Improved Column Lifetime with Thermally Stable Polymer Columns for Oligonucleotide Ion-Pair RP HPLC

Application Note

Authors

J Massi and L Lloyd
Agilent Technologies, Inc.

Introduction

HPLC is widely used for the purification and analysis of oligonucleotides due to its selectivity, stability, and robustness. Oligonucleotide separations can be very demanding, often requiring separations based on small changes in size or sequence. It has previously been shown that the resolution of oligonucleotide separations can be dramatically improved by increasing run temperatures.[4] In Application Note 5990-7765EN we have also shown that polymeric columns give the best performance under these conditions, due to their ability to withstand much higher temperatures with alkali eluents than silica or hybrid particle columns.[1-4] In this note, the useable lifetimes and resolving capabilities of polymer, hybrid, and traditional silica columns for oligonucleotide separations are examined.

Agilent Technologies

Materials and Methods

Column A:	PLRP-S 100Å, 3 µm polymeric HPLC column, 50 x 4.6 mm (Part number PL1512-1300)
Column B:	125Å, 2.5 µm hybrid silica-methylsiloxane C18 column, 50 x 4.6 mm
Column C:	1000Å, 3 µm Thermally stable bonded silica C18 column, 50 x 3.0 mm
Column D:	100Å, 5 µm Traditional silica C18 column, 50 x 4.6 mm
Mobile Phase:	Eluent A: 100 mM Triethylammonium acetate (TEAA) in HPLC-grade water Eluent B: 100 mM TEAA in 25:75 Acetonitrile:water
Flow rate:	1.0 mL/min or 0.45 mL/min, see Figure 1
Temp:	60 ºC or 80 ºC
Detection:	UV, 254 nm

The thermal stability and lifetime of the columns was evaluated. Columns were subjected to 25 minute-long gradient cycles at either 80 °C (PLRP-S) or 60 °C (all other columns). The resolution (R values) of poly(dT)$_{19\text{-}24}$ and 29/30 mer samples were calculated after every 100 gradient cycles. Columns were considered dead when R values dropped by more than 25%.

Results and Discussion

Figures 1 and 2 show the results of the lifetime studies. In Figure 1, the resolution as a function of the number of runs (gradient cycles) for the four columns is plotted. It is immediately evident from this plot that the performance, as measured by the resolution factor, decreases most dramatically, as would be expected, with the traditional reversed phase silica column. There is some improvement in column lifetime for the thermally stable bonded reversed phase silica. The hybrid silica-methylsiloxane column exhibits a further improvement. However, the polymeric column, PLRP-S, showed no deterioration in performance after 1600 gradient cycles at even higher temperature of 80 °C.

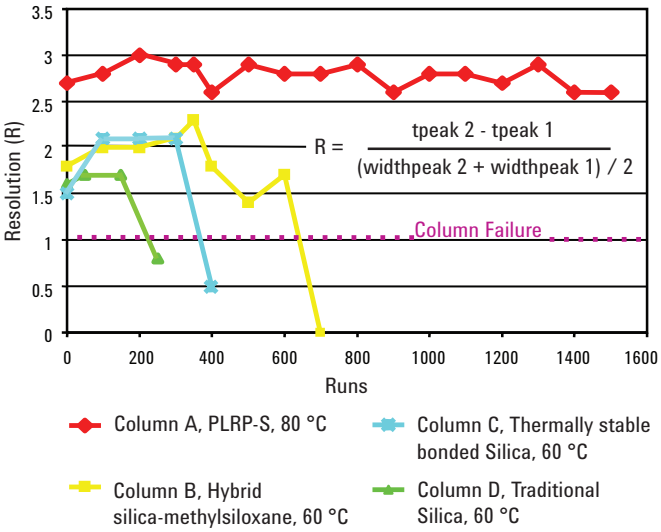

Figure 1. Column lifetimes at elevated temperature.

Figure 2 shows the chromatograms of both samples, the poly(dT) $_{19\text{-}24}$ and the 29/30 mer pair, at the beginning of the stability trial and at the point of failure. Column A, the PLRP-S column, showed no decrease in performance after 1500 runs at 80 °C. Column B, the hybrid silica-methylsiloxane C18 column, gave acceptable resolution of the samples for 600 runs, and then died catastrophically between 600 and 700 runs. No chromatograms were available at 700 runs for Column B, as the column died due to media breakdown and caused large pressure increases and silica leakage past the inlet frit. This catastrophic failure was observed with three different Column Bs. Column C, the thermally stable bonded silica C18 column, gave unacceptable R values by 400 runs, and Column D, the traditional silica C18 column, gave unacceptable R values by 250 runs.

At the end of the lifetime experiment the column inlet ends were removed to assess the state of the media and to identify the cause of the column failure. The photographs in Figure 2 show the state of the inlet packed bed. The PLRP-S packed bed was not disrupted and there was no void or channel formation, but the three other columns all voided.

Column A: PLRP-S 100Å, 3 µm, 4.6 mm x 50 mm

Runs before failure: Did not fail. Shown after 1500 runs.

