Fast impurity profiling of synthetic oligonucleotides with the Agilent 1290 Infinity LC System and Agilent 6530 Accurate-Mass QTOF LC/MS

Application Note

Drug Discovery

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Abstract

This Application Note demonstrates the use of the Agilent 1290 Infinity LC System coupled with an Agilent 6530 Accurate-Mass QTOF LC/MS System, providing both UV and MS detection. This provides for the separation and relative quantification of a complex mixture of a synthetic oligonucleotide and its impurities together with their identification via accurate mass measurement.

Introduction

Gene regulation by short DNA[1] or RNA[2] molecules is a natural biological pathway. Short nucleic acids modulate gene expression at the transcriptional or the translational level, while other oligonucleotides fold to three-dimensional structures and act as enzymes in order to activate or deactivate molecular events in living organisms. Recognition of their wide range of biological actions has driven modern biopharmaceutical research into the therapeutic value of oligonucleotides.

Therapeutic oligonucleotides are prepared by chemical synthesis[3] and while their size and biological function vary, the requirement for their quality control is the same. The quality control of synthetic oligonucleotides requires a high resolution chromatographic method in order to separate the desired oligonucleotide from fragments generated as a consequence of the automated synthesis and post synthetic process. Liquid chromatography coupled to UV and MS detection allows both relative quantification of the main component and its impurities (UV) and their identification via accurate mass determination (MS). In this Application Note, we demonstrate the utility and high performance of Agilent's 1290 Infinity LC system coupled to the high mass accuracy Agilent 6530 Accurate-Mass QTOF LC/MS System for the analysis of a crude synthetic DNA example. There have been similar studies regarding the analysis of other nucleotides using other LC/MS systems, and these were used as a basis for this study.[4]

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Experimental

Equipment

- Agilent 1290 Infinity LC System, comprised of an Agilent 1290 Infinity Pump with Integrated Degasser, Agilent 1290 Infinity Autosampler, Agilent 1290 Infinity Thermostatted Column Compartment, Agilent 1290 Infinity Diode Array Detector.
- Agilent 6530 Accurate-Mass QTOF LC/MS System.

UHPLC Method

Column	Agilent ZORBAX Eclipse Plus C18 2.1 × 150 mm, 1.8 µm
Mobile Phase	A: 200 mM HFIP + 8 mM TEA in water B: MeOH
Column Temperature	30 °C
Flow rate	0.6 mL/min
Injected Volume	20 µL

Gradient

Time	%B
0	15
9	23
9.1	95
9.9	95
Stop time	10 min, post time 1 min
DAD	259 nm, BW 4

QTOF Acquisition Method

4 GHz, MS1 only, 2 Scans/second, mass range 100-3200 *m/z*, negative polarity

Source	Drying gas 8 L @ 300 °C Sheath gas 11 L @ 400 °C Nebulizer 45 psi

Sample: A synthesized 20 mer oligonucleotide with its impurities from sequence breaks during synthesis.

Result and discussion

The main component, together with its byproducts, were detected by UV absorbance at 259 nm. Impurities could be determined down to 0.2 % (Figure 1). The base sequences were identified from their accurate mass as discussed below and are annotated in Figure 1.

Figure 2 demonstrates that the UV (259 nm) response was linear over the range 160 pg/µl to 100 ng/µL (3.2 ng to 2 µg on-column) and the LLOQ was 160 pg/µl with S/N=13. This was sufficient to provide quantification of the desired oligonucleotide.

