9.2 mm/sec was achieved at 395 bar, whereas a backpressure of over 700 bar was necessary on the 1.7 µm column to obtain the same 9.2 mm/sec. It required 955 bar to obtain a linear velocity of 10.5 mm/sec with a 1.7 µm column. It therefore took 2.4x greater backpressure to obtain a 13 % increase in linear velocity compared to the 2.5 µm column, and this small reduction in analysis time could only be achieved using specialized HPLC equipment.

Figure 4: The linear velocities that are obtainable on columns packed with various sized packing materials at different pressure ranges are illustrated. All columns were 50 mm in length and tested at 60 °C, using 65 % acetonitrile. Columns were only run to the stated column pressure limit. Uracil was used as the void volume marker.

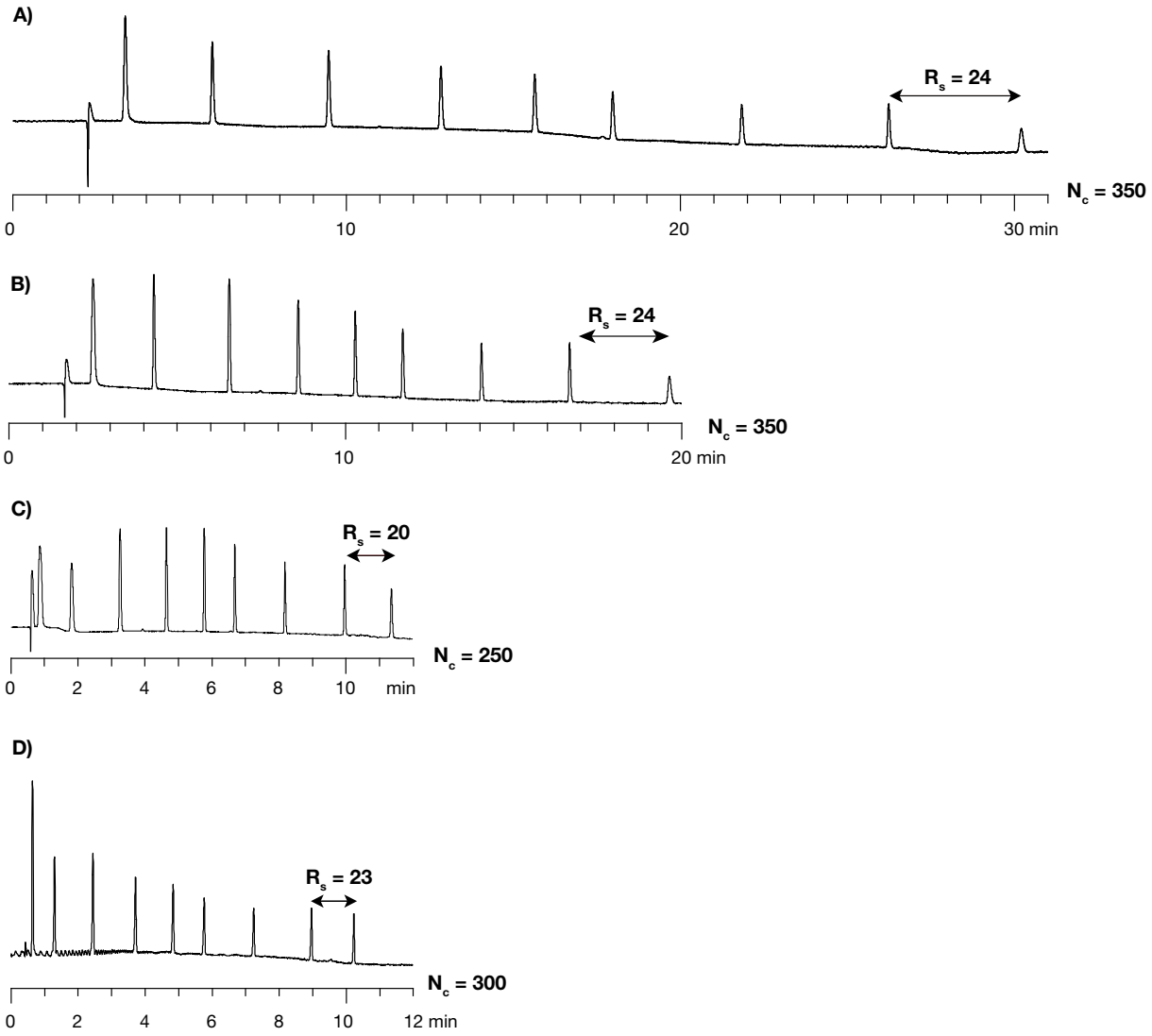


The differences demonstrated between the 2.5 µm and sub 2 µm media at this juncture have been performed under isocratic conditions. While these are useful guides, isocratic separations represent a small portion of the overall separation methods in routine use. Because peak compression and peak stacking occurs in gradient separations, the differences between sub 2 µm column performance and 2.5 µm column performance is even further blurred.

One of the primary driving forces to the use of smaller packing materials is the desire to decrease HPLC run times, without a significant sacrifice in the overall separation power. In ***Figure 5***, a mixture of nine ketones is separated using gradient conditions with decreasing particle size columns from 5 µm to 2.5 µm. Decreasing the media particle size increases the number of plates generated per meter of column. This increase in plates allows for the subsequent decrease in the column length while maintaining the overall column efficiency (***Equation 1***). It is possible to decrease the column length from 250 mm to 100 mm, without decreasing the resolution between the last two peaks by utilizing a column packed with 2.5 µm material (***Figure 5D***), instead of a column packed with 5 µm material (***Figure 