Application Note

Biologics and Biosimilars

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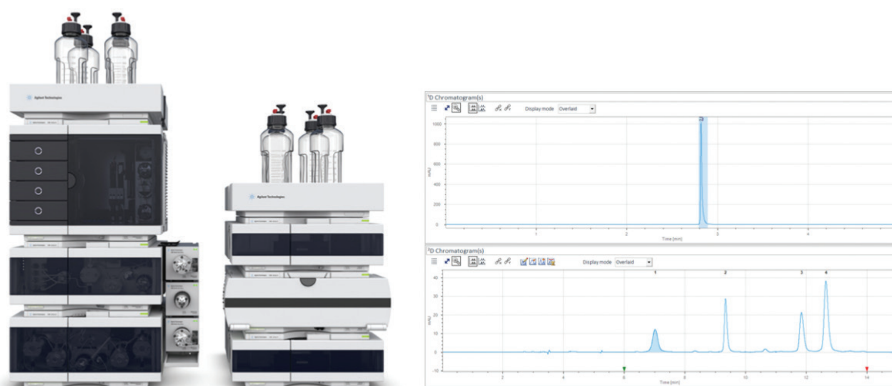
Direct Analysis of In-Process Oligonucleotides Without Manual Purification

Ion-pair reversed-phase LC analysis with online desalting using Agilent InfinityLab 2D-LC Solutions



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Abstract

Oligonucleotides synthesized using phosphoramidite chemistry are typically analyzed and purified using ion-pair reversed-phase liquid chromatography (IP-RPLC) and anion-exchange chromatography. The high salt content of anion-exchange purification fractions impairs the ability of oligonucleotides to participate in ion pairing during IP-RPLC analysis. This leads to the need for sample desalting prior to IP-RPLC analysis, which is typically performed manually using centrifugal filters.

This Application Note shows the direct 2D-LC analysis of oligonucleotides in solution with high salt content, with online desalting performed in the first dimension (^{1}D) followed by IP-RPLC analysis in the second dimension (^{2}D). In this setup, the application of 2D-LC increases the workflow speed and avoids manual sample preparation.

Introduction

In molecular biology and molecular diagnostics, the availability of standard and modified synthetic oligonucleotides is required during polymerase chain reaction (PCR), gene silencing, and related techniques[1]. In addition, synthetic oligonucleotides are gaining increasing importance as therapeutic agents for various diseases such as cancer and viral diseases[2]. Oligonucleotide-based pharmaceuticals include antisense oligonucleotides, short interfering RNAs (siRNAs), and aptamers. Antisense oligonucleotides and siRNAs both prevent protein synthesis by preventing mRNA translation. Aptamers form aptamer-protein complexes, thereby inhibiting the protein's biological function[3].

Oligonucleotides are commonly synthesized using phosphoramidite chemistry[2,4]. Achievable purities are typically greater than 70 %, and common impurities present after synthesis include oligonucleotides with deleted or extended sequences as well as incompletely deprotected products, oligonucleotides with a loss of a purine base, and other degradation products[2]. Analytical separation and purification of synthesized deprotected oligonucleotides is typically performed using ion-pair reversed-phase liquid chromatography (IP-RPLC) and anion-exchange chromatography[2,4].

Anion-exchange purification fractions typically contain high concentrations of salt, for example, sodium chloride (NaCl) or sodium bromide (NaBr.) When analyzing these fractions using IP-RPLC, the high salt levels impair the ability of the oligonucleotides to participate in ion pairing, and thereby lead to changes in retention time, peak splitting, and breakthrough[5]. To enable successful IP-RPLC analysis of anion-exchange purification fractions, samples need to be desalted prior to analysis[5], which is commonly performed manually.

This Application Note shows the direct 2D-LC analysis of oligonucleotides in solution with high salt content, comparable to anion-exchange purification fractions obtained after oligonucleotide synthesis. The ^{1}D of the 2D-LC analysis is used for online desalting, before IP-RPLC analysis in the ^{2}D. In this setup, the application of 2D-LC enables time savings and avoids manual sample preparation. Avoiding manual sample preparation steps can improve reproducibility and avoid sample loss during sample preparation.

Experimental

Equipment

The Agilent 1290 Infinity II 2D-LC System comprised the following modules:

- Two Agilent 1290 Infinity II High Speed Pumps (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with cooler (option #100)
- Two Agilent 1290 Infinity II Multicolumn Thermostats (G7116B)
- Two Agilent 1290 Infinity II Diode Array Detectors (G7117B) with Max-Light cartridge cell 10 mm (G4212-60008)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2D-LC valve, active solvent modulation (G4243A)
- Two Agilent 1290 Infinity Valve Drives (G1170A) with multiple heart-cutting valves (G4242-64000) equipped with 40 µL loops

Software

Agilent OpenLab CDS ChemStation Edition version C.01.08 [210] with 2D-LC software version A.01.04 SR1.

