

Characterization of Synthetic Oligonucleotides Using Agilent LC/MS Systems

The characterization of synthetic oligonucleotides using integrated Agilent 6000 Series Q-TOF and Ion Trap LC/MS platforms is described. The superior sensitivity and mass accuracy of the LC/MS platforms enabled rapid identification of oligonucleotide products and process-related impurities, and the technology is ideally suited to the support and optimization of oligonucleotide synthesis.

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Introduction

Synthetic oligonucleotides have emerged as promising therapeutic agents for the treatment of a variety of diseases, including viral infections and cancer. Several classes of nucleic acids, such as anti-sense oligonucleotides, small interfering RNAs (siRNAs) and aptamers, are being investigated for therapeutic applications. While the different types of oligonucleotides work by distinct mechanisms of action, all are designed to modulate the expression of the targeted gene.

Over the past twenty years, the method of choice for the chemical synthesis of oligonucleotides has been the phosphoramidite four-step process, which utilizes the reaction of deoxynucleoside phosphoramidites with a solid phase-tethered nucleoside or oligonucleotide.¹⁻³ The four-step cycle of detritylation, coupling, capping and oxidation/thiolation is repeated until all the bases have been

added to generate the desired full-length product. Although coupling efficiencies are typically very high (98-99%) using this approach, the overall yield of the product decreases as the number of cycles increase. Incomplete capping of coupling failures result in a series of deletion sequences, with single- (n-1) and double-nucleotide deletions (n-2) constituting the major impurities in solid-phase oligonucleotide synthesis. Additional impurities may result from incomplete detritylation and incomplete oxidation/thiolation as well as from the starting materials and post-synthesis processing. The impurities that arise must be removed as completely as possible to ensure that the quality and drug performance of the full-length product are not compromised. For therapeutic use, oligonucleotide purity is generally expected to be greater than 90%.



Crude synthetic oligonucleotides are typically purified by reversed-phase high performance liquid chromatography (RP-HPLC) or strong anion-exchange chromatography (SAX-HPLC). The optimal technique depends on the structure and length of the oligonucleotide, the presence or absence of the trityl group, and the nature of any modifications. Synthetic oligonucleotide purification is particularly challenging because of the small differences in size, charge and hydrophobicity between the full-length product and impurities, which often co-elute. Moreover, chromatography techniques alone cannot unequivocally identify oligonucleotides or impurities. Liquid chromatography (LC) coupled with electrospray ionization mass spectrometry (ESI-MS) is increasingly being used for characterization and identification of oligonucleotides.

As oligonucleotide drug discovery advances, efforts to develop more efficient, scalable and cost-effective synthesis and purification methods have intensified. LC/MS is an important characterization tool for oligonucleotide synthesis, enabling identification of process-related impurities and subsequent elucidation and optimization of process chemistries. Agilent 1200 Series LC platforms seamlessly couple with Agilent's 6000 Series MS systems to deliver superior LC performance and exceptional mass accuracy and sensitivity for optimal LC/MS characterization of oligonucleotides. In this note, we demonstrate the use of LC/MS to characterize the synthesis of three classes of oligonucleotides using Agilent 6000 Series Q-TOF and Ion-Trap platforms.

Experimental

1. Instrumentation

Agilent 1200 SL HPLC system was integrated with either an Agilent 6520 Accurate Mass Q-TOF or 6330 Ion Trap MS platform for LC/MS analysis.

2. LC/MS Parameters for Agilent 6330 Ion Trap MS Instrument

(i) LC settings

Column: Waters X-Terra C18 (2.1 x 50 mm)
 Buffer A: 5 mM NH₄OAc, pH = 7.0 – 8.0
 (pH adjusted with ammonia)
 Buffer B: MeOH
 Flow rate: 0.2 mL/min.
 Column temperature: 50°C

Gradient:	Time	%B
	0.0	0.0
	1.0	0.0
	28.0	30.0
	29.0	85.0
	30.0	0.0
	33.0	0.0
5 min post time at 100% A		

Some degree of chromatographic separation was achieved for large failures from the full length product (observed on crude samples), however, closely related impurities, N-1,N-2, N-3, N-4, N-1,N+1 etc., co-eluted with the full length product, manifesting as one large peak.

