Application Note

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Rapid and Cost-Effective Desalting of HPLC-Purified Oligonucleotides

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Abstract

Oligonucleotide purification by HPLC is a common technique used to dramatically increase purity by removing related impurities that may otherwise interfere with analysis. The most commonly used approaches are anion exchange chromatography or ion-pair reversed-phase (IP-RP) chromatography. Fractions generated from these techniques result in solutions that either contain high salt and buffer concentrations or ion-pair reagents. An extremely effective method of desalting such fractions, either to perform buffer exchange, reduce salt concentration, or to remove ion-pair reagents, is to use a centrifugal gel filtration device such as Agilent AdvanceBio Spin columns. In this application note, we demonstrate the speed and effectiveness of such an approach when compared to other traditional techniques such as ethanol precipitation.

Introduction

We have previously demonstrated the effectiveness of using a superficially porous reversed-phase HPLC column for semipreparative purification of synthetic oligonucleotides.[1] IP-RP chromatography is the leading method for analyzing and purifying oligonucleotides on both small and large scales. One of the main drawbacks when analyzing oligonucleotides using IP-RPLC, especially combined with mass spectrometry (LC/MS), is the need for ion-pair reagents like triethylammonium acetate (TEAA). These ion-pair reagents improve chromatographic retention and separation of oligonucleotides by neutralizing their charge and making them more hydrophobic. However, this process makes coupling with MS rather challenging. It is possible to switch from TEAA to a hydrophobic amine ion-pair reagent with hexafluoroisopropanol (HFIP) in place of acetic acid. However, more recently, ion-pair-free methods have been developed.[2]

Fractions collected from a reversed-phase purification may still contain TEAA, which can still cause problems with MS analysis. TEAA can accumulate in the MS system, leading to contamination, signal suppression, and long cleaning times. Ion-pair reagents can form adducts with oligonucleotides, complicating spectral interpretation and mass accuracy. One of the most commonly used methods of removing the ion-pair reagent prior to analysis is ethanol precipitation. In this process, TEAA ion-pair reagent is exchanged with sodium ions and the oligonucleotide is precipitated with cold ethanol. However, residual TEAA and acetonitrile in the fractions will prevent precipitation and must be first removed by evaporation. This step is rather time consuming and is often done at elevated temperature, which can lead to damage of the purified oligonucleotide as well as significant loss of yield.

A more convenient solution is to use centrifugal gel filtration for desalting and buffer exchange prior to LC/MS analysis. AdvanceBio Spin columns are designed specifically for desalting and cleanup of oligonucleotides and they can be very effective for removing ion-pair reagents, small molecule impurities, and salts. This approach has the advantage of minimizing the time required for sample preparation and data processing when compared with other sample cleanup by ethanol precipitation (see Figure 4).

Experimental

Reagents and chemicals

Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). All other reagents (including ion-pair reagents and buffer salts) were HPLC-grade or higher.

Sample preparation

The sample under analysis is a crude all-2'-O-methylated 22-mer oligonucleotide provided by a customer.

Centrifugal gel filtration was performed using AdvanceBio Spin columns (part number 1980-1105).

Mobile phase preparation

Reversed-phase conditions: Agilent InfinityLab acetonitrile (ACN) gradient grade for LC (part number 5191-5100) was used.

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 or triethylamine to reach pH 8.65.

Size exclusion conditions: Sodium phosphate buffer (150 mM) at pH 7.0, 220 nm wavelength was used to allow detection of both oligonucleotide and TEAA simultaneously.

Ion-pair-free reversed-phase conditions: Ammonium bicarbonate (20 mM) was prepared from high-purity reagents and used without further pH adjustment. Agilent InfinityLab ultrapure LC/MS grade methanol (part number 5191-5111-001) was used. Bio solvent bottles (part number 9301-6028) were used for both solvent A and solvent B during the experiment.