Columns

- Agilent PLRP-S 100 Å, 2.1 × 50 mm, 3 µm (p/n PL1912-1300)
- Agilent AdvanceBio Oligonucleotide, 2.1 × 50 mm, 2.7 µm (p/n 659750-702)

Chemicals

All solvents were LC grade. Methanol was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Ultrapure Lab Water System equipped with a Millipak 0.22 µm membrane point-of-use cartridge (Millipore, Merck (Darmstadt, Germany)). Ammonium acetate and 1,1,1,3,3,3-hexafluoro-2-propanole (HFIP) were purchased from Merck (Darmstadt, Germany). Triethylamine (TEA) and ammonia solution were obtained from Fluka (Steinheim, Germany) and VWR (Darmstadt, Germany), respectively.

Samples and sample preparation

The oligonucleotide resolution standard, RNA, and DNA sample were dissolved in water. The resulting stock solutions were further diluted 1:1 with either water or 2 M NaCl solution to obtain solutions with identical concentrations of oligonucleotide in water and in 1 M NaCl. The oligonucleotide solutions in 1 M NaCl were prepared to mimic anion-exchange purification fractions containing a high salt level.

The following procedure was used for manual desalting of the oligonucleotides in 1 M NaCl solution using Microcon YM-3 centrifugal filter units with a NMWCO of 3 kDa (Millipore, Merck (Darmstadt, Germany)):

- Oligonucleotide solution (200 µL) in 1 M NaCl was transferred to the sample reservoir and centrifuged for 30 minutes at 14,000 g to concentrate the oligonucleotide and elute the salt.
- For washing, 200 µL of water were transferred to the sample reservoir and centrifuged for 30 minutes at 14,000 g.
- The resulting washed concentrate was transferred to a clean vial by placing the sample reservoir upside down in the vial and centrifuging for three minutes at 1,000 g.
- The concentrate was reconstituted to approximately 200 µL with water.

Oligonucleotide resolution standard (p/n 5190-9028):

14 mer: rCrArCrUrGrArArUrArCrCrArArU

17 mer: rUrCrArCrArCrUrGrArArUrArCrCrArArU

20 mer: rUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU

21 mer: rGrUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU

RNA sample (RNA/2'-OMethyl mix; synthesized by Agilent NSAD):
5'-GuGcCaAcCuGaUgCaGcU-3', upper case: RNA, lower case: OMethyl

DNA sample (fully thiolated; synthesized by Agilent NSAD):
5'-ugcaCCCTGGATACCauuu-3', upper case: DNA, lower case: OMethyl

Methods

One-dimensional analysis

Parameter	Value	
Column	AdvanceBio Oligonucleotide, 2.1 × 50 mm, 2.7 µm	
Solvent	A) 400 mM HFIP + 15 mM TEA in water B) Solvent A/methanol (50:50 v:v)	
Gradient	**Oligonucleotide resolution standard:** 0 minutes – 16 %B 7 minutes – 30 %B 13 minutes – 34 %B 14 minutes – 100 %B Stop time: 18 minutes Post time: 3 minutes	**RNA and DNA sample:** 0 minutes – 16 %B 10 minutes – 70 %B 10.5 minutes – 100 %B Stop time: 14.5 minutes Post time: 3 minutes
Flow rate	0.4 mL/min	
Temperature	60 °C	
Detection	260/4 nm, reference 360/100 nm, 20 Hz	
Injection	Injection volume: 5 µL Sample temperature: 10 °C Needle wash: 3 seconds in water:acetonitrile (90:10)	

2D-LC analysis

<table>
<tr><th colspan="3">First dimension</th></tr>
<tr><td>Column</td><td colspan="2">PLRP-S, 2.1 × 50 mm, 3 µm</td></tr>
<tr><td>Solvent</td><td colspan="2">A) 50 mM Ammonium acetate in water, adjusted to pH 7 with ammonia
B) Methanol</td></tr>
<tr><td>Gradient</td><td colspan="2">0 minutes – 2 %B
2 minutes – 2 %B
3 minutes – 70 %B
5 minutes – 70 %B
5.1 minutes – 2 %B
This gradient was repeated three times to minimize carryover.

