Application Note

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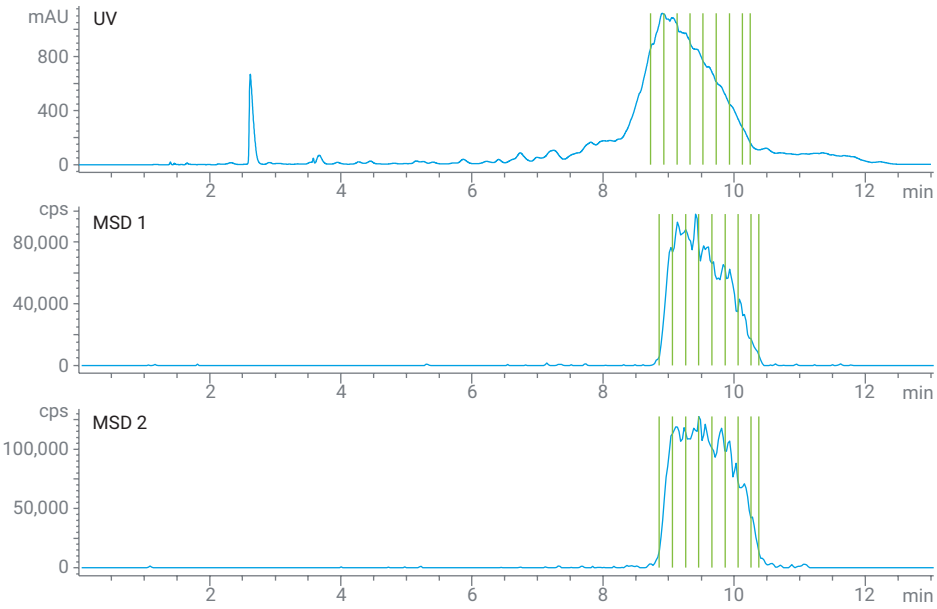
# Fast and Selective Purification of Oligonucleotides Using Preparative HPLC/MS and Software Support

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## Abstract

Synthetic oligonucleotides with fewer than 100 nucleotides are typically analyzed and purified using high-performance liquid chromatography (HPLC). For higher selectivity, mass-selective detection (MSD) is often desired, although this technique requires high-purity, costly reagents such as hexafluoroisopropanol (HFIP). This application note describes a purification method that is based on dibutylamine (DBA) and tris(hydroxymethyl)aminomethane (TRIS), and still allows fraction collection triggered by an MSD signal. With software support and Agilent Poroshell HPH-C18 superficially porous particle columns, the target compounds were collected faster and with higher purity, compared to conventional methods.

## Introduction

In recent years, synthetic oligonucleotides (ONs) such as aptamers, guide RNA, small interfering RNA, and antisense ONs have moved into the focus of life science and diagnostics research. These molecules typically exhibit chain lengths of fewer than 100 nucleotides, and can thus be analyzed using ion pair-reversed phase (IP-RP) HPLC. This technique has also been successfully applied for the purification of ONs.[1] For higher sensitivity in analyses with MSD, triethylamine (TEA) and 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 note presents a preparative purification method using DBA and TRIS for ion-pairing and pH adjustment. DBA has already been shown to yield higher resolution than TEA with short-chained ONs[2], while TRIS serves to substitute expensive HFIP.

## Experimental

### Instrumentation

All experiments were carried out on an Agilent 1290 Infinity II Autoscale Preparative LC/MSD System comprising the following modules:

- Agilent 1290 Infinity II Preparative Binary Pump (G7161B)
- Agilent 1260 Infinity II Quaternary Pump (G7111B) with active seal wash and active inlet valves (options 030 and 032)
- Agilent 1290 Infinity II Preparative Open-Bed Sampler/Collector (G7158B) with 5 mL preparative sample loop (option 241)
- Agilent 1260 Infinity II Multiple Wavelength Detector (G7165A) with 0.3 mm preparative flow cell (option 084)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with 10 mm standard flow cell (option 018)
- Agilent 1260 Infinity II Preparative Valve-Based Fraction Collector (G7166A)
- Agilent 1290 Infinity II MS Flow Modulator (G7170B)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-position/14-port valve (G4738A)
- Agilent 1290 Infinity II Preparative Column Compartment (G7163B)
- Agilent 1260 Infinity II Delay Coil Organizer (G9324A) with knitted delay coils for 15 to 40 mL/min (210)
- Agilent LC/MSD XT (G6135B)

