Impurity Analysis of a 22mer DNA Phosphorothioate

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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 related to sample degradation.

Phosphorothioate oligos represent unique challenges. These oligos are characterized by the replacement of one or multiple phosphates in the phosphodiester bond with a sulfur group. Although this results in both increases in stability (i.e. nuclease resistance) and efficacy for the oligo, analytically it makes the separation and characterization more difficult. The thioate modification introduces a stereoisomer, which differs slightly in physicochemical properties and typically leads to peak broadening by standard IP-RPLC methods. Further, characterization and quantitation of an oxidized thioate group is often desired, and this separation can be highly sequence dependent. This is in addition to the standard impurity analysis of n-1, n-2 separation that is necessary for any synthetic oligonucleotide manufactured by solid phase synthesis. Here we highlight the separation of a 22mer DNA phosphorothioate to illustrate the importance of chromatographic separation for proper MS characterization. Notably, we show the separation of n-1 and so-called PS/PO oxidized variant.

Figure 1 shows the separation of the 22mer thioate. The UV chromatogram shows two earlier eluting impurities, labeled as peaks 1 and 2 in the inset. **Figure 2** highlights the full-scan TOF-MS data for the main peak, as well as deconvoluted spectrum. With thioates, these earlier eluting impurities are typically n-1 or n-2 failure sequences, However, these can also be the oxidized "PO" variant.

The deconvoluted spectrum for impurity 1 (**Figure 3a**) predominantly represents the oxidized PO variant. There is some spectral overlap with a presumed n-1 impurity due to a co-elution of the two peaks. **Figure 3b** reveals both n-1 and n-2 impurities. The deconvolved mass indicates a delta mass of ~329 Da when compared to main peak, likely representing the target sequence minus a guanosine residue. Further characterization of the other earlier impurity peaks failed to yield sufficient spectral quality for deconvolution, though these are likely other short mer impurities.

Here we have demonstrated the capabilities of separating closely eluting impurities of a DNA thioate on the bioZen™ 2.6 µm Oligo column. PS/PO and n-1 impurities were identified. Although LC optimization could further improve the separation of impurities, this method shows proof of concept in characterization, while also highlighting the importance for chromatographic performance in impurity analysis of phosphorothioate oligos.

LC Conditions

Column:	bioZen 2.6 µm Oligo
Dimension:	150 x 2.1 mm
Part No.:	00F-4790-AN
Mobile Phase:	A: 100 mM HFIP and 4 mM TEA in Water
	B: 100 mM HFIP and 4 mM TEA in Methanol
Gradient:	5-30 % B, 14 minutes
Flow Rate:	0.3 mL/min
Injection:	1 µL
Temperature:	60°C
Detection:	UV @ 260 nm (Figure 1)
	TOF-MS (Figures 2 and 3)
Sample:	22mer DNA Phosphorothioate

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Figure 1. Impurity Profile of DNA Phosphorothiaote

