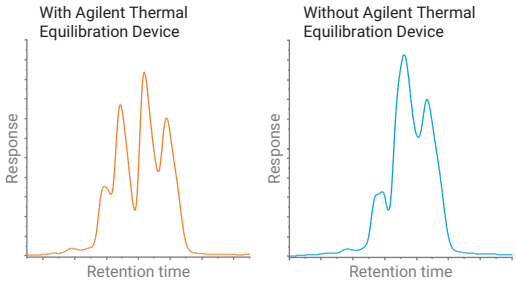
Application Note

Biopharma/Pharma

The Agilent Thermal Equilibration Device Increases Resolution for Oligonucleotide Analysis

Combining the Agilent 1290 Infinity II Bio LC System with the Agilent Thermal Equilibration Device for High-Performance Oligonucleotide Analysis



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Abstract

Due to advances in oligonucleotide synthesis and modification, modern DNA- or RNA-based biopharmaceuticals can make characterization more demanding because of their increased complexity. This application note demonstrates that the Agilent Thermal Equilibration Device (TED) combined with the biocompatible Agilent 1290 Infinity II Bio LC is an excellent setup for in-depth analysis of oligonucleotides. Using the TED with a high-temperature LC method, the peak resolution and analysis speed can be significantly increased. Product and process impurities can be separated and analyzed. These results show that oligonucleotide analysis can significantly benefit from the TED.

Introduction

Recent approvals of several oligonucleotide-based biopharmaceuticals highlighted the enormous potential of cell and gene therapy as new modalities for myriad indications.[1] Typically comprised of 18 to 30 nucleotides, these biopharmaceuticals operate through various molecular mechanisms designed to interfere with genetic information processing. A wide range of chemical modifications can be introduced to these structures to increase bio-availability, ribonuclease resistance, and potency. Replacement of the phosphate group by phosphorothioate and modification of the 2' hydroxyl position of the ribose are just two examples.[2] However, increasing the diversity of these oligonucleotides puts extra pressure on the thorough characterization of the therapeutics in terms of selectivity and robustness. Typical techniques for analyzing oligonucleotides are hybridization techniques such as ELISA (enzyme-linked immunosorbent assay) or PCR (polymerase chain reaction). These techniques are highly sensitive but lack specificity compared to alternative approaches such as LC or LC/MS.[3] The two major chromatographic methods for oligonucleotides analysis are anion exchange chromatography (AEX) and ion-pair reversed-phase chromatography (IP-RP). In particular, IP-RP can be used for mass verification, quantitative impurity assessment, and identification. A critical factor in LC method development is the complete denaturation of the duplex oligonucleotide of interest to decrease unwanted tailing and introduce resolution of the single strands. This can be achieved with high-pH mobile phases or high column temperatures (>60 °C), especially when using IP-RP chromatography. This application note investigates the benefits of the TED with the 1290 Infinity II Bio LC for the analysis of oligonucleotides with temperature sensitive LC methods.

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System comprised the following modules:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) equipped with a Standard Flow Quick-Connect Bio Heat Exchanger (G7116-60071) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (VWD) (G7114B), equipped with a Bio Micro Flow Cell VWD, 3 mm, 2 µL, RFID (G1314-60189)

Software

Agilent OpenLab CDS version 2.6 or later

Column

Generic C18 column, 2.1 × 50 mm, 4.0 µm connected with Generic C18 column, 2.1 × 100 mm, 4.0 µm

Chemicals

Fresh ultrapure water was obtained from a Milli-Q Integral system (Millipak, Merck-Millipore, Billerica, MA, USA). LC/MS-grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany), triethylamine (TEA) from Sigma-Aldrich (Steinheim, Germany), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) from Acros Organics (New Jersey, USA).

Sample preparation

A modified siRNA oligonucleotide (20 bp, provided by Johnson & Johnson) was dissolved in ultrapure water with a concentration of 0.5 mg/mL.

Table 1. LC method for oligonucleotide analysis with the Agilent 1290 Infinity II Bio LC and Agilent Thermal Equilibration Device.

Parameter	Value
Solvent	A) 0.07% TEA in 60 mM aqueous HFIP solution B) 70:30 (v:v) methanol:acetonitrile
Gradient	Time (min) %A %B 0 100 0 20 85 15 25 30 70 26 30 70 28 100 0 34 100 0
Flow Rate	0.300 mL/min
Temperature	75 °C with and without two Thermal Equilibration Devices installed
UV Detection	260 nm
Injection	Injection volume: 3 µL Sample temperature: 10 °C Wash: 30 s with water (flush port)

Results and discussion

Retention time stability, selectivity, or resolution can be altered by temperature fluctuation in the column oven of LC systems. Depending on the laboratory setup, these temperature shifts can occur, for example, due to air conditioning variations, especially with high-temperature applications. To demonstrate the positive influence of the TED, a demanding IP-RP method analyzing a therapeutic oligonucleotide was chosen (Table 1). Mobile phase compositions were based on triethylamine (TEA) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) dissolved in water and a methanol/acetonitrile mixture in the second eluent. As already mentioned, oligonucleotide analysis can benefit from elevated column temperature.