### Columns

- **Analytical column:** Agilent InfinityLab Poroshell HPH-C18, 3 × 100 mm, 2.7 µm (part number 695975-502)
- **Preparative column:** Agilent InfinityLab Poroshell HPH-C18, 21.2 × 150 mm, 4 µm (part number 670150-702)

### Software

- Agilent OpenLab CDS ChemStation edition for LC and LC/MS Systems, Rev. C.01.10 [287] or later versions
- Agilent Automated Purification Software for OpenLab ChemStation, revision A.01.08 [043] or later versions

### Chemicals and solvents

HPLC gradient grade acetonitrile (ACN), tris(hydroxymethyl)aminomethane (TRIS) >99.9%, analytical grade hydrochloric acid (37%), HPLC grade dibutylamine (DBA), and hexylamine (HA) were purchased from VWR (Darmstadt, Germany). Analytical grade hexafluoroisopropanol (HFIP) was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipore Sigma, Darmstadt, Germany).

### Samples

Two DNA ON samples of 30 to 50 bases in length were provided by a customer. Sample concentrations were 3.1 mM in 20 mM PBS for the short ON, and 1.6 mM in water for the long ON. Both samples were injected without dilution or sample preparation.

Before undergoing purification, both samples were analyzed using an HPLC/MS separation method (HA/HFIP method, see Table 1). This method is a typical quality control procedure, yielding the purity of the synthesized product and the target mass to be used 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 and column chemistry. For this purpose, 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 can be used since only a minor part of the flow is transported to the MSD using an active splitter and volatile make-up solvent.

The prime choice for analyses of ONs up to 100 nucleotides is a column of the Agilent AdvanceBio Oligonucleotide series. These columns are based on Agilent InfinityLab Poroshell technology, and feature superficially porous particles (SPP) with High pH (HPH) coating. Along with compatibility with high pH and temperature, every batch of AdvanceBio Oligonucleotide columns undergoes quality control for the separation of oligonucleotides to achieve n/(n – 1) separation.

As the AdvanceBio Oligonucleotide columns were not available in suitable dimensions, all separations in this application note were conducted using InfinityLab Poroshell HPH-C18 columns. Similar to the AdvanceBio oligonucleotide columns, Poroshell HPH-C18 columns are robust in a pH range from 2 to 11 and provide sharp peaks, owing to their SPP. With the addition of columns of 21.2 mm inside diameter to the InfinityLab Poroshell family, high-resolution analytical methods can now be transferred to preparative conditions more easily. Using the same stationary phase on both analytical and preparative scales reduces the need for method adjustments, and increases confidence in the method transfer.

## Method settings

**Table 1.** Chromatographic conditions for analytical runs, HA/HFIP method.

| Parameter | Analytical Runs |
|---|---|
| Mobile Phase | A) Hexylamine 15 mM + HFIP 200 mM in $H_2O$ (pH ca. 8.3)<br>B) Methanol |
| Flow Rate | 0.8 mL/min |
| Gradient | Time (min) %B<br>0 50<br>7 71<br>8 100<br>9 100<br>9.5 50 |
| Stop Time | 11 min |
| Injection Volume | 2 µL |
| Sampler Method Preset | Preset 1: Polar sample matrix<br>180 µL loop solvent |
| Temperature | Ambient |
| UV Detection | 260 nm<br>10 Hz data rate |
| MS Detection | negative scan<br>*m/z* 500 to 3,000 |

