Increasing Throughput in LC/MS Analysis by Using 1.9μm Particle Packed Columns

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Abstract

The work presented in this poster demonstrates the concept that columns packed with 1.9 μm particles run with fast gradients can be used to achieve very short run times in LC/MS analysis, allowing increased productivity without compromising the quality of the data. The availability of alternative column chemistries in 1.9 μm media is shown to facilitate method selectivity improvements.

Introduction

One of the challenges of LC/MS methods is speed, as the demand for high throughput sample analysis becomes more crucial. The analysis time is determined by the chromatographic method, namely column size, flow rate and elution mode. Using short columns with fast gradients and high flow rates can reduce run time, but very often at the expense of chromatographic efficiency and therefore resolution, sensitivity and overall method performance. Columns packed with conventional 5 and 3 μm particles have a limited flow rate range for which separation efficiency is optimal. Outside this range, performance is compromised. The flow rate range for optimum chromatographic efficiency is wider for smaller sub-2 μm particles. This provides the opportunity to use columns packed with small particles at high flow rates to improve sample turnaround, but without sacrificing efficiency and therefore separation performance. The trade-off is column backpressure which increases proportionally to the inverse of the square of particle diameter, and proportionally with mobile phase flow rate. In this instance specialized HPLC equipment may be required to handle pressures above 400 bar. Columns packed with 1.9 μm particles provide very high efficiency separations and therefore a short column (50mm and below) provides enough separation power for samples which are not too complex, with the added advantage of not exceeding the pressure limits of conventional HPLC systems. The ability of enhancing analysis speed by using very fast gradients relies on a pump capable of delivering such fast gradients to the column in real time, i.e., a pump with a low dwell volume.

Changes in reversed phase HPLC (RP-LC) selectivity are generally obtained by changing the mobile phase composition or column packing. However, solvent and buffer type choices are restricted in LC/MS since these need to balance the chromatographic separation requirements with the ionization efficiency. Generic mobile phases, which provide good detection signal are generally chosen first, and therefore the analyst is left with another parameter, column chemistry, to adjust the selectivity of the chromatographic separation. High performance sub-2 μm column packings in a range of functionalities for selectivity screening or for fine-tuning complex separations need to be available to enable fast method development.

Methods

- Columns - Hypersil GOLD™ 5 μm, 200 x 2.1 mm; Hypersil GOLD 3 μm, 200 x 2.1 mm; Hypersil GOLD 1.9 μm, 200 x 2.1 mm; Hypersil GOLD 1.9 μm, 100 x 2.1 mm; Hypersil GOLD 1.9 μm, 50 x 2.1 mm; Hypersil GOLD aQ™ 1.9 μm, 50 x 2.1 mm; Hypersil GOLD PFP 1.9 μm, 50 x 2.1 mm (Thermo Electron Corporation, Bellefonte, PA).
- LC and MS systems - Finnigan™ Surveyor™; Finnigan LCQ™ Deca (Thermo Electron Corporation, San Jose, CA)
- U-HPLC system: Accela™ (Thermo Electron Corporation, San Jose, CA)
- Mobile phase compositions, gradients, flow rates, solutes, temperatures and detection details are specified on each figure.
- Method transfer to smaller column geometries packed with smaller particles (Figures 1 to 3), was performed using the following equations for flow rate and gradient time adjustment:

a) Adjust flow rate (keep reduced linear velocity constant between original and new method)

Equation 1 $F_2 = F_1 \times (d_{c2}^2 / d_{c1}^2) \times (d_{p1} / d_{p2})$

F_1 - original flow rate; F_2 - new flow rate (mL/min)
d_{c1} - original column; d_{c2} - new column ID (mm)
d_{p1} – original particle size; d_{p2} – new particle size (μm)

b) Keep initial and final mobile phase composition, adjust gradient time in proportion to change in flow rate and column volume

Equation 2 $t_{g2} = t_{g1} \times (V_{02} / V_{01}) \times (F_1 / F_2)$

t_{g1} – gradient time in original method; t_{g2} - gradient time in new method (min)
V_{01} – original column volume; V_{02} – new column volume (mL)
F_1 - original flow rate; F_2 - new flow rate (mL/min)

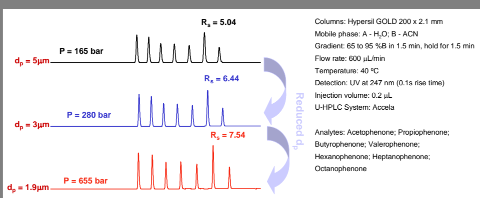
Equation 3 $V_0 = 0.68 \times \pi \times r^2 \times L$

V_0 – column void volume (mL); L – column length (cm); r – column radius (cm)

Results

I) Column - Particle Size

FIGURE 1. Effect of reducing column particle size (d_p) on chromatographic resolution (R_s) and column backpressure (P), whilst maintaining flow rate and gradient constant.

