

APPLICATIONS

Effect of Temperature on Single Stranded Oligonucleotide Analysis

Ivan Lebedev, Dani Xing, Brian Rivera
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501, USA

Overview

Therapeutic oligonucleotides represent a recent breakthrough in the pharmaceutical industry. However, characterization of oligos, specifically by ion-pair reversed phase liquid chromatography (IP-RPLC), can be quite challenging. Oligos are manufactured by solid phase synthesis, where nucleotides are added in a step-wise manner. As such, impurities such as n-1 and n+1 must be characterized, and this may require extensive method development to optimize. Further, it may be necessary for characterization and quantitation of other closely related impurities, such as oxidation of phosphorothioates.

Another challenge is the variation in chromatography seen with oligo analysis. Retention times, peak shapes, and peak area recovery are often variable from injection to injection in any given sequence. One assumed cause of these variations is intramolecular interactions that compromise chromatographic performance. As such, high temperatures, exceeding 60 °C, are often implemented to improve separation. Although this is a requirement when working with double-stranded oligos such as siRNAs, there is still a question of whether this is also necessary for single stranded oligos. Here we present the effect of temperature on two oligos, a 5' conjugated oligo and a phosphorothioate, and how it might be implemented for method development of single stranded oligonucleotides.

Figure 1 illustrates the effect of increasing the method running temperature in 5 °C increments. Retention time for the 5'-Amino C12 oligonucleotide decreases as temperature increases, which is common for any chromatographic method. Often, efficiency and peak shapes are improved at higher temperatures, though that is not observed in this example. Further, in comparing the 45 °C and 65 °C impurity profiles as shown in the insets show little to no differences. One might conclude then that selectivity is not being effected.

To better confirm selectivity changes, mass spectrometry is necessary to identify and characterize impurity peaks. A 22 mer DNA Phosphorothioate, with an unknown sequence was run by high resolution MS. The measured mass for the thioate was 6772.6 Da. The oligo was then run at different temperatures to observe any changes in selectivity for the impurity profiling.

In comparing methods run at 60 and 70 °C (**Figure 2**), as expected, there was a marked decrease in retention for the main peak with the higher temperature running conditions. However, both methods gave similar impurity profiles, with the three earlier eluting impurities observed. The deconvoluted mass spectrum confirmed the same elution order; Impurity Peak 1 was 6443.4 Da, Peak 2 was 6468.4 Da, and Peak 3 was 6170.8 Da. Deconvoluted spectra for Peak 1 with both running conditions are shown in **Figure 3**. Interestingly, Peak 1 and 2 are likely both n-1 impurities, with Peak 1 being target sequence minus guanosine, and Peak 2 being target sequence minus thymidine.

In summary, temperature is often utilized for oligonucleotide analysis. However, for single stranded oligos, there may not be a benefit to running at temperatures exceeding 60 °, as increases in temperature may not improve chromatography nor effect selectivity as one might observe with other macromolecules.

LC Conditions

- Columns:** bioZen™ 2.6 µm Oligo
Dimension: 100 x 2.1 mm (Figure 1) [00D-4790-AN](#)
150 x 2.1 mm (Figures 2,3) [00F-4790-AN](#)
Mobile Phase: Figure 1:
A: 12.5 mM HFIP, 4 mM TEA in Water
B: 12.5 mM HFIP, 4 mM TEA in Methanol
Figures 2,3:
A: 100 mM HFIP, 4 mM TEA in Water
B: 100 mM HFIP, 4 mM TEA in Methanol
Gradient: 5-30 % B in 14 minutes (Figure 1)
25-95 % B in 15 minutes (Figures 2,3)
Flow Rate: 0.3 mL/min
Injection: 1 µL
Temperature: As noted in Figures
Detection: UV @ 260 nm (Figure 1,2)
TOF-MS (Figure 3)
Sample: 5'-Amino C12 Oligo (Figure 1)
DNA 22 mer Phosphorothioate (Figure 2,3)