(ii) MS settings

Source:	ESI in negative mode
Capillary:	5000 V
Skimmer:	-40.0 V
End Plate Offset:	-500 V
Cap Exit:	-116.7 V
Oct RF:	200.0 Vpp
Oct 1 DC:	-12.00 V
Oct 2 DC:	-4.00 V
Lens 1:	5.0 V
Lens 2:	60.0 V
Nebulizer:	20 psi
Dry Gas:	12 L/min
Dry Gas Temp:	340°C
Trap Drive:	146.2
Scan:	1000 – 2200 <i>m/z</i>
Mode:	standard enhanced 50-2200 <i>m/z</i> , 8,100 <i>m/z</i> /sec

3. LC/MS Parameters for Agilent 6520 Accurate Mass QTOF Platform

(i) LC settings

Column:	Waters XBridge C18, 2.1 x 50 mm x 2.5 µm dp
Solvent A:	200 mM HFIP + 8.1 mM TEA at pH 7.7
Solvent B:	MeOH
Flow:	0.2 mL/min
Gradient:	95:5 (A:B) to 65:35 over 30 min
Stop time:	30 min
Post time:	6 min
Injection Vol:	4 µL of a 1 mg/mL solution in purified water

Sample temp: Ambient
Column Temp: 40°C
Detection: DAD 220-320 nm, single wavelength
extracted at 260 nm (no reference used)
Detector flow cell: 5 µL, 6 mm path

(ii) MS settings

Source: ESI in negative mode with dual spray for reference mass solution
Dry gas: 9.0 L/min
Dry Temp.: 350°C
Nebulizer: 35 psi
Mass range: 500-3000
Fragmentor: -200 V
Skimmer: -75 V
Capillary: 3500 V
Collision energy: N/A, system operated in MS mode

4. Data Analysis

Agilent MassHunter Qualitative Analysis software was used for Q-TOF derived MS data. Bruker analysis software was used for data obtained using the ion-trap MS.

Results and Discussion

LC/MS Analysis of Single Strand RNA

The synthetic route of manufacture of therapeutic RNA is typically more complex than that of DNA, due to the need to protect the 2'-hydroxyl group in the ribose. The close proximity of a protected 2'-hydroxyl to the internucleotide phosphate presents problems, both in terms of formation of the internucleotide linkage and in the removal of the 2'-protecting group

once the oligoribonucleotide is synthesized. In addition, the internucleotide bond in RNA is far less stable than that in DNA.

LC/MS was implemented to characterize the synthesis of a 20-mer single strand RNA. Using ion-pair HPLC on an Agilent 6520 Q-TOF LC/MS platform, the full-length oligonucleotide and a series of deletion sequences were separated and identified (**Figures 1a and b**).

LC/MS Analysis of Modified RNAs

Nucleic acid aptamers exhibit three-dimensional structure that specifically binds to target proteins and inhibit their activity. RNA aptamers are highly susceptible to degradation by nucleases. RNA aptamers with 2'-fluoro modifications at the pyrimidine residues are more stable and functional in the SELEX process and exhibit greater nuclease resistance. The synthesis of these highly structured nuclease-resistant molecules is challenging, particularly those with longer sequences.

A synthetic 30-mer RNA containing 2'-fluoro modifications and a 5' C6-amino linker was analyzed using ion-pair HPLC on an Agilent 6330 Ion Trap LC/MS system. Multiply charged ions representing the 5th, 6th and 7th charge states were observed in the raw ESI mass spectrum (**Figure 2a**). An expanded view of the 6th charge state shows deletion sequences and an N+1 coupling failure as well as M-94 and M-20 oligonucleotide species resulting from post-synthesis deprotection (**Figure 2b**). The M-94 and M-20 impurities were detected and identified at levels as low as 0.1%. **Figure 2c** shows that excellent linearity was achieved in the range of 0.1 – 20% of the full length concentration. The full length oligo was used as a proxy for closely related impurities.