5A***). This column length reduction ultimately leads to a major reduction in analysis time from 30 minutes to 10 minutes. If this same separation is performed using a 100 mm long column packed with 3 µm material (***Figure 5C***), there is a loss of resolution between the last peaks of 20 %, showing that the 3 µm 100 mm long column represents less separation power than the 2.5 µm column in gradient elution mode.

Also shown in ***Figure 5*** is the peak capacity (N_c) for each column under the chromatographic conditions shown. Peak capacity is a measure of the number of chromatographic peaks that can be resolved in a given window of time by a particular column.

Figure 5: Gradient separations of a mixture of ketones, ranging from C_3 to C_{16}. The columns were A) 250 x 4.6 mm packed with 5 µm material, B) 150 x 4.6 mm packed with 3 µm material, C) 100 x 3.0 mm packed with 3 µm material, and D) 100 x 2.0 mm packed with 2.5 µm material.



In the examples shown in ***Figure 5***, it is evident that one can afford to lose some of the resolving power in an effort to decrease analysis time. If analysis time reduction is the primary concern, then the column can be further shortened from 100 mm to 50 mm as demonstrated in ***Figure 6***. Reducing the column length also decreases the back pressure required to operate the column, thereby allowing for increases in flow rate, which can be used to even further shorten the analysis time. ***Figure 6*** shows that by reducing the column length and increasing the flow rate, a further 5 fold reduction in analysis time can be achieved. The reduction in analysis time, however, does come at the expense of a 50 % loss in resolution but the overall resolution is still sufficient to allow for accurate peak integration.

Figure 6: Gradient separations of a mixture of ketones, ranging from C_3 to C_{16}. The columns were A) 100 x 2.0 mm packed with Luna 2.5 µm C18(2) material, run at 0.65 mL/min B) 50 x 2.0 mm packed with Luna 2.5 µm material, run at 0.65 mL/min and C) 50 x 2.0 mm packed with Luna 2.5 µm C18(2) material run at 1.0 mL/min.