**Table 2.** Chromatographic conditions of analytical and preparative runs, DBA-TRIS method.

| Parameter | Analytical Runs | Preparative Runs |
|---|---|---|
| Mobile Phase | A) TRIS-HCl, pH 8.3, 75 mM DBA in 7.5% ACN<br>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 calculated by the software | |
| Injection Volume | 2 µL | 1,000 µL |
| Sampler Method Preset | Preset 1: Polar sample matrix<br>180 µL loop solvent | Preset 1: Polar sample matrix<br>800 µL loop solvent |
| Temperature | Ambient | Ambient |
| UV Detection | 260 nm<br>10 Hz data rate | |
| MS Detection | Negative scan<br>*m/z* 500 to 3,000 | Negative scan<br>*m/z* 500 to 3,000<br>EIC picked by the software based on target mass |
| Split Ratio to MSD | Full flow | 500:1 (mode M1)<br>Active from 12 to 24 min |
| Fraction Collection | Not applicable | Peak-based fraction mode, UV combined with MSD signal by logical AND<br>UV threshold: 10 mAU<br>UV upslope: 2 mAU/s<br>UV downslope: 1 mAU/s<br>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 to 3,000 *m/z* |
| Target Mass (*m/z*) | Short ON: 2,674.7; 2,139.8<br>Long ON: 2,718.0; 2,264.1 |
| Ion Species | $[M-H]^-$, $[M-2H]^{2-}$, $[M-3H]^{3-}$ |

## Results and discussion

Both ON samples were successfully separated on an analytical scale using an HPLC/MS method, and the analytical path of the 1290 Infinity II Autoscale Preparative LC. The optimized gradient from 50 to 71 %B separated the full-length product (FLP) from the incomplete ON fragments. Figure 1 displays a UV chromatogram and the TIC of the short ON separation. The long ON separation is displayed in Figure 2. The long ON appears to be less pure than the short ON. Nevertheless, both samples were separated enough to identify the respective FLP. Based on these analytical separations, the mass spectra at the elution time of the FLP were extracted. The integrated deconvolution tool in OpenLab ChemStation calculated the molecular weight of the FLPs and connected the signals in the spectrum with a charge number (Figures 3 and 4). The identified multiply-charged ions could then be used to trigger fraction collection in the preparative runs.

To purify the two ONs, the method was changed to use mobile phases containing DBA as the ion-pair reagent, and TRIS as buffer. Although TRIS is not MS-compatible, the target ONs were detected and collected by MSD signals in the preparative run. This was achieved by active splitting of the full flow, and delivering the split flow to the MS with a compatible make-up solvent. The flow rate was adjusted to 25 mL/min, reflecting the increased inside diameter and particle size of the preparative column. The gradient was also changed: a linear gradient for analytical scouting was created by the Automated Purification Software. Likewise, a focused preparative gradient for optimized separation was calculated by the software, based on the selection of a target peak in the analytical results.

