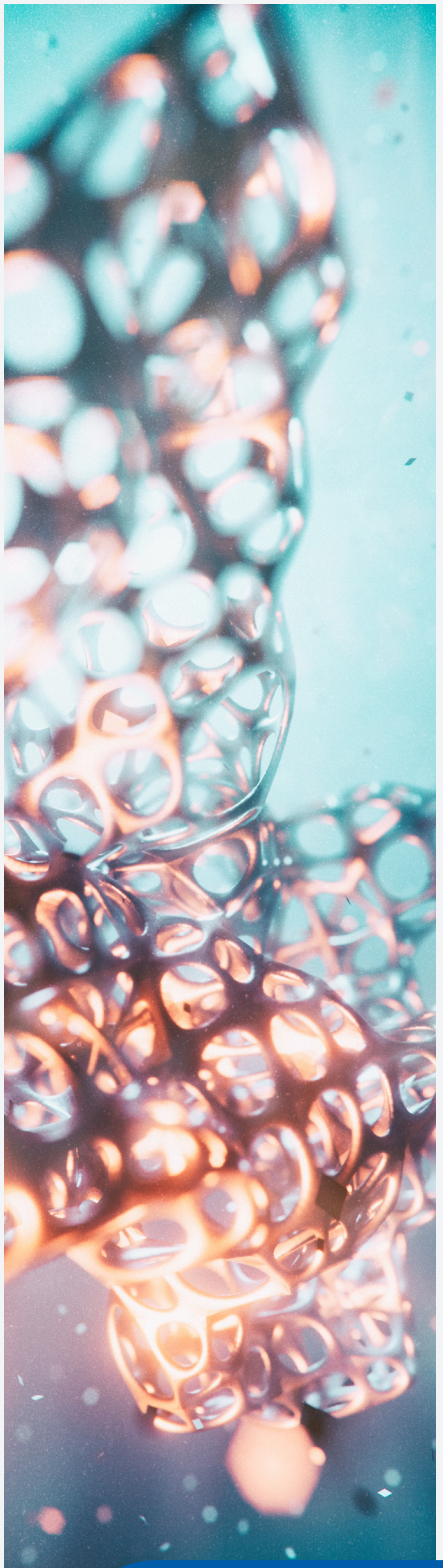
WHITE PAPER

Mobile Phase Optimization for the Analysis of Synthetic Oligonucleotides by Ion-Pair Reversed Phase Liquid Chromatography

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Introduction

Therapeutic oligonucleotides represent a recent breakthrough in the pharmaceutical industry. Improved reliability to synthetic routes coupled with new drug delivery technologies has given a heightened level of interest in these clinically interesting targets. However, characterization of oligos, specifically by ion-pair reversed phase liquid chromatography (IP-RPLC), can be quite challenging. Most oligos are manufactured by solid phase synthesis, where nucleotides are added in a step-wise phase, leading to characterization of the commonly associated *n-1* and *n+1* impurities. However, even with ion-pair to facilitate the analytical separation, for full characterization of process and product related impurities for synthetic oligonucleotides, extensive method development and mobile phase optimization is required.[1]

Further complicating the analysis of oligonucleotides are the modifications to confer stability against nucleases or to improve complementary strand annealing and single mismatch pair recognition. The most critical of oligo modifications is the replacement of the non-bridging oxygen of the phosphodiester backbone with a sulfur group, referred to as a phosphorothioate. This so-called PS chemistry minimizes hydrolysis by endo- and exonucleases, thus improving stability. However, it does lead to an introduction of diastereomers. Unless stereospecificity is controlled, thioation of the oligo yields *2n* isomers where *n* is the number of thioate linkages. Interestingly, this leads to physicochemical changes resulting in differences in efficacy as well as stability depending on either the R or S chirality of anti-sense phosphorothioate oligonucleotides. However, this difference in chemistry often lead to band broadening by IP-RPLC, as the differences in hydrophobicity are insufficient for an appreciable separation.[2]

Further, PS oligos can be oxidized readily and this needs to be characterized for metabolite identification and stability-indicating methods downstream. Oxidations, being inherently more polar, do separate by IP-RPLC but typically only partially, as an earlier eluting pre-peak. With some oligos, such as anti-sense oligonucleotides (ASOs), the oligo is fully thioated, i.e. each phosphodiester bond is fully sulfurized. This leads to many potential oxidations, and the chromatographic separation depends on the number and the position of oxidations. Often, it may be easier to rely on high resolution mass spectrometry to identify oxidized thioates, though spectral overlap from other process related impurities may complicate the deconvolution.