Stop time: 19 minutes
Post time: Off
Stop time and post time were set in the ^{2}D pump to allow extension of the run time until completion of ^{2}D analysis of all cuts.</td></tr>
<tr><td>Flow rate</td><td colspan="2">0.4 mL/min</td></tr>
<tr><td>Temperature</td><td colspan="2">60 °C</td></tr>
<tr><td>Detection</td><td colspan="2">260/4 nm, reference 360/100 nm, 20 Hz
To protect the flow cell from pressure pulses originating from valve switches, a pressure release kit (G4236-60010) was installed between the ^{1}D DAD and the 2D-LC valve.</td></tr>
<tr><td>Injection</td><td colspan="2">Injection volume: 5 µL
Sample temperature: 10 °C
Needle wash: 3 seconds in water:acetonitrile (90:10)</td></tr>
<tr><th colspan="3">Second dimension</th></tr>
<tr><td>Column</td><td colspan="2">AdvanceBio Oligonucleotide, 2.1 × 50 mm, 2.7 µm</td></tr>
<tr><td>Solvent</td><td colspan="2">A) 400 mM HFIP + 15 mM TEA in water
B) Solvent A/methanol (50:50 v:v)</td></tr>
<tr><td>Temperature</td><td colspan="2">60 °C</td></tr>
<tr><td>Detection</td><td colspan="2">260/4 nm, reference 360/100 nm, 20 Hz</td></tr>
<tr><th colspan="3">2D-LC</th></tr>
<tr><td>2D-LC mode</td><td colspan="2">Heart-cutting</td></tr>
<tr><td>Flow rate</td><td colspan="2">0.4 mL/min</td></tr>
<tr><td>Sampling table</td><td colspan="2">Time-based heart-cutting was set up using the ^{1}D chromatograms as reference chromatograms.
Oligonucleotide resolution standard: 2.79 minutes
RNA sample: 2.88 minutes
DNA sample: 3.04 minutes</td></tr>
<tr><td>ASM</td><td colspan="2">ASM capillary: 5500-1300 (0.12 × 85 mm)
ASM enabled (ASM factor: 5)
Flush sample loop 3.0 times (1.54 minutes)</td></tr>
<tr><td>^{2}D gradient</td><td>Oligonucleotide resolution standard:
0.00 minutes – 10 %B
1.54 minutes – 10 %B
1.55 minutes – 16 %B
8.55 minutes – 30 %B
14.55 minutes – 34 %B
15.55 minutes – 100 %B

^{2}D gradient stop time: 19.55 minutes
^{2}D cycle time: 22.55 minutes</td><td>RNA and DNA sample:
0.00 minutes – 10 %B
1.54 minutes – 10 %B
1.55 minutes – 16 %B
11.55 minutes – 70 %B
12.05 minutes – 100 %B

^{2}D gradient stop time:
16.05 minutes
^{2}D cycle time: 19.05 minutes</td></tr>
</table>

Results and discussion

Figure 1 shows the one-dimensional IP-RPLC analysis of the oligonucleotide resolution standard in water (Figure 1A) and in 1 M NaCl (Figure 1B). Figure 1B shows clearly that the high salt level in the injected oligonucleotide solution impairs the ability of the oligonucleotides to participate in ion pairing, leading to peak splitting and breakthrough, as visible from the large injection peak.

Manual desalting of the oligonucleotide resolution standard in 1 M NaCl using centrifugal filters avoids peak splitting and breakthrough, as seen from the IP-RPLC analysis of the desalted oligonucleotide solution shown in Figure 2. Manual desalting, however, is labor-intensive and, with the employed centrifugal filters, takes approximately 75 minutes. Furthermore, a change in the intensity ratio of the individual oligonucleotides is observed after manual desalting, which can most probably be attributed to partial loss of the smaller oligonucleotides.

Heart-cutting 2D-LC using the 1290 Infinity II 2D-LC System with active solvent modulation enables the direct analysis of oligonucleotides in solution with high salt content. The ^{1}D of the 2D-LC analysis is used for online desalting, followed by IP-RPLC analysis in the ^{2}D.