Equipment and materials

Additional equipment needed:

- Centrifuge with swing-out rotor
- Variable volume pipette
- Centrifugal evaporator (for traditional sample preparation comparison)

Instrumentation

Preparative equipment: An Agilent 1290 Infinity II preparative LC system, used for the purification of the sample, was composed of the following modules.

- Agilent 1290 Infinity II preparative binary pump (G7161B)
- Agilent 1260 Infinity II preparative fraction collector (G7157A)
- Agilent 1290 Infinity II preparative column compartment (G7163B)
- Agilent 1260 Infinity II diode array detector (G7165A)

IP-RP analytical equipment: An Agilent 1290 Infinity III LC system was composed of the following modules.

- Agilent 1290 Infinity III high-speed pump (G7120A)
- Agilent Infinity III multisampler with sample thermostat (G7167B)
- Agilent 1290 Infinity III multicolumn thermostat (G7116A)
- Agilent 1290 Infinity III diode array detector (G7165A) with an Agilent InfinityLab Max-Light cartridge cell 10 mm (G7117-60020)

SEC analytical equipment: An Agilent 1260 Infinity III bio-inert LC system with the following configuration was used for the analysis of fractions after SPE.

- Agilent 1260 Infinity III bio-inert pump (G5654A)
- Agilent 1260 Infinity III bio-inert multisampler (G5668A)
- Agilent 1260 Infinity III multicolumn thermostat (G7116A) with bio-inert heat exchanger
- Agilent 1260 Infinity III variable wavelength detector (G7165A) with 10 mm standard flow cell

LC/MS equipment:

- Agilent 1290 Infinity II high-speed pump (G7132A)
- Agilent 1290 Infinity II multisampler (G7167B) with Agilent InfinityLab thermostat
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7115A) with a standard flow cell
- Agilent 6530 quadrupole time-of-flight LC/MS system (G6530C)

Software and data processing

- Agilent OpenLab software suite, version 2.8
- Agilent MassHunter acquisition software for instrument control, version 10
- Agilent MassHunter Qualitative Analysis software, version 10

Method conditions

Table 1. Method conditions.

Parameter	Value
Preparative HPLC Conditions	
Column	Agilent AdvanceBio oligonucleotide, 21.2 × 150 mm, 4 µm (p/n 671150-702)
Mobile Phase	Eluent A: 100 mL TEAA + 900 mL water Eluent B: 100 mL TEAA + 900 mL acetonitrile
Flow Rate	21.2 mL/min
Column Temperature	Room temperature
Injection Volume	500 µL (20 mg/mL)
Total Run Time	60 min
Analytical HPLC Conditions: Fraction Analysis	
Column	Agilent AdvanceBio oligonucleotide, 4.6 × 150 mm, 2.7 µm (p/n 653950-702)
Mobile Phase	Eluent A: 100 mL TEAA + 900 mL water Eluent B: 100 mL TEAA + 900 mL acetonitrile
Flow Rate	1.0 mL/min
Column Temperature	60 °C
Injection Volume	5.0 µL
Total Run Time	30 min
Analytical HPLC Conditions: Ion-Pair Removal	
Column	Agilent AdvanceBio SEC 130 Å, 4.6 × 300 mm, 2.7 µm (p/n PL1580-5350)
Mobile Phase	150 mM sodium phosphate pH 7.0
Flow Rate	0.350 mL/min
Column Temperature	30 °C
Injection Volume	5.0 µL
Total Run Time	15 min
Analytical HPLC Conditions: Ion-Pair-Free Reversed-Phase LC/MS	
Column	Agilent AdvanceBio oligonucleotide, 2.1 × 50 mm, 2.7 µm (p/n 659750-702)
Mobile Phase	Eluent A: 20 mM ammonium bicarbonate Eluent B: methanol
Flow Rate	0.8 mL/min
Column Temperature	75 °C
Injection Volume	5.0 µL
Total Run Time	8 min

Results and discussion

With a simple linear gradient containing 0.1 M TEAA as ion-pair reagent, a crude synthetic modified RNA sample was purified with fractions eluting in a region of approximately 15% ACN (see Table 1) collected in eight fractions of approximately 1.5 mL (Figure 1). IP-RP chromatography was also carried out on the individual fractions to determine which fractions should be pooled (Figure 2). For conditions, please see the "Experimental" section.