Other modifications worth mentioning are 2' modifications of the ribose, which also confer stability against nuclease degradation. This includes the so-called constrained nucleotides, such as the locked nucleic acid, which confers both high affinity and stability. Ultimately, these modifications lead to some additional hydrophobicity which effect their physicochemical properties, thus their behavior by RPLC.

With suggested identification thresholds at 1.0-1.5 %, optimization of chromatographic separation of oligonucleotides is paramount for characterization of these closely related impurities.[3] With IP-RPLC being a primary method for oligo impurity profiling, method development needs to expand beyond column selection and gradient program, which is the common approach for small molecule impurity assay. Indeed, an undervalued and critical aspect to oligo separations is mobile phase optimization. In this white paper, alkylamine ion-pair and perfluorinated alcohol optimization are shown to be critical method parameters for chromatographic and mass spectrometric performance for characterization of a variety of oligonucleotides.

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Ion-Pairing Agents and Their Role in Oligo Electrospray Desorption of Oligonucleotides

To understand the importance of acidic modifier and alkylamine in driving the separation of oligonucleotides, it is first prudent to discuss the fundamentals of electrospray desorption of oligonucleotides. Indeed, it is the ion-pair and the acidic modifiers themselves that not only drive the chromatographic separation but the electrospray ionization efficiency.

As a review, ion-pairing agents are commonly used to facilitate the retention of otherwise polar, charged compounds. With oligos being negatively charged, ion-pairs commonly used for oligo RPLC are alkylamines, as primary or secondary amines have a positive charge under most LC conditions. Triethylamine (TEA) is a commonly used ion-pairing agent, but as discussed later, there are other options that may have benefits.

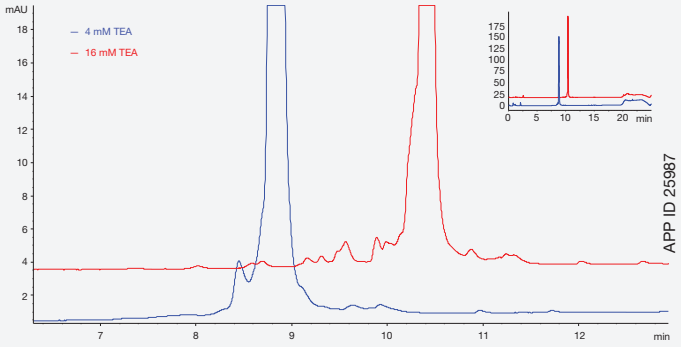
In addition to the alkylamine ion-pair, an acidic modifier is used to buffer mobile phase and to ensure LC conditions are more reasonable for oligos that require high temperature for proper LC separation. Triethylammonium acetate buffers with an acetonitrile gradient is the most common for LC purification of DMT-off (i.e. unprotected) oligos. However, ammonium acetate has been shown to be deleterious to the ionization efficiency of oligos. The seminal work by Gilar and colleagues demonstrated the advantage of using a hexafluoroisopropanol (HFIP) to improve the ion-pairing effectiveness of TEA.[4] Bartlett, et al was the first to formalize the mechanism for electrospray desorption for oligos.[5] HFIP has a boiling point of 58.2 °C and during droplet formation, the pH is lowered with the TEA or other alkylamine donating a proton to water. This facilitates the oligo taking on negative charges at the surface of the droplet as it enters the gas phase.

Considerations for Ion-Pair

Although different types of alkylamine have been explored for improvements in ionization efficiency, the most common ion-pairing reagent used for oligo IP-RPLC methods remains TEA at varying concentrations, though sometimes as high as 16mM based on Gilar's initial work. Indeed, a relatively high concentration of ion-pairing reagent is necessary for optimizing chromatographic performance and separation. **Figure 1** shows a comparison of two different concentrations of TEA (4 mM vs 16 mM) in the mobile phase. Conditions use the same column packing and dimensions, along with relatively low concentration of HFIP. The gradient program was also identical between the two methods, with methanol as the strong solvent. Peak heights and selectivity are improved considerably with the higher concentration of ion-pair.