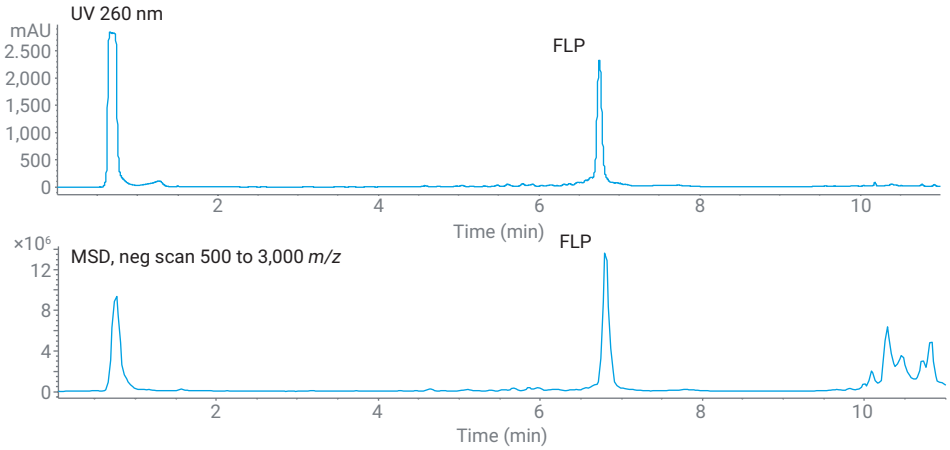


**Figure 1.** Separation of the short ON using HA/HFIP and an optimized gradient.

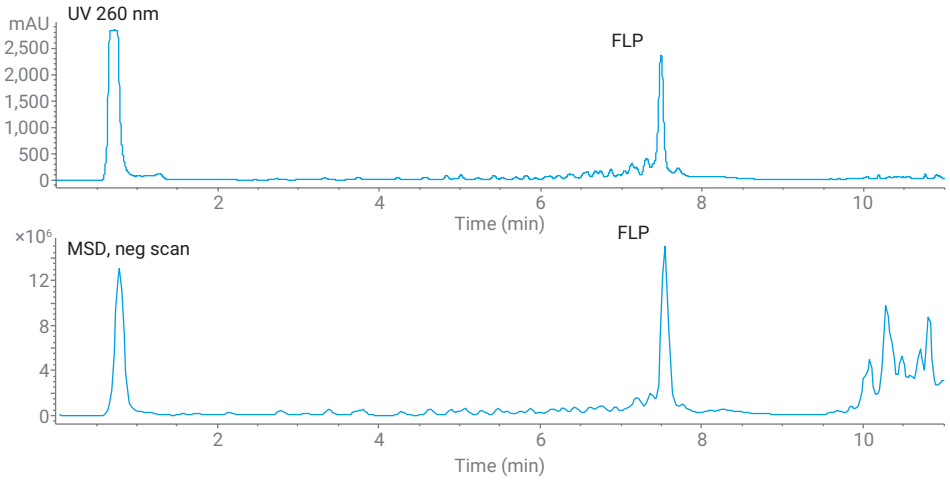


**Figure 2.** Separation of the long ON using HA/HFIP and an optimized gradient.

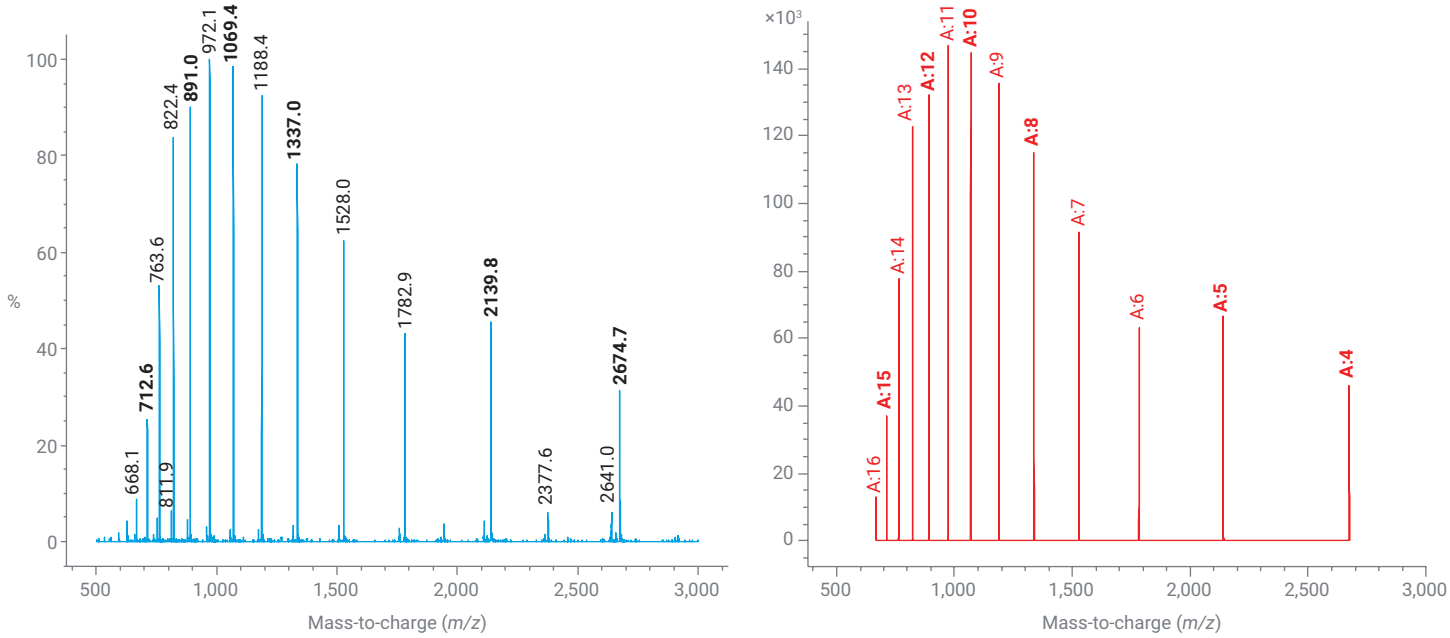


**Figure 3.** Spectrum and multiply-charged ions (A: charge number) of the short ON, calculated by