Application Note

Biopharma/Pharma

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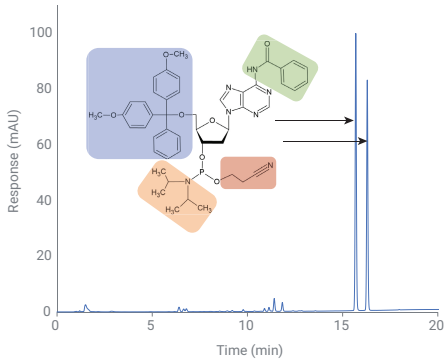
Trusted Answers

Analyzing Raw Material for Oligonucleotide Synthesis

Flexible and robust method development for the analysis of nucleoside phosphoramidites with the Agilent 1290 Infinity II Bio LC System

Suitable for Agilent
1290 Infinity III LC



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Abstract

As the first step of the biopharmaceutical production process, the raw material of any synthesis needs to be analyzed thoroughly for the best quality products. In the case of oligonucleotides, these raw materials are called nucleoside phosphoramidites and act as building blocks in the subsequent DNA synthesis. In this application note, we developed LC methods based on the biocompatible Agilent 1290 Infinity II Bio LC. By coupling the LC with the Agilent 6545XT AdvanceBio LC/Q-TOF, several impurities could be identified by accurate mass, and method development could be easily carried out by the four-channel Agilent 1290 Infinity II Bio Flexible Pump, which showed excellent retention time and area precision. Based on this, additional method development was carried out to decrease LC run time by 66%, retaining the outstanding performance, and method compatibility experiments were performed, showing seamless method transfer from the conventional Agilent 1290 Infinity II LC. Taken together, these results show that the 1290 Infinity II Bio LC is the perfect choice for the robust and versatile analysis of raw materials for oligonucleotide synthesis.

Introduction

The chemical synthesis of DNA oligonucleotides has been one of the enabling technologies for modern molecular biology.[1] Combined with the recent approval of several oligonucleotide-based biopharmaceuticals[2], there is an increased need for reliable and robust analytical methods across the manufacturing and production chain of oligonucleotides. Nucleoside phosphoramidites are considered the gold-standard building blocks in DNA synthesis technology due to the quick and easy removal of the relevant protection groups.[3] Natural nucleosides are rich in reactive sites like hydroxyl (-OH) and amino ($-NH_2$) groups. These functional groups are modified in the phosphoramidite building blocks. Figure 1 shows the structural formula of the 5'-DMT-deoxy adenosine 3'-phosphoramidite molecule with four distinct modifications: A dimethoxytrityl (DMT) group (blue) protecting the 5'-hydroxyl group of the desoxyribose, the diisopropylamino (orange) and the 2-cyanoethyl (red) group modifying the phosphoramidite moiety, and a benzoyl (green) group protecting the amino group in the adenine base. After modification, the phosphoramidites of the corresponding DNA nucleoside like deoxyadenosine (dA), deoxyguanosine (dG), deoxycytidine (dC), and deoxythymidine (dT) act as raw material for the subsequent automated oligonucleotide synthesis with multiple synthesis cycles consisting of distinct deprotection, coupling, oxidation, and capping steps.[1] However, the purity and impurities of the raw material need to be closely monitored and identified to minimize sequence impurities and improve coupling efficiencies.

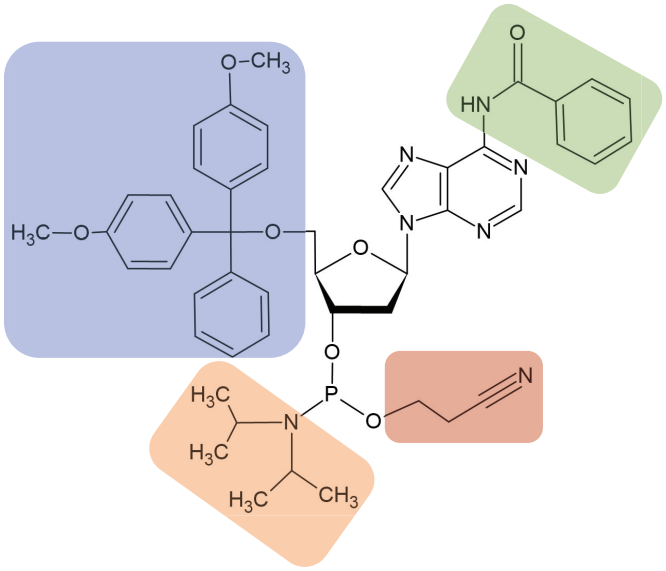

Figure 1. Schematic overview of the modified groups in the 5'-DMT-deoxy adenosine 3'-phosphoramidite molecule. Blue: dimethoxytrityl (DMT) group. Green: benzoyl group. Orange: diisopropylamino group. Red: 2-cyanoethyl group.