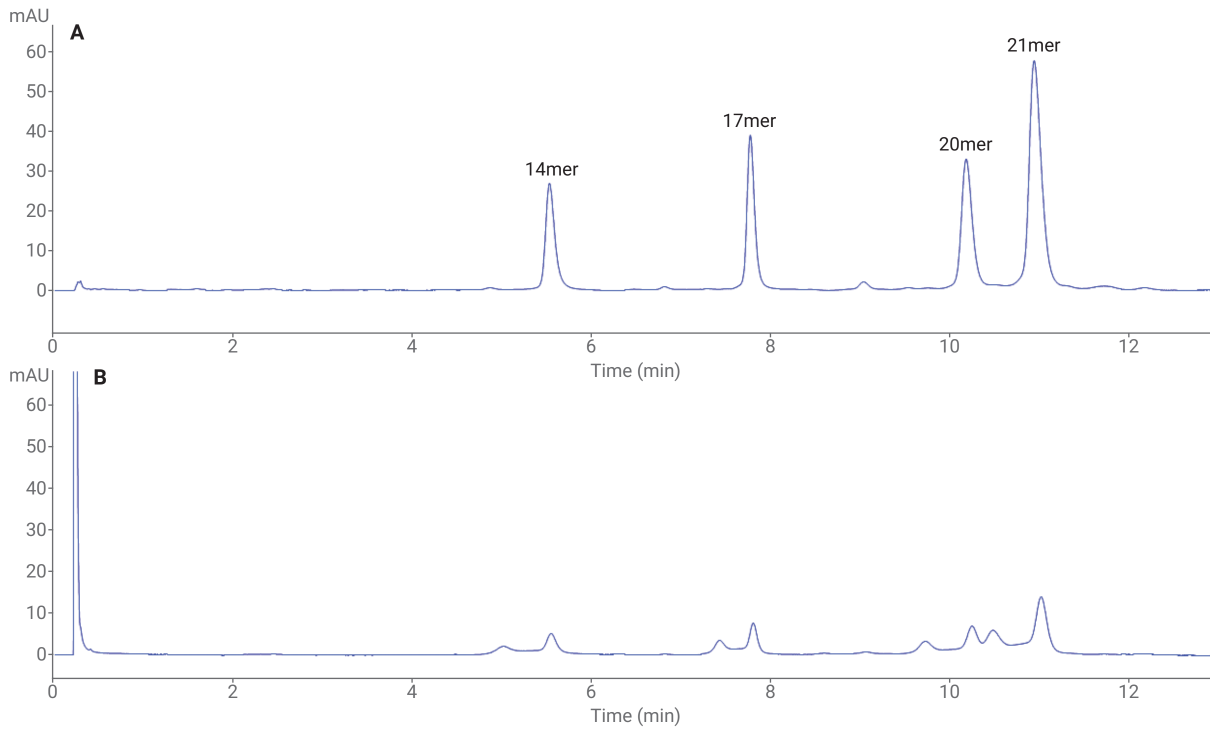

Figure 1. One-dimensional IP-RPLC analysis of the oligonucleotide resolution standard; (A) oligonucleotide resolution standard in water; (B) oligonucleotide resolution standard in 1 M NaCl; chromatograms obtained after blank run subtraction.

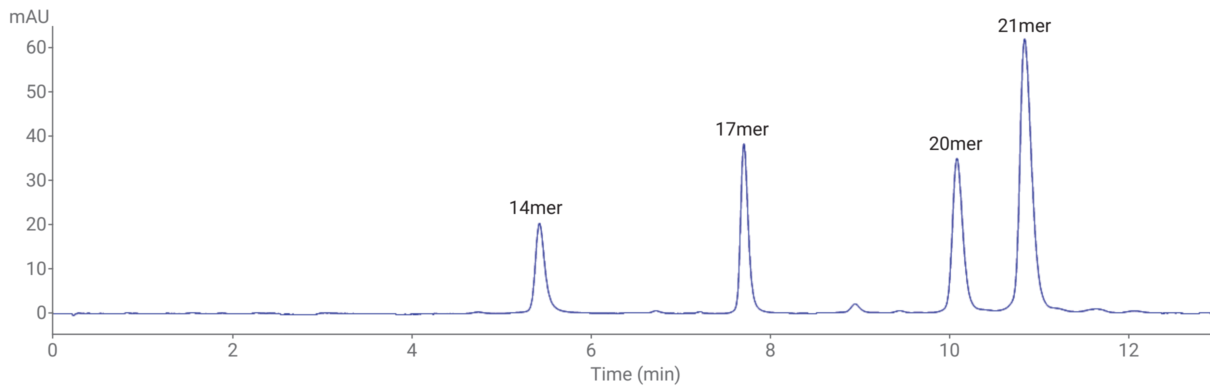

Figure 2. One-dimensional IP-RPLC analysis of the oligonucleotide resolution standard in 1 M NaCl after manual desalting using centrifugal filters; chromatogram obtained after blank run subtraction.

Figures 3 and 4 show the analysis of the oligonucleotide resolution standard in water and in 1 M NaCl, respectively. In the ^{1}D, the oligonucleotides are effectively retained on the PLRP-S column while the salt is eluted to waste. The oligonucleotides are then eluted from the ^{1}D as a single peak and transferred to the ^{2}D IP-RPLC analysis. In the ^{2}D, successful IP-RPLC analysis of the oligonucleotides is achieved. The 2D-LC analysis shows good retention time and area precision in both ^{1}D and ^{2}D separations, as shown in the insets in Figures 3 and 4 (retention time and area precision from six consecutive runs).