deconvolution. The calculated molecular weight of the FLP was 10,704 Da. Ions used as fraction triggers are highlighted in bold font.

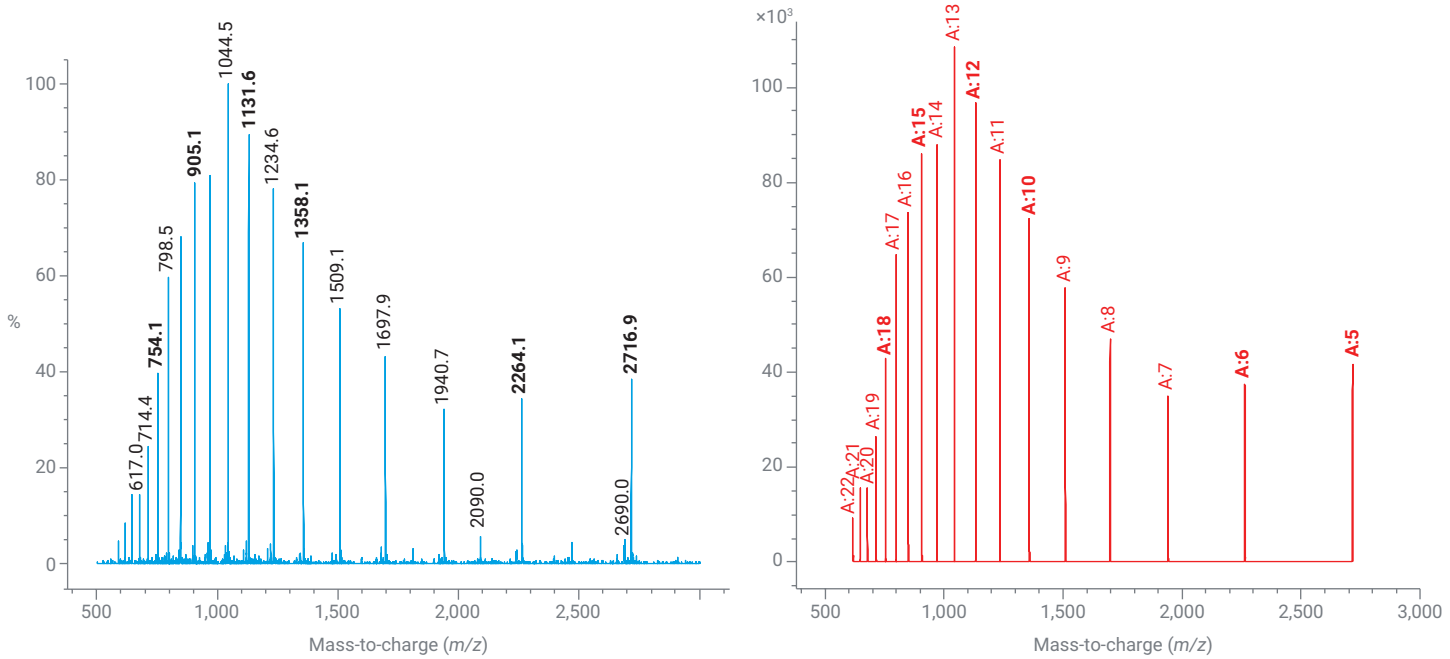


**Figure 4.** Spectrum and multiply charged ions (A: charge number) of the long ON calculated by deconvolution. The calculated molecular weight of the FLP was 13,592 Da. Ions used as fraction triggers are highlighted in bold font.