From a 500 µL injection of 20 mg/mL crude RNA (approximately 10 mg injected), eight fractions were collected and the seven fractions with highest purity and yield were pooled. The estimated purity of the full-length product is about 75%, with the combined fractions containing approximately 7.5 mg.

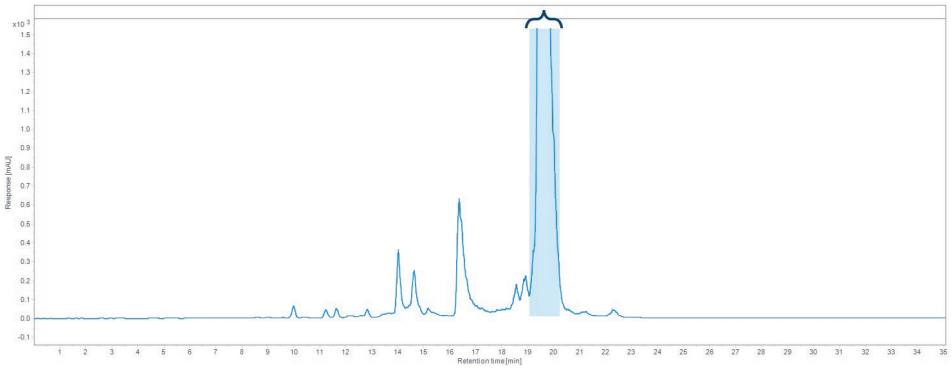

Figure 1. Preparative chromatogram of the crude synthetic oligonucleotide using an Agilent AdvanceBio oligonucleotide 4.0 µm column. The highlighted integration (light blue) corresponds to the full-length product (FLP), with fractions collected at 1.5 mL intervals during the elution of the main peak. A total of eight fractions were collected.

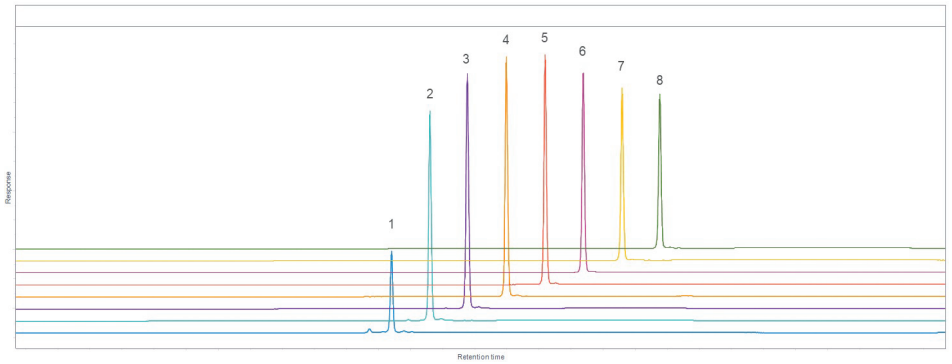

Figure 2. Chromatogram overlay (UV 260 nm) of the reanalysis of eight fractions collected from the fraction collector. The individual fractions show that the peak concentration is not uniform across the peak, as would be expected.

To determine the effectiveness of the sample preparation techniques for TEAA removal, a size exclusion chromatography (SEC) method was used. With detection at 220 nm, both the oligonucleotide and the TEAA could be separated and quantified based on peak area (Figure 3).