For this reason, a temperature of 75 °C was used in the LC method. A therapeutic, modified siRNA oligonucleotide with 20 base pairs was used as an analyte. To investigate the impact of the TED, analyses were performed with and without two TEDs installed. Figure 1 shows two overlaid chromatograms with both experimental setups.

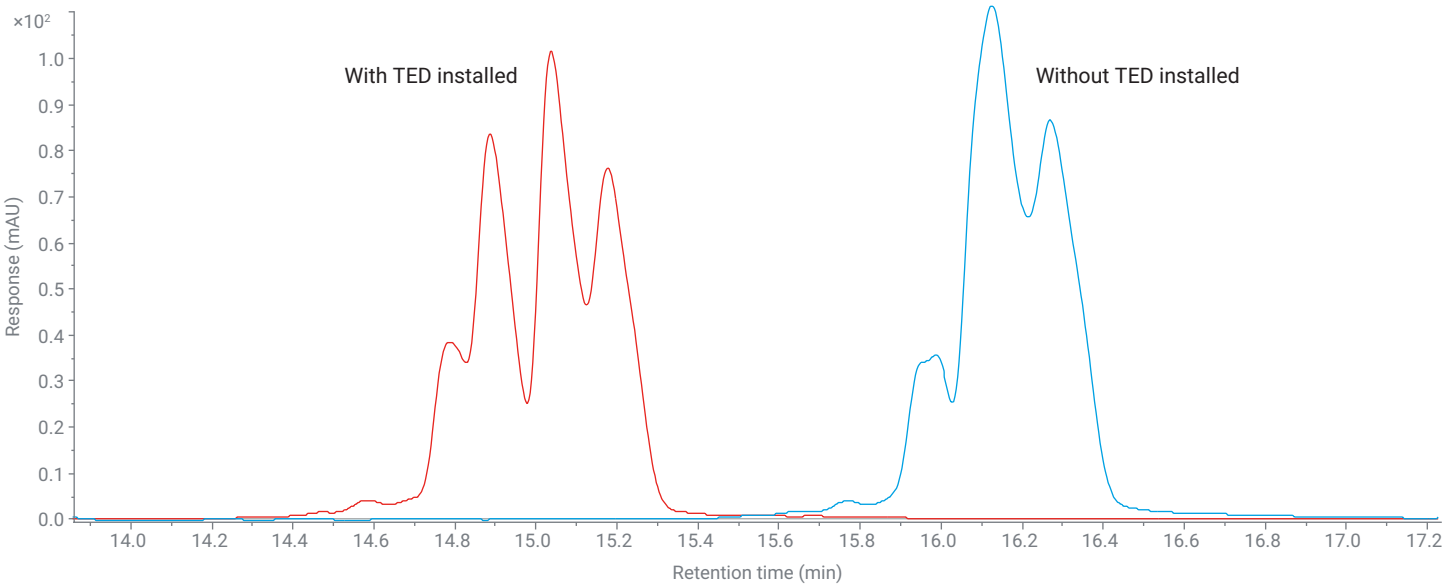

Figure 1. Overlaid chromatograms of IP-RP analysis of an siRNA oligonucleotide at 75 °C with an Agilent Thermal Equilibration Device (TED) installed (red) and without the TED (blue).

Comparing both chromatograms, it becomes evident that the TED significantly increases the resolution between the prominent peaks. Only three peaks can be distinguished without the TED installed, whereas four peaks can be seen with the TED. The peaks also elute approximately 8% earlier when using the TED, which can positively impact the productivity of the corresponding laboratory.

Using the TED, the single-strand peaks and their corresponding impurities can easily be separated (Figure 2). Four distinct peaks represent the product (due to isomerization), and the impurities elute before and after the main peaks. These impurities can consist of shortmers, longmers, oxidized products, depurinated species, or other process impurities.