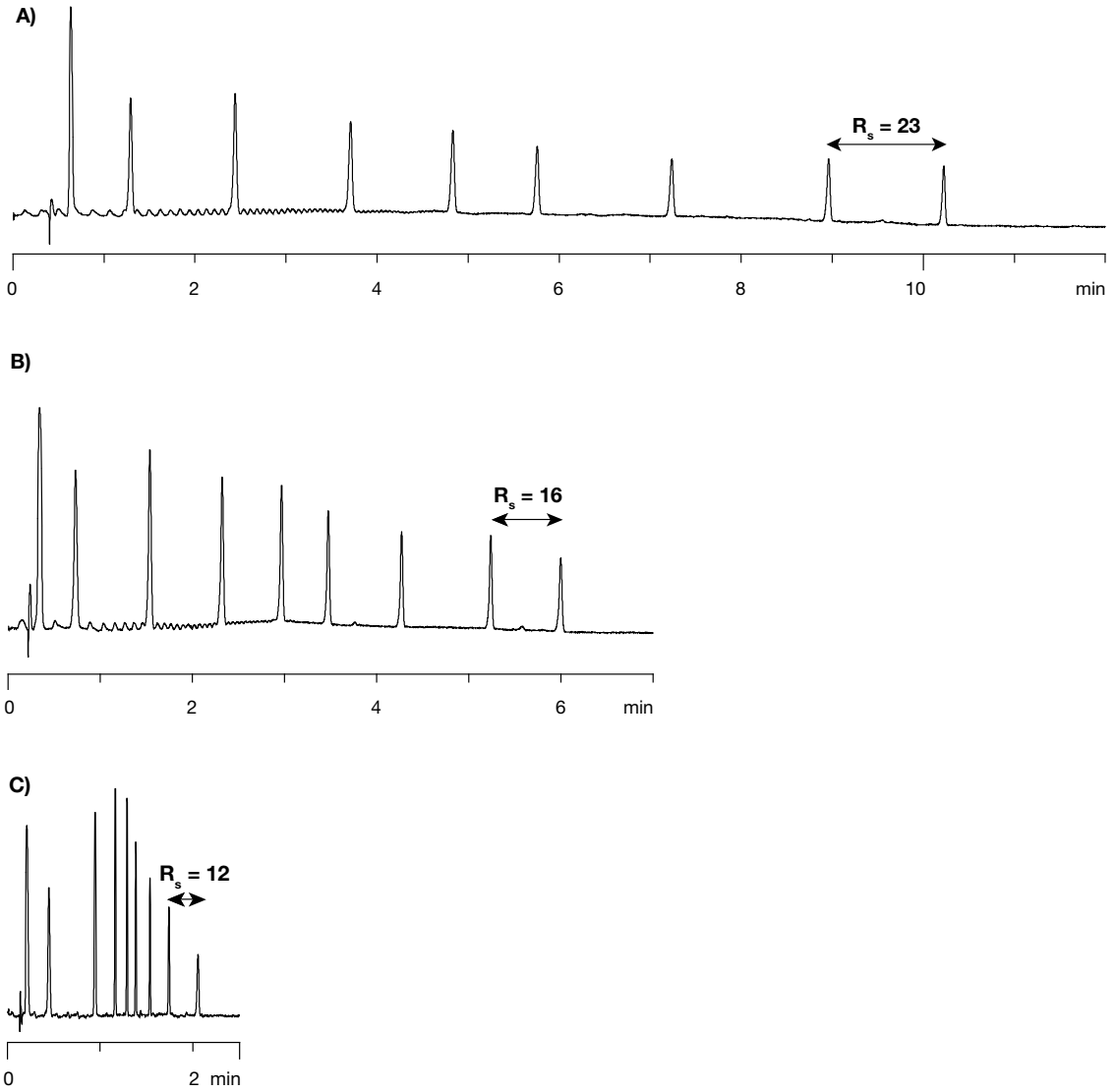
