Application Note

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Superficially Porous Columns for Semi-Preparative Purification of Synthetic Oligonucleotides

Method development and scale-up using same analytical LC instrumentation

Authors

Andrea Angelo P. Tripodi and
Andrew Coffey
Agilent Technologies, Inc.

Abstract

Synthetic oligonucleotides inevitably contain many impurities due to limitations in the efficiency of each chemical reaction used during manufacture. They include deletion sequences (missing one or more nucleotides) and reaction by-products often arising during the final deprotection or cleavage steps. It is often advantageous to be able to use ion pair reversed-phase chromatography for small scale purification (up to a few milligrams) without having to switch to large-scale purification columns and instrumentation. The latest column developments include columns packed with superficially porous particles that provide excellent performance and resolution without compromising column loading. This application note determines the optimum method conditions for the purification of a synthetic oligonucleotide and for scaling up to a 10 mm internal diameter column using the same analytical LC instrument.

Introduction

Several classes of nucleic acids, such as antisense oligonucleotides, small interfering RNAs (siRNAs), and aptamers are being investigated for therapeutic applications. They are formed by a repeating nucleotide unit containing a ribose sugar, a purine or pyrimidine base, and a phosphate linkage.[1] They may also contain a variety of chemical modifications to improve their stability against enzymatic degradation, including 2'-O methoxylation and phosphothiolation.

Before semi-preparative purification, the sample was analyzed by HPLC to understand its impurity profile. The sample was then subjected to various method optimization steps using an analytical column packed with the same stationary phase as contained in the semi-preparative column to simplify the scale-up process.

The column used for this application note uses superficially porous particles. Such particles offer significant advantages, with short diffusion path lengths into the porous outer layer, giving sharper peaks for higher resolution. However, the particle diameter means the operating pressures can be considerably lower without dramatically reducing the overall capacity of the stationary phase.

The work also highlights the ability to use the same analytical LC instrumentation and software for crude analysis, semi-preparative purification, and fraction analysis. Using the same LC instrument, including the heated column compartment, simplifies the scale-up procedure.

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher.

Sample: The sample to be purified was a crude all-2'-O-methylated 22-mer oligonucleotide provided by a customer.

Sample preparation

Different concentrations of RNA sample were diluted in mobile phase A containing 0.1 M TEAA.

Mobile phase preparation

A stock solution of 1 M TEAA was prepared using glacial acetic acid and triethylamine. The pH was adjusted to suit the analysis with either glacial acetic acid (pH 7.0) or triethylamine (pH 8.65).

Equipment and materials

All experiments were carried out on an Agilent 1290 Infinity II analytical system comprising the following modules:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler with sample thermostat (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7117C) with a 10 mm InfinityLab max-light cartridge cell (G7117-60020)
- Agilent 1260 Infinity bio-inert analytical fraction collector (G5664A)

Method conditions

HPLC Conditions	
Column	Analytical columns: AdvanceBio Oligonucleotide 2.7 µm, 4.6 × 150 mm (653950-702) AdvanceBio Oligonucleotide 4 µm, 4.6 × 150 mm (693971-702) Semi-preparative columns: AdvanceBio Oligonucleotide 4 µm, 10 × 50 mm (639750-702) AdvanceBio Oligonucleotide 4 µm, 10 × 150 mm (633750-702)
Mobile Phase	Eluent A) 100 mL 1M TEAA + 900 mL water Eluent B) 100 mL 1M TEAA + 900 mL ACN
Gradient	As described
Flow Rate	As described
Column Temperature	As described
Injection Volume	As described

Results and discussion

To ensure the oligonucleotide will bind to a reversed-phase HPLC column, it is necessary to use a suitable ion pair reagent. Triethylamine acetate (TEAA) is often used; however, a variety of methods are described in the literature. It is important to ensure that the concentration of ion pair reagent is constant during the gradient by preparing the aqueous and organic mobile phase using a stock solution of 1 M TEAA (see Experimental for details). By dissolving 1 mol acetic acid in water, then adding 1 mol triethylamine and allowing it to dissolve completely, the pH of the stock solution could be adjusted as required.