Initial attempts at using cold ethanol to precipitate a 0.5 mL sample of the pooled fraction failed. It was therefore necessary to use forced drying to evaporate the excess of water and acetonitrile and repeat the process. The next sample was dried down for 120 minutes with a gentle nitrogen stream at elevated temperature of approximately 40 °C. The sample was then reconstituted in 0.2 mL of 3 M sodium acetate solution and transferred to a 10 mL centrifuge tube, followed by addition of 0.4 mL chilled (–20 °C) ethanol. After thorough mixing, the sample was placed in a freezer for 30 minutes. The solution was placed in a centrifuge and spun at 13,000 rpm for 10 minutes. After the ethanol was carefully decanted, the pellet was re-dispersed in chilled ethanol and the process repeated. Finally, the pellet was rinsed with chilled ethanol and spun once more at 13,000 rpm for 10 minutes. The pellet was allowed to dry in air for 30 minutes. The dried pellet was then dissolved in 200 µL of mobile phase prior to analysis. The small amount of liquid meant that evaporation time was not too long.

Centrifugal gel filtration was carried out by taking an even number of AdvanceBio Spin columns, removing the end caps, and decanting the shipping solvent to waste. Water (1 mL) was added to each tube, and the tubes spun at 1,000 g for 2.5 minutes as per the use instructions. A second wash with water was performed before the columns were placed into collection tubes and 1 mL sample solution added. After centrifugation, the desalted samples were transferred to vials for analysis. The entire process using AdvanceBio Spin columns took a little over 10 minutes and reduced the amount of sample preparation time by 96% (Figure 4).

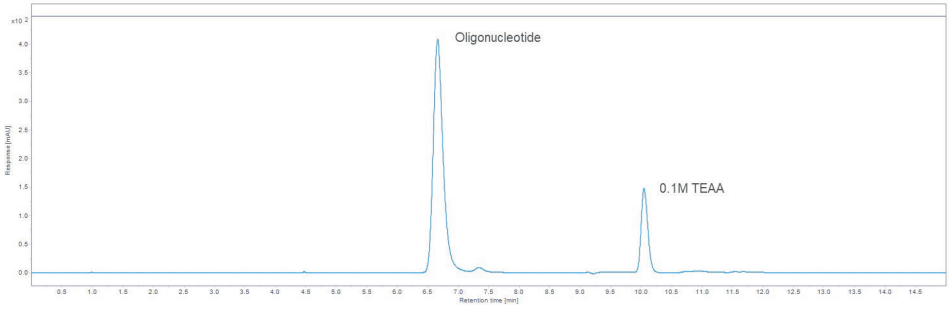

Figure 3. SEC chromatograms of the synthetic oligonucleotide showing the presence of the ion-pair reagent TEAA prior to desalting.

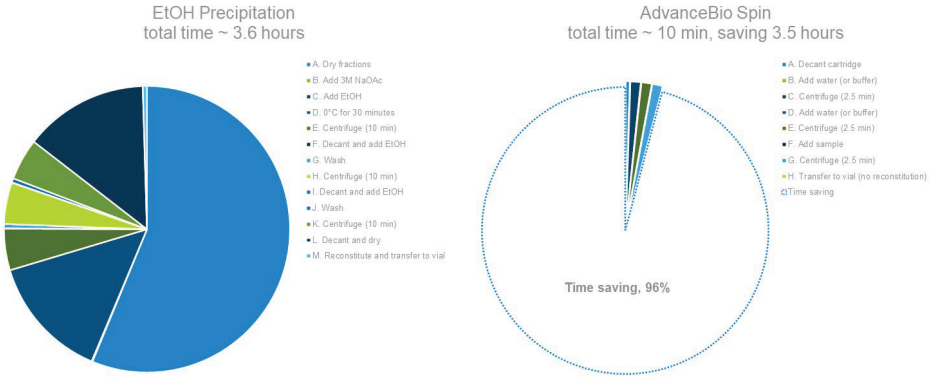

Figure 4. Time comparison: Agilent AdvanceBio Spin versus ethanol precipitation.