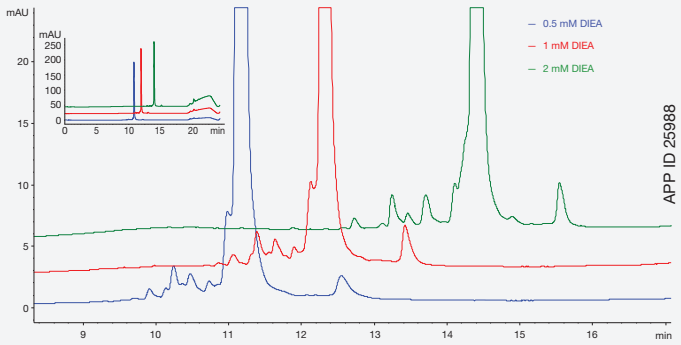
In addition to concentration of alkylamine, the type of ion-pair may also improve chromatographic separation, as using a more hydrophobic alkylamine should provide more retention than TEA. **Figure 2** shows the chromatographic performance when modulating the concentration of N,N-diisopropylethylamine (DIEA), for a bridged nucleic acid. With concentrations as low as 0.5 mM DIEA, chromatographic performance is acceptable. Increasing the concentration of DIEA incrementally shows an improved separation and improved peak shapes for lower level impurities. In any case, the 2 mM concentration would be a much more suitable method if the intent is to maximize chromatographic performance while minimizing concentration of ion-pair.

Figure 1. Comparison of triethylamine concentration on chromatographic performance for a single stranded DNA oligonucleotide. In addition to increased retention, pre-peak separation improves and reveals several earlier eluting impurities.



Column: Biozen® 2.6 µm Oligo
Dimensions: 100 x 2.1 mm
Part No.: 00D-4790-AN
Mobile Phase: A: 12.5 mM HFIP & TEA (as indicated) in Water
B: 12.5 mM HFIP & TEA (as indicated) in Methanol
Flow Rate: 0.3 mL/min
Gradient: 5-30 % B in 14 min
Temperature: 65 °C
Detection: UV @ 260 nm

Figure 2: Comparison of N,N-diisopropylethylamine concentrations, showing not only that increasing alkylamine can improve impurity profile, but as little as 0.5 mM DIEA can yield reasonably good chromatography.



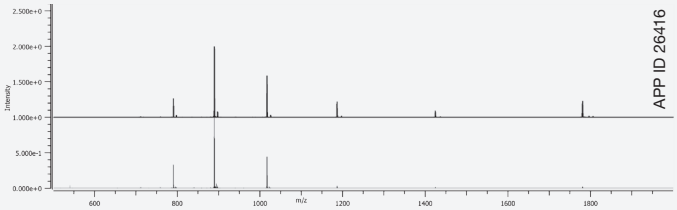
Column: Biozen 2.6 µm Oligo
Dimensions: 100 x 2.1 mm
Part No.: 00D-4790-AN
Mobile Phase: A: 12.5 mM HFIP & DIEA (as indicated) in Water
B: 12.5 mM HFIP & DIEA (as indicated) in Methanol
Flow Rate: 0.3 mL/min
Gradient: 5-30 % B in 14 min
Temperature: 65 °C
Detection: UV @ 260 nm

There are two primary considerations for LC-MS when analyzing oligonucleotides. The first is the impact alkylamine has on charge envelope. As with all biopolymers, when analyzed by LC-MS oligos take on a multiply charge. Oligos commonly take on relatively high charge states as the phosphodiester backbone has the potential for each to take on a negative charge entering the gas phase. As such, charge states as high as -13 for a 20mer can be observed. This may prove somewhat beneficial for Multi-Reaction Monitoring (MRM) methods using nominal mass instrumentation. However, lower charge states can be used for specificity as well as to simplify spectrum. This has been postulated to be due to be the preservation of secondary structures associated to a duplex.[6] Presumably for single stranded oligos, this may be

related to intramolecular interactions which would otherwise inhibit the oligo from taking on too many charges, thus yielding lower charge states and a wider charge state distribution.