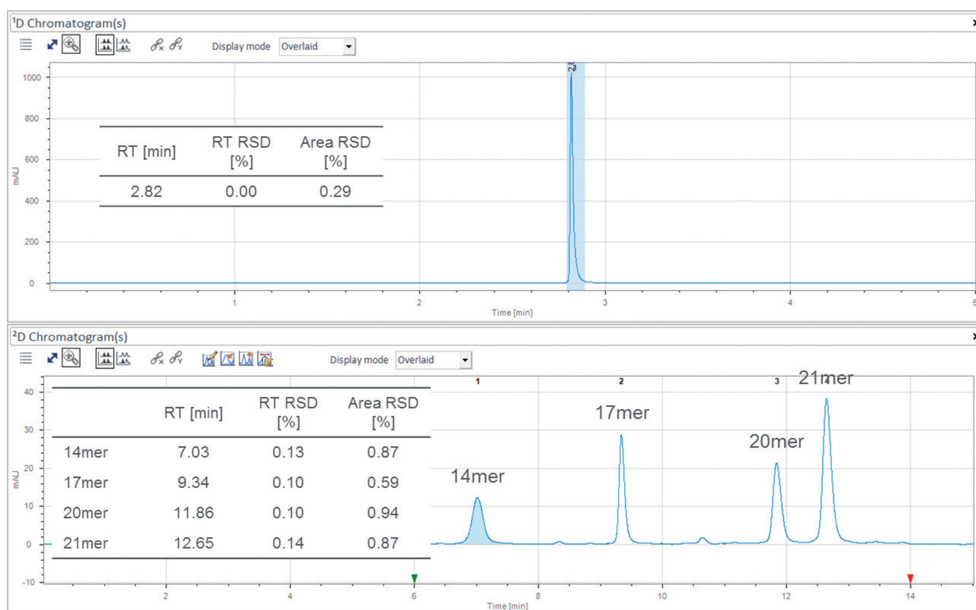

Figure 3. 2D-LC analysis of the oligonucleotide resolution standard in water; A) ^{1}D chromatogram; B) ^{2}D chromatogram; retention time and area precision from six consecutive runs; chromatograms obtained after blank run subtraction.

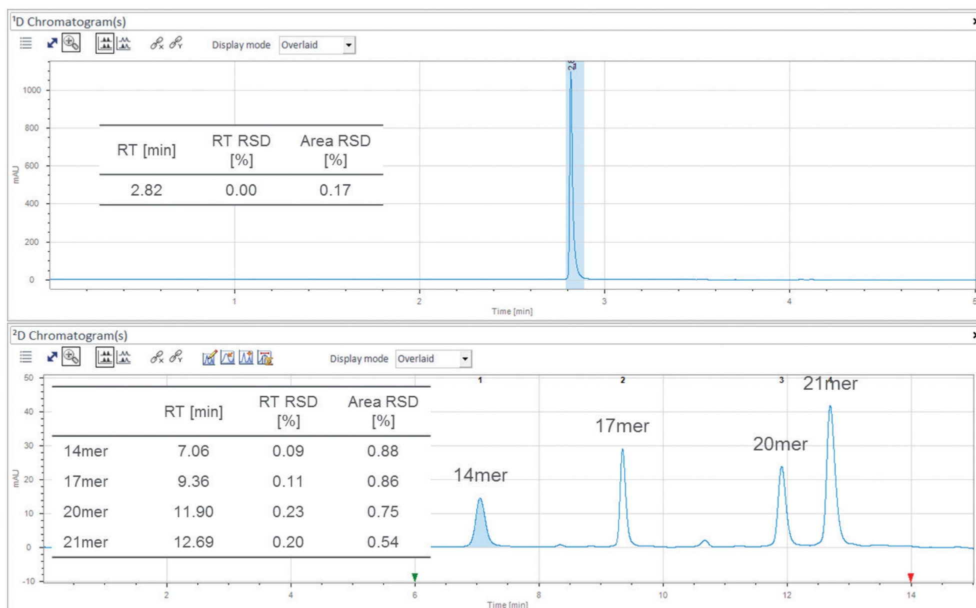

Figure 4. 2D-LC analysis of the oligonucleotide resolution standard in 1 M NaCl; A) ^{1}D chromatogram; B) ^{2}D chromatogram; retention time and area precision from six consecutive runs; chromatograms obtained after blank run subtraction.

Compared to the one-dimensional IP-RPLC method, the 2D-LC method, comprising online desalting in the [1]D and IP-RPLC in the [2]D, takes approximately 4.5 minutes longer, but requires no sample preparation. The manual desalting required for one-dimensional IP-RPLC analysis, however, takes approximately 75 minutes. Considering the required time for sample preparation and analysis of a sample, the 2D-LC method reduces the required time from approximately 96 minutes to 26 minutes for the given protocols. This is equivalent to a more than threefold increase of the workflow speed. In addition, the 2D-LC method eliminates the need for sample preparation, which reduces manual labor.

Figures 5 and 6 show the one-dimensional IP-RPLC analysis of the RNA sample and the DNA sample, respectively. When injecting the RNA sample in 1 M NaCl, the high salt content impairs the ability of the oligonucleotides to participate in ion pairing, as seen from the peak splitting and breakthrough observed in Figure 5B. Manual desalting of the RNA sample in 1 M NaCl enables successful IP-RPLC analysis (Figure 5C). For the DNA sample in 1 M NaCl, the effect of the high salt level of the injected solution is less pronounced, here only a small breakthrough peak is observed (Figure 6B).