The analytical chromatograms of the DBA/TRIS separation are depicted in Figures 5 and 6. Note that the aim of the analytical gradient was not to provide the highest resolution, but rather to determine the solvent conditions at the retention time of the target peak. To initiate this calculation, the user selects the peak in the chromatogram and clicks **Assign As Target**. The Automated Purification Software then calculates not only the elution point of the target peak, but generates an entire gradient to optimize the separation of the target. This gradient can be fine-tuned, e.g., to control whether the target should elute in the center of the focused gradient, or rather at the end, to allow more time for early-eluting impurities. The gradient that was applied to purify the short ON is depicted in Figure 7.

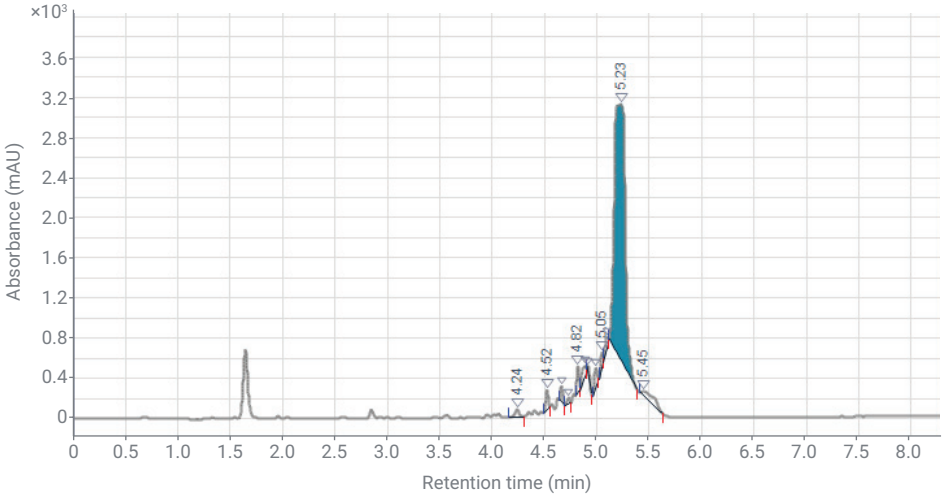


**Figure 5.** Separation of the short ON using DBA/TRIS and a generic gradient. The FLP is highlighted in blue.

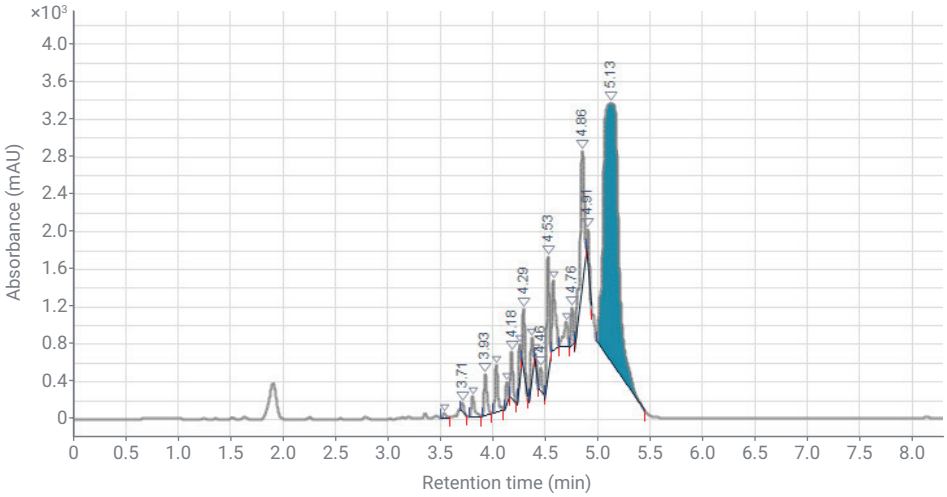


**Figure 6.** Separation of the long ON using DBA/TRIS and a generic gradient. The FLP is highlighted in blue.

Before starting the purification run with the focused gradient, target masses to trigger fraction collection need to be defined. Two target masses were picked for each ON, which represent $[M-4H]^{4-}$ and $[M-5H]^{5-}$ for the short ON, and $[M-5H]^{5-}$ and $[M-6H]^{6-}$ for the long ON (see Figures 3 and 4). By activating the automated trigger on double- and triple-charged target masses, the collection would also be triggered on $[M-8H]^{8-}$, $[M-10H]^{10-}$, $[M-12H]^{12-}$, and $[M-15H]^{15-}$ for the short ON, and $[M-10H]^{10-}$, $[M-12H]^{12-}$, $[M-15H]^{15-}$, and $[M-18H]^{18-}$ for the long ON. Figure 8 shows the preparative separation and fraction collection of the short ON. Eight time slices were collected and successfully triggered by the MSD signal. The overlay with the UV signal (fraction ticks corrected for different delay times) demonstrates the selectivity gain by the MSD. If only the UV signal had been used to trigger fractions, the collection would have started too early, and many of the impurities would have been collected. Triggering based on the MSD signal reduces the number of fractions to be screened, pooled, and dried, which will result in purer fractions and a faster overall purification process. The purification run for the long ON yielded similar results, with 10 collected fraction slices (not shown). By separating the collection into slices of predefined duration or volume, it is possible to re-analyze single fractions, and pool those that contain the product and fulfill purity requirements.[3]