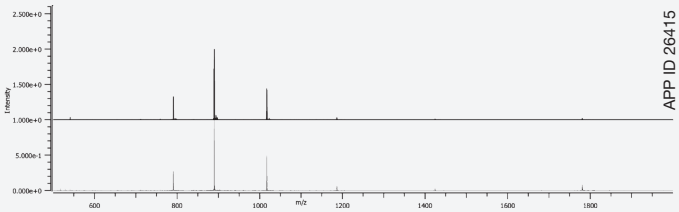
Figure 3 show spectra for a 20mer phosphorothioate, comparing to the charge envelope with TEA and hexylamine as ion-pairs. Axes have been normalized for comparison. Also, note that concentrations compared are not equivalent; 1 mM HA and 4 mM TEA. That said, as shown in **Figure 4**, the concentration of TEA plays little impact in the overall charge envelope; even as high as 16 mM, the charge envelope is still limited to -9 through -7. The more hydrophobic HA yields more of the lower charge states. Again, this could be useful for improving sensitivity and/or specificity of the MS method. As such, if the intent is to modulate charge envelope for improved specificity or simplification of spectra, more hydrophobic alkylamines like HA or octylamine might be advantageous.

Figure 3. Normalized spectra comparing charge envelope with different alkylamine ion-pair - triethylamine (bottom) and hexylamine (top). Hexylamine leads to wider charge state distribution, perhaps due to a minimal effect to any intramolecular interactions to the oligo, thus reducing charge state. The shifting of charge state distribution might be useful for improving method specificity or for simplifying spectra for characterization.



Column: Biozen® 2.6 µm Oligo
Dimensions: 100 x 2.1 mm
Part No.: 00D-4790-AN
Mobile Phase A: 12.5 mM HFIP, 1 mM TEA in Water
12.5 mM HFIP, 4 mM HA in Water
Mobile Phase B: 12.5 mM HFIP, 1 mM TEA in Methanol
12.5 mM HFIP, 4 mM HA in Methanol
Flow Rate: 0.3 mL/min
Gradient: 5-75% B in 14 minutes
Temperature: 65 ˚C
Detection: TOF-MS

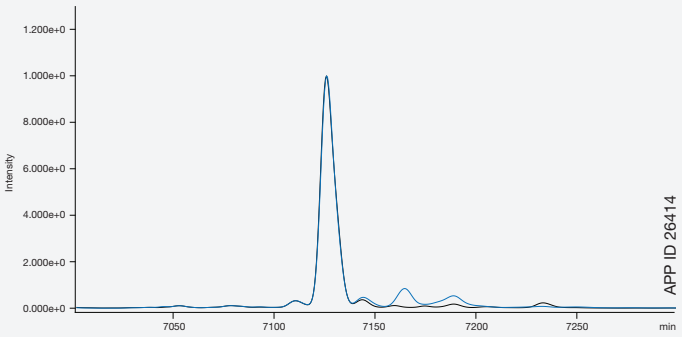
Figure 4. Normalized spectra comparing concentrations of TEA - 16 mM (bottom) and 1 mM (top). Minimal impact to charge state distribution, with only a nominal effect observed to the charge state distribution.



Column: Biozen 2.6 µm Oligo
Dimensions: 100 x 2.1 mm
Part No.: 00D-4790-AN
Mobile Phase A: 12.5 mM HFIP, 16 mM TEA in Water
12.5 mM HFIP, 1 mM TEA in Water
Mobile Phase B: 12.5 mM HFIP, 16 mM TEA in Methanol
12.5 mM HFIP, 1 mM TEA in Methanol
Flow Rate: 0.3 mL/min
Gradient: 5-75 % B in 14 min
Temperature: 65 ˚C
Detection: TOF-MS

The other impact that type of alkylamine can have is on cation adduct formation. Akali metals such as sodium and potassium are attracted to the negatively charged phosphodiester and phosphorothioate backbone of oligos, negatively impacting ESI sensitivity.[7] **Figure 5** shows deconvoluted spectrum comparing two differences in concentrations for TEA, 16 mM vs 1 mM. This example shows differences in adduct formation, with a significant reduction in potassium adduct (7164.6 Da deconvoluted mass) and sodium/potassium adduct (7188.8 Da deconvoluted mass). There are some differences between types of alkylamine adduct formation (data not shown) though it is unclear if this is a result of differences in charge envelope and deconvolution artifacts, or if there is indeed a difference in adduct formation due to the alkylamine used. In either case, it is thought that the alkylamine ion-pair, rather than the sodium cation, preferentially binds to the phosphodiester backbone during the ESI process. Thus, adding more ion-pair, typically 10-16 mM, minimizes the cationic adduction.