Figure 1 shows the gradient elution of the crude oligonucleotide sample, prepared at a concentration of 10 mg/mL, using the analytical 4.6 × 150 mm column packed with 4.0 µm particles. Separation was carried out using mobile phase pH 7.0 and mobile phase pH 8.65. The gradient used was 5 to 35% B in 30 minutes.

Under closer scrutiny, the separation at pH 8.65 clearly shows better resolution of several impurity peaks, and was chosen as the optimal mobile phase composition.

Elevated temperature is often used for oligonucleotide analysis because it enables inter- and intramolecular secondary interactions to be overcome, leading to sharper peaks. This is illustrated in Figure 2, which compares the separation at 25 °C and 60 °C. At higher temperatures, mobile phase viscosity is also reduced, which can further benefit resolution while lowering operating pressures. AdvanceBio Oligonucleotide columns are suitable for operation at both elevated pH and elevated temperatures, but not all reversed-phase columns are able to tolerate such harsh conditions.

To scale up from analytical 4.6 mm id columns to 10 mm id semi-preparative columns on the same LC instrument, it was decided to perform the separation at lower flow rates to avoid problems with backpressure from the use of analytical capillaries on the LC instrument.

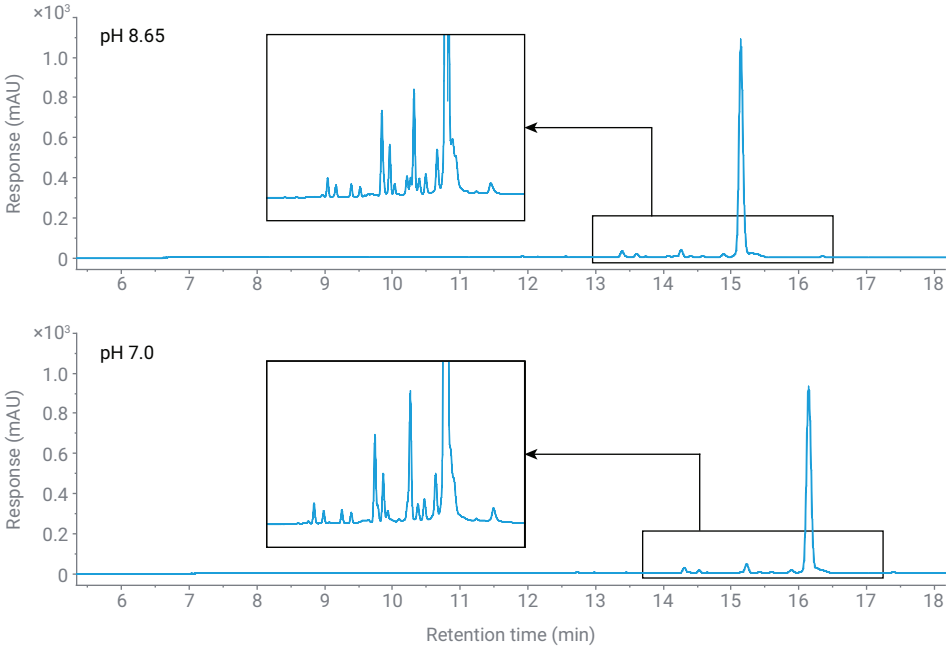


Figure 1. Analytical LC-UV chromatograms (260 nm) showing the pH optimization for the RNA oligonucleotide sample on a 4.6 × 150 mm, 4 µm Agilent AdvanceBio Oligonucleotide column.