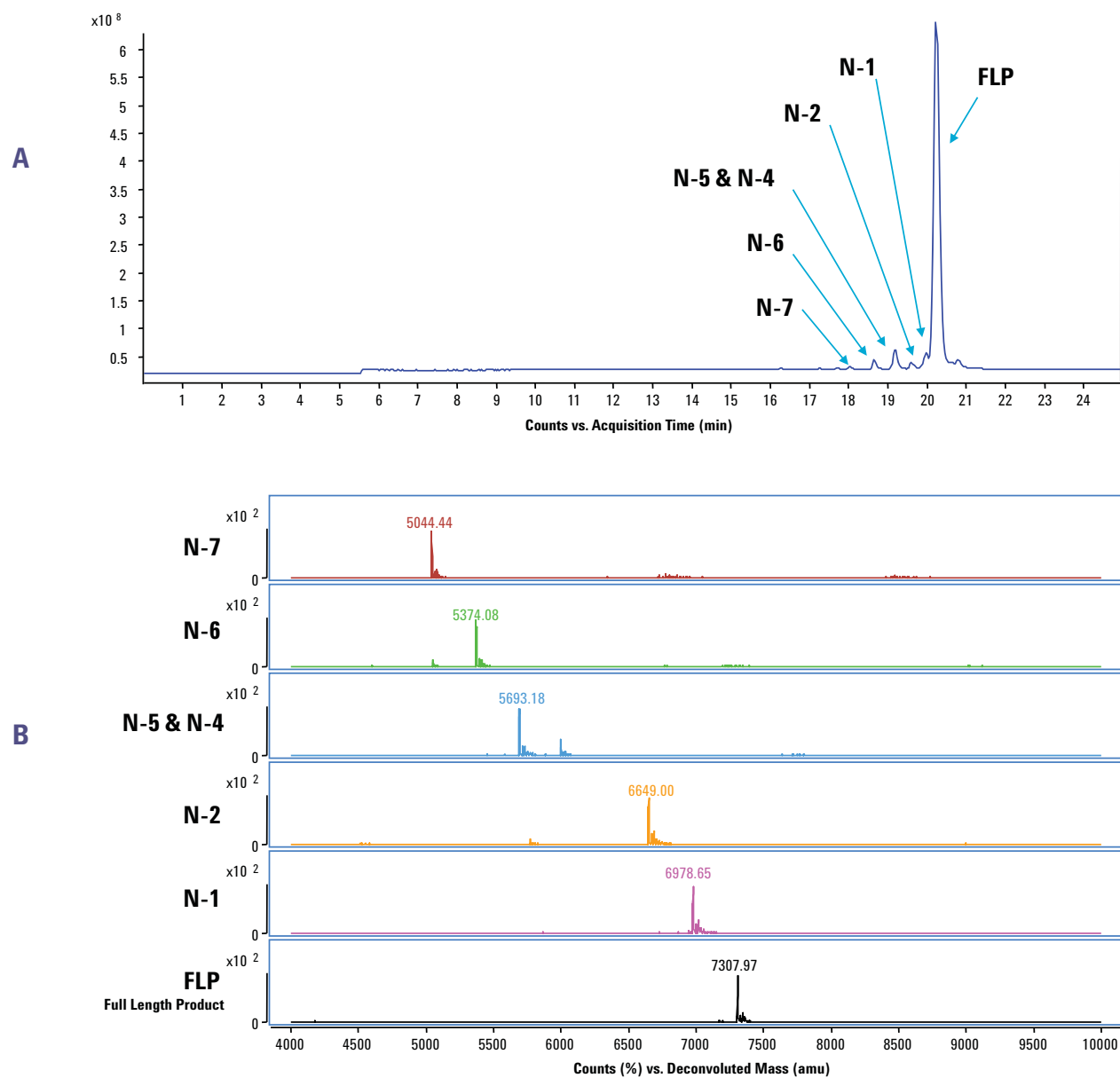


Figure 1. LC/MS analysis of a synthesized 20-mer single strand RNA using an Agilent 6520 Q-TOF instrument. **(a)** The total ion chromatogram and **(b)** deconvoluted ESI mass spectrum show the full-length product and a series of deletion sequences. Ion-pair HPLC separation was performed on a C18 column in HFIP/TEA/MeOH at 40°C. Flow rate = 0.2 mL/min.