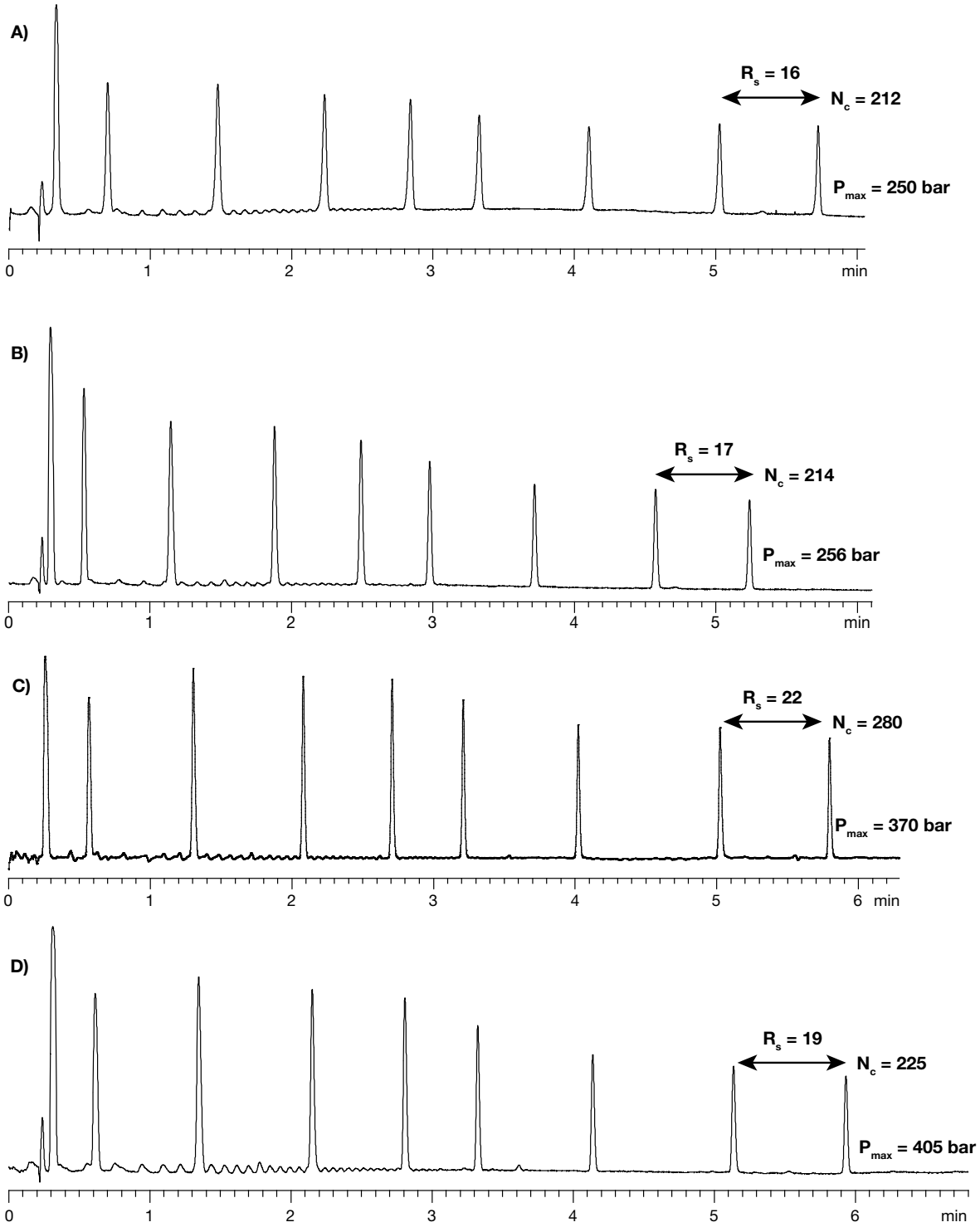