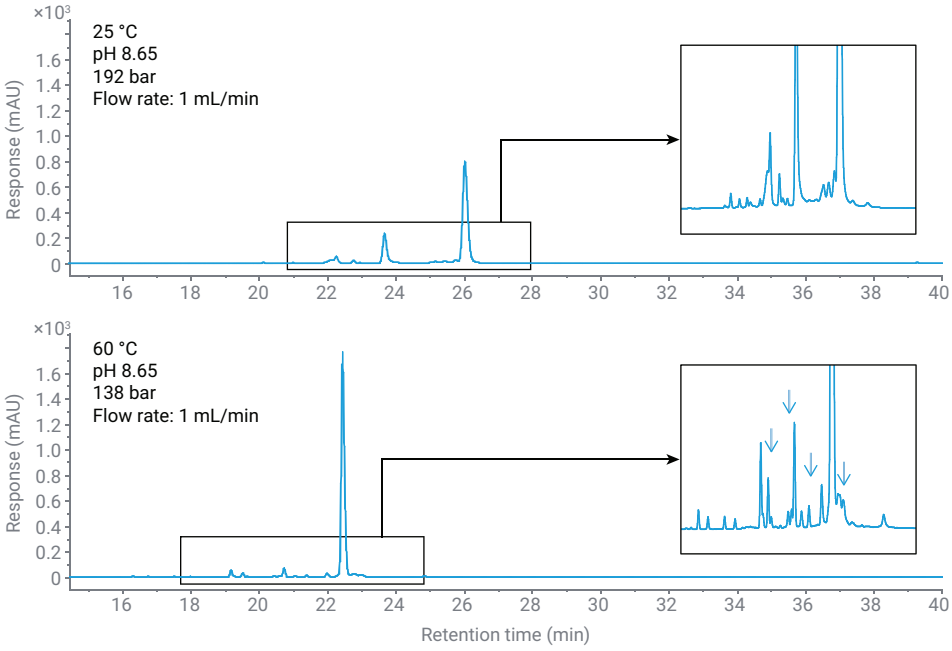


Figure 2. Analytical LC-UV chromatograms (260 nm) showing the temperature optimization for the RNA oligonucleotide sample on a 4.6 × 150 mm, 4 µm Agilent AdvanceBio Oligonucleotide column.

Figure 3 shows the separation performed on the 4.6 mm id analytical column (packed with 4 µm particles) operated at 0.42 mL/min compared to the 10 mm id semi-preparative column operated at 2.0 mL/min (i.e., both columns were operated at the same linear velocity of approximately 150 cm/hr).

Prior to performing the semi-preparative separation on a 10 × 150 mm column, the separation was also conducted on a 10 × 50 mm column. The results shown in Figure 4 clearly indicate a comparable result with the separation being controlled by the gradient profile rather than the column length. The operating pressure revealed that the columns and instrument functioned well