Following both desalting cleanups, the recovered solutions were analyzed by SEC and compared to the original solution shown in Figure 3. The solution recovered from the AdvanceBio Spin column cleanup contained a small amount of residual TEAA, but almost 90% had been removed. Ethanol precipitation was very effective at removing TEAA, but there were considerable losses in yield of the oligonucleotide (Table 2).

Although it may be possible to reduce losses during the ethanol precipitation approach (by extending the time taken for precipitation or by increasing the duration of centrifugation), it is also possible to reduce the amount of residual TEAA for the AdvanceBio Spin column approach by passing the solution through a second device with a minimal amount of oligonucleotide loss (Figures 6 and 7).

Using the ion-pair-free reversed-phase approach, the desalted oligonucleotide solution was analysed by LC/MS. As many chromatographers will know only too well, triethylamine is particularly pervasive and can be detected in LC/MS experiments for many days to come, long after the source of the triethylamine has been removed. The desalted oligonucleotide fraction was analysed first and compared to the non-desalted fraction still containing 0.1 M TEAA.

Using the extracted ion chromatogram for 102.1–102.3 *m/z* (the expected *m/z* for protonated triethylamine is 102.19), it is apparent that the triethylammonium ions elute from the reversed-phase column immediately. However, in the case of the non-desalted fraction, triethylammonium ions continue to bleed from the column for an extended period of time (Figure 7), potentially causing ion suppression and loss of signal.

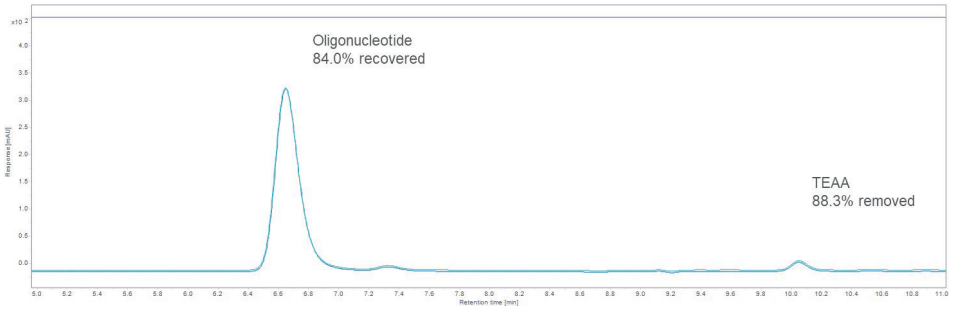

Figure 5. Reduction in TEAA with a single pass through an Agilent AdvanceBio Spin column.

Table 2. Reduction in oligonucleotide yield from ethanol precipitation compared to using an Agilent AdvanceBio Spin column.

	Injection Volume (µL)	Peak Area (mA.s)	Area (µg)	Quantity (µg)	Total Amount (mg)	Recovery (%)
Initial Concentration 7.5 mg/7.5 mL	5	4,641.1	928.2	–	–	–
After AdvanceBio Spin Cleanup	5	3,921.8	–	4.2	–	84.5
After Ethanol Precipitation	0.5	8,086.5	–	8.7	3.5	46.5

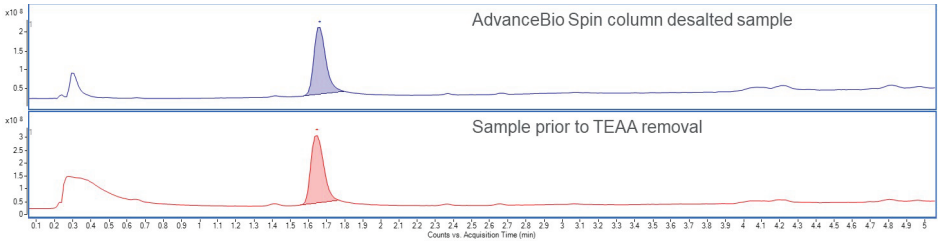

Figure 6. Total ion count chromatogram of RNA sample prior to and after TEAA removal by Agilent AdvanceBio Spin column cleanup.