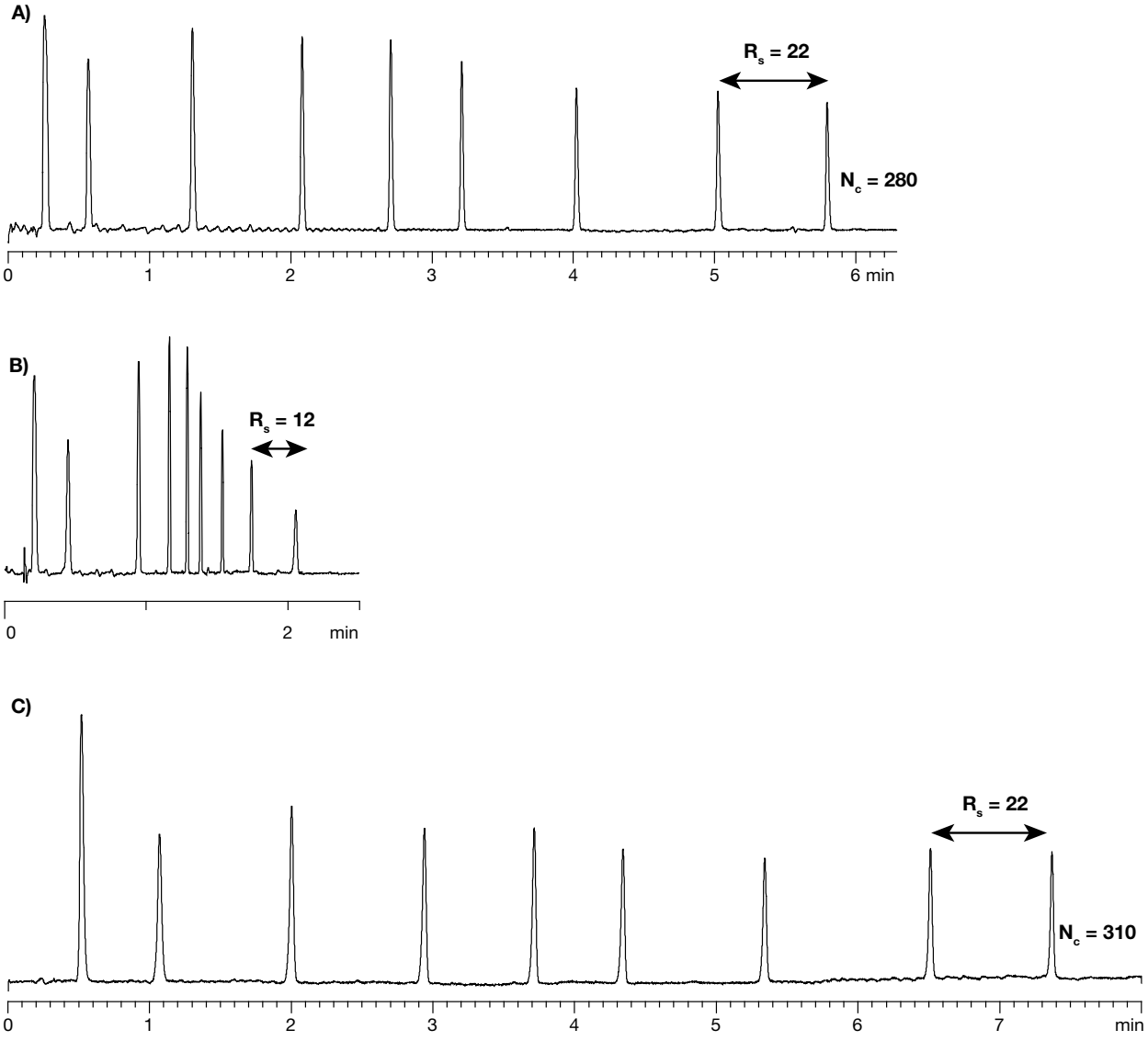
Figure 6 demonstrates that by reducing the particle size to 2.5 µm it was possible to dramatically decrease the analysis time. The separation obtained with a 2.5 µm media is compared to several different sub 2 µm media separations (***Figure 7***). The sub 2 µm media columns gave resolution values between 17-22 for the last peaks and peak capacities between 214-280. The average values for these sub 2 µm columns are a resolution of 19, a peak capacity of 240, with an average backpressure of 350 bar. The column packed with 2.5 µm material gave a resolution of 16 with a peak capacity of 212 at only a backpressure of 250 bar (33 % less). This shows that the 2.5 µm materials achieves 84 % of the resolution and 90 % of the peak capacity compared to the sub 2 µm media, but at only 68 % of the backpressure of sub 2 µm columns.