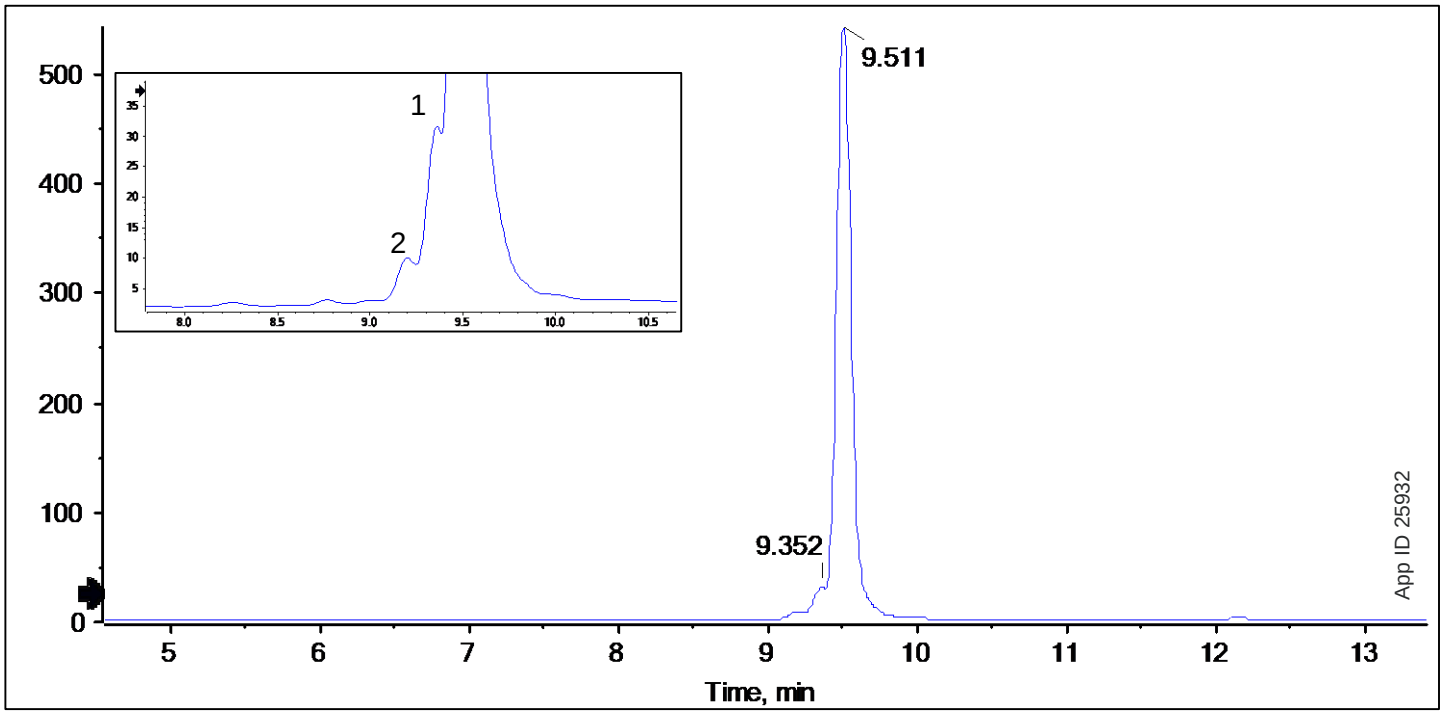


Figure 2. Full Scan, Deconvoluted Spectrum, Main Peak

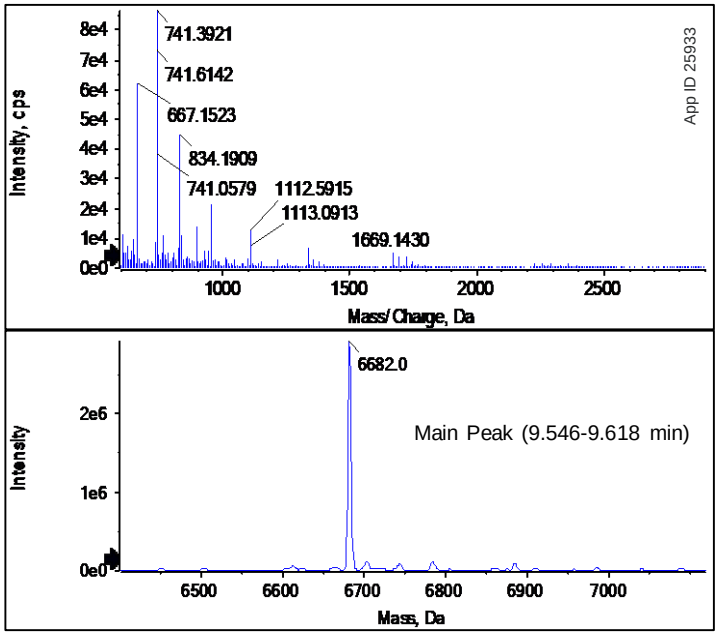
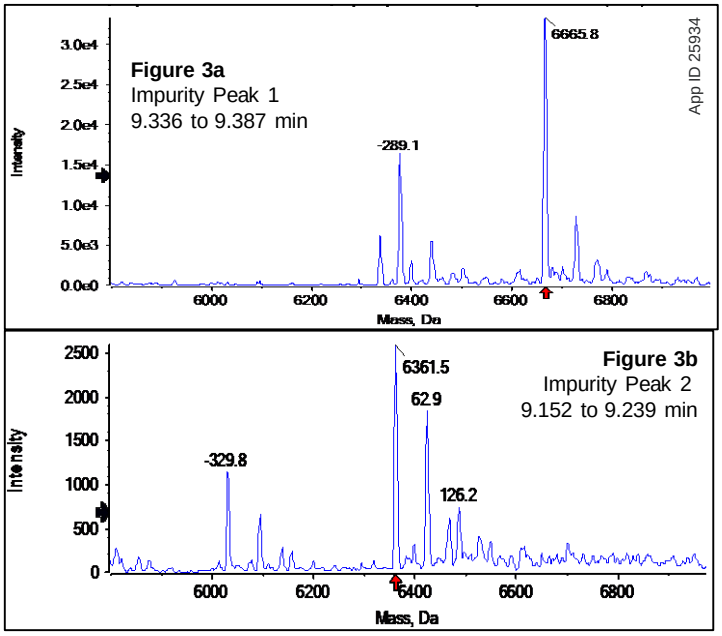


Figure 3. Deconvoluted Spectra, Impurity Peaks



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