Use Temperature to Enhance Oligonucleotide Mass Transfer and Improve Resolution in Ion-Pair RP HPLC

Application Note

Authors

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Introduction

Growth in the field of genomics has increased the need for pure synthetic oligonucleotides, and for ways in which to analyze these oligonucleotides and oligonucleotide products of various genomic assays (i.e. genotyping, methylation analysis, HPLC). HPLC is widely used for the purification and analysis of these oligonucleotides due to its selectivity, automation, and robustness.[1],[2] Oligonucleotide separations are very demanding, often requiring separations based on small changes in size or sequence. Separations become increasingly difficult as the size of the oligonucleotide increases. In order to improve resolution to meet these demanding requirements it is necessary to look at ways of improving the mass transfer, including the use of monoliths, non-porous particles, small porous particles and elevated temperatures. Monoliths and non-porous particles have the disadvantage of reduced loading capacity, and so oligonucleotide separations are increasingly being performed using small particles at elevated temperatures.[1],[2],[3]

After oligonucleotide de-protection, removal of the dimethoxytrityl group (DMT) from the 5′ hydroxyl end, ion-pair reversed phase HPLC is often the method of choice for oligonucleotide analysis and purification, as unlike ion exchange chromatography, the eluents are volatile and mass spec compatible. However, traditional silica-based HPLC columns are chemically unstable at elevated temperatures, at alkaline pH, and also with the ion-pairing agents typically needed for oligonucleotide separations. Silica columns tend to dissolve quickly under these conditions, and can contaminate purified fractions with silica and bonded phase. In order to address these problems, research has focused on the development of advanced hybrid organic-inorganic methylsiloxane-silica media and advanced polystyrene-divinylbenzene polymer materials.[4], [5] Neither silica-based or hybrid particle columns are recommended for use at temperatures over 70 °C. Polymeric polystyrene-divinylbenzene HPLC columns can withstand temperatures far in excess of this. In this example, the effect of temperature on the resolution of oligonucleotide separations is investigated and compared on reversed phase silica, hybrid, and polymeric HPLC columns.

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Materials and Methods

Temperature dependence of oligonucleotide separations was evaluated on four different types of columns, using two different samples and a range of temperatures. The reversed phase columns are described below. They were comprised of a polymeric PLRP-S, a hybrid-particle, a thermally stable bonded silica, and a silica-based column. All columns, except the PLRP-S, have temperature limits near 60 °C or below. Therefore, only the PLRP-S column was investigated at temperatures above 60 °C. The first sample (Figure 1) is a poly(dT) oligonucleotide ladder (19 to 24 mer). The second sample (Figure 2) is a 29/30 mer oligonucleotide pair of random sequence, representing the (n) and (n-1) products of an oligonucleotide synthesis. This separation is the same as would be required in a typical (n)/(n-1) oligonucleotide purification.

Column A:	PLRP-S 100Å, 3 µm, PS/DVB HPLC column, 50 x 4.6 mm (Part number PL1512-1300)
Column B:	2.5 µm, 125Å 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:	35 °C, 60 °C, or 80 °C
Detection:	UV, 254 nm

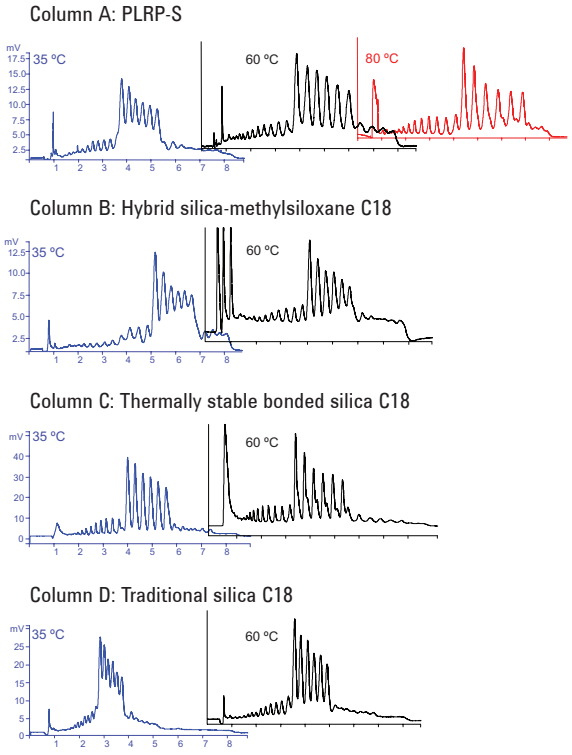

Figure 1. Poly(dT) 19-24 ladder.
50 pmol of poly(dT) ladder was injected in a 10 µL sample volume. Gradients varied with each column and temperature, but all gradient slopes were the same. A gradient of 5% change in buffer B over 6 minutes, plus a 2 minute equilibration was used. All flow rates were 1 mL/min, with the exception of column C, which was 0.45 mL/min, due to the smaller diameter of the column.