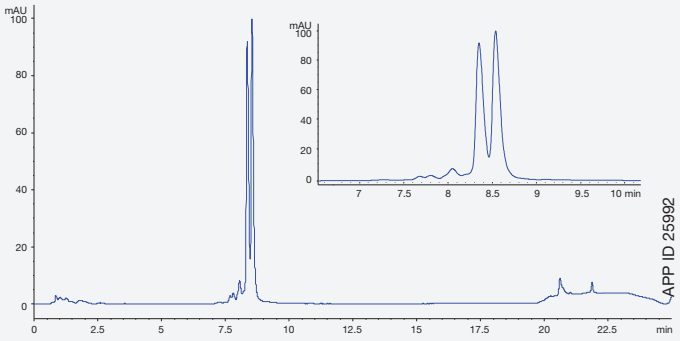
Figure 5. Comparison of deconvoluted spectra for LC-MS analysis of phosphorothioate using different concentrations of ion-pair (Blue - 1 mM TEA, Gray -16 mM TEA). Significant reduction (15x) in cationic adduction of the potassium ion was observed (7164.6 Da deconvoluted mass). A reduction in sodium/potassium adduct (7188.8 Da deconvoluted mass) was also observed.



Column: Biozen 2.6 µm Oligo
Dimensions: 100 x 2.1 mm
Part No.: 00D-4790-AN
Mobile Phase A: TEA + 12.5 mM HFIP in Water
Mobile Phase B: TEA + 12.5 mM HFIP in Methanol
Flow Rate: 0.3 mL/min
Gradient: 25-75% B in 14 minutes
Temperature: 65 ˚C
Detection: TOF-MS

One final note on ion-pair is that some consideration should be made for single stranded oligos and duplex oligos. In general, more hydrophobic alkylamines are optimal for improved chromatography and specificity with ionization (i.e. lower charge states). However, some additional considerations should be made for duplex oligonucleotides. **Figure 6** shows a 21mer double-stranded RNA using TEA as the ion-pairing reagent. The concentration of 12.5 mM HFIP is used and a reasonably shallow gradient of approximately 1 % B per column volume. The relatively low concentration of 4 mM TEA provides sufficient retention of the siRNA, with good resolution of sense and anti-sense strand. Although not baseline, this would be acceptable to obtain high quality spectral data if characterizing each strand separately by high resolution MS.

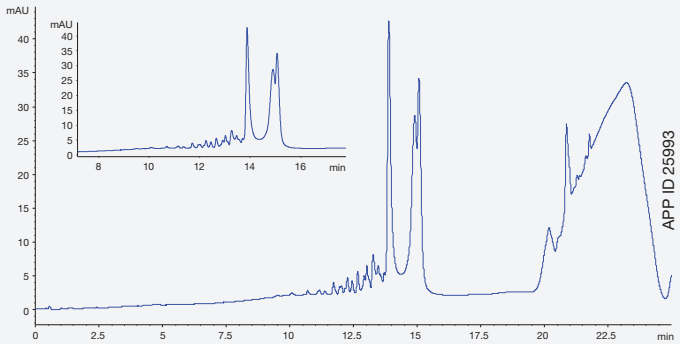
Figure 6. Separation of duplex RNA, with 4 mM triethylamine. Complementary strands are separated sufficiently, as shown in inset, for LC-MS applications to minimize spectral overlap.



Column: Biozen® 2.6 µm Oligo
Dimensions: 100 x 2.1 mm
Part No.: 00D-4790-AN
Mobile Phase A: 12.5 mM HFIP, 4 mM TEA in Water
Mobile Phase B: 12.5 mM HFIP, 4 mM TEA in Methanol
Flow Rate: 0.3 mL/min
Gradient: 5-30% B in 14 minutes
Temperature: 65 ˚C
Detection: UV @ 260

However, as in **Figure 7**, the separation of the duplex RNA with a more hydrophobic ion-pairing agent (DIEA) yields errant results with the duplex RNA. Although it might appear that the separation is improved because of the many earlier eluting impurities, this result was not repeatable. Further, more hydrophobic ion-pairing agents yielded worse chromatography, similar to the analysis of a duplex oligo when one does not run at sufficiently high temperature exceeding melting temperature (data not shown). As such, using triethylamine modulating concentration and optimizing gradient program is likely the more prudent approach for double stranded nucleic acids.