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System coupled to the Agilent 6545XT AdvanceBio LC/Q-TOF comprised the following modules:

Agilent 1290 Infinity II Bio LC System:

- Agilent 1290 Infinity II Bio Flexible Pump (G7131A)
- 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.
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6545XT)

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

Agilent 1290 Infinity II LC:

- Agilent 1290 Infinity II Flexible Pump (G7104A)
- Agilent 1290 Infinity II Multisampler (G7167A) with Sample Thermostat (option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) equipped with a Standard Flow Quick Connect Heat Exchanger (G7116-60015) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (VWD) (G7114B), equipped with a Micro Flow Cell VWD, 3 mm, 2 µL, RFID.

Software

- Agilent MassHunter workstation data acquisition (B.09.00 or later)
- Agilent MassHunter qualitative analysis (10.0 or later)

Column

Agilent ZORBAX RRHD Eclipse Plus C18, 95 Å, 2.1 × 100 mm, 1.8 µm (part number 959758-902)

Chemicals

InfinityLab Ultrapure LC/MS acetonitrile (part number 5191-4496) and the InfinityLab Ultrapure LC/MS water (part number 5191-4498) were used. Ammonium acetate and acetic acid were obtained from VWR (Darmstadt, Germany).

Sample preparation

Authentic 5'-O-DMT 2' deoxyadenosine phosphoramidite (dA) raw material for the synthesis of oligonucleotides was provided by the Agilent Nucleic Acid Solutions Division. The injection concentration was 1 mg/mL phosphoramidite in acetonitrile.

Table 3. Source and MS parameters for the impurity analysis of 5'-DMT-deoxy adenosine 3'-phosphoramidite raw material with the Agilent 1290 Infinity II Bio LC.

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOF
Gas Temperature	320 °C
Drying Gas Flow	8 L/min
Nebulizer	35 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
VCap	3,500 V
Nozzle Voltage	1,000 V
Fragmentor	140 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Acquisition Mode	Positive, extended (*m/z* 10,000) mass range
Mass Range	*m/z* 25 to 10,000
Acquisition Rate	1 spectrum/sec
Reference Mass	*m/z* 121.050873, *m/z* 922.0098

Table 1. Method A for the impurity analysis of 5'-DMT-deoxy adenosine 3'-phosphoramidite raw material with the Agilent 1290 Infinity II Bio LC.

Parameter	Value
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 95 Å, 2.1 × 100 mm, 1.8 µm
Solvent	A) Acetonitrile B) Water C) 500 mM ammonium acetate, pH 5.5
Gradient	Time (min) A (%) B (%) C (%) 0.00 50 48 2 1.00 50 48 2 15.00 90 8 2 15.01 90 8 2 18.00 90 8 2 18.01 50 48 2 25.00 50 48 2
Flow Rate	0.200 mL/min
Temperature	20 °C with thermal equilibration devices installed
UV Detection	VWD: 236 nm, 10 Hz/MS: see Table 2
Injection	Injection volume: 2 µL Sample temperature: 4 °C Wash: 3 s with 90% acetonitrile/10% water (flush port)

Table 2. Method B with optimized run time for the impurity analysis of 5'-DMT-deoxy adenosine 3'-phosphoramidite raw material with the Agilent 1290 Infinity II Bio LC.

Parameter	Value
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 95 Å, 2.1 × 100 mm, 1.8 µm
Solvent	A) Acetonitrile B) Water C) 500 mM ammonium acetate, pH 5.5
Gradient	Time (min) A (%) B (%) C (%) 0.00 50 48 2 0.33 50 48 2 5.00 90 8 2 5.01 90 8 2 6.00 90 8 2 6.01 50 48 2 8.33 50 48 2
Flow Rate	0.600 mL/min
Column Temperature	20 °C with thermal equilibration devices installed
UV Detection	VWD: 236 nm, 10 Hz
Injection	Injection volume: 2 µL Sample temperature: 4 °C Wash: 3 s with 90% acetonitrile / 10% water (Flush Port)

Results and discussion

HPLC method development and method transfer into different business units, like early process development and quality control, can be tedious and cumbersome. However, owing to the versatility that the 1290 Infinity II Bio Flexible Pump lends to the 1290 Infinity II Bio LC System, method development can be convenient and robust. To showcase this, an impurity analysis LC method for the 5'-DMT-deoxy adenosine 3'-phosphoramidite raw material was developed by employing a ternary gradient on the four-channel Infinity II Bio Flexible Pump. Due to the corrosion-resistant flow path of the 1290 Infinity II Bio LC, highly concentrated buffers like 500 mM ammonium acetate (pH 5.5) can be used routinely for method development, which enables the fast screening of different buffer amounts and types with on-the-fly gradient mixing (Table 1, Method A). The product and three impurities (Table 4) could be identified with accurate mass by coupling the 1290 Infinity II Bio LC with the 6545XT AdvanceBio LC/Q-TOF sequentially, which allowed simultaneous UV and MS analysis of the compounds. Due to an epimerization at the chiral center of the phosphorus atom of the phosphoramidite, two peaks (A and B) are visible for the product, impurity 2 and impurity 3.