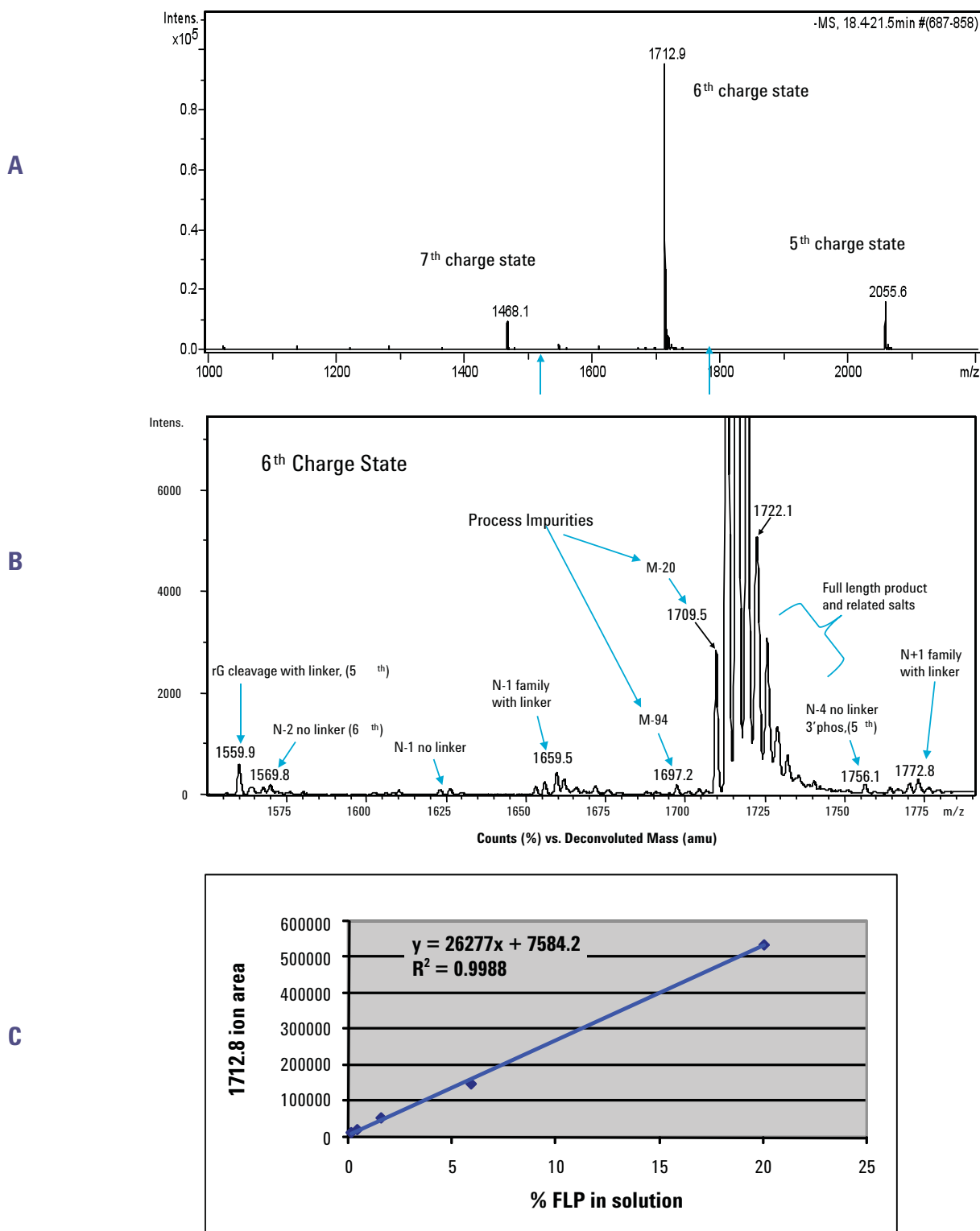


Figure 2. LC/MS analysis of a synthesized 30-mer RNA aptamer using an Agilent 6330 ion trap instrument (negative ion mode). **(a)** ESI-mass spectrum showing multiply charged states. **(b)** Expanded view of the 6th charge state show a variety of process impurities. **(c)** Concentration of the full-length product has a linear relationship with the ion abundance (peak height) of the 6th charge state species. Ion-pair HPLC separation was performed on a C18 column in $\text{NH}_4\text{OAc}/\text{MeOH}$ at 50°C , pH 7.0-8.0. Flow rate: 0.2 mL/min.

LC/MS Analysis of Thiolated Oligonucleotides

Antisense oligonucleotides inhibit expression of a gene by hybridizing to the complimentary target RNA. Thiolated oligonucleotides, or phosphorothioates, constitute a major class of antisense oligonucleotide drugs. These compounds, in which one of the non-bridging oxygens of the phosphodiester bond is substituted with sulfur, are more resistant to degradation by cellular nucleases than oligonucleotides with phosphodiester bonds.

The synthesis of phosphorothioates generates phosphodiester oligonucleotide by-products. Thiolated oligonucleotides

bind more strongly to an anion exchange support than phosphodiester oligonucleotides at most pHs. A crude thiolated DNA 20-mer was characterized using ion-pair LC/MS on an Agilent 6520 Q-TOF system. The deconvoluted ESI-MS spectrum shows a M-16 phosphodiester oligonucleotide species indicating a diester bond at one of the linkages of an otherwise fully thiolated sequence, as well as the n-1 deletion sequence and contaminating adducts (**Figure 3a**). LC/MS of the final lyophilized product using an ammonium acetate/methanol buffer system at 50°C on an Agilent 6330 Ion Trap platform confirmed the presence of the M-16 phosphodiester impurity and n-1 deletion sequence at 0.6% and 0.5%, respectively (**Figure 3b**).