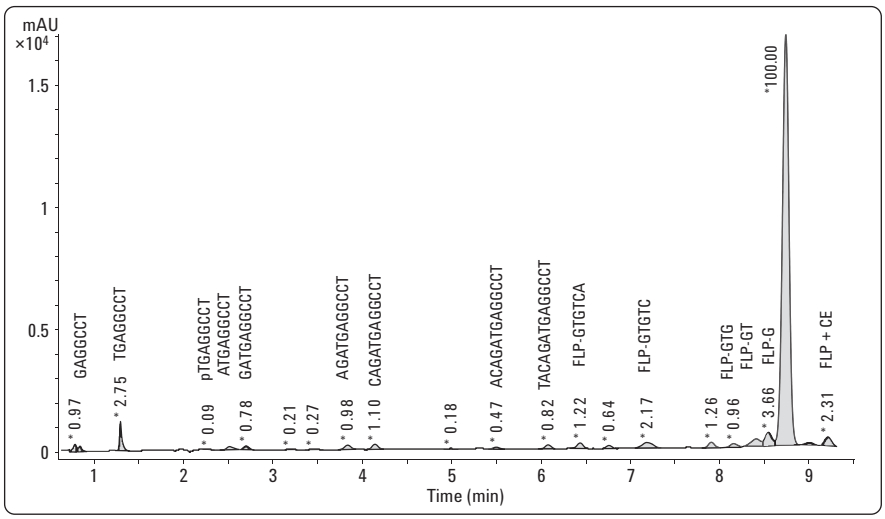

Figure 1
Detection of a synthetic oligonucleotide and its byproducts with a DAD at 259 nm, 2 µg on-column. The annotations show both the peak area percent and base sequence.

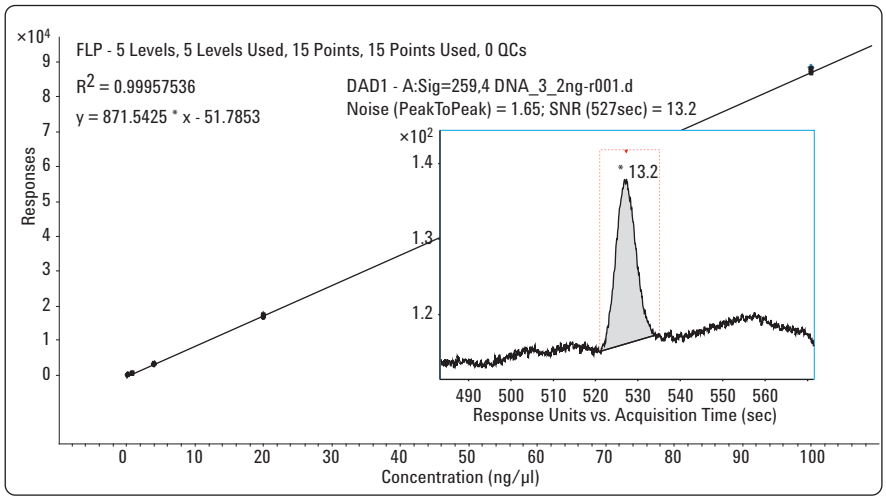

Figure 2
Calibration curve of full length product (FLP (UV 259 nm) (Insert: response obtained for the LLOQ 160 pg/uL).

The Full Length Product (FLP): 5′-d(GTGTCAGTACAGATGAGGCCT)-3′, elutes at 8.6-8.8 minutes (Figure 3) and different charge states can be detected (Figure 4a and b).

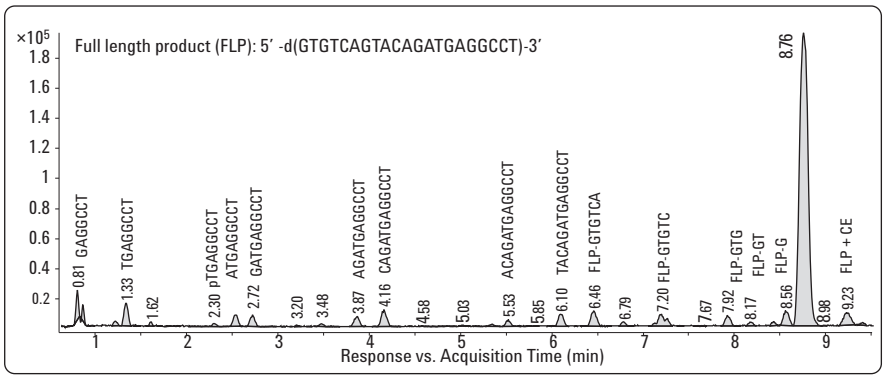

Figure 3
Base peak chromatogram (BPC) 800-2500 *m/z*, 2 µg on-column.
The annotations show RT and base sequence.