Figure 2 shows chromatograms of the raw material separation detected with the VWD equipped with the Bio Micro Flow Cell. The lower chromatogram depicts a zoomed-in view of the impurities with good resolution between the peaks of interest. The analysis of the 3'-DMT-deoxy adenosine 5'-phosphoramidite (impurity 2A/2B) is especially critical since the so-called "reverse amidite" can lead to errors in the subsequent DNA synthesis.

Table 4. Product and impurities identified in the 5'-DMT-deoxy adenosine 3'-phosphoramidite raw material.

Name	Species	Retention Time (min)
5'-DMT-Deoxy Adenosine 3'-Phosphoramidite	Product A	15.69
	Product B	16.29
5'-DMT-Deoxy Adenosine	Impurity 1	6.40
3'-DMT-Deoxy Adenosine 5'-Phosphoramidite (Reverse Amidite)	Impurity 2A	10.89
	Impurity 2B	11.13
5'-DMT-Deoxy Adenosine Phosphoramidate	Impurity 3A	11.41
	Impurity 3B	11.83

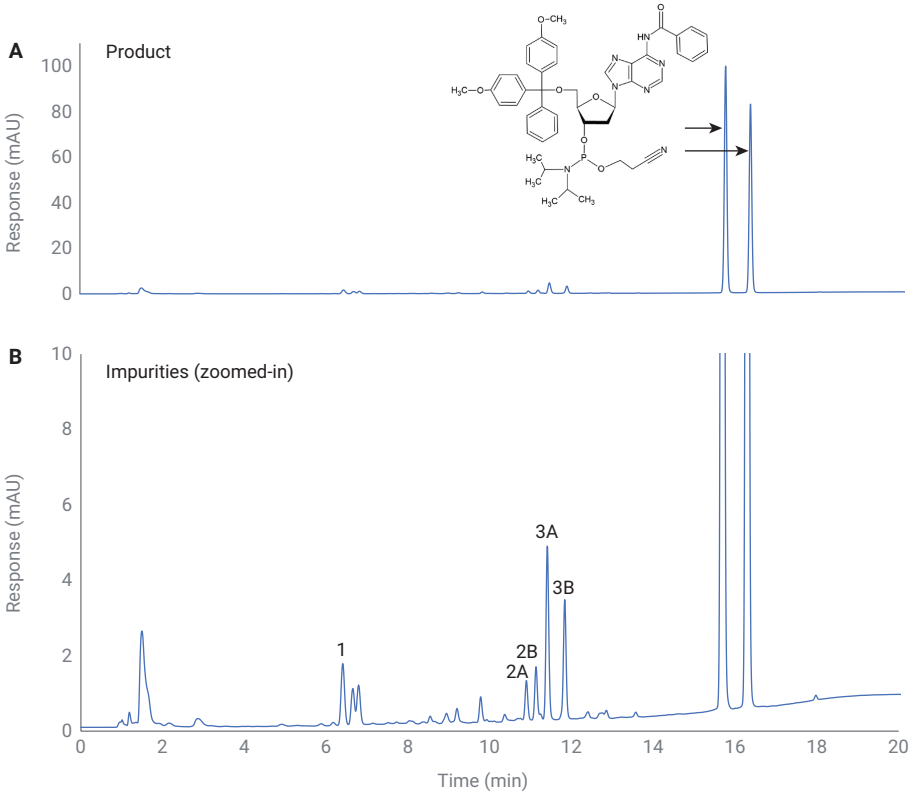

Figure 2. A: Chromatogram of the LC separation (method A) of the raw material with the Agilent 1290 Infinity II Bio LC, highlighting the product. B: Zoomed-in view of the same chromatogram for better visibility of the impurities.