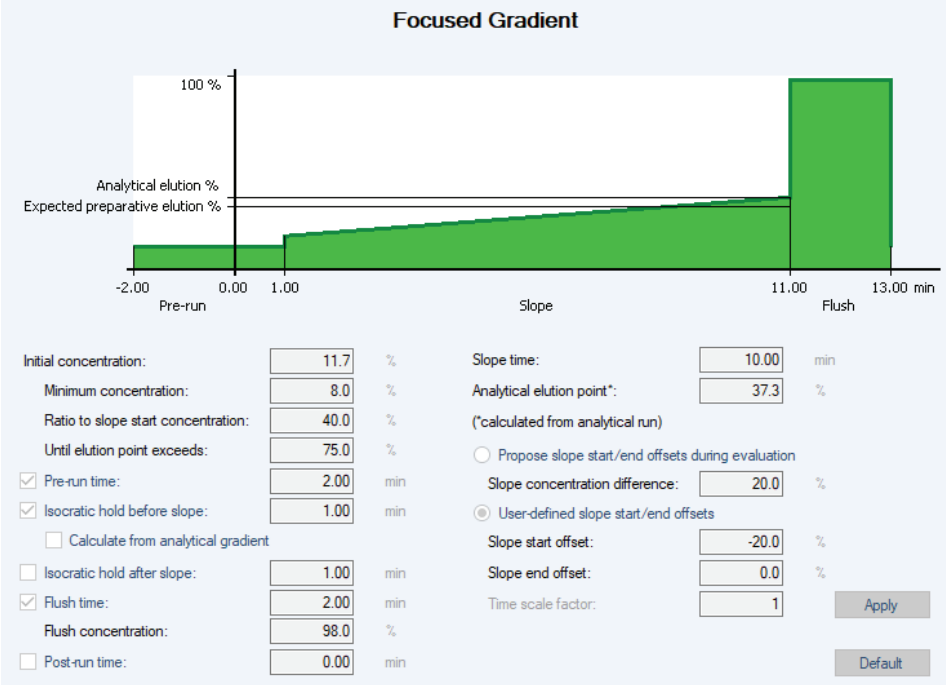


**Figure 7.** Focused gradient profile calculated for the short ON, with options for fine-tuning.

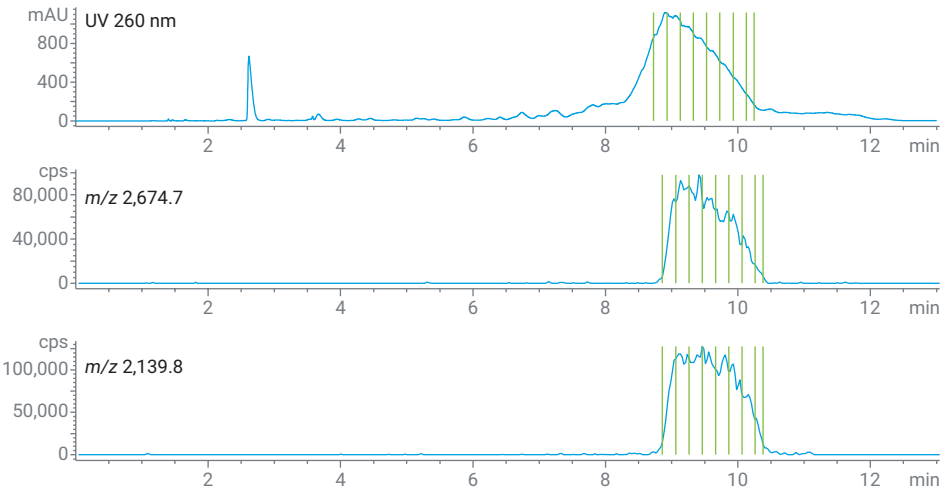


**Figure 8.** Preparative purification run of the short ON, using DBA/TRIS and a focused gradient. Green bars represent time slices of fraction collection.

Fraction re-analysis results of the eight slices collected during the short ON purification run are shown in Figure 9. The first slice (blue trace) contains an impurity, which is visible by the split peak. All other slices are >99% pure, and can be used for the final product pool. The 10 slices collected during the long ON purification run were re-analyzed as well, showing a similar picture: the first slice was partly contaminated with impurities, while the others were >99% pure (not shown). To achieve the highest recovery of the product, more slices with shorter intervals could be collected. If a quantitative re-analysis method is applied, a pooling table listing purity and product content can be created and used to maximize yield for a given purity requirement.[3]

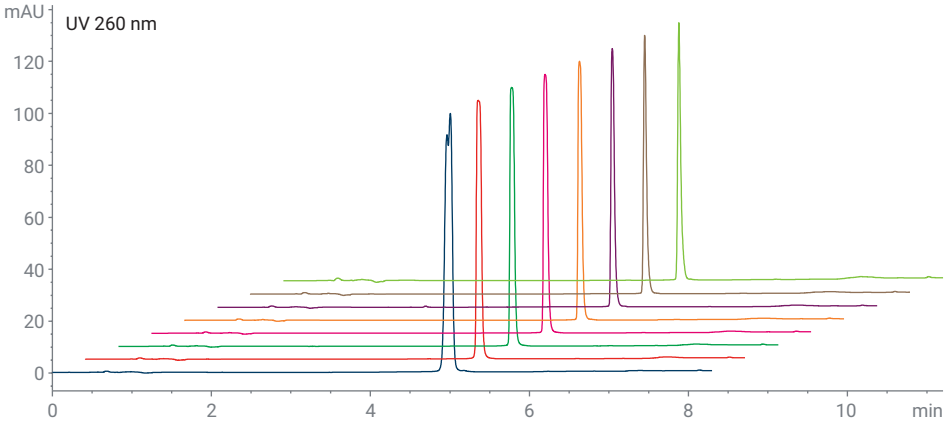


**Figure 9.** Chromatogram overlay of analyses of the eight fractions collected in the short ON purification run.

## Conclusion

Two ON samples of 30 to 50 nucleotides were successfully purified using preparative HPLC with fraction collection triggered by MSD signals. Both samples were analyzed using an MS-compatible method (HA/HFIP), which yielded the mass spectra required to trigger fraction collection selectively. For preparative purification, another method using DBA/TRIS was used, avoiding costly HFIP reagent. Although TRIS is not MS-compatible, a suitable make-up solvent in the split flow to the MSD enabled mass-based fraction collection with high selectivity and confidence. Automated Purification Software turned a generic analytical gradient into an optimized focused gradient to shorten the preparative runs and get the pure compounds faster. Fraction slices of both ONs were successfully collected, allowing re-analysis and selective pooling of fractions that met purity requirements. All runs were conducted using InfinityLab Poroshell HPH-C18 columns, which provide fast and easy scale-up from analytical to preparative applications.

## Acknowledgments

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