Figure 7. Duplex RNA separated using DIEA; running conditions are identical to **Figure 6**. Although there may be some separation of earlier eluting impurities, the separation injection to injection is not consistent. This may indicate that that these are not impurities but aberrations in chromatography due to the nucleic acids maintaining their secondary structure.

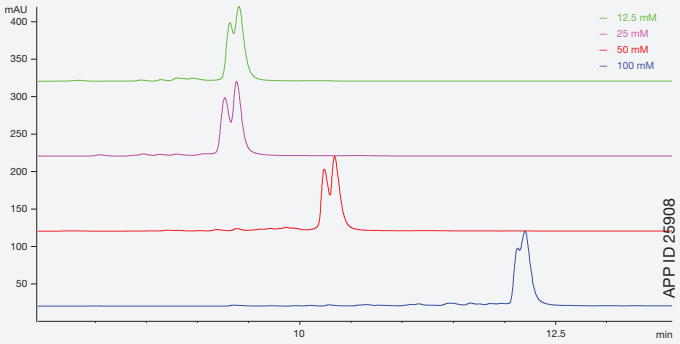


Column: Biozen 2.6 µm Oligo
Dimensions: 100 x 2.1 mm
Part No.: 00D-4790-AN
Mobile Phase A: 12.5 mM HFIP, 2 mM TEA in Water
Mobile Phase B: 12.5 mM HFIP, 2 mM TEA in Methanol
Flow Rate: 0.3 mL/min
Gradient: 5-30 % B in 14 min
Temperature: 55 ˚C
Detection: UV @ 260

Hexafluoroisopropanol Concentration Effect on Chromatography and ESI Efficiency

Although there has been extensive work on alkylamine ion-pair and their effect on LC-MS separation and sensitivity, there has not been as much focus on HFIP as the acidic modifier, nor has there been many published data on other perfluorinated alcohols. Basiri and colleagues has demonstrated the use of hexafluoro-2-methyl-2-propanol (HFMIP), with some benefits with the use of more hydrophobic alkylamines.[6] Otherwise, there has been minimal work exploring how HFIP concentration can impact LC separation and MS sensitivity. As mentioned previously, HFIP is used as an acidic modifier for oligonucleotide LC-MS analysis and facilitates ionization leading to a better signal in the mass. However, the use of relatively high concentrations (100-400 mM or 1-4 %) were mainly to improve the ion-pair effectiveness.[8] Because of the minimal solubility of alkylamines in HFIP and other perfluorinated alcohols, alkylamines more effectively facilitate the hydrophobic retention of polar oligos. With modern particles being more hydrophobic than first generation pH-stable silica, the need for 400 mM HFIP (essentially the highest concentration soluble in aqueous buffers) may not be necessary. Further, as increases in ion-pair typically result in superior chromatography and spectral data, decreases in HFIP will allow for higher concentrations and/or different types of alkylamine. Nonetheless, with HFIP critical for ESI droplet desorption, a minimum amount is still required. However, mobile phase composition with less than 100 mM is not common practice.

Figure 8 siRNA separating using different concentrations of HFIP (Blue - 100 mM, Red - 50 mM, Magenta - 25 mM, Green - 12.5 mM). Note the decrease in retention time as HFIP is decreased, though both 12.5 and 25 mM have similar retention.

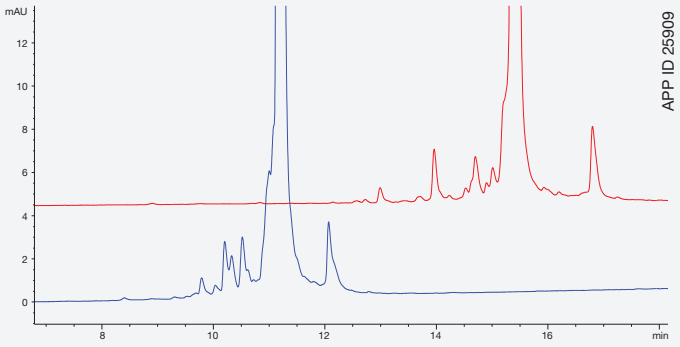


Column: Biozen 2.6 µm Oligo
Dimensions: 100 x 2.1 mm
Part No.: 00D-4790-AN
Mobile Phase A: HFIP as indicated, 4 mM TEA in Water
Mobile Phase B: HFIP as indicated, 4 mM TEA in Methanol
Flow Rate: 0.3 mL/min
Gradient: 5-30 % in 14 minutes
Temperature: 55 ˚C
Detection: UV @ 260

