Fast and Selective Purification of Oligonucleotides Using Preparative HPLC/MS

Florian Rieck, Ronald Guilliet, Gina Black
Agilent Technologies, Inc.

PREP2022
POSTER #

Agilent
Trusted Answers

Introduction

Synthetic oligonucleotides (ONs) have recently moved into the focus of life science and diagnostics research. ONs with chain lengths of fewer than 100 nucleotides are frequently analyzed using ion pair-reversed phase (IP-RP) HPLC. This technique has also been successfully applied for their purification[1,2]. For higher sensitivity in analyses with mass-selective detection (MSD), triethylamine (TEA) and hexafluoroisopropanol (HFIP) are considered the standard ion-pair reagents. Scaling up methods to preparative conditions, however, requires large amounts of costly HFIP, which can be a limiting factor.

This application presents a preparative purification method using dibutylamine (DBA) for ion-pairing and tris(hydroxy-methyl) aminomethane (TRIS) as buffer. DBA has already been shown to yield higher resolution than TEA with short-chained ONs[3], while TRIS serves to substitute expensive HFIP.

Experimental

Instrumentation

Agilent 1290 Infinity II Autoscale Preparative LC/MSD System

Columns: Agilent InfinityLab Poroshell HPH-C18, 3 × 100 mm, 2.7 µm (p/n 695975-502); Agilent InfinityLab Poroshell HPH-C18, 21.2 × 150 mm, 4 µm (p/n 670150-702)

Software

Agilent OpenLab CDS ChemStation Edition, C.01.10 [287], with Automated Purification Software, A.01.08 [043]

Chemicals and Solvents

Acetonitrile (ACN), DBA, hexylamine (HA), and methanol were HPLC grade. TRIS was >99.9%; hydrochloric acid (37%) and HFIP were analytical grade. Fresh ultrapure water was used.

Samples

Two DNA ON samples of 30 to 50 bases in length were provided by a customer.

Both samples were analyzed using an HPLC/MS separation method (HA/HFIP method, see table 1) to determine the purity and the target masses for purification. To enable an optimized scale-up by the Automated Purification Software, the analytical and preparative methods need to use the same mobile phase. Therefore, another method was applied (DBA/TRIS method, see table 2), which does not require the costly HFIP reagent at the high flow rate used for purification. This second method is not compatible with mass spectrometry, as the TRIS buffer is not volatile. Nevertheless, mass-based fraction collection was used as only a minor part of the flow is transported to the MSD using an active splitter and volatile make-up solvent.

Experimental

Table 1. Chromatography conditions of HA/HFIP method.

Parameter	Analytical Runs
Mobile Phase	A: HA 15 mM + HFIP 200 mM in H2O (pH ca. 8.3) B: Methanol
Flow Rate	0.8 mL/min
Gradient	0 min 50 %B 7 min 71 %B 8 min 100 %B 9 min 100 %B 9.5 min 50 %B
Stop Time	11 min
Injection Volume	2 µL
Sampler Method Preset	Preset 1: Polar sample matrix, 180 µL loop solvent
Temperature	Ambient
UV Detection	260 nm, 10 Hz data rate
MS Detection	negative scan: m/z 500 to 3,000

Table 2. Chromatography conditions of DBA/TRIS method.

Parameter	Analytical Runs	Preparative Runs
Mobile Phase	A) TRIS-HCl, pH 8.3, 75 mM DBA in 7.5% ACN B) TRIS-HCl, pH 8.3, 75 mM DBA in 80% ACN	
Flow Rate	0.8 mL/min	25 mL/min
Gradient	Scouting and focused gradients were calculated by the software	
Injection Volume	2 µL	1000 µL
Sampler Method Preset	Preset 1; 180 µL loop solvent	Preset 1; 800 µL loop solvent
Temperature	Ambient	
UV Detection	260 nm, 10 Hz data rate	
MS Detection	negative scan: m/z 500 to 3,000	negative scan: m/z 500 to 3,000 Target EIC picked by the software
Split Ratio to MSD	Full flow	500:1 (mode M1), active: 12–24 min
Fraction Collection	Not applicable	Peak-based, UV + MSD (AND logic) UV threshold: 10 mAU UV upslope: 2 mAU/s UV downslope: 1 mAU/s MSD threshold: 2,000 cps

Table 3. MSD spray chamber and fraction collection settings.

Parameter	Value
Makeup Solvent	0.1% formic acid in methanol: water (70:30)
Makeup Flow	1.5 mL/min
Ionization Source	Agilent Electrospray (ESI) Source
Nebulizer Pressure	40 psig
Drying Gas Temperature	350 °C
Drying Gas Flow	13.0 L/min
Capillary Voltage	–3,000 V
Scan Range	500–3,000 m/z
Target Mass (m/z)	Short ON: 2,674.7; 2,139.8, Long ON: 2,718.0; 2,265.1
Ion Species	$[M-H]^-$, $[M-2H]^{2-}$, $[M-3H]^{3-}$

Results and Discussion

Analytical Runs and Deconvolution

Both ON samples were separated using the analytical path of the 1290 Infinity II Autoscale Preparative LC/MSD. The optimized gradient from 50 to 71 %B separated the full-length product (FLP) from the incomplete ON fragments. Figure 1 displays UV and MSD signals of the long ON separation. The mass spectra of the FLP were extracted and deconvoluted by the software to provide the molecular mass of the ONs and trigger ions for fraction collection in the preparative runs.