Ten consecutive injections were analyzed to further investigate the 1290 Infinity II Bio LC performance, and retention time and area precision values were calculated (Table 5). Excellent retention time RSD values between 0.015% and 0.050% for the product and impurities were determined, showcasing the outstanding performance of the 1290 Infinity II Bio Flexible Pump when deploying a challenging ternary gradient separation. Additionally, the 1290 Infinity II Bio LC's performance in terms of area precision was also excellent, with an average relative standard deviation of 0.269%. To further evaluate the method compatibility of the 1290 Infinity II Bio LC with the 1290 Infinity II LC, the previous experiments were also run on the latter. Table 5 shows that the absolute retention times on both LC systems only deviated with an average of 0.686%, which shows excellent method transferability from the 1290 Infinity II LC.

Time is a critical resource in modern laboratories, especially for routine analysis in the quality control of biopharmaceuticals and their raw materials. Hence, further method development was employed to reduce the run time of the LC analysis. Because of the high-pressure rating of up to 1,300 bar for the 1290 Infinity II Bio LC System, high flow rates and small particles can be used to optimize performance and run time. By increasing the flow rate to 0.6 mL/min and keeping the gradient volume constant, the LC method was shortened by 66% to only 8.33 minutes (Table 2, Method B). Even though the run time was decreased, the excellent performance remained with high retention time and area precision (Figure 3 and Table 6), rendering the 1290 Infinity II Bio LC the perfect fit for versatile and robust analytical method development across the production chain of oligonucleotides.

Table 5. Retention time and area precision values for the compounds analyzed with method A and absolute retention time deviations compared between the Agilent 1290 Infinity II Bio LC and Agilent 1290 Infinity II LC.

Species	Retention Time RSD (%)	Area RSD (%)	Retention Time Deviation 1290 Bio LC Versus 1290 LC (%)
Product A	0.017	0.113	0.07
Product B	0.015	0.118	0.19
Impurity 1	0.050	0.211	1.53
Impurity 2A	0.026	0.314	0.85
Impurity 2B	0.020	0.539	0.76
Impurity 3A	0.022	0.209	0.73
Impurity 3B	0.021	0.378	0.67

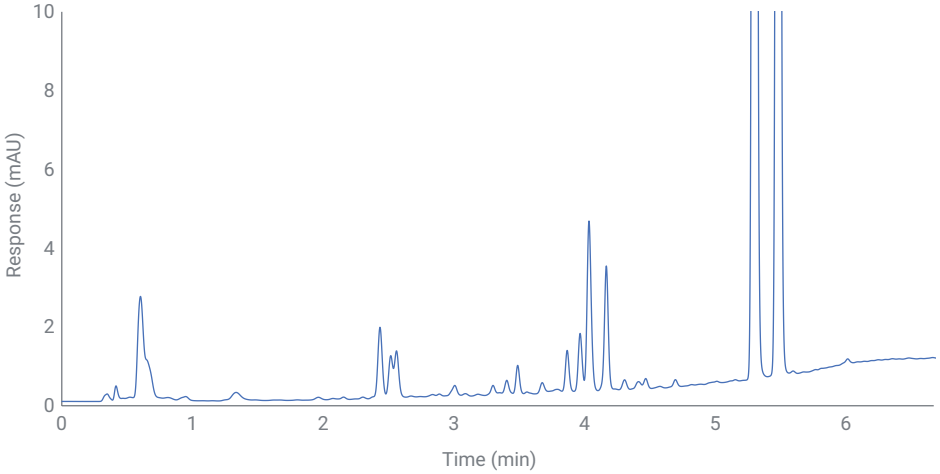

Figure 3. Zoomed-in view of the LC separation with shorter run time (method B) of the raw material with the Agilent 1290 Infinity II Bio LC.

Table 6. Retention time and area precision values for the compounds analyzed with method B.

Species	Retention Time (min)	Retention Time RSD (%)	Area RSD (%)
Product A	5.30	0.042	0.180
Product B	5.48	0.40	0.185
Impurity 1	2.43	0.115	0.214
Impurity 2A	3.86	0.079	0.557
Impurity 2B	3.96	0.066	0.986
Impurity 3A	4.03	0.065	0.355
Impurity 3B	4.16	0.059	0.301

Conclusion

Even though the DNA synthesis technology based on phosphoramidites has been around since the 1980s, streamlining related analytical challenges is still an important task. In this application note, we showed the ability of the Agilent 1290 Infinity II Bio LC with Flexible Pump to perform impurity analysis on the authentic raw material of the 5'-DMT-deoxy adenosine 3'-phosphoramidite building block with the highest confidence in process development and quality control environments. Thanks to the versatility and high performance of the Agilent 1290 Infinity II Bio Flexible Pump combined with the entire 1290 Infinity II Bio LC System's biocompatible flow path, excellent retention time and area precision were achieved, and seamless method compatibility was observed. That is why the 1290 Infinity II Bio LC System is a future-proof choice for challenging analyses of biomolecules.

References

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