Void: None

Column B: Hybrid silica-methysiloxane C18, 4.6 mm x 50 mm

Runs before failure: 600

Void: 1 mm

Column C: Thermally stable bonded silica C18, 3.0 mm x 75 mm

Runs before failure: 400

Void: 5.5 mm

Column D: Silica C18, 4.6 mm x 50 mm

Runs before failure: 250

Void: 4.5 mm

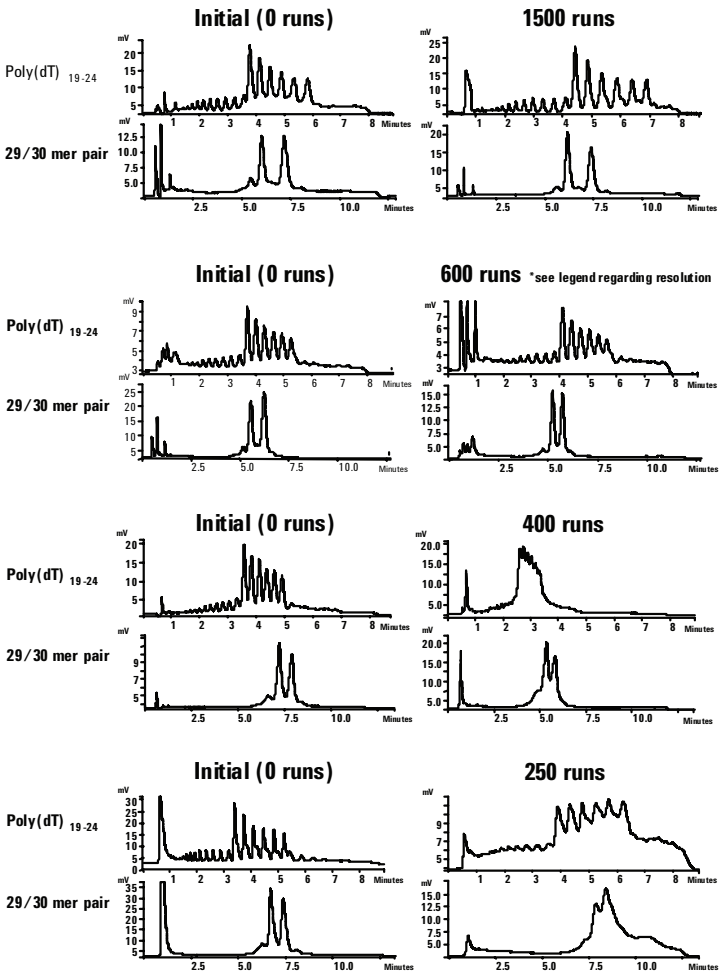

Figure 2. Separation of poly(dT)$_{19-24}$ and 29/30 mer oligonucleotide pair. 50 pmol of each oligonucleotide was injected in a 10 µL sample volume. The gradient was a 5% change in buffer B over 10 minutes with a 2 minute equilibration.

Conclusions

For high performance oligonucleotide separations, using ion-pair reversed phase HPLC, it is necessary to run at elevated temperatures to denature the oligo and improve the mass transfer, and hence resolution. Although the modern RP-silica based materials are more stable, operating at elevated temperatures will accelerate failure of the column packed bed, stripping of the alkyl functional group and dissolution of the silica base particle.

All silica-based materials run at elevated temperatures with eluents routinely used for oligonucleotide analysis and purification exhibited significant breakdown of the stationary phase. This phase dissolution adversely affects column performance, by either reducing the resolving power of the column, or by plugging the column. This not only represents a problem for column lifetimes, an obvious economic disadvantage, but can also mean potential contamination of purified oligonucleotide fractions with silica and bonded phase. Hybrid silica-methylsiloxane materials offer better lifetimes than traditional silica-based materials, but are still prone to breakdown and contamination of fractions. Only the polymer media, PLRP-S, has the chemical and thermal stability necessary to be able to run routinely under the conditions needed to achieved the maximum resolution for synthetic oligonucleotide analysis at elevated temperature.

The PLRP-S polymeric HPLC column offered more than double the lifetime of even the most stable reversed phase silica or silica–polymer composite columns. This represents a considerable economic advantage, especially in a high throughput environment.

References

[1] Hoogendoorn, B., Owen, M.J., Oefner, P.J., Williams, N., Austin, J. and O'Donovan, M.C. 1999. Genotyping Single Nucleotide Polymorphisms by Primer Extension and High Performance Liquid Chromatography. *Human Genetics* 104:89-93.

[2] El-Maarri, O., Herbiniaux, U., Walter, J. and Oldenburg, J. 2002. A Rapid, Quantitative, Non-Radioactive Bisulfite-SNuPE-IP RP HPLC Assay for Methylation Analysis at Specific CpG Sites. *Nucleic Acids Research* 30(6):e25.

[3] Gilar, M., Fountain, K.J., Budman, Y., Neue, U.D., Yardley, K.R., Rainville, P.D., Russell, R.J. and Gebler, J.C. 2002. Ion-Pair Reversed-Phase High-Performance Liquid Chromatography Analysis of Oligonucleotides: Retention Prediction. *Journal of Chromatography A* 958:167-182.

[4] Massi, J., Lloyd, L., Use temperature to enhance oligonucleotide mass transfer and improve resolution in ion-pair RP HPLC. *Application Note 5990-7765EN*

[5] Lloyd, L.L, Millichip, M.I, and Mapp, K.J., Rigid Polymerics: the Future of Oligonucleotide Analysis and Purification. *Journal of Chromatography A* 1009 (2003) 223-230

www.agilent.com/chem

Published in UK, May 17, 2011

5990-7764EN

Agilent Technologies