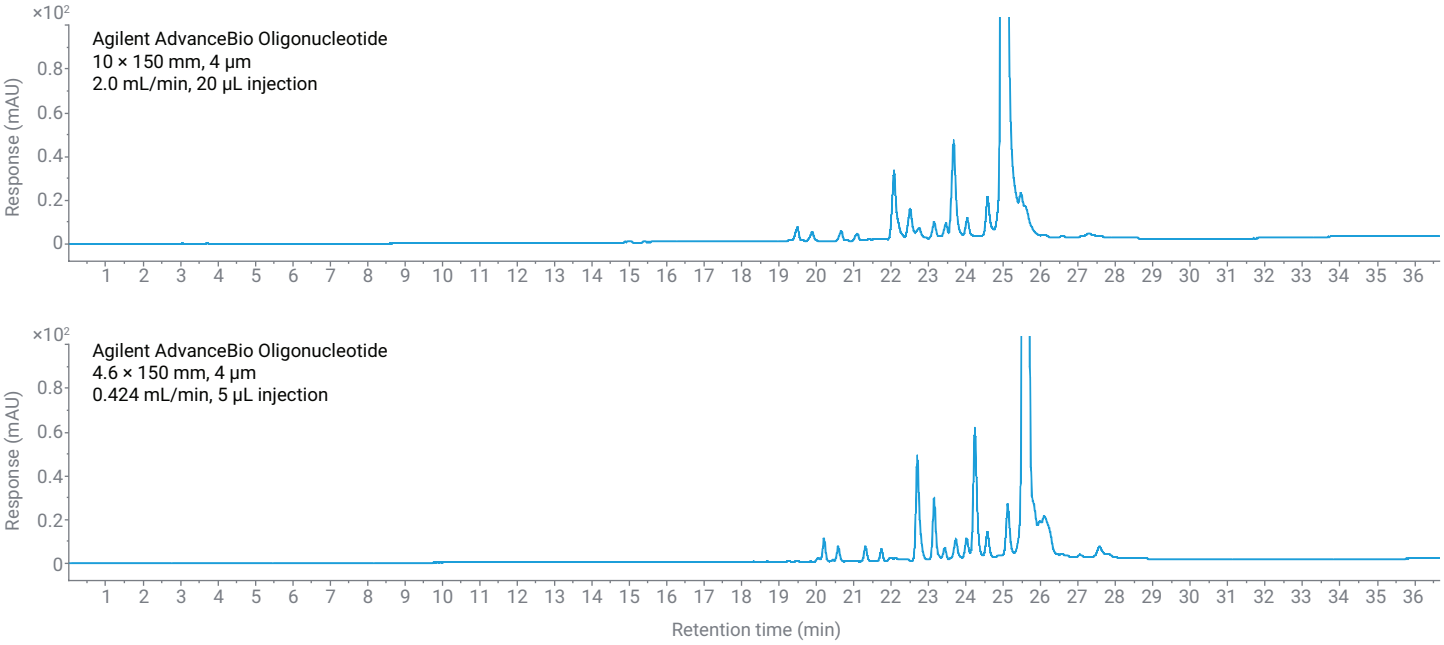


Figure 3. Analytical and semi-preparative LC-UV chromatograms showing the same linear velocity separation for the RNA oligonucleotide sample.

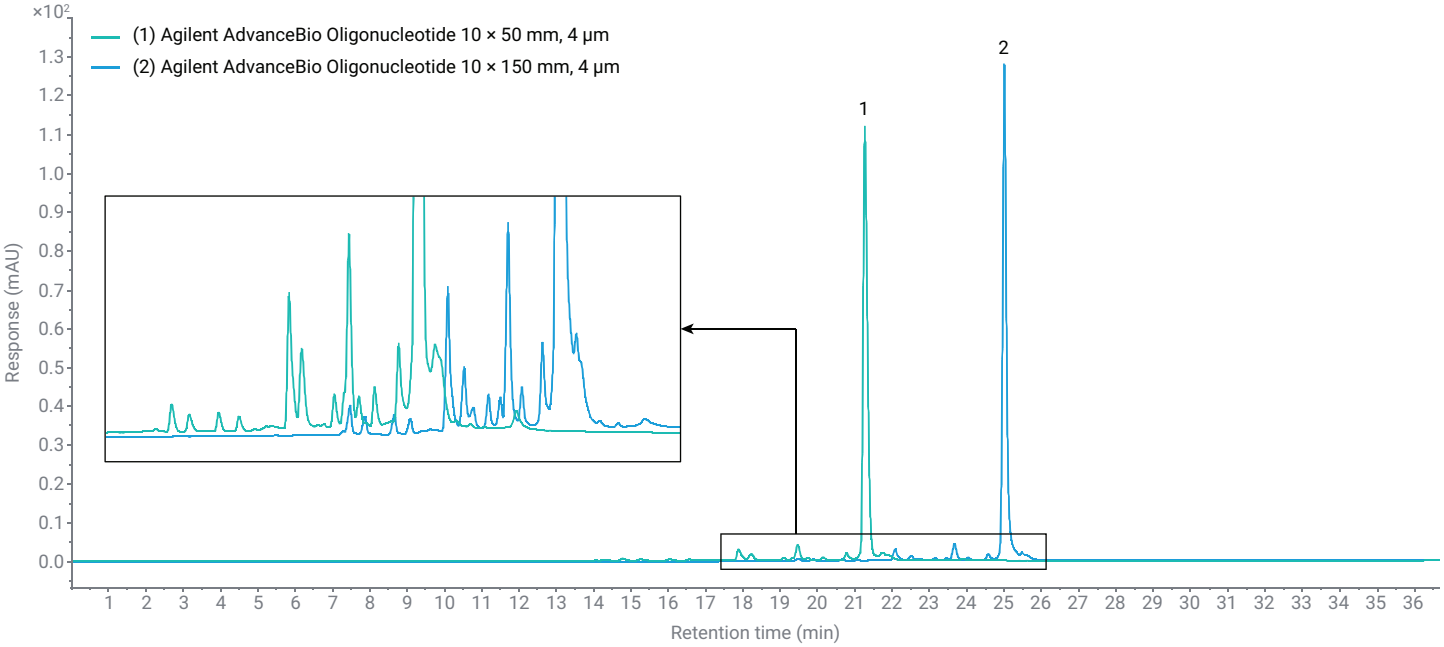


Figure 4. Semi-preparative LC-UV chromatograms (260 nm) comparison showing high reproducibility when using the same gradient profile.