Figure 7: Gradient separations of a mixture of ketones, ranging from C_3 to C_{16}. The columns were A) 50 x 2.0 mm packed with Luna 2.5 µm C18(2) material, run at 0.65 mL/min B) 50 x 2.1 mm packed with a 1.9 µm material, run at 0.65 mL/min, C) 50 x 2.1 mm packed with a 1.8 µm material run at 0.65 mL/min and D) a 50 x 2.1 mm column packed with a 1.7 µm material run at 0.6 mL/min. The gradient slope was held constant in all cases.



Because the 2.5 µm media has substantially reduced backpressure, one can manipulate the column architecture for the specific goals of the separation. In ***Figure 8A***, the best gradient separation achieved using a sub 2 µm material is shown. If the separation goal is to decrease analysis time, the flow rate (linear velocity) can be increased because the 2.5 µm material requires lower backpressure. This increased speed is achieved while maintaining pressures compatible with traditional HPLC equipment, and the run time can be reduced almost 3 fold, as shown in ***Figure 8B***. If the separation requires more resolution, then the column length can be increased and the column operated at higher flow rate since the 2.5 µm media generates less backpressure compared to the sub 2 µm media (***Figure 8C*** shows increased resolution and peak capacity with only a minor increase in the overall runtime).

Figure 8: Gradient separations of a mixture of ketones, ranging from C_3 to C_{16}. The columns were A) 50 x 2.1 mm packed with a 1.8 µm material, run at 0.65 mL/min B) 50 x 2.0 mm packed with Luna 2.5 µm C18(2) material, run at 1.0 mL/min and C) 100 x 2.0 mm packed with Luna 2.5 µm C18(2) material run at 0.8 mL/min.



3.2 Conclusion

Plate height versus linear velocity plots for columns packed with materials ranging from 1.7 µm to 3 µm showed that the highest performance came from columns packed with 1.8 µm and 2.5 µm materials. When these results were compared to the expected number of plates that should be generated, it was found that columns packed with the available sub 2 µm materials generated only about sixty percent of the expected theoretical plates, whereas a column packed with 2.5 µm material generated nearly ninety percent of the expected theoretical plates. When sub 2 µm columns were compared with a 2.5 µm column in isocratic elution, it was shown that the 2.5 µm column produced ninety percent of the theoretical plates at seventy percent of the backpressure compared to a column packed with sub 2 µm media.

When these columns were compared in gradient elution mode, the 2.5 µm column produced 84 % of the resolution and 90 % of the peak capacity at less than 70 % of the backpressure. It was demonstrated that the lower backpressure generated in the 2.5 µm column allowed this column to operate at the same linear velocities as the sub 2 µm columns but with backpressures compatible with conventional HPLC equipment. Furthermore, because of the significantly lower backpressure, the column length could be optimized for the particular separation needs, whether that is overall analysis time (shorter column) or separation power (longer column). The 2.5 µm column allows one to use existing HPLC systems to obtain most of the benefits that sub 2 µm columns can provide.

3.3 References

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Advantages of 2.5 µm for increasing the speed of analysis while maintaining high efficiency

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