Figure 1. Effect of Temperature On 5'-Amino C12 Oligonucleotide

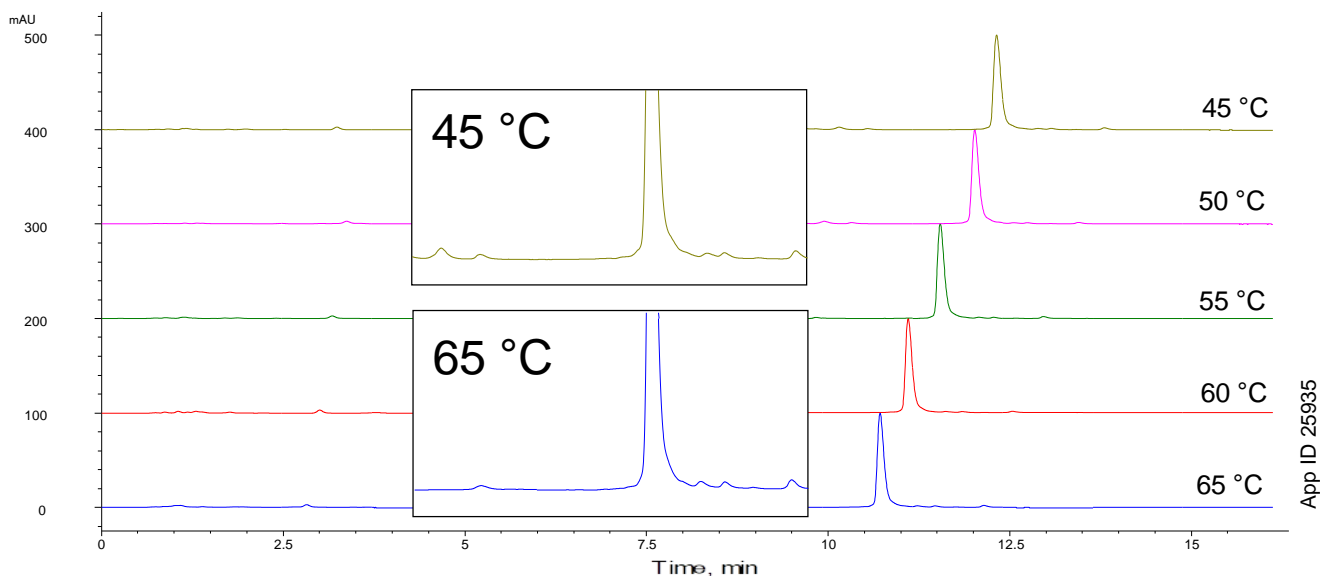


Figure 2. Impurity Profile of 22 mer DNA Phosphorothioate

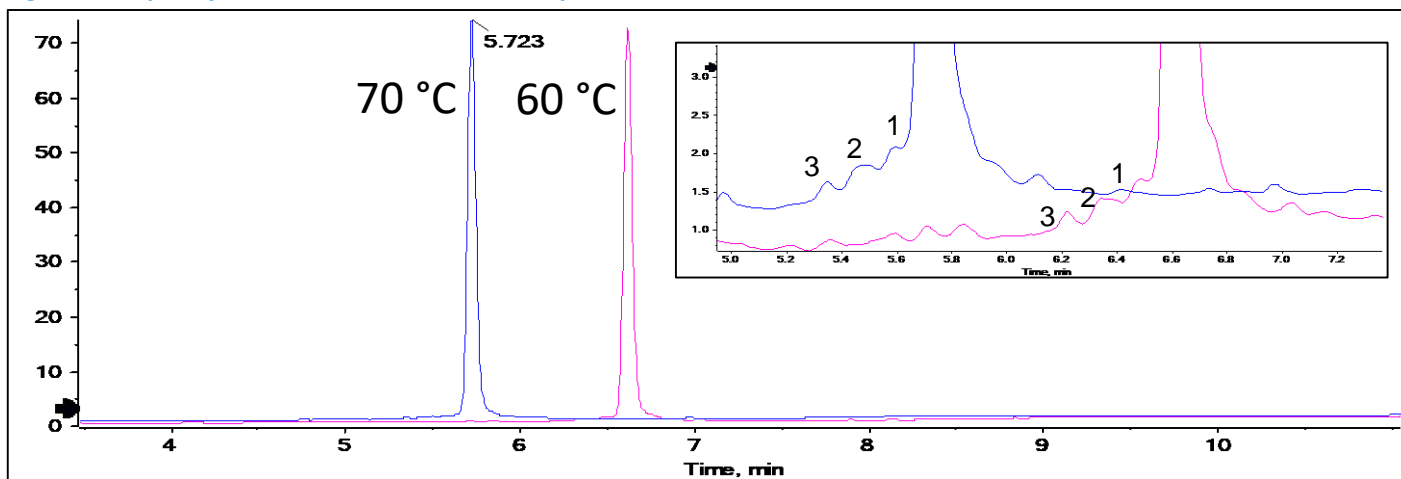
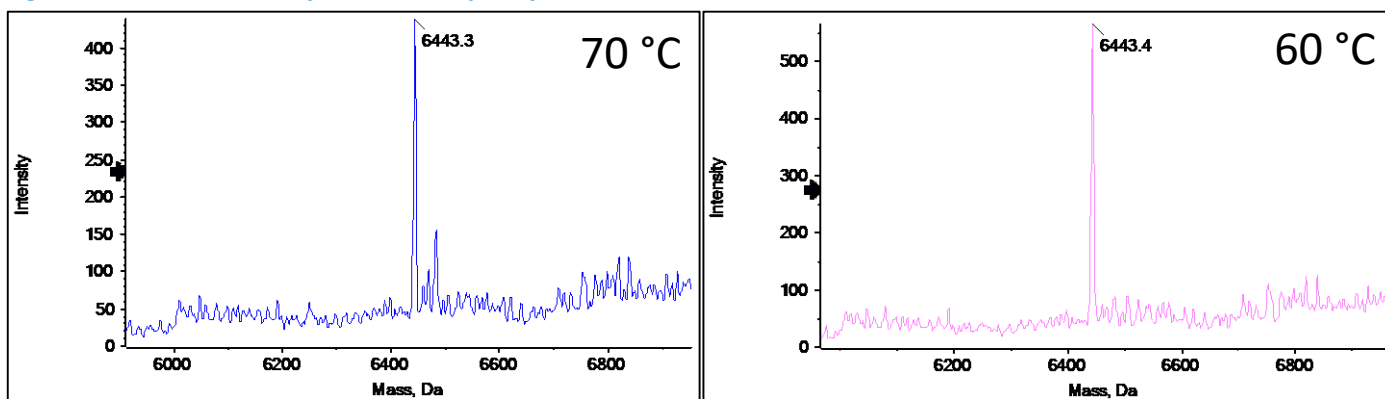