at 2.0 mL/min with only a change to the pre-column heat exchanger. This was changed to a high flow rate version to ensure the mobile phase could be heated to 60 °C without any problems. The longer 150 mm column has an increased capacity and was used for further work.

The default maximum injection volume for this instrument is 20 µL. To inject a larger volume of sample, the standard injection loop was replaced with larger size allowing us to inject 40 µL (per injection). Rather than reconfigure the instrument for larger injections (which would compromise the performance with analytical separations), repeated injections were made onto the column (2 × 40 µL or 4 × 40 µL) before starting the separation gradient. Figure 5 shows the semi-preparative separation together with the time-based fractions collected for subsequent analysis without the need for instrument reconfiguration.

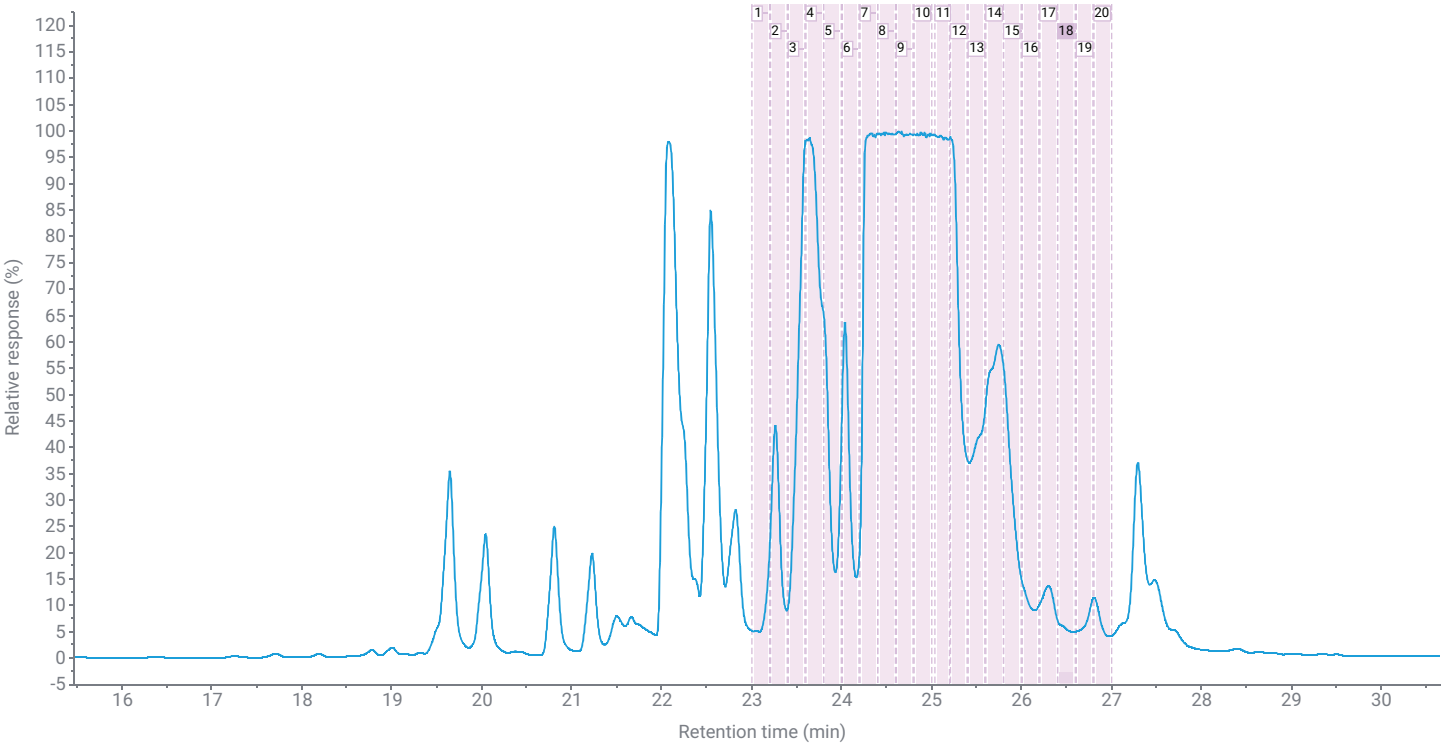
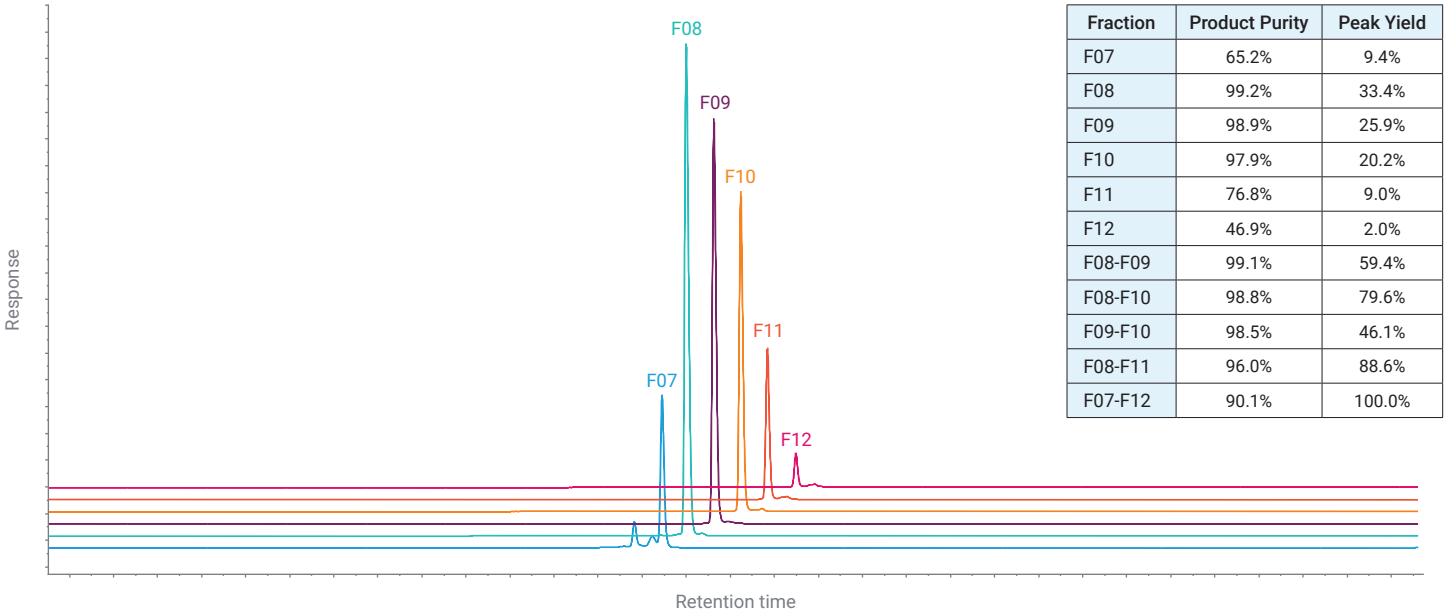


Figure 5. Chromatogram (UV 260 nm) of a 160 µL (3.2 mg on column) injection on the semi-preparative column. Purple bars represent fraction collection of 0.2-minute time slices.

After replacing the semi-preparative column with a 2.7 µm 4.6 × 150 mm analytical column, the individual fractions were analyzed. Figure 6 shows a stacked overlay of the six fractions covering the main peak, together with the yield and purity information illustrating the effect of combining fractions. Figure 7 shows the information in a graphical format. Although the main peak in the semi-preparative chromatogram appears to have the same concentration throughout the fractions, this is because the detector is saturated. The individual fractions show the peak concentration is not uniform across the peak, as would be expected.