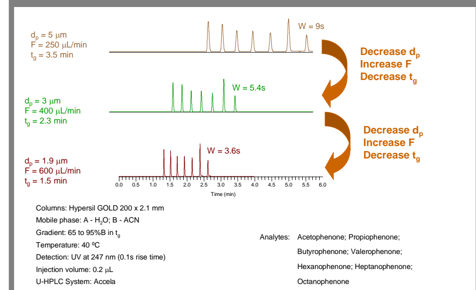


Reducing particle size (d_p) from 5 to 1.9 μm increases chromatographic resolution (R_s) by 50%.

To demonstrate how sub-2μm particles can be used to increase either chromatographic resolution or to reduce analysis time, a separation of seven phenones was performed on 200 x 2.1 mm columns packed with 5, 3 or 1.9 μm particles. In Figure 1 the analysis was performed under the same experimental conditions on the the three columns, therefore maintaining run time constant. The reduction in particle size provides an improvement in efficiency (peaks are narrower) which is evident by the higher resolution between peaks 6 and 7. The trade–off is column backpressure, which is inversely proportional to the square of particle diameter, requiring specialized HPLC equipment to handle pressures above 400 bar.

Small particles perform well at high flow rates; on Figure 2 the separation on a 200 x 2.1mm, 5 μm column was transferred to a 3 μm and then to a 1.9 μm column. The flow rates and gradient times were adjusted using Equations 1 and 2 respectively (in Methods section), to obtain equivalent separations, with similar chromatographic resolution. On the 1.9 μm column the equivalent separation is completed in half of the time, with chromatographic peaks that are two and a half times narrower.

FIGURE 2. Effect of particle size (d_p) on analysis time and chromatographic peak width (W), at baseline, when flow rate (F) and gradient time (t_g) are scaled geometrically.

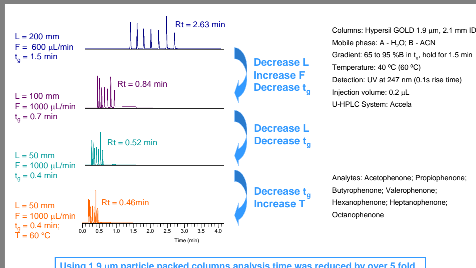


Reducing particle size (d_p) from 5 to 1.9 μm reduces run time by over 2 fold and reduces chromatographic peak width by 2.5 fold.

II) Column - length

In the separation above there is still full resolution of all the sample components. To make the analysis time faster, a shorter column length can be utilized; this represents a balance between run time and chromatographic resolution. The chromatograms in Figure 3 illustrate the transfer of the method from a 200 mm length to a 100 mm, and then to a 50 mm length column, all packed with 1.9 μm particles. On the first step as column length is reduced, flow rate can be increased; because both flow rate and column volume change gradient time is adjusted proportionally (Equations 2 and 3). On the second step column length is reduced again, and a proportional reduction in gradient time was made. Another experimental parameter that can be used to reduce analysis time is separation temperature; on the final step the column temperature was increased from 40 to 60 ºC. Overall, analysis time was reduced by five fold.

FIGURE 3. Effect of column length (L), flow rate (F) and separation temperature (T) on analysis time (Rt). t_g is gradient time.