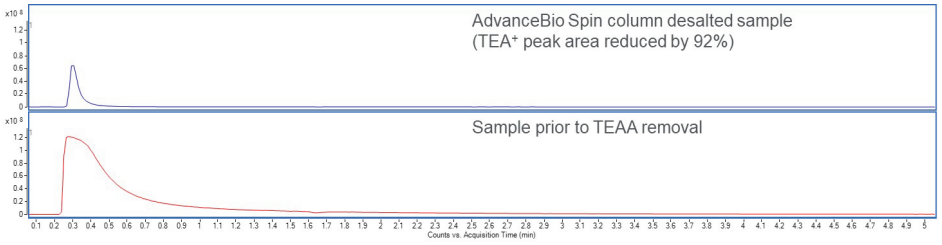

Figure 7. Extracted ion count chromatogram of triethylammonium ions (102.2 *m/z*) before desalting and after second Agilent AdvanceBio Spin column cleanup.

Extraction of the mass spectrum for the peak eluting at ~1.7 minutes gives a series of peaks most notably from $[M+5]^{5+}$ (1467.13 *m/z*) to $[M+10]^{10+}$ (734.09 *m/z*) ion species (Figure 8A). In particular, ions corresponding to triethylammonium ions are absent. The deconvoluted mass spectrum (Figure 8B) clearly shows the accurate mass of the full-length product (FLP) with a range of sodium and potassium adducts present due to either contaminants in the ammonium bicarbonate or ions from glassware.

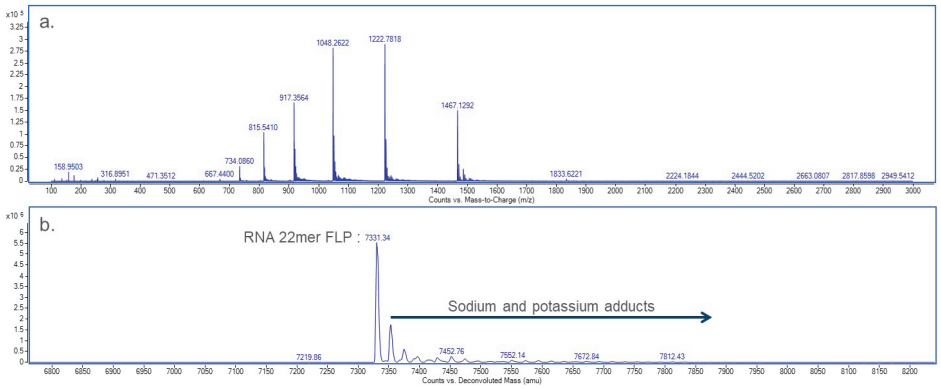

Figure 8. Mass spectrum and deconvoluted mass spectrum of RNA sample after Agilent AdvanceBio Spin column cleanup.

Conclusion

In this application note, we have demonstrated the effectiveness and significant time savings that can be made using Agilent AdvanceBio Spin columns for desalting and buffer exchange of purified oligonucleotide fractions. This ability is particularly important when it is necessary to remove excess ion-pair reagents prior to LC/MS analysis, helping to protect the instrument and reduce unnecessary downtime by eliminating the need for cleaning residual ion-pair reagent from the system.

References

1. Tripodi, A.; Coffey, A. Superficially Porous Columns for Semi-Preparative Purification of Synthetic Oligonucleotides. *Agilent Technologies application note*, publication number 5994-7478EN, **2024**.
2. Bertram, L.; Hsaio, J. Analysis of Oligonucleotides Using an Ion Pairing-Free Reversed Phase Method with TOF LC/MS. *Agilent Technologies application note*, publication number 5994-8013EN, **2024**.

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