In experiments to explore optimal concentration of acidic modifier, lower HFIP typically provided superior separation of earlier eluting impurities and overall better peak shapes and peak heights. This is somewhat counterintuitive, since the decrease in overall retention typically adversely impacts chromatography. Nonetheless, improvements in chromatographic performance can be observed in **Figure 8**, decreasing HFIP from 100 mM to 25 mM HFIP showed an improved separation of complementary strands of a siRNA, with some loss of resolution from 25 to 12.5 mM. This effect was observed with multiple oligonucleotides, irrespective of length, chemical modifications, and thioation.

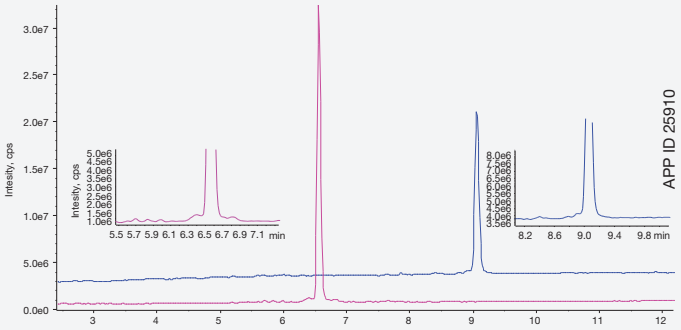
Although in some cases dramatic, there is a nominal effect observed with BNA, as seen in **Figure 9**, with separation and impurity profiles being relatively similar. Even though the impurity profile of BNA by LC-UV might not show a marked improvement, the differences are more apparent when the oligos are analyzed by high-resolution mass spectrometry. Decreasing the HFIP improves ionization efficiency 3x, as shown in differences in peak height in **Figure 10**. Interestingly, TICs do show the later eluting peaks with the 12.5 mM HFIP concentration have similar spectral quality as the main peak (data not shown); but they differ enough in physicochemical properties to retain longer by IP-RPLC. Being that this is a thioated oligo, these may be R/S isomers of full-length product, though extensive work beyond LC and mass spectrometry might be necessary to determine identity. Note that MS spectra may reveal more process-related and product related impurities; LC-UV should still be utilized for quantitation, so both should be considered. Further optimization with acidic modifiers might be exploring other perfluorinated alcohols such as HFMIP.

Figure 9 LC-UV chromatogram (260 nm) for BNA phosphorothioate oligo, Red - 100 mM HFIP, Blue- 12.5 mM HFIP. Somewhat nominal effect on separation, with slight improvements in pre-peak separation.



Column: Biozen® 2.6 µm Oligo
Dimensions: 100 x 2.1 mm
Part No.: 00D-4790-AN
Mobile Phase A: 100 mM HFIP, 4 mM TEA in Water
12.5 mM HFIP, 4 mM HA in Water
Mobile Phase B: 100 mM HFIP, 4 mM TEA in Methanol
12.5 mM HFIP, 4 mM HA in Methanol
Flow Rate: 0.3 mL/min
Gradient: 5-30 % B in 14 min
Temperature: 65 ˚C
Detection: UV @ 260