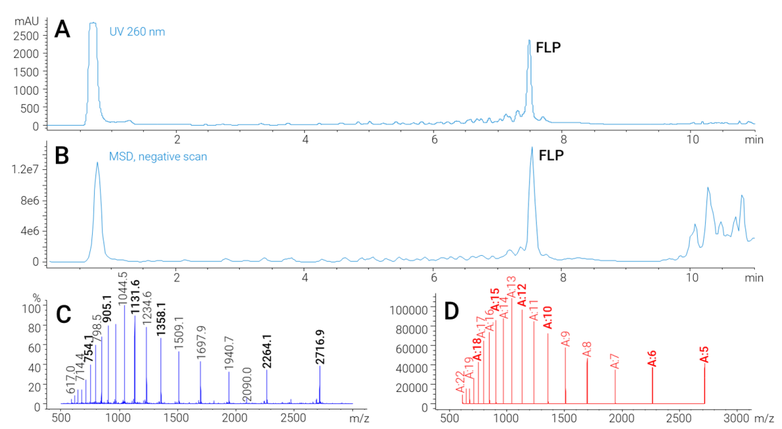

Figure 1. Separation of the long ON, HA/HFIP method. A: UV chromatogram, B: MSD full scan, C: deconvoluted spectrum of FLP, D: multiply charged ions of FLP. Trigger ions in bold font.

Results and Discussion

Purification Runs

To purify the two ONs, the method was changed to DBA/TRIS. Automated Purification Software created a linear gradient for analytical scouting and–based on target peak selection in this analytical run–calculated a focused gradient for purification. With this optimized, shortened gradient, fractions were collected selectively using target masses. The purification of the long ON is shown in figure 2. Purification of the short ON produced similar results. Collected fractions were re-analyzed and showed purities of typically > 99%, see figure 3.

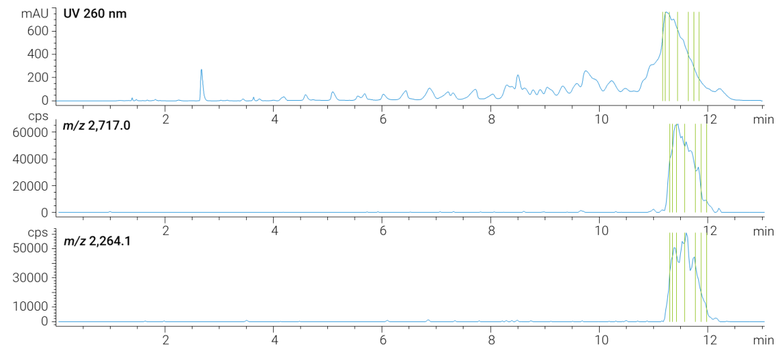

Figure 2. Mass-based purification run of the long ON.

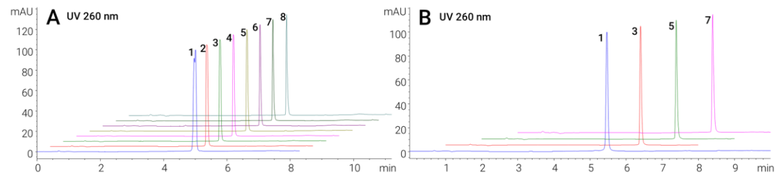

Figure 3. Fraction reanalysis of the short (A) and long ON (B). Fractions are numbered and sorted by increasing retention.

Conclusions

- Two ON samples of 30–50 nucleotides were successfully purified using preparative HPLC with MS trigger
- Analyses by an MS-compatible method yielded the mass spectra required to trigger fraction collection selectively
- Purification was done using DBA/TRIS, avoiding costly HFIP
- Active splitting enabled the use of TRIS with an MSD
- Automated Purification Software turned a generic analytical gradient into an optimized focused gradient, shortened the preparative runs, and collected the pure compounds faster
- InfinityLab Poroshell HPH-C18 columns provided fast and easy scale-up from analytical to preparative applications

Acknowledgments

This work was supported by the Swedish Knowledge Foundation through the KKS SYNERGY project "Improved Methods for Process and Quality Controls using Digital Tools" (grant number 20210021). The authors gratefully acknowledge Olle Stålberg, PhD, of Qiagen DNA Synthesis for supplying the oligonucleotide samples.

References

(1) Catani, M. *et al.* Oligonucleotides: Current Trends and Innovative Applications in the Synthesis, Characterization, and Purification. *Biotechnology Journal* **2020**, *15*, 8.

(2) Purification of Single-Stranded RNA Oligonucleotides Using High-Performance Liquid Chromatography. *Agilent Technologies application note*, publication number 5994-3514EN, **2021**.

(3) Evaluation of Different Ion-Pairing Reagents for LC/UV and LC/MS Analysis of Oligonucleotides. *Agilent Technologies application note*, publication number 5994-2957EN, **2021**.

DE93176643