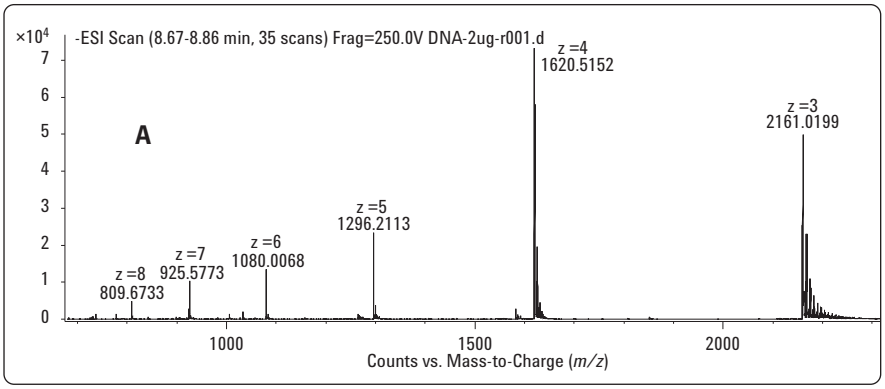

Figure 4a
Mass spectrum of the FLP at 8.76 minutes. Annotations show charge state and *m/z*.

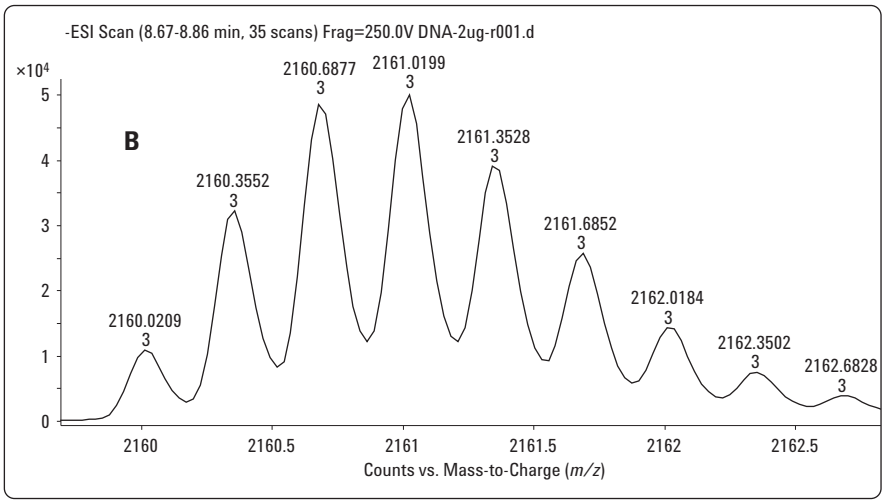

Figure 4b
Zoomed mass spectrum of the z-3 charge state of the FLP. Annotations show charge state and *m/z*.

The calculated average mass using maximum entropy deconvolution was 6486.28 (Figure 5) and the monoisotopic mass calculated by resolved isotope deconvolution was 6483.108 (Figure 6). It was possible to confirm the mass of the main product and to assign the impurities as shown in Figure 1 and Figure 3.

Conclusion

This Application Note demonstrates the separation of a crude synthetic oligonucleotide mixture after synthesis and final cleavage from the solid support. The Agilent 1290 Infinity LC System coupled with the Agilent 6530 Accurate-Mass QTOF LC/MS System can be used to simultaneously quantify and identify impurities down to lower nanogram levels and relative levels of 0.2% of the main compound. While UV detection allows quantification down to a LLOQ of 3.2 ng on-column, the accurate mass measurement allows the highly accurate determination of the average and monoisotopic mass and the base sequence of the synthesized oligonucleotide and process-related impurities.