Figure 3. Deconvoluted Spectra For Impurity Peak 1



APPLICATIONS

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Australia

t: +61 (0)2-9428-6444
auiinfo@phenomenex.com

Austria

t: +43 (0)1-319-1301
anfrage@phenomenex.com

Belgium

t: +32 (0)2 503 4015 (French)
t: +32 (0)2 511 8666 (Dutch)
beinfo@phenomenex.com

Canada

t: +1 (800) 543-3681
info@phenomenex.com

China

t: +86 400-606-8099
cninfo@phenomenex.com

Denmark

t: +45 4824 8048
nordicinfo@phenomenex.com

Finland

t: +358 (0)9 4789 0063
nordicinfo@phenomenex.com

France

t: +33 (0)1 30 09 21 10
franceinfo@phenomenex.com

Germany

t: +49 (0)6021-58830-0
anfrage@phenomenex.com

India

t: +91 (0)40-3012 2400
indiainfo@phenomenex.com

Ireland

t: +353 (0)1 247 5405
eireinfo@phenomenex.com

Italy

t: +39 051 6327511
italiainfo@phenomenex.com

Luxembourg

t: +31 (0)30-2418700
nlinfo@phenomenex.com

Mexico

t: 01-800-844-5226
tecnicomx@phenomenex.com

The Netherlands

t: +31 (0)30-2418700
nlinfo@phenomenex.com

New Zealand

t: +64 (0)9-4780951
nzinfo@phenomenex.com

Norway

t: +47 810 02 005
nordicinfo@phenomenex.com

Poland

t: +48 (12) 881 0121
pl-info@phenomenex.com

Portugal

t: +351 221 450 488
ptinfo@phenomenex.com

Singapore

t: +65 800-852-3944
sginfo@phenomenex.com

Spain

t: +34 91-413-8613
espinfo@phenomenex.com

Sweden

t: +46 (0)8 611 6950
nordicinfo@phenomenex.com

Switzerland

t: +41 (0)61 692 20 20
swissinfo@phenomenex.com

Taiwan

t: +886 (0) 0801-49-1246
twinfo@phenomenex.com

United Kingdom

t: +44 (0)1625-501367
ukinfo@phenomenex.com

USA

t: +1 (310) 212-0555
info@phenomenex.com

🌐 **All other countries/regions**
Corporate Office USA
t: +1 (310) 212-0555
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