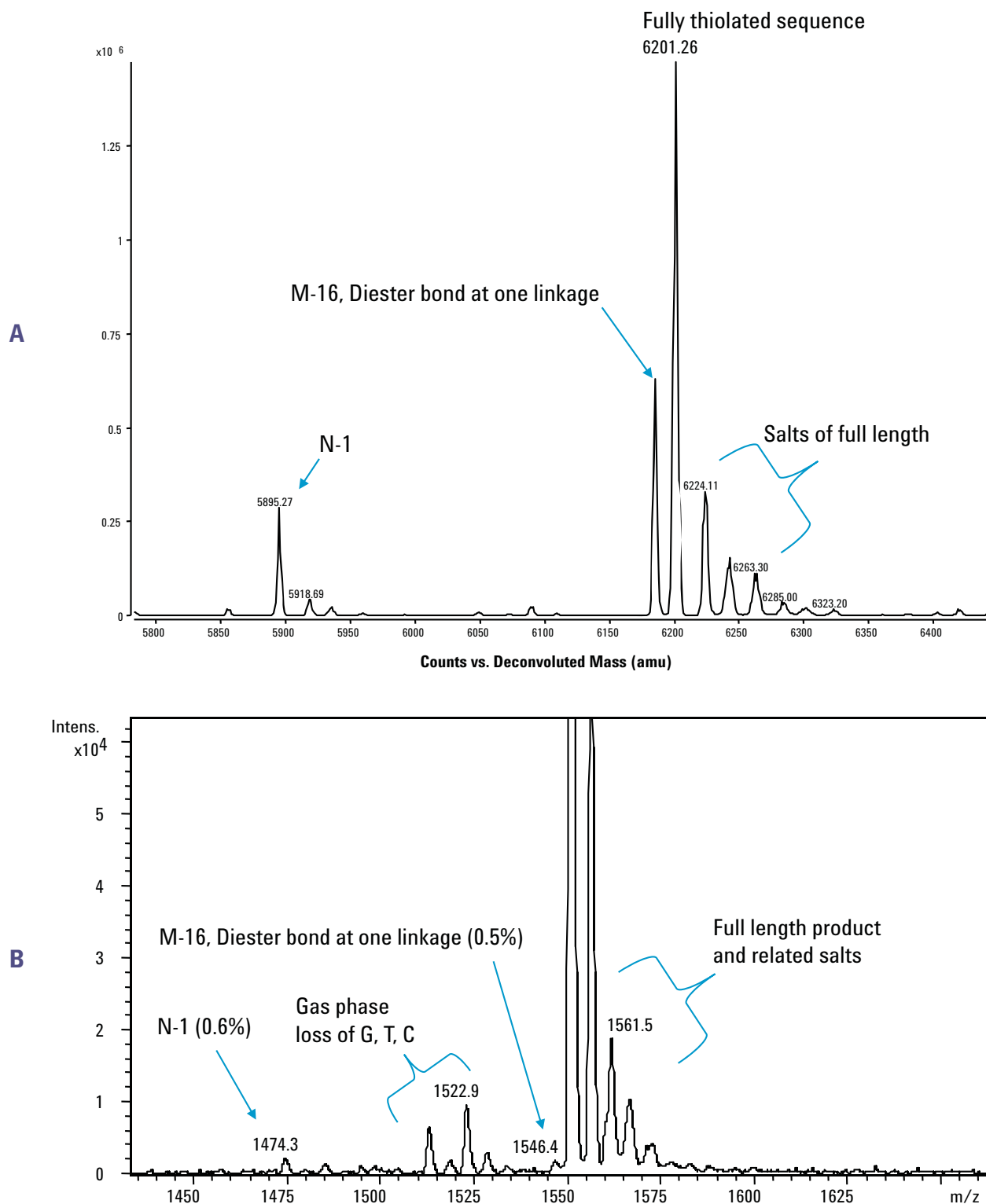


Figure 3. LC/MS analysis of a synthesized thiolated 20-mer DNA. **(a)** Deconvoluted ESI mass spectrum showing a M-16 phosphodiester species (6201.26 Da), n-1 deletion (5895.27 Da) and various salt adducts. **(b)** ESI mass spectrum (4th charge state) of the final lyophilized phosphorothioate product obtained using an Agilent 6330 Ion Trap LC/MS platform showing the presence of low levels of the M-16 phosphodiester and n-1 failure. Ion-pair HPLC was performed on a C18 column in $\text{NH}_4\text{OAc}/\text{MeOH}$ at 50°C , pH 7.0-8.0. Flow rate: 0.2 mL/min.

Conclusions

LC/MS characterization and quantitation of synthetic oligonucleotides has been demonstrated using integrated Agilent Q-TOF and Ion Trap LC/MS systems. High resolution ion-pair HPLC methods using MS-compatible mobile phases were developed to separate full length products from impurities and to allow for direct MS analysis of eluents. The high sensitivity of the LC/MS systems enabled identification of low levels process impurities and elucidation of process chemistry. LC/MS is an ideal analytical tool for the characterization and optimization of oligonucleotide synthesis.

References

1. Letsinger, R. L.; Lunsford, W. B. *J. Am. Chem. Soc.* 1976, 98, 3655-3661.
2. Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* 1981, 22, 1859-1862.
3. Matteucci, M. D.; Caruthers, M. H. *J. Am. Chem. Soc.* 1981, 103, 3186-3191.

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