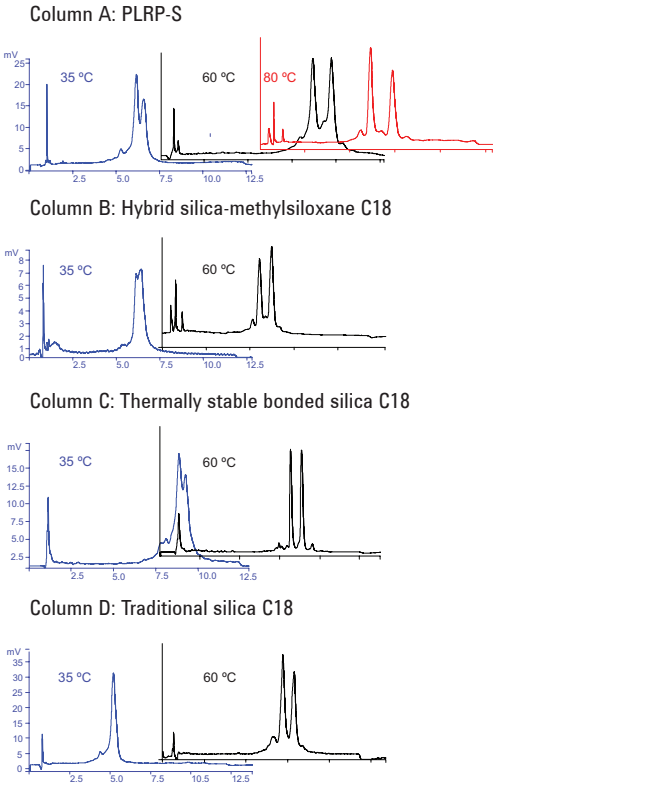

Figure 2. 29/30 mer oligonucleotide pair.
50 pmol of each oligonucleotide was injected in a 10 µL sample volume. The gradient was a 5% change in buffer B over 10 minutes with a 2 minute equilibration.

Results and Discussion

Figures 1 and 2 illustrate that with the four columns evaluated the resolution and selectivity improve dramatically with elevated temperature. In fact, temperature can make the difference between no resolution and base line resolution. As seen in Figure 1, the best separation of the poly(dT) oligonucleotide ladder (19 to 24 mer) at 35 °C is achieved with column C, thermally stable bonded silica C18. However, by increasing the temperature to 80 °C superior performance is seen with the PLRP-S column. The best separation of the 29/30 mer sample (Figure 2), is achieved with the PLRP-S at 35 °C, but again is significantly improved by increasing the temperature to 80 °C. The improvement in resolution and selectivity with higher temperature is due to improved mass transfer of the denatured oligonucleotides between the stationary and mobile phases.[3] The small particle, small pore PLRP-S column gives the best performance due to its high surface area, permeability, and its enhanced thermal stability, enabling its use at 80 °C.

Conclusions

Improvements in the resolution and selectivity of synthetic oligonucleotide ion-pair reversed phase HPLC can be achieved by increasing the analysis temperature. All four columns showed improved oligonucleotide resolution at high temperature due to the improvement in mass transfer. However, the small particle, small pore polymeric PLRP-S column gave the best overall performance for the separation of the 29/30 mer sample at 35 °C, and of both this sample and the poly(dT)19-24 ladder sample at 80 °C. The use of small particle polymeric HPLC column media not only has advantages for the improvements in mass transfer due to the use of the small particles, but the stability obviously has implications not only for resolution at elevated temperatures, as shown here, but also for column lifetimes. Its chemical and thermal stability is much greater than the silica-based and hybrid materials, which will exhibit phase dissolution over time, so reducing the efficiency and resolving power of the column and column lifetime. The effect of temperature on column lifetime is reported in Application Note 5990-7764EN.

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Published in UK, May 17, 2011

5990-7765EN

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