References

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2. A. Fire, S. Xu, M. Montgomery, S. Kostas, S. Driver, C. Mello, *Nature* 1998, 391 (6669), 806–11.
3. S. L. Beaucage, M. H. Caruthers, *Tetrahedron Lett.* 1981, 22, 1859-1862.
4. T. Kreutzian, M. Waldheim, A. Jackson, M. Hoppe, V. Mokler, D. Eidenmueller, K. Hill, Agilent Publication Number 5990-3539EN.

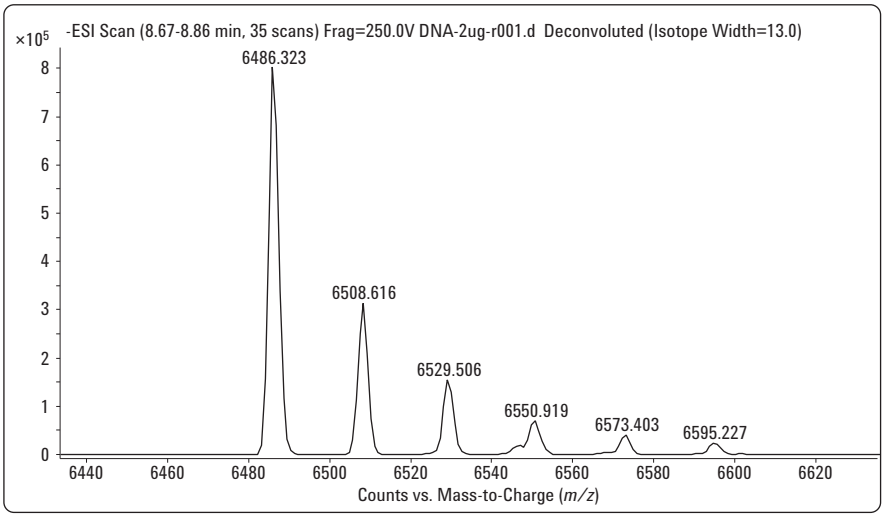

Figure 5
Maximum Entropy deconvoluted mass spectrum of FLP, calculated average mass 6486.203 a.m.u.

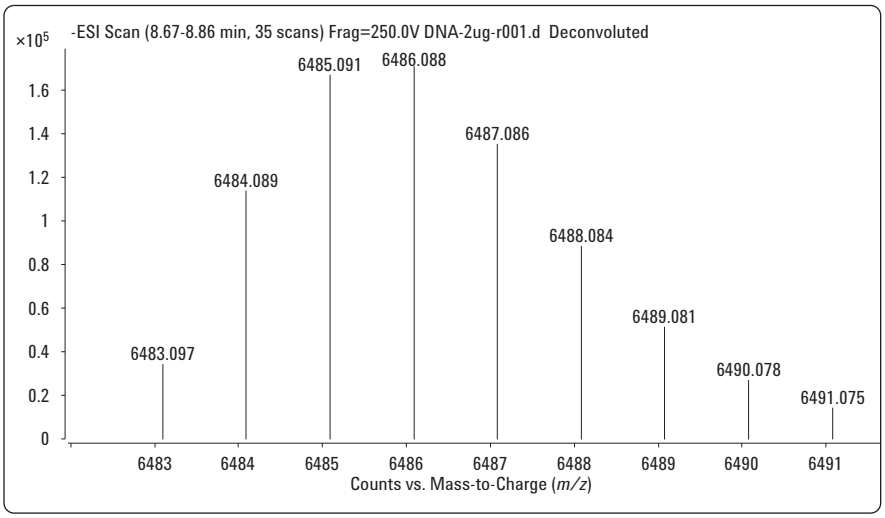

Figure 6
Resolved isotope deconvoluted mass spectrum of FLP, calculated monoisotopic mass 6483.116 a.m.u.

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