Fraction	Product Purity	Peak Yield
F07	65.2%	9.4%
F08	99.2%	33.4%
F09	98.9%	25.9%
F10	97.9%	20.2%
F11	76.8%	9.0%
F12	46.9%	2.0%
F08-F09	99.1%	59.4%
F08-F10	98.8%	79.6%
F09-F10	98.5%	46.1%
F08-F11	96.0%	88.6%
F07-F12	90.1%	100.0%

Figure 6. Chromatogram overlay (UV 260 nm) of the reanalysis of six fractions collected from the fraction collector. The table (inset) indicates the purity and yield of the main peak, including the effect of pooling different fractions.

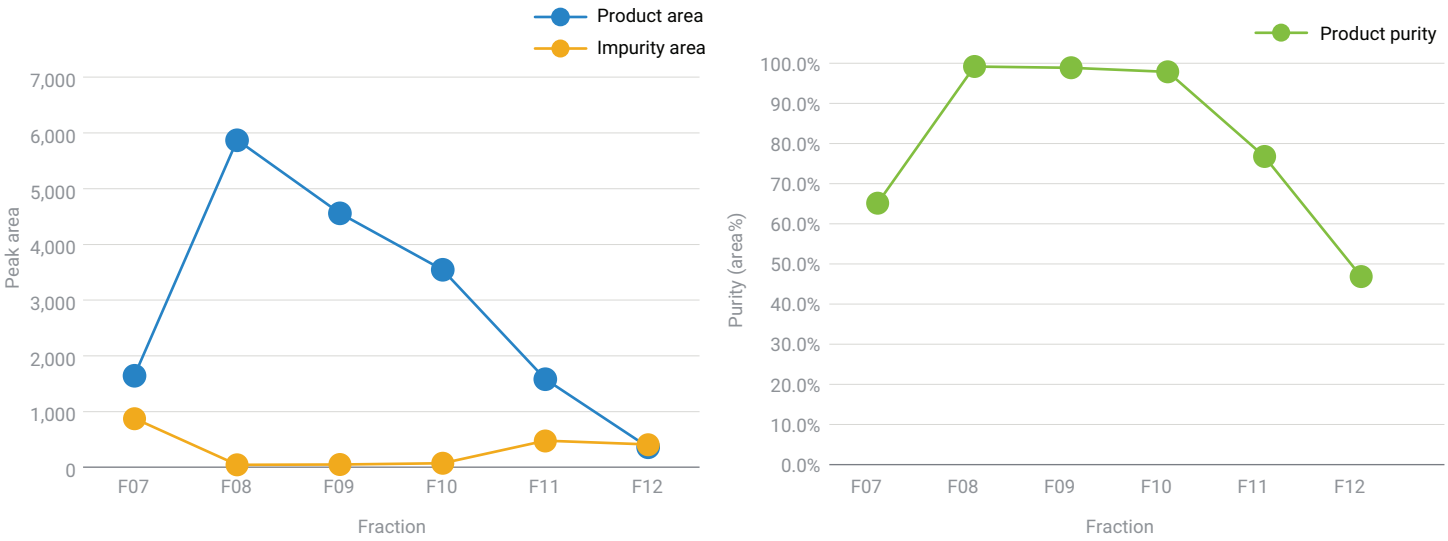


Figure 7. Graphical summary of fraction composition.

Conclusion

This application note demonstrates optimized method conditions for the purification of an RNA sample under ion pair reversed-phase chromatography. Moreover, a fast and easy scale-up method from analytical to semi-preparative scale was successfully achieved using a 10 mm id column, enabling higher sample loading. Finally, the use of the same Agilent 1290 Infinity II Analytical LC System throughout, coupled to an autosampler/fraction collector, allowed collection of the target peak in time slices and re-analysis of the pool of fractions for purity.

Reference

1. Goyon, A.; Fan, Y.; Zhang, K. Chapter 10 - Analysis of Oligonucleotides by Liquid Chromatography. *In* Handbooks in Separation Science, Liquid Chromatography (Third Edition); v2; Fanali, S.; *et al.* ed.; 2023; pp. 357–380.

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