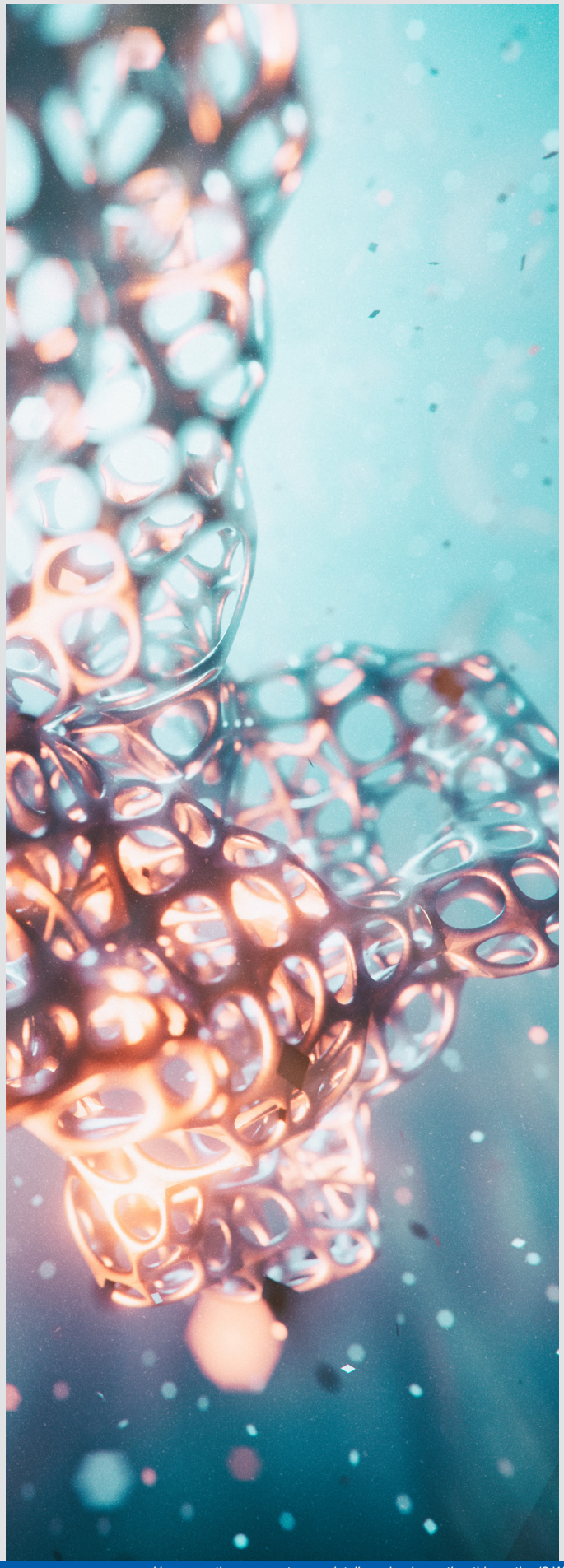
Figure 10 Effect of ionization efficiency for BNA phosphorothioate, TOF-MS (Pink -12.5 mM, Blue -100 mM). Peak height for lower concentration of HFIP is 3.6e7, for 100mM, peak height is 1.8e7.



Column: Biozen 2.6 µm Oligo
Dimensions: 100 x 2.1 mm
Part No.: 00D-4790-AN
Mobile Phase A: 100 mM HFIP, 4 mM TEA in Water
12.5 mM HFIP, 4 mM HA in Water
Mobile Phase B: 100 mM HFIP, 4 mM TEA in Methanol
12.5 mM HFIP, 4 mM HA in Methanol
Flow Rate: 0.3 mL/min
Gradient: 5-30 % B in 14 min
Temperature: 65 ˚C
Detection: UV @ 260

Summary

In summary, ion-pair reversed phase is a powerful technique for the characterization and quantitation of synthetic oligonucleotides, which have a myriad of process and product related impurities. Alkylamines such as triethylamine can effectively separate various oligos, though others such as N,N-diisopropylethylamine may confer benefits for improved chromatographic separation. Higher concentrations of alkylamine, up to 16 mM, might improve not only LC separation but spectral data, as higher concentrations of alkylamine minimize cation adduct formation during electrospray ionization. Special considerations for alkylamines should be made for double-stranded oligonucleotide modalities, wherein the effect of the alkylamine on secondary structure of the oligo may compromise the chromatographic separation and thus overall repeatability of the method. Although less explored than alkylamine ion-pair, hexafluoroisopropanol is the most common acidic modifier used for LC-MS of oligos. Chromatographic performance is often improved by decreasing HFIP concentration, with acceptable or even optimal chromatography being obtained at 12.5 mM. Even more compelling is the improved ionization with the decrease in HFIP yielding significantly higher peak heights, facilitating characterization and quantitation.



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