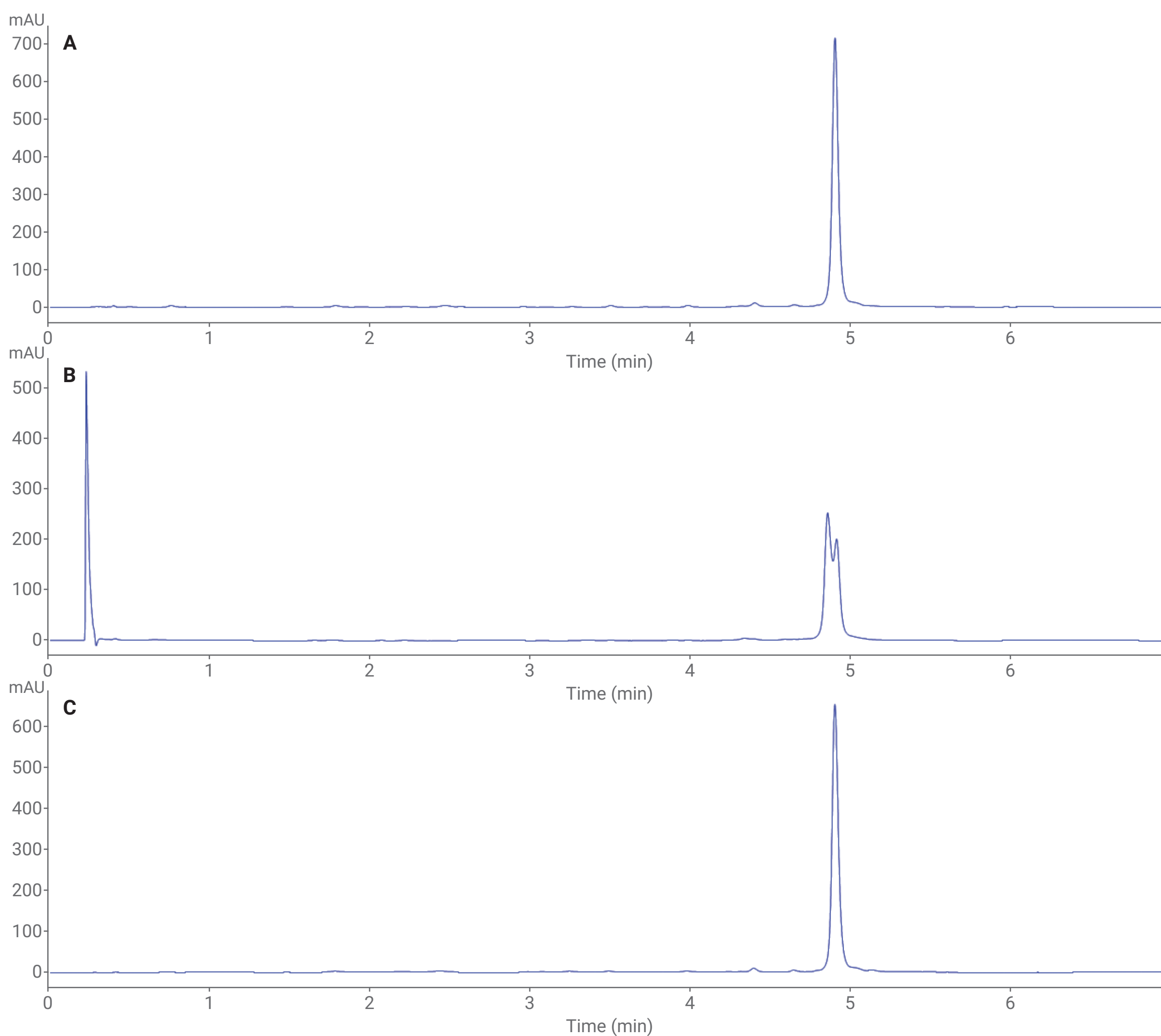

Figure 5. One-dimensional IP-RPLC analysis of the RNA sample; A) RNA sample in water; B) RNA sample in 1 M NaCl; C) RNA sample in 1 M NaCl after manual desalting using centrifugal filters; chromatograms obtained after blank run subtraction.