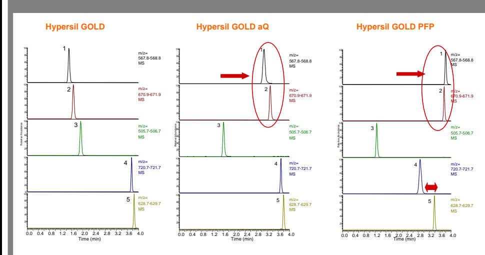


Using 1.9 μm particle packed columns analysis time was reduced by over 5 fold

III) Column - Selectivity

Method development in LC/MS using small particle technology is fast since run times can be under 1 to 2 minutes. A way of further reducing method development time is to have available a range of column chemistries that can be screened using generic mobile phases [1]. C18 like selectivity (Hypersil GOLD), polar endcapped C18 (Hypersil GOLD aQ) and perfluorinated (Hypersil GOLD PFP) columns were used to separate a mixture of protease inhibitors (anti-HIV drugs) and a mixture of aromatic amines (Figures 4 and 5 respectively). In the first instance, several changes in elution order are observed with both the aQ and the PFP phases. For the aromatic amines, the elution order of the o-toluidine and 4,4-oxydianiline on the PFP is reversed. The polar functional group used to endcap Hypersil GOLD aQ provides an additional interaction mechanism by which polar compounds can be retained and resolved. Hypersil GOLD PFP has a pentafluorophenyl ligand that provides extra selectivity of molecules which contain several nitro, hydroxyl, carboxyl or other polar groups that may not be well retained or resolved on alkyl chain phases. The introduction of a fluorine group in the alkyl stationary phase causes significant changes in the solute-stationary phase interactions. The carbon-fluorine bond is more polar than the carbon-hydrogen bond, which explains the extra selectivity and retention observed for compounds containing halogens and other polar groups. The extra rigidity of the perfluorinated ring may explain why a high degree of shape selectivity for isomers can be observed.

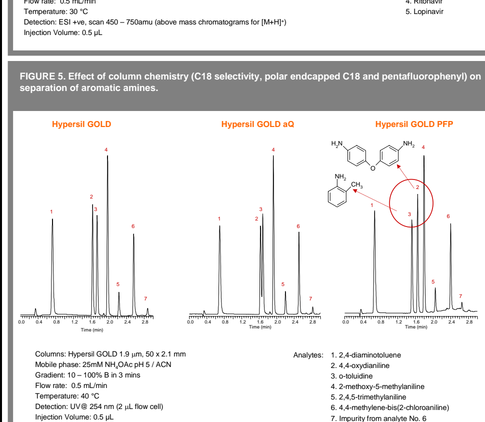
FIGURE 4. Effect of column chemistry (C18 selectivity, polar endcapped C18 and pentafluorophenyl) on separation of protease inhibitors.



Columns: Hypersil GOLD 1.9 μm, 50 x 2.1 mm
Mobile phase: H_2O + 0.1% Formic Acid / ACN + 0.1% Formic Acid
Gradient: 0 – 2.2 mins @ 35% B, then to 100 % B @ 4 mins
Flow rate: 0.5 mL/min
Temperature: 30 °C
Detection: ESI +ve, scan 450 – 750amu (above mass chromatograms for $[M+H]^+$)
Injection Volume: 0.5 μL

Analytes: 1. Amprenavir
2. Nelfinavir
3. Saquinavir
4. Ritonavir
5. Lopinavir

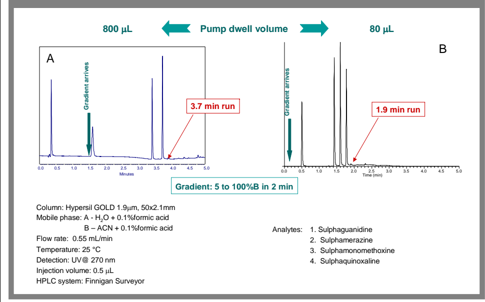
FIGURE 5. Effect of column chemistry (C18 selectivity, polar endcapped C18 and pentafluorophenyl) on separation of aromatic amines.



IV) Gradient

The pump dwell volume is extremely important when running high speed applications using ballistic gradients, typical of high throughput separations on small particle packed columns. Pump dwell volume affects the time it takes for the gradient to reach the head of the column. In Figure 6 the same 2 minute gradient was run on a pump with a 800 μl dwell volume, and a pump with a 80 μl dwell volume. The chromatograms are very different: for chromatogram A, it was necessary to introduce an isocratic hold at the end of the 2 minutes gradient to allow elution of the analytes. In these conditions the pump dwell volume can double the run time, and it also impacts on column re-equilibration at the end of the run.

FIGURE 6. Effect of pump dwell volume during fast gradient elution.



Conclusions

The work presented in this poster demonstrates that:

- Reducing column particle size from 5 to 1.9 μm can increase chromatographic resolution by 50% or more.
- Using short columns packed with 1.9 μm particles can provide very fast separations, with very narrow peak widths and therefore high peak capacities.
- 1.9 μm Hypersil GOLD column chemistries provide selectivity differences to facilitate method development under generic mobile phase conditions.
- Selection of correct LC hardware is crucial to enable fast gradients and to fully utilize the benefits of small particle technology.

References

(1) L. Pereira, C. Blythe, H. Ritchie, "Column selectivity screening in LC/MS Method development", Poster presented at Pittcon 2006, Orlando , FL

Additional Information

For additional information, please browse our *new* Chromatography Resource Centre which can be accessed from: www.thermo.com/columns.

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