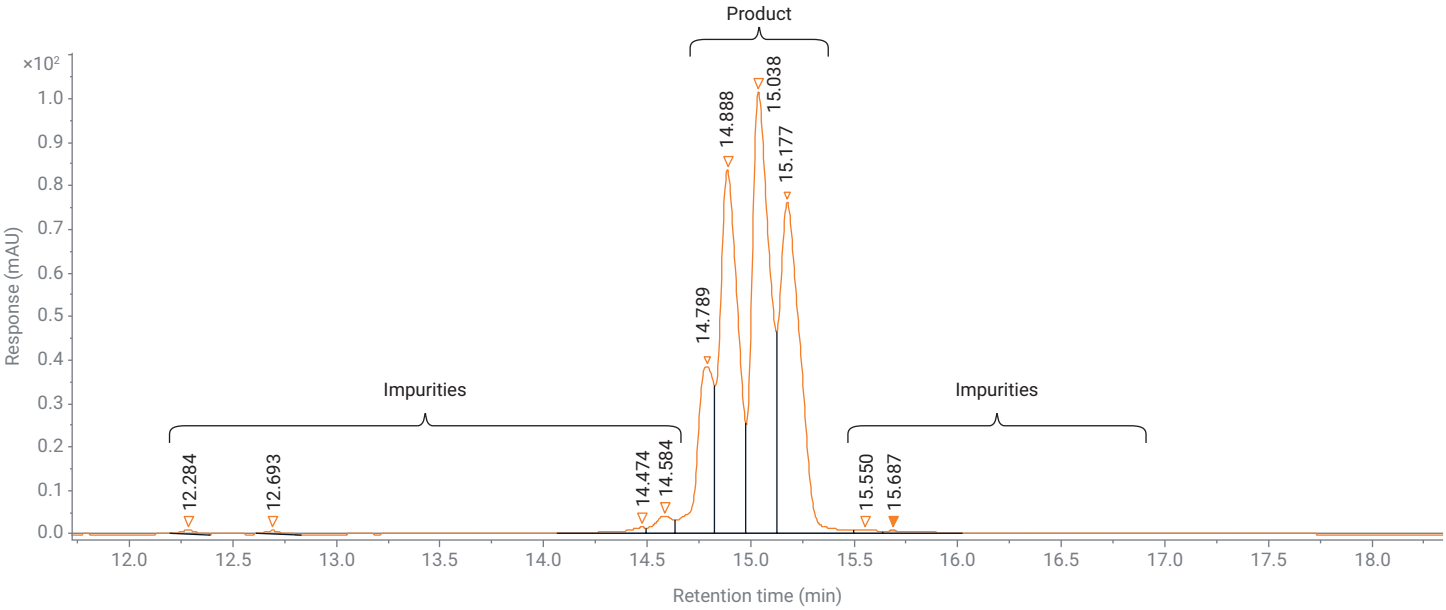

Figure 2. Product and impurities of an siRNA oligonucleotide analyzed with an Agilent Thermal Equilibration Device (TED) installed.

Due to the increased resolution, product peaks and impurities can easily be integrated, and area percentages can be calculated (Table 2).

Table 2. Example of impurity analysis of an siRNA oligonucleotide depicting retention time and area percentage.

Compound	Retention Time (min)	Area (%)
Impurities	<14.789	2.6
Product	14.789	11.1
	14.888	26.3
	15.038	31.4
	15.177	27.6
Impurities	>15.177	1.0

To quantify the increased resolution when using the TEDs, peak resolution (R_s) values between the different product peaks and the first significant impurity were calculated (Figure 3). All values were above 0.5, rendering this LC method suitable for impurity percent analysis. By adding MS detection, this method could also be used for product and impurity identification and Quantitative Analysis, due to the excellent separation.

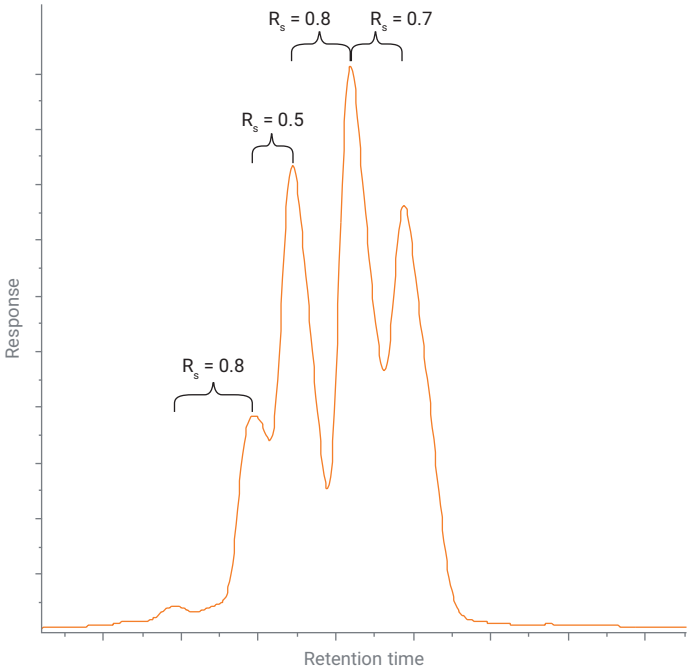

Figure 3. Peak resolution values between product peaks and first impurity peak of an siRNA oligonucleotide analyzed with an Agilent Thermal Equilibration Device (TED) installed.

Conclusion

Oligonucleotide-based therapeutics have seen increased relevance in the past years. However, analytical methods for in-depth characterization still have room for improvement. This application note shows that the combination of the iron-free flow path of the Agilent 1290 Infinity II Bio LC and the increased temperature stability and performance when using an Agilent Thermal Equilibration Device are an excellent strategy to improve resolution and speed of analysis when analyzing modified DNA oligonucleotides. Corresponding impurities can be sufficiently separated and integrated, rendering this LC method suitable for QC analysis of biopharmaceuticals based on oligonucleotides.

References

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