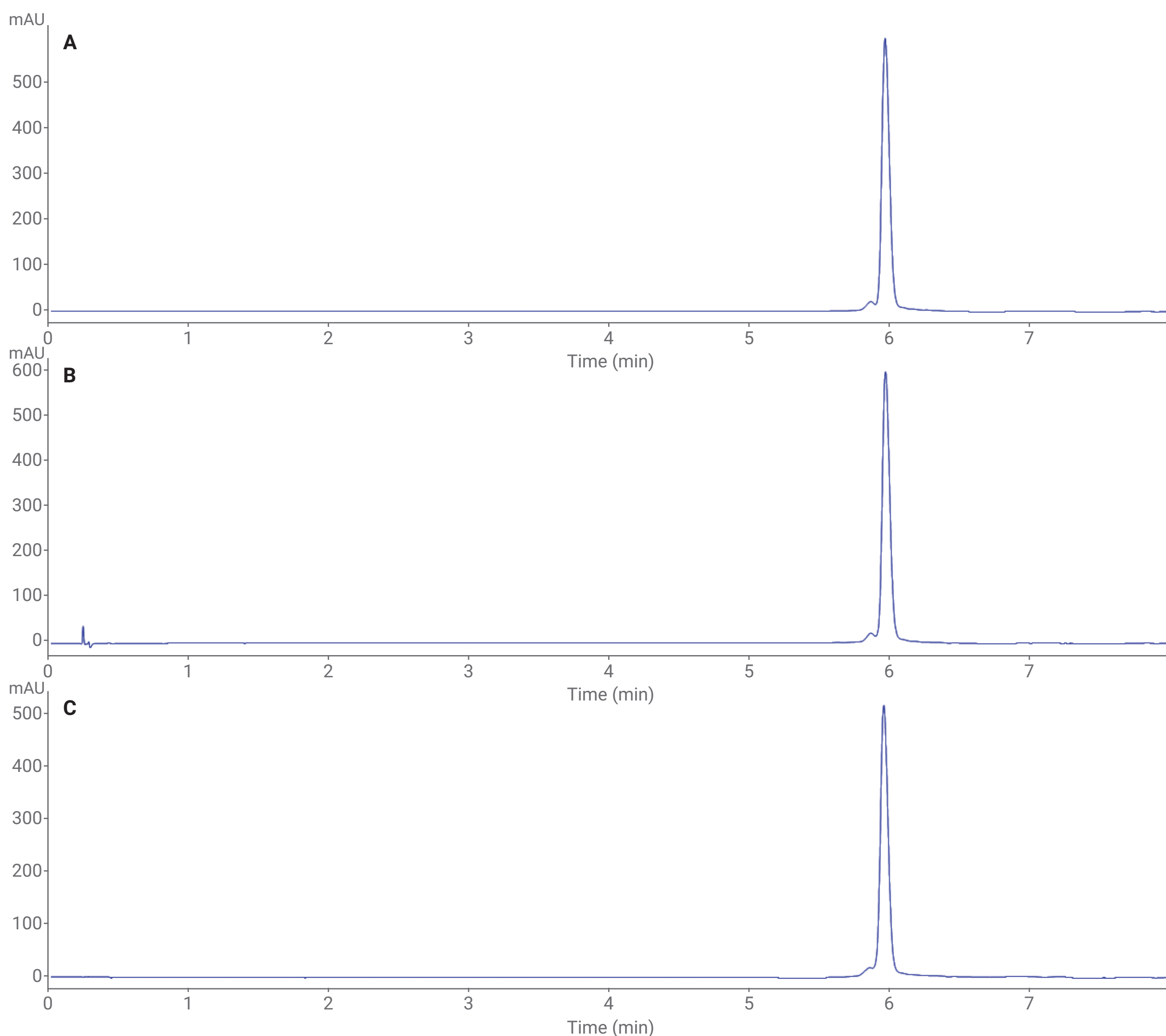

Figure 6. One-dimensional IP-RPLC analysis of the DNA sample; A) DNA sample in water; B) DNA sample in 1 M NaCl; C) DNA sample in 1 M NaCl after manual desalting using centrifugal filters; chromatograms obtained after blank run subtraction.

Figures 7 and 8 show the direct 2D-LC analysis of the RNA sample and the DNA sample in 1 M NaCl, respectively. Effective retention and desalting of the RNA and DNA is achieved in the ^{1}D followed by successful IP-RPLC analysis in the ^{2}D. In the ^{2}D, the separation of several impurities from the main compound is observed, which can be seen in the insets in the ^{2}D chromatograms of Figures 7 and 8, showing a zoom close to the baseline. Based on peak area of the main peak and the detected impurities obtained after 2D-LC analysis of the RNA and the DNA sample in 1 M NaCl, a purity of approximately 96 % can be calculated for both samples. This value is in accordance with the purity that can be calculated in the same manner from the one-dimensional analysis after manual desalting using centrifugal filters.

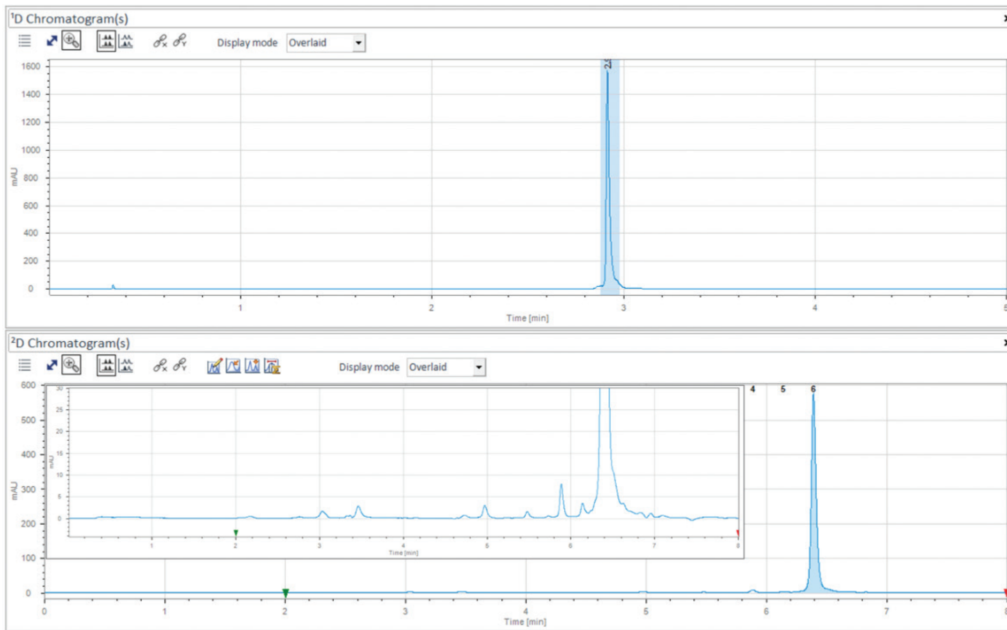

Figure 7. 2D-LC analysis of the RNA sample in 1 M NaCl; A) ¹D chromatogram; B) ²D chromatogram, the insert in the ²D chromatogram shows a zoom close to the baseline; chromatograms obtained after blank run subtraction.

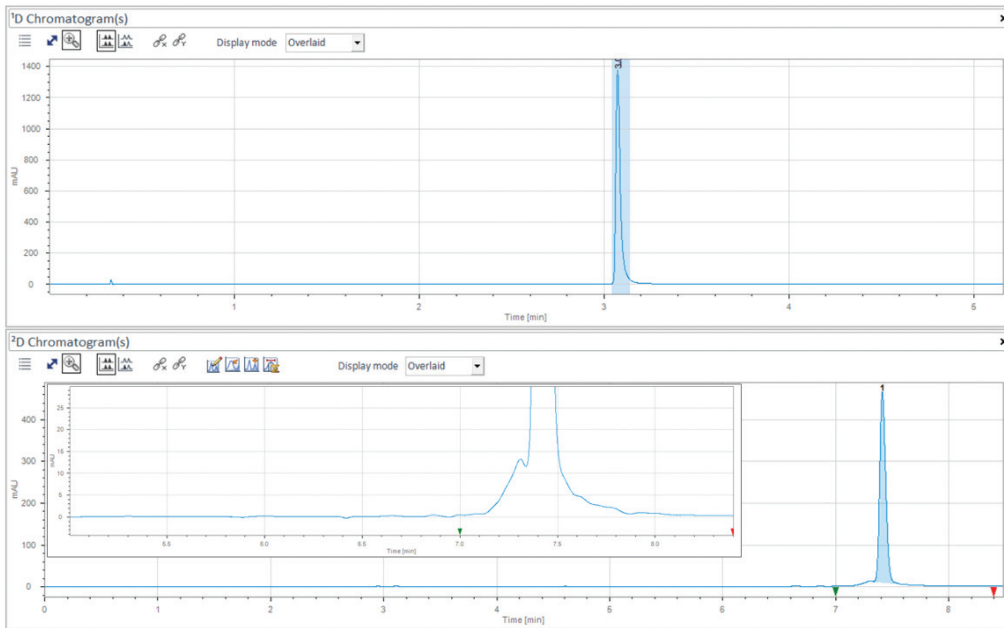

Figure 8. 2D-LC analysis of the DNA sample in 1 M NaCl; A) ¹D chromatogram; B) ²D chromatogram, the insert in the ²D chromatogram shows a zoom close to the baseline; chromatograms obtained after blank run subtraction.

Conclusion

Heart-cutting 2D-LC analysis using the 1290 Infinity II 2D-LC System with active solvent modulation enables the direct analysis of oligonucleotides in solution with high salt content, such as anion-exchange purification fractions obtained following oligonucleotide synthesis. In the ^{1}D, effective online desalting is achieved followed by IP-RPLC analysis in the ^{2}D. Compared to manual desalting using centrifugal filters followed by one-dimensional IP-RPLC analysis, the 2D-LC method enables a more than threefold increase of the workflow speed. In addition, manual labor is reduced since the need for sample preparation is eliminated.

References

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